



Description and Validation of New Therapeutical Targets to Prevent Neurodegeneration and Cognitive Deficits in Huntington's Disease

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Universitat de Barcelona

**DESCRIPTION AND VALIDATION OF NEW THERAPEUTICAL
TARGETS TO PREVENT NEURODEGENERATION AND COGNITIVE
DEFICITS IN HUNTINGTON'S DISEASE**

Doctoral degree of Biomedicine in the Facultat de Medicina de la Universitat de Barcelona.
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Sílvia Ginés Padrós

Programa de Doctorat de Biomedicina

“L’espurna del canvi comença en un mateix,

la força dels somnis és l’arma dels rebels”

Seguirem lluitant-Els Catarres

A la meva mare

Al meu pare

A la meva Tuki

Al Gerard

Per les hores escatimades

RESUM

INTRODUCCIÓ

La malaltia de Huntington (MH) és un desordre neurodegeneratiu caracteritzat per la disfunció i mort neuronal de regions específiques del cervell. La regió més afectada és l'estriat (nuclis caudat i putamen en humans), tot i que en estadis més avançats de la malaltia s'ha descrit una atròfia i pèrdua neuronal del còrtex cerebral i hipocamp (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). La temprana disfuncionalitat de les neurones hipocampals i corticals es creu crítica per restablir les deficiències cognitives i de memòria en aquesta patologia. La malaltia s'hereta de forma autosòmica dominant i és causada per la mutació del gen *IT15*, localitzat en el braç curt del cromosoma 4 (4p.16.3), que codifica per la proteïna anomenada huntingtina (htt). Aquesta mutació va ser identificada l'any 1993 com una expansió de repeticions del triplet CAG que codifiquen per una regió poliglutamínica (poliQ) a l'extrem N-terminal de la proteïna htt (350KDa) (HDCRG, 1993). En individus sans, el nombre de repeticions oscil·la de 6 a 35; quan el nombre de repeticions d'aquest triplet és superior a 40, l'individu desenvoluparà la malaltia. Les primeres manifestacions de la malaltia solen produir-se als 35 anys d'edat conduint a la mort 15-20 anys després de l'aparició dels símptomes (Bates, 2003; Martin and Gusella, 1986). La simptomatologia inclou disfunció motora, associada majoritàriament a l'atròfia estriatal, acompanyada de trastorns cognitius i emocionals associats a l'afectació corticoestriatal i hipocampal que són de manifestació primerenca, fins i tot prèvia a la simptomatologia motora. Aquestes alteracions cognitives i emocionals constitueixen un dels pilars discapacitants en aquesta patologia, per això al llarg d'aquesta Tesi doctoral proposem un estudi dual que ens permeti definir diverses estratègies terapèutiques dirigides al tractament d'ambdues simptomatologies: motora i cognitiva.

Si bé es coneix que aquesta mutació és la causant de la malaltia, avui en dia no es coneixen els mecanismes cel·lulars i moleculars responsables de la disfunció i mort neuronal en la MH. Diversos estudis han postulat que la pèrdua de funció de la proteïna *wild-type* i/o el guany de funció de la proteïna mutada (mhtt) juguen un paper clau en el desenvolupament de la malaltia. Així s'ha descrit que l'expressió de la proteïna huntingtina mutada resulta en l'alteració de diversos processos cel·lulars i

moleculars, tals com l'agregació proteica, alteracions en el sistema ubiquitina-proteosoma, desregulació en la maquinària transcripcional així com en la remodelació de la cromatina, alteracions en la síntesi proteica, reducció del suport tròfic, alteracions en les vies de senyalització intracel·lulars, alteració en la homeòstasis del calci, dany mitocondrial, excitotoxicitat, activació de caspases, alteracions en les interaccions proteïna-proteïna i alteració en la circuiteria neuronal (Cattaneo et al., 2005; Zuccato and Cattaneo, 2009). En aquesta Tesis ens hem centrat en estudiar alguns dels mecanismes moleculars implicats en la mort neuronal, així com en els dèficits cognitius i alteracions en la plasticitat sinàptica produïda per la presència de la huntingtina mutada, mitjançant l'estudi de les alteracions produïdes en: 1) **maquinària transcripcional**, 2) **suport neurotròfic**, 3) **canvis estructurals en les sinapsis excitadores**, 4) **senyalització de proteïnes cinasa i fosfatasa** i 5) **formació d'heteròmers entre receptors acoblats a proteïnes G**.

1. Maquinària transcripcional

Diferents estudis han postulat que l'alteració en la maquinària transcripcional té lloc de forma primerenca en la MH (Cha, 2007; Dunah et al., 2002; Luthi-Carter et al., 2002; McCampbell et al., 2000; Steffan et al., 2000). Treballs previs han demostrat que la via CBP (*CREB-binding protein*)/CREB (*cAMP-response element binding protein*) es troba compromesa en la regió estriatal de diferents models cel·lulars i animals de la MH (Gines et al., 2003; Nucifora, Jr. et al., 2001; Sugars et al., 2004; Wyttenbach et al., 2001). Així, s'ha observat que l'alteració en aquesta via deguda a la presència de la huntingtina mutada, resulta en una disminució de l'expressió de diversos gens regulats per aquesta via (Augood et al., 1997; Luthi-Carter et al., 2000; Zuccato et al., 2010). CBP exerceix una paper dual en el control de la transcripció gènica mediada per CREB, mitjançant el seu paper com 1) co-activador del factor de transcripció CREB, i 2) com a proteïna amb activitat histona acetilasa que afavoreix la relaxació de la cromatina. En ambdues situacions, CBP promou la transcripció mediada per CREB de diversos gens (Chan and La Thangue, 2001). Tot i les evidències publicades de la participació de CREB i CBP com a reguladors clau en promoure la transcripció de gens implicats en processos cognitius (Alarcon et al., 2004; Benito and Barco, 2010; Chen et

al., 2010;Silva et al., 1998;Valor et al., 2011), el paper de la via CBP/CREB en els dèficits cognitius en la MH no ha sigut estudiat. Per això, en aquesta Tesis ens hem proposat estudiar el paper de la via CBP/CREB en els dèficits cognitius presents a la MH.

2. Suport tròfic

Les alteracions en el suport neurotròfic en la MH ha sigut un dels mecanismes més estudiats en la recerca de candidats terapèutics per evitar la disfunció i mort neuronal. La senyalització mediada per les diferents neurotrofines exerceix un paper clau en l'activació de cascades que promouen supervivència i mort neuronal (Bibel and Barde, 2000). Entre elles, la neurotrofina BDNF (*brain-derived neurotrophic factor*), considerada un regulador clau en la supervivència neuronal i en processos sinàptics (Arancio and Chao, 2007;Liu et al., 2008), es troba significativament reduïda en diferents regions cerebrals de models murins així com en pacients afectats per la MH (Zuccato and Cattaneo, 2007;Zuccato et al., 2008). Estudis previs han demostrat que aquesta alteració en els nivells de BDNF regula l'inici i severitat de la simptomatologia motora i cognitiva així com dels dèficits observats en plasticitat sinàptica en models murins de la MH (Canals et al., 2004;Giralt et al., 2009;Lynch et al., 2007). De fet, tractaments destinats a restablir els nivells d'expressió de BDNF han donat resultats prometedors en la reversió de la simptomatologia en models murins (Gharami et al., 2008;Giralt et al., 2009;Simmons et al., 2009;Simmons et al., 2011). No obstant, és important tenir en compte que la correcta funció d'aquesta neurotrofina es troba estretament lligada a la òptima expressió dels seus receptors de membrana (TrkB i $p75^{\text{NTR}}$) a través dels quals promourà els estímuls pertinents de supervivència o mort neuronal. De fet, una reducció significativa en els nivells d'expressió del receptor TrkB han estat observats en diversos models cel·lulars i murins i en pacients afectats de la MH (Gines et al., 2006;Gines et al., 2010;Zuccato et al., 2008). Per altra banda, BDNF pot interaccionar i senyalitzar via el receptor $p75^{\text{NTR}}$, a través del qual s'ha observat que pot potenciar o reduir la funció del receptor TrkB o bé actuant independentment induint fenòmens apoptòtics (Baker and Reddy, 1996;Ip et al., 1993;MacPhee and Barker, 1997). En un estudi previ, es va demostrar que mentre els nivells del receptor TrkB es trobaven disminuïts en mostres de caudat de pacients de la MH, els nivells de

p75^{NTR} es trobaven incrementats (Zuccato et al., 2008). Aquests resultats suggereixen que l'alteració en el suport neurotròfic en la MH pot donar-se no només per una disminució en els nivells de BDNF, sinó també degut a un desequilibri en l'expressió dels receptors TrkB i p75^{NTR}, afectant així les subseqüents cascades de senyalització. Per això, al llarg d'aquesta Tesis ens hem proposat estudiar com el desequilibri en l'expressió dels receptors TrkB i p75^{NTR} contribueix en la major vulnerabilitat estriatal enfront estímuls excitotòxics, així com en la presència de dèficits cognitius en la MH.

3. Canvis estructurals en les sinapsis excitadores

En els darrers anys s'ha proposat que la disfunció neuronal precedeix a la degeneració, essent així la major responsable de l'aparició de símptomes cognitius en aquesta malaltia (Levine et al., 2004). De fet, en pacients de la MH els dèficits cognitius tals com alteracions en funcions executives, aprenentatge de procediment, aprenentatge de tasques motores, canvis d'estratègia i de planificació apareixen prèviament a l'aparició dels moviments coreics i la mort neuronal, característiques de la MH (Hahn-Barma et al., 1998; Ho et al., 2003; Lawrence et al., 1998; Lawrence et al., 2000; Paulsen et al., 2001; Rosenberg et al., 1995; Schneider et al., 2010). Aquests estudis, entre d'altres, donen suport a la hipòtesi que la disfunció en la via corticoestriatal precedeix la degeneració estriatal (Cepeda et al., 2007; Cybulska-Klosowicz et al., 2004; Sotrel et al., 1993; Spires et al., 2004). No obstant, es desconeixen els mecanismes moleculars implicats en aquesta disfunció corticoestriatal. Kalirina-7 és una proteïna Rho-GEF expressada en neurones de diferents regions cerebrals. Diversos estudis han demostrat la seva participació clau en el manteniment i formació d'espines dendrítiques. Mitjançant estudis *in vitro* i *in vivo*, s'ha observat que la disminució o falta d'aquesta proteïna contribueix a l'alteració en la densitat de sinapsis excitadores (Ma et al., 2003; Ma et al., 2008a; Ma et al., 2008b; Penzes and Jones, 2008), així com en l'aparició de dèficits cognitius (Sommer and Budreck, 2009; Xie et al., 2010). En aquesta Tesis ens hem proposat caracteritzar els possibles dèficits en la via corticoestriatal dels nostres models murins de la MH i avaluar la participació de Kalirina-7 en la disfunció corticoestriatal de la MH.

4. Proteïnes cinasa i fosfatasa

L'alteració en l'activitat cinasa i fosfatasa juntament amb l'alteració en la seva senyalització intracel·lular, és subjecte de molts estudis enfocats a buscar diferents dianes farmacològiques. Cdk5 (*Cyclin-dependent kinase 5*) és una serina/treonina cinasa, l'activitat de la qual es troba principalment restringida al sistema nerviós on s'expressen els seus co-activadors p35 i p39. Tot i que aquesta cinasa és essencial pel correcte desenvolupament del cervell, diverses malalties neurodegeneratives presenten una activació sostinguda d'aquesta cinasa en neurones (Cruz and Tsai, 2004; Dhariwala and Rajadhyaksha, 2008; Tsai et al., 2004). Estudis previs del nostre grup han demostrat una incrementada activitat de Cdk5 en la regió estriatal de la MH i mitjançant l'ús d'un model cel·lular estriatal han demostrat que aquesta activitat aberrant de Cdk5 produïda per l'expressió de la huntingtina mutada confereix una major vulnerabilitat de les cèl·lules estriatals enfront estímuls neurotòxics (Paoletti et al., 2008). Paral·lelament, diferents estudis han senyalat el paper fonamental que diverses proteïnes cinasa, incloent la cinasa Cdk5, i fosfatasa juguen en la modulació de la plasticitat sinàptica com a resultat del processament d'informació. Degut a que prèviament s'ha descrit que l'aberrant activitat de la cinasa Cdk5 en la MH juga un paper determinant en la major vulnerabilitat estriatal enfront estímuls neurotòxics, i les darreres evidències de la seva implicació en modular processos cognitius (Barnett and Bibb, 2011; Cheung et al., 2006; Hawasli et al., 2007; Hawasli and Bibb, 2007; Hawasli et al., 2009; Lai and Ip, 2009), en aquesta Tesis ens proposem estudiar el paper de la cinasa Cdk5 en l'aparició dels dèficits cognitius en la MH.

5. Heteròmers de receptors acoblats a proteïna G

Una alterada senyalització dopaminèrgica ha estat proposada com a un procés clau en la mort cel·lular produïda en la MH (Chen et al., 2003; Chen et al., 2004; Paoletti et al., 2008). De fet, tot i la disminució en els nivells d'expressió dels receptors de dopamina a membrana, la presència de huntingtina mutada incrementa la sensibilitat del receptor, promovent així un increment en la seva senyalització (Spektor et al.,

2002). Diferents treballs han demostrat que mitjançant l'ús d'antagonistes i/o altres compostos que disminueixen la senyalització dopaminèrgica, es redueix l'aparició de símptomes motors, mentre que l'estimulació dopaminèrgica n'exacerba la simptomatologia (Mestre et al., 2009; Tang et al., 2007). El conjunt d'aquests estudis suggereix que estratègies terapèutiques que promoguin la reducció en la senyalització dopaminèrgica podrien ser òptimes pel tractament o retard de l'aparició de la simptomatologia en la MH. No obstant, l'ús general d'antagonistes del receptor D1 presenta una sèrie de limitacions, entre les quals cal destacar una disminució en l'activitat locomotora espontània (Gimenez-Llort et al., 1997). Un possible mecanisme que permet modular l'activitat d'un receptor sense bloquejar-ne completament la seva activitat, és via la modulació dels complexos que es formen entre diferents receptors. De fet, recentment, s'ha demostrat que el receptor D1 (D₁R) forma heteròmers amb el receptor d'histamina H3 (H₃R) (Ferrada et al., 2008; Ferrada et al., 2009; Moreno et al., 2011). Ambdós receptors són receptors acoblats a proteïnes G (GPCR: *G-protein coupled receptors*). En diversos treballs s'ha demostrat que lligands del H₃R modulen la senyalització dopaminèrgica via D₁R, mitjançant fenòmens de *cross-talk* i *cross-antagonism* (Ferrada et al., 2009; Moreno et al., 2011). En aquesta Tesis, ens hem proposat estudiar el paper d'aquest heteròmer D₁R-H₃R en la MH i el seu possible paper com a diana terapèutica tant en els dèficits cognitius com en la mort neuronal.

En resum, l'objectiu d'aquesta Tesi doctoral és descriure i validar noves estratègies terapèutiques comuns a les alteracions motores i cognitives en la MH.

RESUM DE RESULTATS OBTINGUTS I DISCUSSIÓ

1. Contribució de la via CBP/CREB en els dèficits cognitius de la MH

Entre els mecanismes moleculars pels quals la mhtt és capaç d'induir la mort o disfunció cel·lular, la desregulació del sistema transcripcional està críticament involucrat. En aquest aspecte, estudis previs han demostrat que la disminució de CBP

esta associada a la degeneració estriatal en models de la MH (Jiang et al., 2006; McCampbell et al., 2001; Nucifora, Jr. et al., 2001; Taylor et al., 2003). En el nostre treball hem volgut avaluar el possible paper dual que pot estar jugant CBP en la MH. L'objectiu és dissenyar noves teràpies que impliquin al mateix temps una millora en l'afectació estriatal (sovint motora) i l'afectació hipocampal i cortical (més relacionada amb processos cognitius i memòria). Degut a que ja s'ha descrit la importància funcional de CBP en la regió estriatal, en aquest treball vam proposar-nos estudiar la contribució de la via CBP/CREB en els dèficits cognitius en la MH. En el nostre treball hem descrit per primera vegada dèficits de memòria espacial i de reconeixement en ratolins heterozigots knock-in Hdh^{Q7/Q111} associats a una disminució dels nivells de CBP així com d'acetilació de la histona 3 en la regió hipocampal. A més, hem demostrat una alteració selectiva en la transcripció de gens implicats en memòria, tals com c-fos, Arc i NR4A2 dependents de l'activitat CBP/CREB. De manera significativa, l'administració d'un inhibidor general d'histones deacetilases (Trichostatin A, TSA) és capaç de rescatar els dèficits en memòria dels ratolins Hdh^{Q7/Q111}. Aquests resultats impliquen la disfunció de CBP en els dèficits cognitius observats en els ratolins MH i suggereixen que la inhibició de les histones deacetilases poden representar una nova estratègia terapèutica pel tractament dels dèficits de memòria presents en aquesta patologia.

2. Estudi dels mecanismes implicats en l'alteració del suport neurotròfic en la MH

2.1 Estudi del paper de p75^{NTR} en la major vulnerabilitat estriatal en la MH

Les teràpies basades en l'administració de BDNF han estat proposades pel tractament de la MH. No obstant, tot i la disminució dels nivells de BDNF, la reducció dels nivells de receptors de membrana tals com TrkB també han estat descrits com a possibles contribuents en l'alteració del suport neurotròfic en la MH (Gines et al., 2006; Gines et al., 2010; Zuccato et al., 2008). Al llarg d'aquesta Tesi doctoral hem volgut estudiar el paper del receptor p75^{NTR} en la neurodegeneració estriatal i la seva possible contribució en els processos cognitius en la MH. En un primer estudi hem analitzat els nivells del receptor p75^{NTR}, conegut per la seva capacitat de modular la

supervivència neuronal mediada per TrkB. Els nostres resultats mostren un desequilibri en els nivells de p75^{NTR}/TrkB en l'estriat de ratolins knock-in homozigots per la MH (Hdh^{Q111/Q111}) i en ratolins R6/1, així com en mostres de putamen d'individus afectats per la MH. El desequilibri entre els nivells de p75^{NTR}/TrkB no afecta l'activació de TrkB mediada per BDNF de vies de pro-supervivència com ara les vies d' Akt o Erk, però indueix l'activació de cascades apoptòtiques tals com JNK. A més, els nostres resultats mostren que en les cèl·lules mutades (mhtt), els alts nivells de p75^{NTR} i els nivells disminuïts de TrkB impliquen la pèrdua de protecció per BDNF davant l'excitotoxicitat mediada per NMDA. En aquest treball hem vist que la manca de neuroprotecció per BDNF està associada a una disminució de fosforilació d' Akt que correlaciona amb l'increment dels nivells proteics de la fosfatasa PP1. Tots aquests resultats demostren que en les cèl·lules estriatals, el desequilibri entre els nivells de p75^{NTR}/TrkB produït per la presència de la mhtt altera la neuroprotecció per BDNF i contribueix a la major vulnerabilitat de les cèl·lules estriatals en la MH. Basant-nos en aquestes dades, hipotetitzem que una possible estratègia terapèutica que moduli l'expressió d'ambdós receptors (TrkB i p75^{NTR}) o de la seva senyalització podria significar una millora en les teràpies neuroprotectores en la MH.

2.2 Estudi de la modulació de p75^{NTR} en els dèficits cognitius de la MH

Si bé hem demostrat amb l'anterior treball, la implicació de p75^{NTR} en la neurodegeneració estriatal, no hi ha cap estudi centrat a estudiar la possible contribució del receptor p75^{NTR} en la patologia cognitiva de la MH. Antecedents bibliogràfics indiquen que el receptor p75^{NTR} juga un paper important en la densitat d'espines dendrítiques, així com en processos d'aprenentatge i memòria. Concretament, un increment en l'expressió d'aquest receptor promou dèficits en espines dendrítiques i afectació cognitiva (Greferath et al., 2000; Woo et al., 2005; Zagrebelsky et al., 2005). Així doncs, el primer objectiu d'aquest segon treball fou analitzar l'expressió del receptor p75^{NTR} en la regió hipocampal i cortical de models murins de la MH així com en mostres hipocampals i corticals d'individus afectats per la MH. Els nostres resultats mostren un increment significatiu en els nivells del receptor p75^{NTR} en l'hipocamp, però no en l'escorça, de la MH. Per tal d'avaluar si la

normalització dels nivells de p75^{NTR} en el context de la MH implicava una millora dels processos cognitius, vam generar un nou model animal, Hdh^{Q7/Q111}:p75^{+/-}, que expressava la mhtt i uns nivells normalitzats de p75^{NTR}. Per tal d'avaluar la funció cognitiva, vam realitzar les següents tasques dependents d'hipocamp: *NOLT*, *NORT*, *T-SAT*, *Passive Avoidance*. Els nostres resultats mostren que la normalització dels nivells de p75^{NTR} restauen els dèficits cognitius observats en els ratolins Hdh^{Q7/Q111}. Aquesta millora cognitiva es troba associada a una millora en la plasticitat i transmissió sinàptica, tal i com mostra el resultat de *LTP* (Long-term potentiation). Degut a la rellevància de la formació i manteniment d'espines dendrítiques en la modulació de la plasticitat sinàptica, ens vam proposar analitzar la densitat d'espines dendrítiques en els ratolins Hdh^{Q7/Q111}:p75^{+/-}. Els resultats mostren un augment significatiu en el nombre d'espines dendrítiques en els ratolins Hdh^{Q7/Q111}:p75^{+/-} comparats amb els ratolins Hdh^{Q7/Q111}. A més, els ratolins Hdh^{Q7/Q111}:p75^{+/-} mostren una recuperació en els nivells proteics de diverses proteïnes sinàptiques alterades en els ratolins Hdh^{Q7/Q111}. Diverses evidències indiquen que p75^{NTR} controla l'activitat de RhoA (Yamashita et al., 1999) i RhoA exerceix un paper clau com a modulador negatiu de la formació d'espines dendrítiques (Nakayama et al., 2000; Tashiro and Yuste, 2008). Els resultats mostren una disminuïda activitat RhoA en animals Hdh^{Q7/Q111}, mentre que els valors d'activitat RhoA es troben restaurats en els animals Hdh^{Q7/Q111}:p75^{+/-}. Finalment, per demostrar que la sobre-expressió de p75^{NTR} mimetitzava els dèficits en memòria i aprenentatge dels ratolins Hdh^{Q7/Q111}, vam procedir a la injecció d'adenovirus, que expressen p75^{NTR}, a la regió hipocampal d'animals Hdh^{Q7/Q7}. Els resultats revelen que la sobre-expressió de p75^{NTR} no només mimetitzava els dèficits cognitius presents en animals Hdh^{Q7/Q111}, sinó que a més mostren una disminuïda activitat RhoA i una disminució en la densitat d'espines dendrítiques. En conjunt, tots aquests resultats estableixen p75^{NTR} com a un excel·lent candidat pel tractament de dèficits cognitius en la MH.

3. Estudi dels mecanismes implicats en l'alteració de la via corticoestriatal de la MH

3.1 Caracteritzar els possibles dèficits de la via corticoestriatal en models murins de la MH i estudiar el paper de la proteïna Kalirin-7 en l'alteració de sinapsis excitadores de la via corticoestriatal de la MH

Si bé la degeneració estriatal és una de les característiques principals de la MH, els mecanismes implicats en aquesta selectiva vulnerabilitat estriatal romanen desconeguts. En aquest aspecte, diversos grups remarquen la importància que pren la via corticoestriatal en la correcta innervació de la regió dorsal del nucli estriat (Cepeda et al., 2007; Cybulska-Klosowicz et al., 2004; Guidetti et al., 2001; Laforet et al., 2001; Sotrel et al., 1993; Spires et al., 2004; Van et al., 2001). En aquest treball hem volgut avaluar si els nostres models murins de la MH presenten una alteració de la via corticoestriatal i quins mecanismes moleculars podrien estar participant en aquesta disfunció. Per tal de conèixer si els nostres models murins de la MH presentaven dèficits d'aprenentatge depenent de la via corticoestriatal, vam procedir a la realització de l'ARTP (accelerating rotarod task procedure) i Strategy shifting o Swimming T-Maze test. Els nostres resultats mostren una temprana (2 mesos d'edat) alteració en l'aprenentatge depenent de la via corticoestriatal observat en dos models murins de la MH: ratolins knock-in Hdh^{Q7/Q111} i ratolins R6/1. De manera important cal destacar que aquestes alteracions s'accentuen a edats més avançades, en les quals encara no s'observen dèficits motors, evidenciant que els dèficits en aprenentatge de tasques motores precedeix les alteracions en la coordinació motora en ambdós models murins de la MH. Per altra banda, els resultats obtinguts mitjançant electrofisiologia demostren que aquesta disfunció temprana correlaciona amb una alteració en la transmissió sinàptica de la via corticoestriatal. Per tal d'estudiar si aquests dèficits en el comportament i en la transmissió sinàptica correlacionaven amb una alteració en les sinapsis excitadores, vam procedir al seu anàlisi mitjançant immunohistoquímica de dos marcadors postsinàptics: Spinophilin i PSD95. Els nostres resultats mostren una disminució significativa de partícules positives per ambdós marcadors en l'escorça cerebral, però no en l'estriat de ratolins Knock-in Hdh^{Q7/Q111} als 2 mesos d'edat, suggerint que l'afectació cortical precedeix a l'afectació estriatal. En canvi, al realitzar l'anàlisi als 8 mesos d'edat, els nostres resultats mostren que tant la regió cortical com

la regió estriatal presenten una disminució significativa en el nombre de partícules positives per spinophilin i PSD95, suggerint que a edats més avançades la regió estriatal participaria també en els dèficits d'aprenentatge observats. A continuació, vam procedir mitjançant la tècnica de Western blot a l'anàlisi dels nivells d'expressió de receptors ionotròpics de glutamat (GluA1, GluN1, GluN2B) així com proteïnes de senyalització i estructurals implicades en processos sinàptics (CamKII, PSD-95, Spinophilin, Shank3, Vglut1, Synaptophysin i Kalirin7). De totes les proteïnes analitzades, tan sols Kalirin-7 (Kal7) mostra una disminució significativa als 2 mesos d'edat en la regió cortical de ratolins Knock-in Hdh^{Q7/Q111} i R6/1, mentre que la regió estriatal no mostra canvis significatius en cap de les proteïnes estudiades. Als 8 mesos d'edat, l'alteració en els nivells de Kal7 es troba acompanyada per l'alteració en altres proteïnes de senyalització i/o estructurals en la regió cortical de models murins de la MH. A més, als 8 mesos d'edat, també s'evidencia una disminució significativa d'algunes de les proteïnes de senyalització i/o estructurals en la regió estriatal. Així doncs, els nostres resultats mostren una específica reducció dels nivells de Kal7 a l'escorça cerebral dels models murins de la MH que correlaciona amb els dèficits d'aprenentatge, l'alteració en la transmissió sinàptica corticoestriatal i la disminució de partícules sinàptiques en l'escorça cerebral a edats temperanes de la MH. La Kal7 és una RhoGTPasa moduladora de l'activitat de Rac1 i RhoG localitzada a la densitat post-sinàptica de neurones excitadores. Així, vam proposar-nos analitzar l'activitat del seu substrat, Rac1. Els nostres resultats demostren una disminució significativa dels nivells d'activitat Rac1 en l'escorça de ratolins Knock-in Hdh^{Q7/Q111} que correlaciona amb la disminució dels nivells de Kal7 observats en aquest model animal de la MH. Finalment, per tal d'avaluar la participació de Kal7 en l'alteració sinàptica de la MH vam procedir a analitzar si la sobre-expressió de Kal7 era capaç de millorar o restaurar el nombre de sinapsis excitadores en un cultiu primari cortical provinent d'embrions wild-type i R6/1. Els nostres resultats mostren que la sobre-expressió de Kal7 reverteix els dèficits en el nombre de sinapsis excitadores d'un cultiu cortical provinent d'embrions R6/1. Aquests resultats demostren que Kal7 participa en l'alteració del control molecular de la plasticitat estructural en la MH i identifiquen Kal7 com a un possible substrat per l'establiment de noves estratègies terapèutiques pel tractament de la disfunció corticoestriatal en la MH.

4. Estudi del paper de la cinasa cdk5 en la presència de dèficits cognitius en la MH

Resultats previs del nostre grup han demostrat una activitat incrementada de Cdk5 en la regió estriatal d'animals Knock-in homozigots ($Hdh^{Q111/Q111}$) de la MH. A més, han demostrat que aquesta aberrant activitat conferia una major vulnerabilitat de les cèl·lules estriatals enfront estímuls excitotòxics, indicant la seva participació en els processos de mort cel·lular deguts a l'expressió de la huntingtina mutada (Paoletti et al., 2008). En aquesta Tesis, ens hem proposat estudiar si aquesta aberrant activitat de Cdk5 participa també en la disfunció neuronal que condueix a la presència de dèficits en aprenentatge i memòria en la MH. La cinasa Cdk5 s'ha vist implicada en diverses patologies que afecten processos cognitius com ara la malaltia d'Alzheimer o la malaltia de Parkinson (Cheung et al., 2006; Lai and Ip, 2009). Degut a que resultats previs ens indiquen una aberrant activitat Cdk5, ens plantegem estudiar si aquesta alterada activitat pot contribuir als dèficits cognitius presents en la MH. Per tal de portar a terme aquest estudi, vam generar un nou model animal transgènic que expressa la huntingtina mutada (mhtt) i alhora presenta heterozigosi en l'expressió de Cdk5 ($Hdh^{Q7/Q111}; Cdk5^{+/-}$). Els nivells totals de la cinasa Cdk5 van ser avaluats observant-se una disminució significativa en els animals $Cdk5^{+/-}$ i dobles mutants: $Hdh^{Q7/Q111}; Cdk5^{+/-}$. A continuació, vam realitzar un estudi exhaustiu de comportament en els nostres animals: $Hdh^{Q7/Q7}, Cdk5^{+/-}$, $Hdh^{Q7/Q111}$ i $Hdh^{Q7/Q111}; Cdk5^{+/-}$ a l'edat de 6 mesos, per tal d'avaluar si la reducció en l'expressió de Cdk5 tenia un efecte en els dèficits cognitius de la MH. Per tal d'estudiar l'aprenentatge dependent de la via corticoestriatal, en aquest treball hem realitzat les següents tasques de comportament: ARTP (Accelerating Rotarod Task Procedure) i l'Strategy Shifting. En ambdues tasques, la modulació genètica dels nivells de Cdk5 mostra una reversió dels dèficits corticoestriatals observats en el ratolí $Hdh^{Q7/Q111}$. Seguidament, ens vam plantejar estudiar els dèficits en processos de memòria espacial i de reconeixement d'objectes mitjançant les tasques de comportament següents: T-SAT, NORT (Novel Object Recognition Test). En tots ells els ratolins $Hdh^{Q7/Q111}; Cdk5^{+/-}$, mostren millores significatives en la realització d'aquestes tasques que involucren la memòria espacial i la memòria d'aprenentatge quan els comparem amb els ratolins $Hdh^{Q7/Q111}$. En conjunt,

aquests resultats suggereixen que la modulació dels nivells de Cdk5 en la MH pot contribuir a la millora dels dèficits cognitius de la MH, convertint així la cinasa Cdk5 en una bona diana terapèutica.

5. Estudi del paper dels heteròmers formats entre el receptor de dopamina D1 (D₁R) i el receptor d'histamina H3 (H₃R) en la mort neuronal i els dèficits cognitius de la MH

La disfunció i mort de neurones de projecció estriatal és una de les característiques neuropatològiques principals en la MH. La regió estriatal no tan sols és diana de les aferències glutamatèrgiques provinents principalment de l'escorça cerebral, sinó que també és diana d'una densa innervació dopaminèrgica provinent principalment de la substància nigra. De fet, diverses evidències bibliogràfiques indiquen la participació del sistema dopaminèrgic en la neuropatologia de la MH (Albin et al., 1990b; Albin et al., 1990a; Andre et al., 2010; Garret et al., 1992; Paoletti et al., 2008; Ross and Tabrizi, 2011; Spokes, 1980; Vonsattel et al., 1985). En estudis previs s'ha descrit que una sobre-activació del D₁R produeix un desequilibri entre les vies directe e indirecte (Chen et al., 2013; Paoletti et al., 2008). A més, diversos treballs han demostrat que reduint la senyalització dopaminèrgica mitjançant l'ús d'antagonistes dels receptor D1, es normalitza la transmissió sinàptica excitadora i inhibidora (Andre et al., 2011). A més, en altres treballs s'ha observat que antagonistes dopaminèrgics redueixen el símptomes motors en pacients i models animals, mentre que l'estimulació dopaminèrgica n'incrementa la simptomatologia (Mestre et al., 2009; Tang et al., 2007). En conjunt, aquests treballs suggereixen que la reducció de la senyalització dopaminèrgica pot resultar en una prometedora estratègia terapèutica en la MH. No obstant, l'ús genèric d'antagonistes D₁R compromet moltes de les funcions fisiològiques de la pròpia senyalització dopaminèrgica, i el bloqueig complet d'aquest receptor causa alteracions importants reduint l'activitat locomotora espontània (Gimenez-Llort et al., 1997). És per això, que en aquest treball ens hem centrat en estudiar com la modulació d'un altre receptor, el receptor d'histamina H3 (H₃R), el qual interactua amb el receptor D1 formant així un complex anomenat

heteròmer (Moreno et al., 2011), pot resultar en una bona diana terapèutica per modular l'excessiva senyalització dopaminèrgica observada en la MH. Els nostres resultats mostren l'expressió i funcionalitat de l'heteròmer format entre D₁R i H₃R en el model cel·lular estriatal de la MH i en l'estriat, hipocamp i escorça cerebral de models murins de la MH en etapes tempranes de la MH. No obstant, la presència d'aquest heteròmer desapareix en etapes més avançades de la malaltia, tant en models murins com en individus afectats per la MH. Enfront la sobre-activació d'aquest heteròmer, els agonistes i antagonistes d'H₃R actuen com a "*molecular brake*", disminuint així la senyalització dopaminèrgica via D₁R. Així, hem observat que davant un estímul dopaminèrgic (mitjançant l'ús d'agonistes del receptor D1) que causa mort neuronal, els lligands del receptor H₃R (agonistes i antagonistes) actuen bloquejant aquesta mort neuronal, en línies cel·lulars i en cultius organotípics realitzats en diverses regions cerebrals de ratolins control i malalts. Per tal d'estudiar si la modulació de la senyalització dopaminèrgica mitjançant l'antagonisme del receptor H₃R, juga un paper important no tan sols en els processos de mort neuronal, sinó també en els dèficits cognitius de la MH, hem realitzat diverses tasques de comportament després d'un tractament crònic amb un antagonista del receptor H₃R, anomenat thioperamide. Així, vam procedir a realitzar el tractament amb thioperamide en edats pre-sintomàtiques on havíem comprovat l'expressió i funcionalitat de l'heteròmer D₁R-H₃R. Al cap d'un mes de tractament, vam procedir a avaluar diferents tasques: ARTP, T-SAT, Open field i NORT, descrites prèviament en els altres treballs realitzats al llarg d'aquesta tesis. Els resultats obtinguts han demostrat que el tractament crònic amb una antagonista del H₃R reverteix els dèficits observats en els animals Hdh^{Q7/Q111} en totes les tasques de comportament realitzades. A més, vam voler estudiar si el tractament amb l'antagonista del H₃R modificava l'expressió de l'heteròmer *in vivo*. Sorprenentment, vam poder observar la presència de l'heteròmer D₁R-H₃R en ratolins Hdh^{Q7/Q111} en edats sintomàtiques després del tractament crònic amb thioperamide, suggerint que el tractament amb l'antagonista de H₃R, preserva la interacció amb el D₁R i modula la senyalització dopaminèrgica implicada en la disfunció neuronal que conduirà a la presència de dèficits cognitius en la MH. El conjunt d'aquests resultats demostren que l'heteròmer D₁R-H₃R juga un paper clau en el control de la senyalització dopaminèrgica en la MH, i el situa com a un candidat

farmacològic prometedor pel tractament dels dèficits cognitius i la mort neuronal en la MH.

En conjunt, els resultats obtinguts en aquesta Tesi Doctoral posen de relleu diferents vies (CBP/CREB, p75^{NTR}, Kalirina-7, Cdk5 i els heteròmers formats entre D₁R-H₃R) amb potencial terapèutic pel disseny de noves estratègies comuns per revertir el processos de mort neuronal i els dèficits motors i cognitius de la MH.

ABBREVIATIONS

3-NP	3-Nitropropionic acid
5-HT	Serotonin
6-OHDA	6-Hydroxydopamine
A	Adenine
AA	Arachidonic acid
AC	Adenylyl cyclase
Ach	Acetylcholine
AChR	Acetylcholine receptor
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
AKAP	A-Kinase anchoring protein
Akt	Serine/Threonine protein kinase C
ALS	Amyotrophic lateral sclerosis
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
Ape1	Apurinic/Apyrimidinic endonuclease 1
APP	Amyloid precursor protein
ARTP	Accelerating rotarod task procedure
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
C	Cytosine
CA	<i>Cornus Ammonis</i>
Ca ²⁺	Calcium
Cables	Cdk5 and Abl enzyme substrate
CAMKs	Ca ²⁺ /calmodulin-dependent protein kinases
cAMP	Cyclic-adenosine monophosphate
CASK	calcium/calmodulin-dependent serine protein kinase
CBP	CREB-binding protein
Cdc-42	Cell division cycle-42 protein
Cdh1	Cadherin1
CDK	Cyclin-dependent kinase
CNS	Central nervous system
CREB	cAMP-response element protein
Crmp2	Collapsin response mediator protein-2
c-Src	Non-receptor tyrosine kinase
CTKD	C-terminal kinase domain
D ₁ R	Dopamine 1 receptor
D ₂ R	Dopamine 2 receptor
DA	Dopamine
DAG	Diacylglycerol
DARPP-32	Dopamine- and cyclic-AMP-regulated phosphoprotein 32
DG	Dentate gyrus
DISC1	Disrupted in schizophrenia 1
Dixdc1	Dix-domain containing 1

ABBREVIATIONS

DLG	PDZ-domain scaffolding protein Discs-large
DNA	Deoxyribonucleic acid
DP	Direct pathway
DRG	Dorsal root ganglion
DUSP	Dual specificity phosphatase
EC	Entorhinal cortex
EGF	Epidermal growth factor
ENK	Enkephalin
ER	Endoplasmatic reticulum
ErbB3	Receptor tyrosine-protein kinase erbB3
ERK 1/2	Extracellular signal-regulated kinase 1/2
FAK	Focal adhesion kinase
G	Guanine
GABA	Gamma-aminobutyric acid
GDNF	Glial cell-derived neurotrophic factor
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GluA1	AMPA subunit GluR1
GPCRs	G protein-coupled receptors
GPe	Globus pallidus pars externa
GPI	Globus pallidus pars interna
GR	Glucocorticoid receptor
GSK-3 β	Glycogen synthase kinase-3 β
GTP	Guanosine triphosphate
H ₃ R	Histamine 3 receptor
HA	Histamine
HATs	Histone acetyl transferases
HD	Huntington's disease
HDACs	Histone deacetylases
HDC	Histidine decarboxylase
Hdh	Huntington's disease gene homolog
HEAT	Huntingtin, Elongation factor3, protein phosphatase 2A and TOR
HIP	Huntingtin-interacting protein
Htt	Huntingtin
iEGs	Immediate early genes
IP	Indirect pathway
IP3	Inositol-1,4,5-triphosphate
IP3R	Inositol (1,4,5)-triphosphate receptor
JNK	c-Jun-N terminal kinase
KA	Kainate
Kal7	Kalirin-7
KO	Knock-out
L-DOPA	L-3,4-dihydroxyphenylalanine
LTD	Long-term depression

LTM	Long-term memory
LTP	Long-term potentiation
M	Months
MAGUKs	Membrane-associated guanylate kinases
MAO	Monoamina oxidasa
MAP1B	Microtubule-associated protein 1B
MAPKs	Mitogen-activated protein kinases
MEK	MAPK/ERK kinase
MEF2	Myocyte enhancer factor-2
mGluR	Metabotropic glutamate receptor
Mhtt	Mutant huntingtin
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MSK1,2	Mitogen- and stress-activated protein kinase-1,2
MSSNs	Medium-sized spiny neurons
MKP	Mitogen-activated protein kinase phosphatase
Munc18	Mammalian uncoordinated-18 protein
NA	Noradrenaline
NAc	Nucleus accumbens
NES	Nuclear export signal
NF	Neurofilament
NF- κ B	Nuclear factor- κ B
NGF	Nerve growth factor
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate acid
NMDAR	NMDA receptor
NMJ	Neuromuscular junction
NORT	Novel Object Recognition test
Nrg	Neurogulin
NSF	N-ethylmaleimide sensitive factor
NT-3 or NT-4	Neurotrophin 3 or Neurotrophin 4
OLT	Object location task
p21(kip1)	Cyclin-dependent kinase inhibitor
p53	Tumor protein 53
p62	p62/Sequestosome 1
p75NTR	p75 neurotrophin receptor
PAK1	p21-activated kinase
Paxilin	Focal adhesion-associated adaptor protein
Pctaire 1	Cyclin-dependent kinase 16 (cdk16)
PD	Parkinson's disease
PDK1	3'-phosphoinositide-dependent kinase-1
PH	Pleckstrin homology
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate

ABBREVIATIONS

PIP2	Phosphatidylinositol-3,4-biphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PIPKI	Phosphatidylinositol(4) phosphate (5) kinase type I gamma
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PLA ₂	Phospholipase A
PLA	Proximity ligation Assay
PLC	Phospholypase C
Plk2	Polo-like kinase
PolyQ	Polyglutamine
PolyP	Polyproline
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP	Perforant path
PPAR γ	Peroxisome proliferator-activated receptor gamma
PRE	Prion-related encephalopathies
Prx2	Peroxiredoxin 2
PSD	Postsynaptic density
PSD-93	Post-synaptic density 93
PSD-95	Post-synaptic density 95
RasGRF	Ras guanine nucleotide releasing factor 2
Rb	Retinoblastoma protein
REST/NRSF	Repressor element-1 silencing transcription factor/Neuron-restrictive silencer factor
Rsk	90-KDa ribosomal S6 kinase
S6K1	S6 kinase 1
SAP	Synaptic associated protein
SC	Schaffer collateral
Ser	Serine
SNARE	SNAP receptor
Snc	Substantia nigra <i>pars compacta</i>
SNr	Substantia nigra <i>pars reticulata</i>
SP	Substance P
SPAR	Spine-associated Rap guanosine triphosphatase activating protein
STAT3	Signal transducer and activator of transcription-3
STEP	Striatal-enriched protein tyrosine phosphatase
STN	Subthalamic nucleus
SUMO	Small ubiquitin-like modifier
Sub	Subiculum
TFs	Transcription factors
TH	Tyrosine hydroxylase
Thr	Threonine
TMN	Tuberomammillary nucleus
TRPV-1	Transient Receptor Potential Vanilloid-1
TSA	Trichostatin A

ABBREVIATIONS

T-SAT	T-maze spontaneous alternation task
Trk	Tropomyosin receptor kinase
Tyr	Tyrosine
ULK1	UNC-51-like kinase
UPS	Ubiquitin proteasome system
VGlut1	Vesicular glutamate transporter 1
W	Weeks
WAVE-1	WASP family verproline-homologous protein-1
wHtt	Wild type huntingtin
YAC	Yeast artificial chromosome

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I. INTRODUCTION

Neurodegenerative diseases are considered as a group of disorders that progressively impairs function of the nervous system due to selective neuronal vulnerability of specific brain regions, synaptic abnormalities and progressive neuronal cell loss. These diseases can be categorized according to the affected regions and the associated clinical manifestations. Although there are many differences in the etiology of these disorders, all of them are incurable and their progression results in debilitating conditions for the patients. The inability to work and socialize associated with the progressive deterioration of cognitive functions leads to a devastating social impact for the patients and families. Finding treatments to delay or cure these neurodegenerative diseases is a goal of increasing urgency for society and scientific community. Thus, the study of the specific mechanisms altered in neurodegenerative diseases and the identification and/or validation of molecular therapeutic targets is crucial in this field. The present Thesis was focused on the study and validation of potential candidates to develop pharmacological strategies to treat or delay motor and cognitive deficits in Huntington's disease.

HUNTINGTON'S DISEASE

1.1 ETIOLOGY

Huntington's disease (HD) is an autosomal dominant, inherited neurodegenerative disorder that was first described in 1872 by George Huntington (April 9, 1850-March 3, 1916), who named the disease Chorea (from the Greek *choreia* that means dance) on account of the dancing propensities of those who are affected by the disease (Huntington, 1982). He observed the inherited property of this clinical manifestation (chorea) in different families from East Hampton (Long Island, New York). In 1983, more than a hundred years after the original description by George Huntington, the *locus* responsible for HD was identified in the chromosome 4p16.3 (Gusella et al., 1983). In 1993, the Huntington's Disease Collaborative Research Group discovered the genetic mutation responsible for the disease, a repetitive DNA element consisting of three nucleotides: C (cytosine), A (Adenine) and G (guanine), in the first exon of the IT15 gene, that encodes for a protein called huntingin (HDCRG,1993). The genetic mutation responsible for the disease is an expanded unstable number of CAG

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trinucleotide repeats within the coding region of the *huntingtin* gene, expressed as an extended polyglutamine tract and localized in the N-terminal sequence of the exon 1 (HDCRG,1993). In normal individuals, the CAG repeat length ranges from 11 to 29 repeats, whereas HD patients showed 40 or more CAG repeats (Duyao et al., 1993). Importantly, there were some individuals with 29 to 34 CAG repeats (intermediate phase), whose are not developing HD pathology but can transmitted the disease to their offspring and lead to a new appearances (Ranen et al., 1995). Furthermore, the individuals with 35 to 40 CAG repeats show incomplete penetrance of the mutant allele and can manifest or not the disease in advanced ages. Several studies showed that there was an inverse correlation between the age of onset of symptoms and the lengthening of the mutation (HDCRG,1993;Duyao et al., 1993;Rubinsztein et al., 1996). Moreover, the earlier onset of the disease is associated with an increased severity and a more rapid symptomatology progression (Beighton and Hayden, 1981;Conneally, 1984). Nonetheless, it is difficult to predict the onset of the disease in the individuals that showed 40-50 CAG repeats (the most cases), due to the interindividual variability. Actually, it has been described that in addition to triplet repeat length, other factors such as environmental factors, genetic factors, or both, could contribute to the age of onset (Alberch et al., 2005;Wexler et al., 2004). The incidence of the disease is variable, depending on the studied population; in Europe and America the prevalence of the mutation is 5-7 cases per 100,000 individuals (Conneally, 1984;Harper, 1992). The most prevalence in the world is in Venezuela, in the area around the Lake Maracaibo. In contrast, the incidence is very low in African and Asian populations (Walker, 2007).

HD patients exhibit severe motor disturbances such as chorea, dystonia, bradykinesia, progressive gait disturbance, oculomotor disturbances, dysarthria and dysphagia, associated with cognitive and psychiatric disturbances. Cognitive deficits include impairments in attention, visuospatial ability, semantic and verbal fluency, and short and long-term memory deficits. Psychiatric disturbances such as depression, irritability and anxiety often appear (50-98% of cases) (Naarding et al., 2001;Paulsen et al., 2001a), and suicide occurs with more frequency than in general population. Motor symptoms usually begin between 35-40 years of age. The course of the disease is progressive and the patient usually dies after 15-20 years of clinical manifestations

(Vonsattel et al., 2008;Vonsattel, 2008). Although there are some symptomatic treatments, there is no effective cure for this devastating disorder, so the deterioration of the patient progresses towards a total dependence state and usually infection or trauma are the cause of death (Folstein et al., 1986;Sturrock and Leavitt, 2010).

1.2 HUNTINGTIN PROTEIN

Huntingtin is encoded by a single gene, which contains 67 exons, being the exon 1 the one containing the mutation (HDCRG,1993). The translated huntingtin is a 348-kDa protein (HDCRG,1993). It is expressed ubiquitously, with highest levels in the testis and in the neurons of the central nervous system, particularly in the neocortex, cerebellar cortex, striatum and hippocampus (Borrell-Pages et al., 2006;DiFiglia et al., 1995;Ferrante et al., 1997;Fusco et al., 1999;Rossi and Cattaneo, 2002;Velier et al., 1998). Huntingtin is a cytoplasmic protein, although a small proportion is also found in the nucleus (Kegel et al., 2002). Thus, within the cell, mammalian huntingtin is associated with a wide-range of organelles, including the nucleus, endoplasmic reticulum, Golgi complex, plasma membrane and mitochondria (Hilditch-Maguire et al., 2000;Hoffner et al., 2002;Kegel et al., 2002;Panov et al., 2002;Strehlow et al., 2007). Furthermore, it is also found within the neurites and synapses, where it has been shown to associate with microtubules, vesicular structures and endosomal compartments (DiFiglia et al., 1995;Hilditch-Maguire et al., 2000;Hoffner et al., 2002;Imarisio et al., 2008;Velier et al., 1998). Both molecular structure as well as this widespread subcellular localization hampers to clarify the data about its structure and function, respectively.

1.2.1 Huntingtin structure

Despite the difficulty to study huntingtin structure because its high molecular weight, several studies contributed to elucidate its structure properties (Fig. 1). Huntingtin is a multidomain protein with a polymorphic stretch of glutamine residues in its N-terminal domain called polyQ region (HDCRG,1993). This polyQ stretch in human huntingtin begins at the 18th amino acid. In unaffected individuals, the polyQ stretch contains up to 35 glutamine residus (HDCRG,1993). In the past ten years, it has been shown that this polyQ tract is a key regulator of huntingtin binding to its partners

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(Harjes and Wanker, 2003). In higher vertebrates, the polyQ region is followed by a polyproline (polyP) stretch, which suggested function may reside in the stabilization of the polyQ tract by keeping it soluble (Steffan et al., 2004; Darnell et al., 2007). Downstream of the polyQ domain, huntingtin is also enriched in consensus sequences called huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1 (HEAT) repeats that are distributed into protein domains and favors protein-protein interactions (Andrade and Bork, 1995; Neuwald and Hirano, 2000). It has been reported a total of 16 HEAT repeats in huntingtin protein that are organized into 4 clusters (Tartari et al., 2008). Other important feature of huntingtin structure is the consensus cleavage sites for proteolytic enzymes (caspases, calpains and aspartyl proteases) that cleave the protein and generate a wide range of fragments (Hermel et al., 2004; Kim et al., 2001; Wellington et al., 1998; Wellington et al., 2002). Although the precise contribution of huntingtin proteolysis to cell functioning is not clear, several studies described diminished mutant huntingtin toxicity as well as a delay on disease onset and progression when caspases and calpains activities are modified and consequently, huntingtin proteolysis reduced (Gafni et al., 2004; Wellington et al., 2000; Wellington et al., 2002). Among other critical sequences in huntingtin structure, a functionally active COOH-terminal nuclear export signal (NES) sequence has been described, indicating that huntingtin protein or huntingtin fragments contribute in transporting molecules from the nucleus to the cytoplasm (Xia et al., 2003). On the other hand, whether huntingtin contains a nuclear localization signal (NLS) is controversial (Bessert et al., 1995; Hackam et al., 1998). Additionally, huntingtin is subjected to several posttranslational modifications, such as ubiquitination, phosphorylation, sumoylation, palmitoylation and acetylation. Ubiquitination of huntingtin at the N-terminus targets huntingtin to the proteasome, controlling its stability, function and intracellular localization, which contribute to maintain huntingtin homeostasis (DiFiglia et al., 1997; Kalchman et al., 1996). The functional relevance of huntingtin phosphorylation has been largely discussed in the literature. An interesting study has mapped the major Htt phosphorylation sites, using a mass spectrometric analysis in the full-length protein (Schilling et al., 2006). Among different kinases, huntingtin phosphorylation by protein kinase B, also called Akt, is crucial to mediate the neuroprotective effects of IGF-1 (Humbert et al., 2002; Rangone et al., 2004; Warby et al., 2005). Cyclin-dependent

kinase 5 (Cdk5) phosphorylation of huntingtin reduces its caspase-mediated cleavage at residu 513 leading to attenuation of aggregate formation and toxicity (Luo et al., 2005). Sumoylation is a highly dynamic posttranslational modification and its outcomes are extremely diverse. Thus, sumoylation of wild-type huntingtin has been described to modulate changes in its localization, stability and activity (Geiss-Friedlander and Melchior, 2007;Steffan et al., 2004). Palmitoylation of huntingtin is conducted by huntingtin interacting protein 14 (HIP14). This modification regulates its localization and function. Importantly, it has been described that the expanded polyglutamine tract in mutant huntingtin reduces its interaction with HIP14 and results in a diminished palmitoylation, which contributes to the formation of inclusion bodies and enhances neuronal toxicity (Yanai et al., 2006). Finally, it has been suggested that huntingtin acetylation could play an important role in modulating its targeting to the macroautophagy pathway. Indeed, it has been reported that when mutant huntingtin is not acetylated, it accumulates and contributes to neurodegeneration (Jeong et al., 2009).

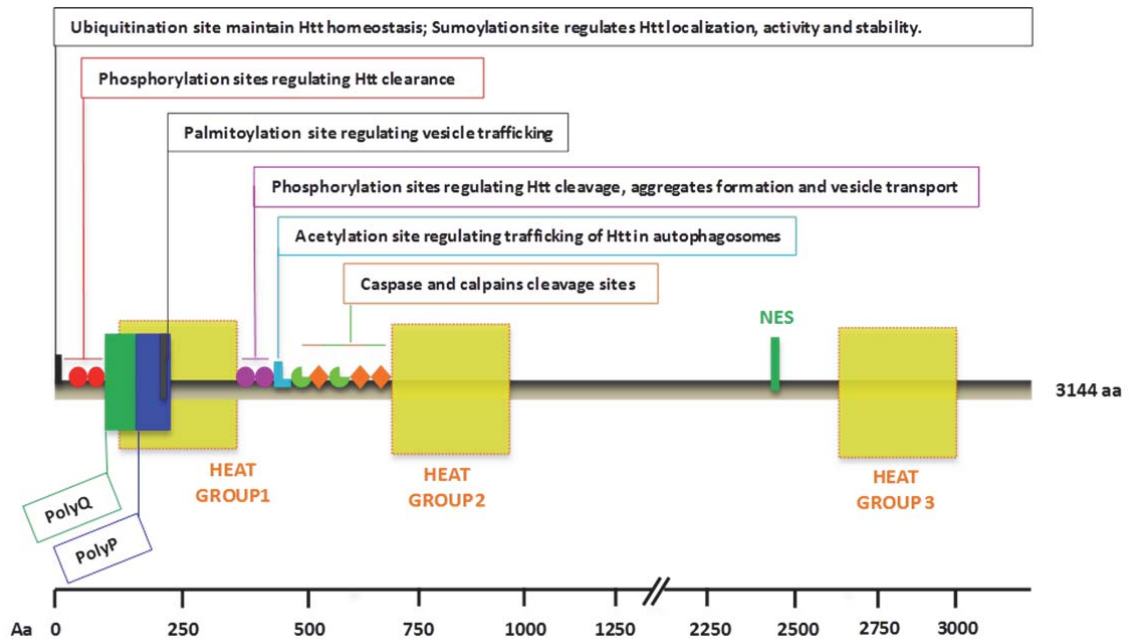


Figure 1. Schematic diagram of huntingtin structure. The diagram represents the huntingtin amino acid sequence with the polyglutamine (PolyQ) and polyproline (PolyP) tracts (regulator of Htt binding to its partners and stabilizer of PolyQ tract, respectively), the HEAT repeat domains and the nuclear exporting signal (NES). The following post-translational modifications are indicated: Ubiquitination, sumoylation, phosphorylation, palmitoylation and acetylation. Figure adapted from (Zuccato et al., 2010).

1.2.2 Huntingtin function

Although the physiological role of huntingtin remains poorly defined due to the large size of the protein that difficult its isolation and analysis (Imarisio et al., 2008), several functions have been proposed (Fig. 2). Huntingtin is essential for normal embryonic development. Indeed, knockout studies in mouse embryos showed abnormal gastrulation at embryonic day 7.5 and lethality around day 8.5, indicating a critical role in early development that precede the emergence of the nervous system (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Interestingly, Zeitlen and colleagues proposed that huntingtin is also involved in apoptosis, as indicated by increased levels of cell death observed in huntingtin knockout animals. The anti-apoptotic role of huntingtin is supported by other studies in which the overexpression of wild-type huntingtin in brain-derived cells protect them from toxic stimuli (Rigamonti et al., 2000; Rigamonti et al., 2001) and protect neuroblastoma and kidney cell lines from death triggered by the mutant protein (Ho et al., 2001). Moreover, cells with huntingtin depletion showed caspase-3 activation and were more sensitive to apoptotic cell death than control cells. By contrast, overexpression of huntingtin blocks apoptosis by physically interacting with caspase-3 resulting in caspase-3 proteolytic activity inhibition (Zhang et al., 2006).

Huntingtin has also been shown to be involved in transcriptional regulation. Thus, huntingtin interacts with a large number of transcription factors and other proteins involved in regulating mRNA production (Borrell-Pages et al., 2006; Harjes and Wanker, 2003; Holbert et al., 2001; Kaltenbach et al., 2007; Kegel et al., 2002; Li and Li, 2004; McCampbell et al., 2000; Steffan et al., 2000). However, how huntingtin influences transcription remains unclear. The most well known mechanism as a transcriptional regulator is in the transcriptional regulation of brain-derived neurotrophic factor (BDNF), an important neurotrophin required for striatal neuronal survival and involved in the activity of corticostriatal synapses (Canals et al., 2001; Zuccato and Cattaneo, 2007).

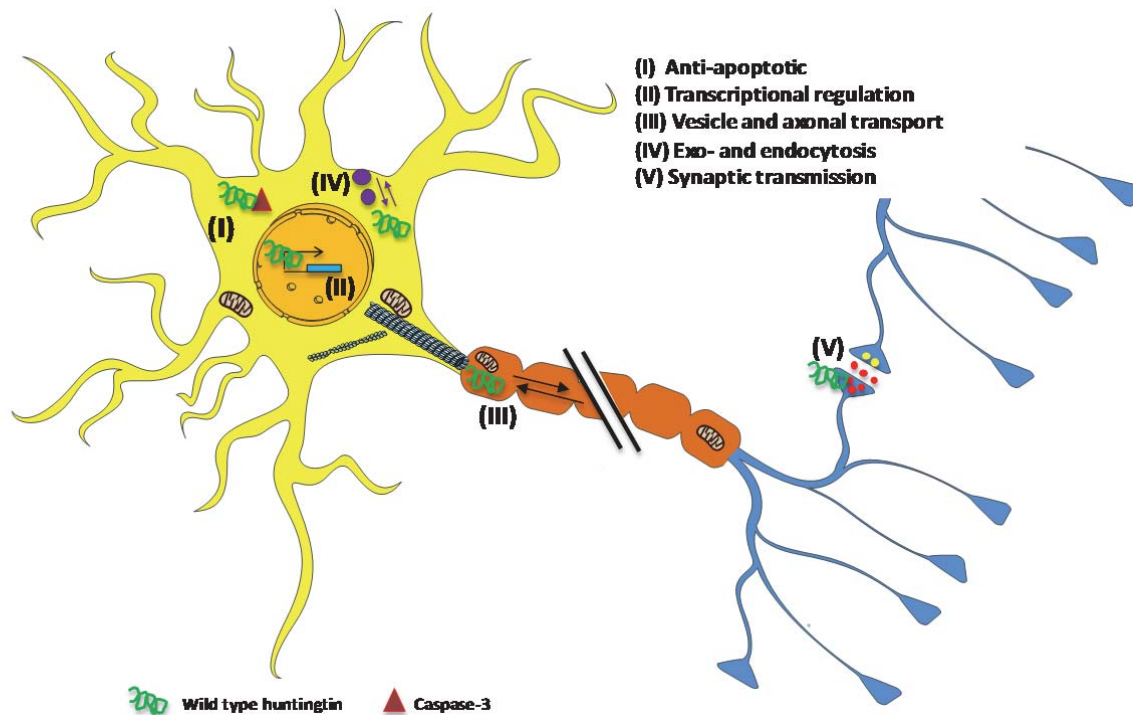


Figure 2. Huntingtin and its normal cellular roles. Proposed cellular functions for wild-type huntingtin. See the text for details.

Huntingtin interacts with the repressor element-1 silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) and maintains it in the cytoplasm, reducing its ability to bind to the neuron-restrictive silencer element (NRSE) and ultimately allowing gene transcription (Zuccato et al., 2003). Supporting this transcriptional role, the overexpression of wild-type huntingtin results in increased BDNF mRNA and protein levels in both *in vitro* and *in vivo* studies (Zuccato et al., 2001), whereas its depletion results in a marked decrease in BDNF mRNA levels (Zuccato et al., 2003; Zuccato et al., 2007). Thus, as a regulator of REST/NRSF, huntingtin acts as a positive transcriptional regulator of NRSE-regulated genes such as BDNF.

Because huntingtin is predominantly found in the cytoplasm of neurons and is enriched in compartments containing vesicle-associated proteins, its participation in regulating vesicle and axonal transport has been studied. Thus, several reports have demonstrated its role in fast axonal trafficking of mitochondria along mammalian neurons (Trushina et al., 2004), and in regulating axonal transport by participating in

the assembly of the motor complex on microtubules (Trushina et al., 2004). In addition to its role as a transcriptional BDNF regulator, huntingtin plays a pivotal role in promoting BDNF vesicular transport, essential in controlling neurotrophic support (del et al., 2006;Gauthier et al., 2004). Actually, huntingtin interacts with several proteins involved in the regulation of exo- and endocytosis (Engqvist-Goldstein et al., 2001;Kalchman et al., 1997;Li et al., 1995;Modregger et al., 2002;Singaraja et al., 2002;Smith et al., 2005;Wanker et al., 1997). Additionally, wild-type huntingtin, mutant huntingtin and huntingtin-interacting proteins are enriched in the synaptic terminals (Nithianantharajah and Hannan, 2013a), where they interact with a wide-range of cytoskeletal and synaptic vesicle proteins (Smith et al., 2005). In this view, wild-type huntingtin directly binds to the SH3 domains of post-synaptic density-95 (PSD-95) protein (Sun et al., 2001;Parsons et al., 2014), an important component of the membrane-associated guanylate kinase (MAGUK) protein family that binds the NMDA and kainate receptors at the postsynaptic density (Fan and Raymond, 2007) and is considered a key molecule in regulating synaptic transmission. Importantly, the overexpression of wild-type huntingtin protects against the neurotoxicity mediated by NMDA or kainate receptors (Sun et al., 2001). Furthermore, huntingtin can also play a role in synapses not only by its interaction with post-synaptic proteins such as PSD-95, but also by interacting with pre-synaptic proteins such as Huntingtin-interacting protein 1 (HIP1) (Parker et al., 2007).

The above data indicate the complexity of wild-type huntingtin functions and highlight the functional relevance of its partners to provide important information for dissecting huntingtin normal functions. Huntingtin mutation affects all these cellular processes by altering protein aggregation, the ubiquitin-proteasome system, transcriptional regulation and chromatin remodeling, protein synthesis, trophic support, signaling pathways, calcium homeostasis, mitochondrial dynamics, NMDA-mediated excitotoxicity, caspase activation, protein-protein interactions and neuronal circuitry. Importantly, it has been suggested that together with this gain of toxic properties, the loss of physiological activities of wild-type protein would contribute to disease pathogenesis (Cattaneo et al., 2005;Zuccato and Cattaneo, 2009).

1.3 NEUROPATHOLOGY

Although huntingtin is expressed ubiquitously in all tissues, the presence of the mutated protein affects differently some areas of the brain compared to others. Thus, a specific and primary cell loss and atrophy in the caudate and putamen nucleus (striatum in rodents) and thinning of the cerebral cortex (particularly layers III, V and VI) have been considered the main neuropathological features of HD (Fig.3) (Reiner et al., 1988; Vonsattel and DiFiglia, 1998). However, neuropathological abnormalities have been detected in other brain areas such as the hippocampus (Herndon et al., 2009; Ille et al., 2011; Morton et al., 2000; Rosas et al., 2003; Spargo et al., 1993), globus pallidus, thalamus, subthalamic nucleus, substantia nigra, white matter, cerebellum and hypothalamus (Kassubek et al., 2004; Politis et al., 2008; Vonsattel and DiFiglia, 1998).

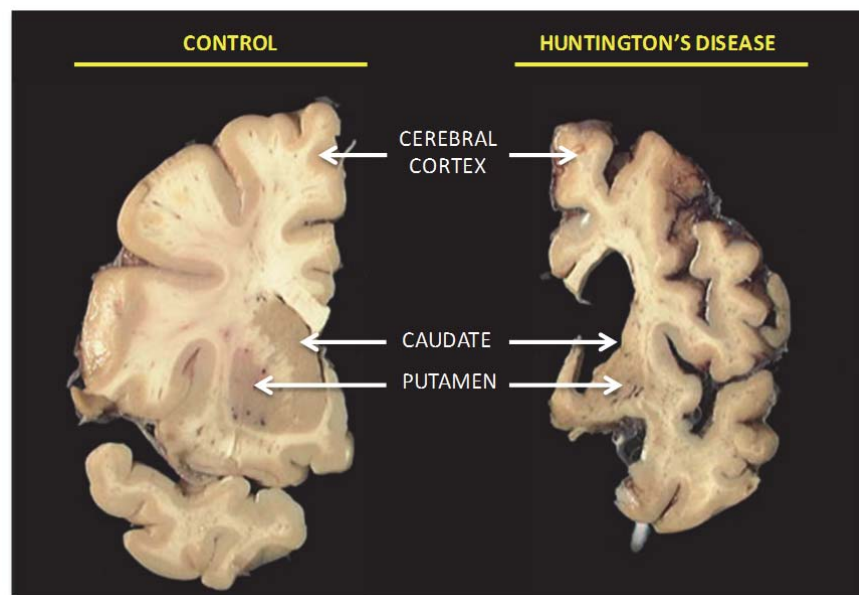


Figure 3. Huntington's disease pathology. Human control (left) and Huntington's disease (right) brain coronal sections showing the degeneration of the caudate and putamen nucleus accompanied by an increased volume of lateral ventricles and a marked cortical atrophy in Huntington's disease brain. Image adapted from <http://hdroster.iu.edu>.

Because the striatum of HD patients exhibit marked variations in the severity of neuropathology, it has been established a system for grading the severity of HD neuropathology. By macroscopic and microscopic criteria, five grades (0-4) in ascending order of severity have been assigned (Vonsattel et al., 1985). Thus, whereas

grade 0 appears indistinguishable from normal brains, neuronal loss can be detected in the caudate nucleus from grade 1 (50%) to grade 4 (95%). In ascending severity, grade 1, grade 2, grade 3 and grade 4 showed a progressive striatal atrophy and neuronal loss, being grade 4, the most severe HD cases. Apart from striatal atrophy and neuronal loss, astrogliosis has also been detected from grade 2 to grade 4 (Vonsattel et al., 1985).

1.3.1 Corticostriatal neuropathology

As reviewed before, HD is characterized by neuronal loss in the striatum and cortex. The striatum is a key component of the basal ganglia, an interconnected set of subcortical nuclei, involved in a variety of functions including the control of voluntary movements, procedural learning, eye movements as well as emotional and cognitive functions (Balleine et al., 2009; Brown et al., 1997). The dorsal division of the basal ganglia includes the striatum (caudate nucleus and putamen), the globus pallidus pars externa (GPe) and interna (GPi), the subthalamic nucleus (STN) and the substantia nigra pars compacta (SNc) and the more ventral pars reticulata (SNr) (Bolam et al., 2000). These structures are associated with motor and associative functions. Additionally, basal ganglia also involves ventral structures associated with limbic functions, such as the ventral striatum or nucleus accumbens, ventral pallidum and ventral tegmental area. Because of its integrative properties and complex circuitry the knowledge of its inputs and outputs represents an important step in interpreting basal ganglia function. The major input to the basal ganglia is derived from the cortex (Bolam et al., 2000; Alexander et al., 1986; Redgrave et al., 2011; Shepherd, 2013). The destination of these incoming axons from the cortex is onto the dendrites of a class of cells localized in the corpus striatum called medium-sized spiny neurons (MSSNs) (Purves et al., 2001, Neuroscience 2nd edition). Thus, cortical information carried by the corticostriatal projections is processed within the striatum, integrated with many other inputs to the basal ganglia and then these “processed information” transmitted to the output nuclei of the basal ganglia (GP and SNr) (Bolam et al., 2000; Alexander et al., 1986; Redgrave et al., 2011; Shepherd, 2013).

In the striatum, MSSNs constitute 90-95% of total neuronal population using GABA as their principal neurotransmitter (Kita and Kitai, 1988), whereas interneurons constitute 5-10% and execute an important role in controlling the excitability of MSSNs (Kreitzer, 2009). In HD neuropathology, medium-size spiny projection neurons are the most affected population and their degeneration occurs progressively (Ferrante et al., 1991; Vonsattel et al., 1985), whereas interneurons are practically not affected (Ferrante et al., 1987b; Ferrante et al., 1987a; Vonsattel et al., 1985). Importantly, a gradient of striatal pathology progressing in a dorsolateral to ventral direction and another in a caudo-rostral direction has been described (Vonsattel et al., 1985). Although all MSSNs are GABAergic, they differ in different aspects including the expression of dopamine and acetylcholine receptor subtypes, peptide content, and their projection targets (Gerfen, 1992a). There are two main populations of GABAergic MSSNs, one of them constitutes the direct pathway that projects to the substantia nigra pars reticulata (SNr) and the internal segment of the globus pallidus (GPi) and mainly expresses dopamine D1 and muscarinic M4 receptors and colocalize with substance P and dynorphin. The other constitutes the indirect pathway that projects to the external segment of the globus pallidus and mainly expresses dopamine D2 receptors and colocalize with enkephalin (Fig.4) (Aizman et al., 2000; Smith et al., 1998; Surmeier et al., 1996).

Importantly, in HD the indirect pathway seems to be affected earlier than the direct pathway (Richfield et al., 1995; Sapp et al., 1995). Because in normal conditions the indirect pathway is involved in the inhibition of neurons in the motor cortex and hence the inhibition of voluntary movements (Albin et al., 1989; Alexander and Crutcher, 1990), the higher vulnerability of these enkephalin-containing MSSNs at early disease stages could cause the hyperkinetic and choreic movements hallmarks of HD. By contrast, the direct pathway has been involved in the initiation of voluntary movements (Albin et al., 1989; Alexander and Crutcher, 1990). Thus, at late stages of the disease, disturbances in MSSNs that co-express substance P and D1 dopamine receptors (Gerfen et al., 1990) could be responsible for the hypokinetic and parkinsonian symptomatology in HD (Fig. 5).

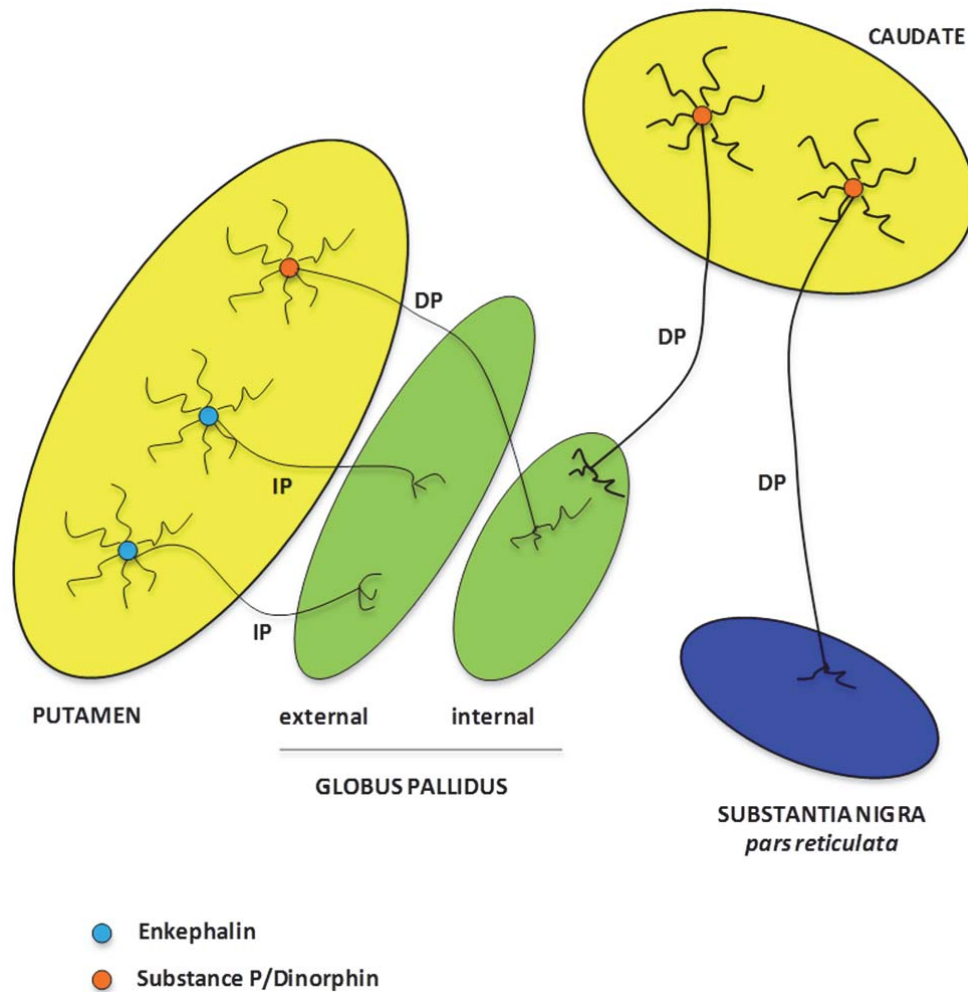


Figure 4. Structure of the basal ganglia. Diagram showing local circuitry of the two main populations of GABAergic MSNs in the striatum. Note (1) the direct pathway (DP) that projects to the substantia nigra *pars reticulata* and GPi and mainly expresses substance P and dynorphin, and (2) the indirect pathway (IP) that projects to the GPe and mainly expresses enkephalin. Image adapted from <http://hdroster.iu.edu>.

The striatum receives massive glutamatergic and dopaminergic innervations. The excitatory glutamatergic input derives mainly from all regions of the cerebral cortex as well as from thalamic nuclei (Fonnum et al., 1981b; Fonnum et al., 1981a; Jones, 1987). Importantly, it was assumed that striatal cell death in HD is due to excitotoxicity caused by aberrant NMDA receptor activation produced by an excessive glutamate release as a cause of mutant huntingtin toxicity (de la Monte et al., 1988; Fan and Raymond, 2007; Martin and Gusella, 1986; Paoletti et al., 2008; Perez-Navarro et al., 2006).

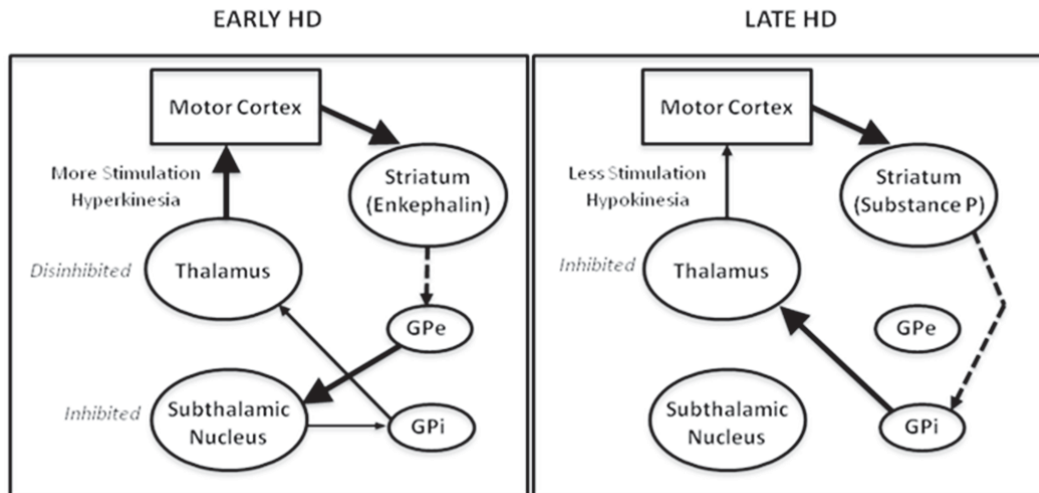


Figure 5. Basal ganglia circuitry in early and late stages of Huntington's Disease. Early in HD, GABAergic neurons in the striatum that project to the external segment of globus pallidus (GPe) degenerate (dashed arrow), resulting in the inactivation of subthalamic nucleus (thick arrow), the consequent disinhibition of the thalamus (thin arrow) and finally the increased stimulation of the motor cortex (thick arrow), responsible for the hyperkinetic phenotype. Later in HD, GABAergic neurons in the striatum that project to the internal segment of the globus pallidus (GPi) degenerate (dashed arrow). This loss reduces the inhibition on GPi neurons and increases their firing (thick arrow), resulting in an enhanced inhibition of the thalamus and decreased stimulation of the motor cortex (thin arrow), responsible for the hypokinetic phenotype. Adapted from (Ramaswamy et al., 2007).

The dopaminergic input comes from the SNc (Gerfen, 1992b). Increasing evidence indicate that dopaminergic system may contribute to HD neuropathology. In the early stages of the disease, alterations in dopamine (DA) stimuli from substantia nigra produce an imbalance in striatal neurotransmission initiating signaling cascades that induce cell death (Garret et al., 1992;Paoletti et al., 2008;Ross and Tabrizi, 2011;Tang et al., 2007). These inputs (glutamatergic and dopaminergic inputs) integrate on MSSNs (Smith and Bolam, 1990), and it has been generally believed that dopaminergic inputs can modify the excitatory responses to glutamate (Cepeda and Levine, 1998;Cepeda and Levine, 2006;Levine and Cepeda, 1998). However, in the past few years, it has become apparent that severe neuronal dysfunction precedes degeneration, being likely the major cause of HD symptoms (Levine et al., 2004), supporting the idea that corticostriatal dysfunction precedes striatal degeneration. Indeed, HD patients at pre-symptomatic stages exhibit alterations in performing tasks that requires shift in strategy (Ho et al., 2003;Lawrence et al., 1998b), in executive functions, in verbal fluency (Hahn-Barma et al., 1998;Paulsen et al., 2001b), in

procedural learning, in planning, and in explicit motor learning (Lawrence et al., 1998b; Rosenberg et al., 1995; Schneider et al., 2010), before motor alterations occur. Moreover, early corticostriatal alterations in HD patients have been further supported by neuroimaging studies in pre-symptomatic-HD subjects, showing impaired corticostriatal functional connectivity measured by BOLD-synchrony between the caudate and the motor cortex (Unschuld et al., 2012). These corticostriatal alterations correlate with decreased activity in the frontal cortex, and putamen regions (Kim et al., 2004a). In middle clinically symptomatic HD patients, executive function, verbal fluency, perceptual speed and reasoning are strongly affected (Backman et al., 1997; Lemièrè et al., 2004), while at more advanced disease stages, a sub-cortical dementia gradually develops, with alterations in several simple and complex cognitive functions involving slow information processing, decreased motivation, depression, apathy and personality changes (Paulsen et al., 1995; Zakzanis, 1998).

Thus, if emotional and cognitive disturbances are the earliest HD manifestations, prior to the appearance of motor symptoms, it is possible that early changes in areas related to these alterations, such as the cerebral cortex, the hypothalamus and the limbic system (Petersen et al., 2005) are responsible for the initial symptomatology in HD. In this view, it has been speculated that cortical changes are fundamental for the onset and progression of the HD phenotype in both humans and animal models (Laforet et al., 2001). Given the controversy on the early role of cortical dysfunction in HD pathology, part of this Thesis has been focused in the study of corticostriatal alterations in HD.

1.3.2 Hippocampal neuropathology

The hippocampus together with the amygdale and the nucleus accumbens form the central axis of the limbic system that play a key role in the formation of episodic, event or declarative memory, spatial learning and awareness, navigation, object recognition and visual memory as well as in executive functions (Bliss and Collingridge, 1993; Burgess et al., 2002; Eichenbaum, 2001; Gruart and Delgado-García, 2007; Kandel and Pittenger, 1999; Kandel, 2001; Kessels et al., 2001; Thierry et al., 2000). In humans, functional imaging studies show that the human hippocampus is activated during

certain kinds of memory tasks, and that damage to the hippocampus results in an inability to form certain types of new memories. Thus, given its importance in maintaining cognitive functions, many studies have been focused in understanding how the hippocampal neuronal network works.

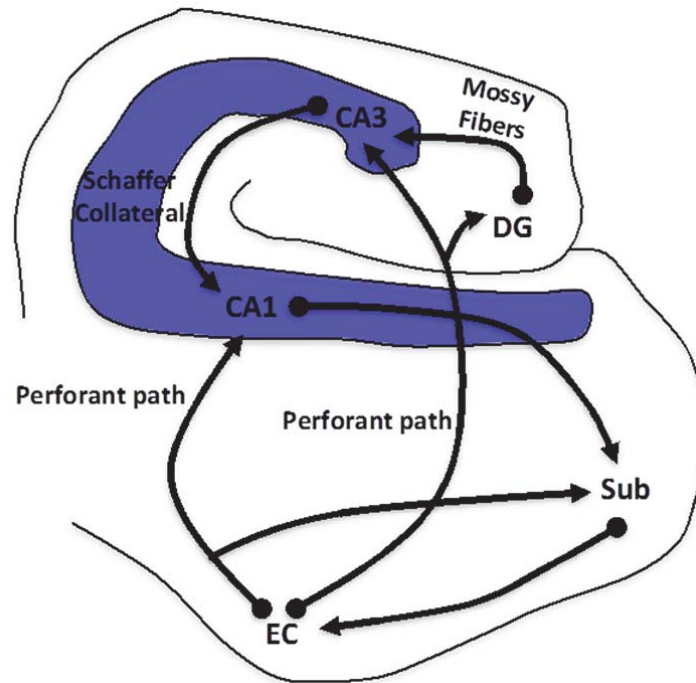


Figure 6. Schematic representation of hippocampal circuitry. Inputs from the Entorhinal Cortex (EC) establish connections with the Dentate Gyrus (DG), CA3 and CA1 via the layer II perforant path. DG sends axons to CA3 via the mossy fibers. CA3 sends axons to CA1 pyramidal cells via the Schaffer Collateral pathway. CA1 sends axons to the subiculum (Sub) that in turns send the main hippocampal output back to the EC.

Because its anatomical properties together with its important implication in the formation and/or retrieval of some forms of memory, long-term potentiation (LTP) and long-term depression has been most thoroughly studied in the mammalian hippocampus (Bliss and Collingridge, 1993; Lynch, 2004). LTP is considered one of the main neural mechanisms by which memory is stored in the brain. In rodents, much of the studies on LTP have focused on the synaptic connections between Schaffer collaterals and CA1 pyramidal cells. Unfortunately, changes in hippocampal connectivity in humans affected by HD are still poorly studied. Although studies in humans mainly focused on the corticostriatal circuitry, there are clear evidences of hippocampal morphological alterations in HD patients such as a reduction in

hippocampal volume (Rosas et al., 2003;Ille et al., 2011;Spargo et al., 1993) and the presence of huntingtin aggregates in the hippocampus from HD patients (Herndon et al., 2009;Morton et al., 2000). Moreover, some cognitive tasks used in human studies such as the evaluation of spatial working memory, spatial recognition memory, object recognition memory, episodic memories and some forms of associative learning, can involve the participation of the hippocampus and temporal lobe structures (Burgess et al., 2002;Clarke et al., 2010;Eichenbaum, 2004;Gruart and Delgado-Garcia, 2007;Kessels et al., 2001;Montaldi and Mayes, 2010). Thus, although no severe deficits in spatial working memory have been shown in pre-symptomatic HD patients, their latency in performing these tasks is higher than in control individuals (Lawrence et al., 1998b;Lawrence et al., 1998a) while recognition memory has been shown to be affected in pre-symptomatic HD-gene carriers (Berrios et al., 2002). Moreover, in early-mild symptomatic HD patients, alterations in associative learning, spatial short-term memory, spatial working memory and recognition memory have been described (Lawrence et al., 2000;Montoya et al., 2006). Importantly, in middle and more advanced disease stages, a global cognitive decline is observed in HD patients (Backman et al., 1997;Lemiere et al., 2004;Paulsen et al., 1995;Zakzanis, 1998). These alterations in cognitive functions involved both corticostriatal and hippocampal integrities. However, it seems that declarative memories more related to hippocampal and cortico-temporal functions are not as altered as procedural learning, more related to corticostriatal integrity. Actually, it has been proposed that hippocampus in HD patients compensate for gradual caudate nucleus dysfunction during route recognition, maintaining close to normal route recognition performance (Voermans et al., 2004). Furthermore, it has been suggested that long-term memory is more prone to be affected in HD than short-term memory (Lemiere et al., 2004;Rohrer et al., 1999;Wilson et al., 1987), indicating a recall deficit more than an impairment in memory storage as occurs in Alzheimer's disease (Rohrer et al., 1999). Given the controversy on the early role of hippocampal dysfunction in HD pathology, one of the aims of this Thesis has been to determine hippocampal alterations in HD and its critical role in the cognitive and synaptic plasticity deficits observed in this devastating disorder.

1.4 HUNTINGTON'S DISEASE MOUSE MODELS

Because Huntington's disease is caused by a single mutation, the generation of genetic animal models of HD represents one of the most important tools to study the molecular mechanisms involved in this devastating disorder.

The discovery of the gene responsible for the disease was not until 1993. Thus, the generation of the first HD transgenic mouse model was not developed until 1996 (Mangiarini et al., 1996). Before that, several research groups worked with toxic models as an approach to mimic the selective striatal degeneration seen in HD. Different disease models were developed by either excitotoxic lesions: 1) intrastriatal administration of kainic acid, a kainate receptor agonist (Coyle and Schwarcz, 1976); 2) intrastriatal injection of quinolinic acid, a NMDA receptor agonist (Beal et al., 1986); or by mitochondrial dysfunction: 3) 3-nitropropionic acid, an inhibitor of the complex II of the mitochondrial respiratory chain (Beal et al., 1993). Although these models represent a good approximation, the lack of expression of mutant largely hampered the study of the pathology.

Genetic models provide an opportunity to test potential treatments that could be translated to human patients. Although the disease has been reproduced in diverse species, the mouse models are the most extensively used. Thus, different genetic mouse models have been generated with differences in their phenotypes as a result of how mutant huntingtin was inserted into the murine genome: 1) Exon-1 transgenic mice: **R6, N171-82Q and the conditional HD94Q-tet off mice**, 2) Full-length transgenic mice: **Yeast artificial chromosome (YAC) and Knock-in mice** (Fig.7).

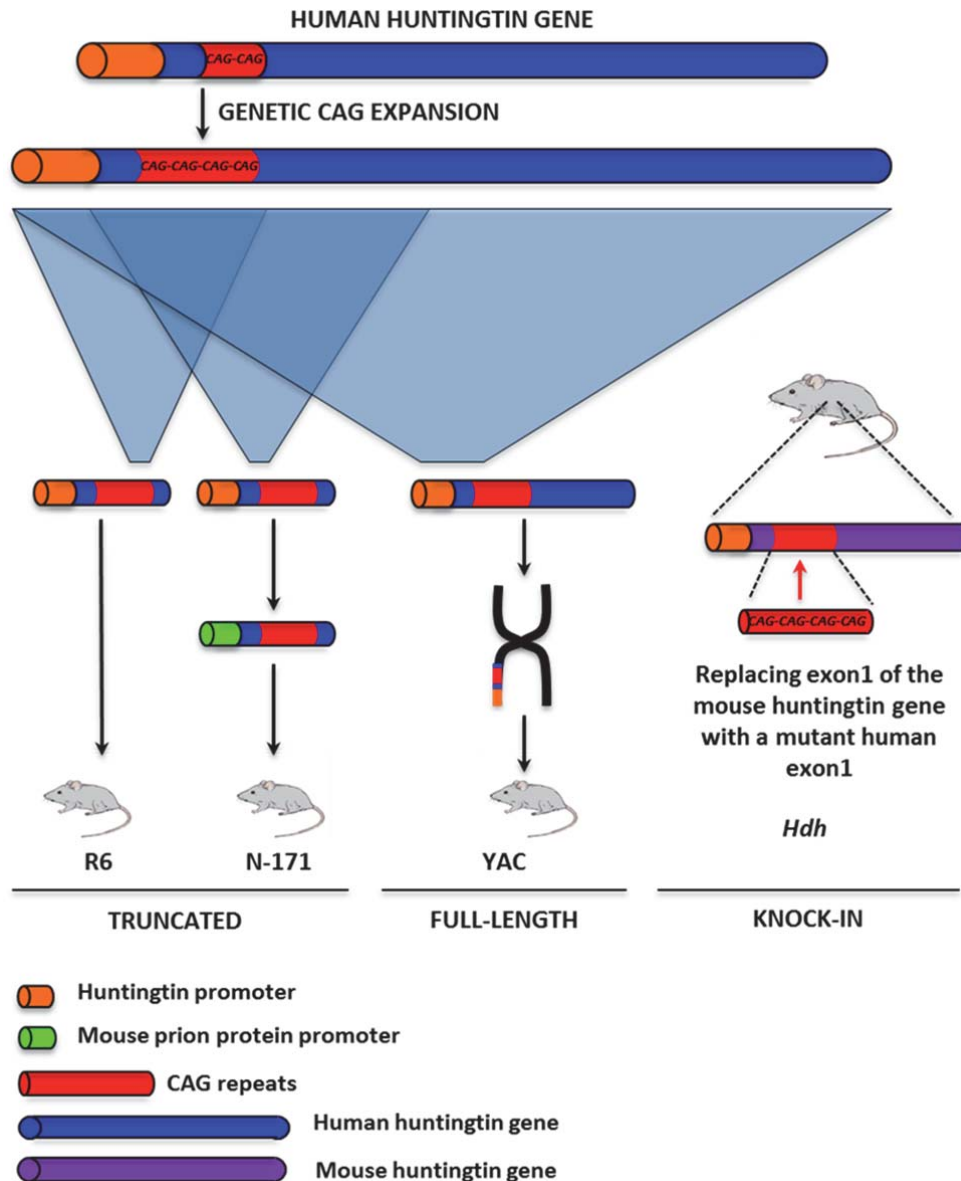


Figure 7. Schematic representation of human huntingtin gene and constructs used for the generation of mouse models of Huntington's disease. See the text for details.

1.4.1 Exon 1 mouse model: R6 and N171-82Q mice

Bates and colleagues were the first in creating a transgenic HD mouse model: R6/1 and R6/2 mice (Mangiarini et al., 1996) that express exon 1 of the human *HD* gene with 116 and 144 CAG repeats, respectively, under the human huntingtin promoter. The differences between both mouse models lie in the number of transgene copies that have been integrated in the genome, and therefore the expression level of the

transgene. Thus, whereas R6/1 mice integrate one single copy of the transgenic fragment resulting in a 31% of transgenic expression relative to the endogenous huntingtin expression, the R6/2 mice integrate in their genome three copies of the transgene with approximately a 75% of expression (Mangiarini et al., 1996). In accordance with human pathology, R6/2 mice develop an earlier onset and more severe symptoms compared to R6/1 mice. Thus, R6/2 mice show weight loss at 7 weeks of age with a lifespan of 14 weeks of age, whereas R6/1 mice exhibit weight loss at 22 weeks of age and their lifespan is around 32-40 weeks of age (Bates et al., 1997; Davies et al., 1997; Mangiarini et al., 1996). Importantly, neuropathological analysis revealed brain weight loss before body weight loss (Davies et al., 1997). The significant reduction in brain volume is the result of individual neuron atrophy rather than neuronal cell death that is minimal in both transgenic mice (Mangiarini et al., 1996; Turmaine et al., 2000). Moreover, in both transgenic mice neuronal nuclear inclusions were found in different brain areas along with dense accumulation of mutant huntingtin in the cytoplasm (Davies et al., 1997). Importantly, neuronal atrophy and the presence of cellular aggregates in transgenic mice occur prior to the initial manifestations of symptomatology (Davies et al., 1997). R6/2 mice also develop astrogliosis at 12 weeks of age (Giralt et al., 2011a). At behavioral level, both R6 mouse lines exhibit progressive motor and cognitive deficits. Thus, R6/2 mice show impaired motor function as early as 6-8 weeks of age (Carter et al., 1999; Giralt et al., 2011a; Mangiarini et al., 1996; Stack et al., 2005) and cognitive decline at 4 weeks of age (Lione et al., 1999), preceding the onset of motor symptoms. On the other hand, R6/1 mice show a later affection of motor and cognitive decline, accordingly with the expression of less CAG repeats, compared to R6/2 mice. Thus, motor deficits have been detected at 14 weeks of age while cognitive decline starts at 12 weeks of age (Giralt et al., 2009; Giralt et al., 2011b; Mangiarini et al., 1996).

Borchelt and colleagues generated a new transgenic mouse named N171-82Q by inserting the first 171 amino acids of the N-terminal fragment of the human huntingtin gene with 82, 44 or 18 glutamines into the mouse genome (Schilling et al., 1999). Mice expressing the N-171 huntingtin with 82Q show a shortened lifespan compared to 18Q, dying prematurely at 16-24 weeks of age (Schilling et al., 1999). Before dying,

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N171-82Q exhibit behavioral abnormalities, including motor alterations such as loss of coordination, tremors, hypokinesia, hind limb claspings accompanied by weight loss at 11 weeks of age (McBride et al., 2006; Schilling et al., 1999). Moreover, these mice show impaired cognition on radial arm water maze and altered reference and working memory as early as 14 weeks of age (Ramaswamy et al., 2007). In mice exhibiting these abnormalities a 25% of neuronal loss and a 20% of cell shrinkage has been detected in the striatum at 16 weeks of age, as well as the presence of intranuclear inclusions and neuritic aggregates in the cortex, striatum, hippocampus, cerebellum and amygdala (McBride et al., 2006; Schilling et al., 1999).

Importantly, the generation of a conditional mouse model that express the N-terminal fragment of the mutant huntingtin containing 94 CAG repeats under an inducible promoter throughout the forebrain, favored the study of Huntington's disease (Yamamoto et al., 2000). This conditional mouse model of HD was generated by using the tetracycline-responsive gene system that allows to turn off the transgene expression with oral administration of tetracycline analogs (Furth et al., 1994; Kistner et al., 1996; Yamamoto et al., 2000). HD94 mice exhibit hind limb claspings at 4 weeks of age, aggregates at 12 weeks of age and reactive astrocytosis in the striatum at 18 weeks of age (Yamamoto et al., 2000). The most important question that solved this model was to determine whether constant mutant huntingtin expression is necessary for the progression of the HD phenotypes. The authors demonstrate that an age in which HD94 mice clearly display a pathological phenotype, the gene-off mice show a significant reversion of aggregate formation, an amelioration of the claspings phenotype, a decrease in reactive astrocytosis and a motor recovery (Yamamoto et al., 2000). Interestingly, another study in 2005 revealed that huntingtin gene silencing can still be beneficial in the late stages of the disease. The authors demonstrate that whereas neuronal loss cannot be reverted in gene-off mice, the additional loss that takes place in gene-on mice was prevented. Moreover, at this advanced disease stages, the total number of huntingtin-containing inclusions is dramatically reverted in gene-off mice while a full recovery of their motor deficits is also evident, suggesting that gene-silencing therapies might work in late disease stages (Diaz-Hernandez et al., 2005).

Mouse model	Construct	Promoter	CAG Repeat size	Aggregates	Motor/cognitive symptoms	Lifespan
R6/2	Insertion of the exon 1 of human <i>HD</i> gene into mouse genome	Human huntingtin	144	3-4.5 W	Mot: 6-8 W C: 4-8 W	13-16 W
R6/1	Insertion of the exon 1 of human <i>HD</i> gene into mouse genome	Human huntingtin	116	9 W	Mot: 14 W C: 12 W	32-40 W
N171-82Q	Insertion of the first 171 aa from the N-terminal fragment of the human <i>HD</i> gene into mouse genome	Mouse prion protein	82	16 W	Mot: 11 W C: 14 W	16-24 W
HD94-tet off	Insertion of a chimeric mouse/human exon 1 fragment with polyQ expansion into mouse genome	CamKIIa-tTA	94	12 W	Mot: 4 W C: Not reported	Normal

Table 1. Genetically modified mouse models of Huntington’s disease: Transgenic mice. Table includes information about the construct insertion, the promoter under which the mutation is expressed, the CAG repeat number, the onset of mutant huntingtin aggregation, the onset of motor and cognitive symptoms, and the lifespan. Weeks of age (W); Motor symptoms (Mot); Cognitive symptoms (C). See the text for references.

1.4.2 Full-length mouse models: YAC and Knock-in mice

In this section we include different mouse models that express the full-length mutant huntingtin. These mice can be classified in: 1) mice in which mutant huntingtin is delivered in a yeast artificial chromosome (YAC) and 2) mice in which a knock-in mutation has been performed to modify the endogenous murine huntingtin (Knock-in). Hayden and colleagues generated a YAC transgenic mouse in 1999, by using the YAC vector system to express the entire human huntingtin gene under the human huntingtin promoter (Hodgson et al., 1999). Several YAC models with different CAG (72 and 128Q) repeat length were generated. Although both 72Q and 128Q exhibit brain atrophy and striatal neuronal loss (Hodgson et al., 1999; Seo et al., 2008), YAC128 mice present the most severe pathology (Slow et al., 2003). Besides striatal and cortical atrophy, YAC128 mice exhibit decreased brain weight loss at 9 months of age compared to wild-type mice associated with decreased striatal volume and decreased cortical volume at 12 months of age (Slow et al., 2003). In contrast to other transgenic HD mice, YAC mice show a significant increase in body weight at 12 months of age

compared to wild-type mice (Pouladi et al., 2010;van Raamsdonk et al., 2006). Importantly, YAC mice show a selective degeneration of medium spiny neurons in the lateral striatum at 12 months of age, associated with the translocation of N-terminal htt fragments into the nucleus (Hodgson et al., 1999;Slow et al., 2003). Moreover, at 18 months of age YAC128 mice exhibit huntingtin-positive inclusions in striatal cells. Interestingly, at early disease stages (3 months) YAC128 mice show a hyperkinetic phenotype compared with wild-type mice, whereas at 6 months of age manifest a hypokinetic phenotype that progress with age, becoming significant by 12 months of age (Slow et al., 2003). Motor assessment demonstrates motor decline as early as 6 months of age in the rotarod test (Slow et al., 2003). In addition, cognitive deficits in the YAC128 mice appear at 7-8.5 months of age (before motor symptoms onset) and progress with age (van Raamsdonk et al., 2005).

Finally, knock-in mouse models represent the most accurate genetic models of the human disease condition because they carry the mutation in its appropriate genomic context. The first knock-in model developed was the chimeric HdhQ lines. They generation consist in replacing the exon 1 of the murine *htt* gene with a mutated exon1 containing either 111 or 92 CAG repeats. Importantly, these lines with large polyglutamine repeats cause a CAG repeat instability, similar to what occurs in humans. Thus, in subsequent mouse generations, the repeat length increases, predisposing them to an earlier onset of symptoms (Wheeler et al., 1999;Wheeler et al., 2000). Importantly, neither HdhQ92 mouse nor HdhQ111 mouse exhibit striatal degeneration. However, at 4.5 months of age both strains show translocation of mutant huntingtin protein to the nucleus and in HdhQ111 mice this nuclear huntingtin appears punctuate (Wheeler et al., 2000). Importantly, although the translocation of mutant huntingtin protein to the nuclei, the appearance of nuclear aggregates do not appear until 14.5 months of age in HdhQ92 and 10 months of age in HdhQ111 (Wheeler et al., 2000). Additionally, at 24 months of age HdhQ111 mice exhibit striatal gliosis. At behavioral level, HdhQ92 mice show an early pathological onset manifested by significant deficits in implicit learning tasks at 4 months of age (Trueman et al., 2007). Importantly, whereas in some tests a progressive phenotype is observed (water maze test), in another test (the grip strength) the early deficits disappeared with age

(Brooks et al., 2012b). Despite moderate pathology in the brain, homozygous HdhQ111 mice show no significant motor symptoms until 24 month of age (Wheeler et al., 1999;Wheeler et al., 2000). Surprisingly, cognitive assessment has not been evaluated in these mice. Thus, although knock-in mice are considered one of the most representative models of HD, the lack of studies attending its behavioral dysfunction difficult their use to test possible therapeutic interventions. Thus, in this Thesis, we have evaluated HD pathology in HdhQ111 model. From HdhQ111 mice, stable striatal cell lines have been generated as an *in vitro* tool to study the molecular mechanisms underlying mutant huntingtin effects. Thus, striatal precursors at embryonic day 14 were immortalized with the simian vacuolating virus 40 Large T antigen resulting in the generation of control STHdhQ7 and mutant STHdhQ111 cellular models (Trettel et al., 2000). Other knock-in mouse models called CAG140 and CAG150, which expresses 140, or 150 CAG repeats, respectively, have been generated (Lin et al., 2001;Menalled et al., 2003). Their generation consists in the insertion of CAG repeats into the mouse huntingtin gene and as in the HdhQ92 or HdhQ111 mouse, the transcriptional regulation of the protein is under the mouse huntingtin promoter. CAG 140 mice showed nuclear and neuropil inclusions in the striatum, cortex, hippocampus and cerebellum starting at 2 months of age worsening thereafter in both size and number. Moreover, these mice display increased locomotor activity at 1 month of age followed by hypoactivity at 4 months of age. At 12 months of age, these mice also exhibit gait abnormalities (Menalled et al., 2003). CAG 150 mice show significant increase in striatal gliosis at 14 months of age as well as the presence of mutant huntingtin aggregates in the nucleus of striatal cells (Yu et al., 2003). Importantly, these knock-in mice exhibit progressive deficits in the rotarod, a clasping phenotype, hypoactivity and gait disturbances being evident at 10 months of age (Brooks et al., 2012a;Lin et al., 2001;Yu et al., 2003). Moreover, CAG 150 mice exhibit cognitive deficits as early as 4 months of age (Brooks et al., 2012a).

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Mouse model	Construct	Promoter	CAG Repeat size	Aggregates	Motor/cognitive symptoms	Lifespan
YAC72	Yeast artificial chromosome expressing the entire human <i>HD</i> gene	Human huntingtin	72	No	Mot: 16 M C: Not reported	Normal
YAC128	Yeast artificial chromosome expressing the entire human <i>HD</i> gene	Human huntingtin	128	18 M	Mot: 6 M C: 7-8.5 M	Normal
Hdh92Q	Replacing exon1 of the mouse <i>huntingtin</i> gene with a mutant human exon1	Mouse huntingtin	92	14.5 M	Mot: 4 M C: 4 M	Normal
Hdh111Q	Replacing exon1 of the mouse <i>huntingtin</i> gene with a mutant human exon1	Mouse huntingtin	111	10 M	Mot: 24 M C: Not reported	Normal
CAG140	Inserting CAG repeats into the mouse <i>huntingtin</i> gene	Mouse huntingtin	140	4 M	Mot: 4-12 M C: Not reported	Normal
CAG150	Inserting CAG repeats into the mouse <i>huntingtin</i> gene	Mouse huntingtin	150	10-14 M	Mot: 10 M C: 4 M	Normal

Table 2. Genetically modified mouse models of Huntington’s disease: Full-length mice. Table includes information about the construct insertion, the promoter under which the mutation is expressed, the CAG repeat number, the onset of mutant huntingtin aggregation, the onset of motor and cognitive symptoms, and the lifespan. Weeks of age (W); Motor symptoms (Mot); Cognitive symptoms (C). See the text for references.

NEURODEGENERATION AND COGNITIVE DYSFUNCTION IN HUNTINGTON’S DISEASE: POSSIBLE UNDERLYING MECHANISMS

HD is caused by a trinucleotide (CAG) repeat expansion in the *huntingtin* gene resulting in an extended polyQ tract that induces a cascade of molecular and cellular changes, including both neuronal dysfunction and neurodegeneration. However, the pathogenic mechanisms whereby mutant huntingtin results in disease symptoms and progression are not fully understood. Thus, the efforts of researches to describe the molecular pathways involved in HD could offer new targets for the development of therapeutics. In this context, it has been determined that the presence of mutant huntingtin alters distinct cellular and molecular mechanisms including protein aggregation, protein-protein interaction, calcium signaling, mitochondrial function,

transcriptional regulation and chromatin remodeling, vesicle transport, neurotransmitters release and receptor activity leading to neurodegeneration, neuronal dysfunction and abnormal synaptic plasticity (Fig. 8) (Labbadia and Morimoto, 2013;Zuccato et al., 2005;Zuccato et al., 2010).

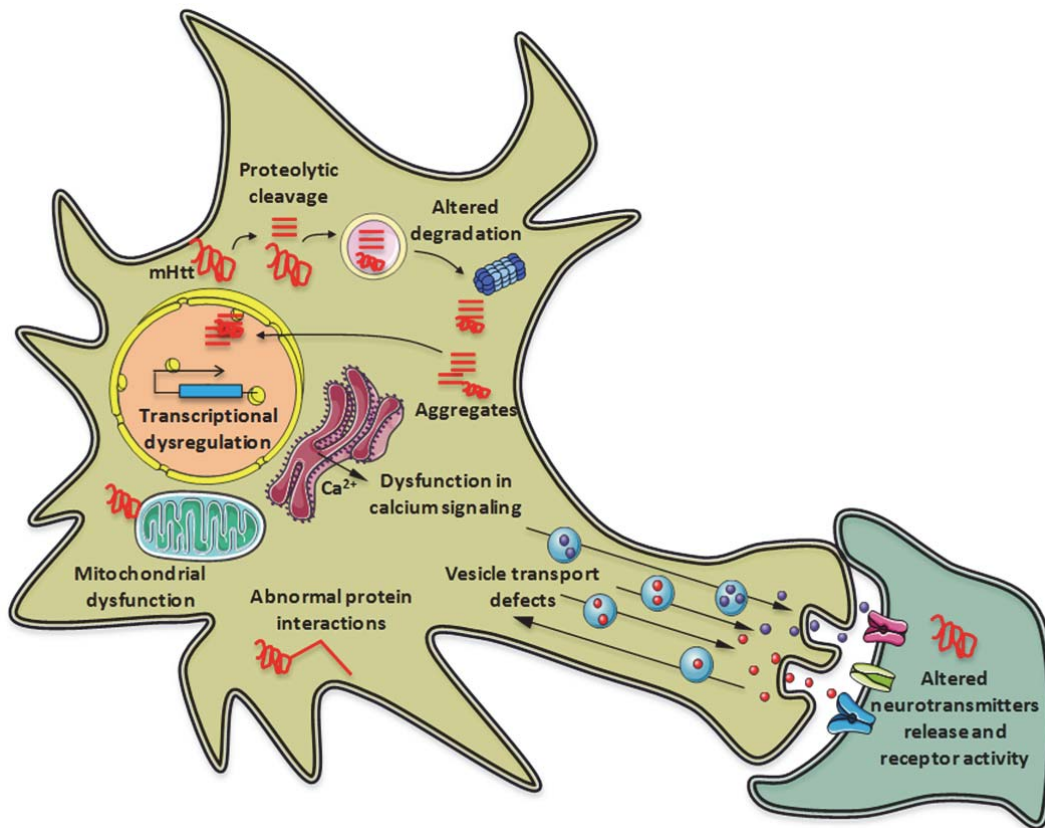


Figure 8. Model of key cellular pathogenic mechanisms in HD. The mutation in huntingtin (mHtt) causes a multiple alteration in cellular pathways. Adapted from (Labbadia and Morimoto, 2013;Zuccato et al., 2010).

Several works indicate that cell death does not occur until late stages of the disease, suggesting that neuronal dysfunction and abnormal synaptic plasticity are likely the main early pathogenic events that finally result in neurodegeneration (Caramins et al., 2003;Levine et al., 2004;Turmaine et al., 2000). Thus, the identification of these early cellular and molecular pathogenic events in HD represents an important hallmark to design new therapeutic approaches to cure or delay the disease progression.

In this Thesis we have studied several underlying mechanisms by which mutant huntingtin could promote neurodegeneration and neuronal dysfunction such as transcriptional deregulation, neurotrophic dysfunction, synaptic impairments, altered kinase and phosphatase signaling and altered receptor activity.

2.1 TRANSCRIPTIONAL DEREGLATION

Several studies have proposed that transcriptional deregulation represents one of the earliest mechanisms involved in HD pathogenesis (Cha, 2007; Dunah et al., 2002; Luthi-Carter et al., 2000; Luthi-Carter et al., 2002b; McCampbell et al., 2000; Steffan et al., 2000). Indeed, a large number of studies have revealed transcriptional abnormalities in HD human brain as well as in *in vivo* and *in vitro* disease models (Desplats et al., 2006; Hodges et al., 2006; Lee et al., 2011; Luthi-Carter et al., 2000; Luthi-Carter et al., 2002a; Marti et al., 2010; Runne et al., 2008). How mutant huntingtin causes this transcriptional deregulation? To solve this important question, different mechanisms have been proposed: 1) Sequestration of positive transcription regulators into mutant huntingtin aggregates, such as TATA-binding protein (Schaffar et al., 2004), specificity protein-1 (Li et al., 2002b) or CREB (cyclic-adenosine monophosphate (cAMP) response element (CRE) binding protein)-binding protein (CBP) (Schaffar et al., 2004; Steffan et al., 2000); 2) Loss of interaction between mutant huntingtin and negative transcriptional regulators such as the repressor element-1 transcription/neuron restrictive silencer factor (NRSE), resulting in REST/NRSF complex nuclear translocation and transcriptional repression of several neuronal-specific genes, including *Bdnf* (Zuccato et al., 2003); 3) Increased activity of histone methylation and ubiquitination and decreased histone acetylation. For instance, it has been reported that mutant huntingtin binds to the acetyltransferase domain of some factors and blocks its activity, such as CBP (Cong et al., 2005; McCampbell et al., 2001; Steffan et al., 2001). Overall, these studies point transcriptional machinery modulation as a key mechanism to achieve novel treatment approaches (Beal and Ferrante, 2004; Cha, 2007; Helmlinger et al., 2006; Luthi-Carter et al., 2003; Serezenina and Luthi-Carter, 2012).

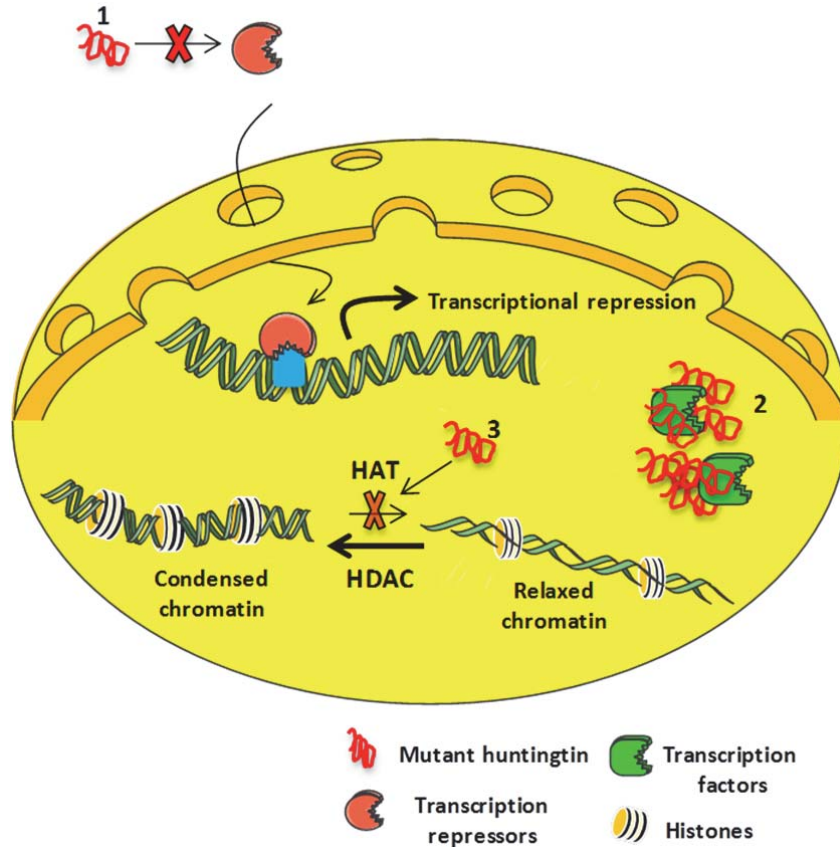


Figure 9. Mechanisms of transcriptional deregulation in Huntington's disease. Mutant huntingtin (mHtt) interferes with transcriptional machinery at different levels: 1) mHtt loss the capacity to bind to transcriptional repressors, allowing them to translocate into the nucleus and repress transcription, 2) mHtt recruits transcription factors in intranuclear inclusions and 3) mHtt interacts with histone acetyltransferases (HATs) resulting in the inhibition of proper histone acetylation and repressing transcription.

2.1.1 cAMP-response element binding protein (CREB) and its co-activator CREB-binding protein (CBP)

CREB is a transcriptional factor that belongs to the bZIP superfamily. Like all bZIP transcriptional factors, CREB family members contain a C-terminal basic domain that mediates DNA binding and a leucine zipper domain that facilitates dimerization (De et al., 1999; Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999). Through the cAMP response element (CRE), CREB is able to bind to DNA, whereas through other domains CREB interacts with co-activators and components of the transcriptional machinery (Foulkes et al., 1991; Gonzalez et al., 1989; Hai et al., 1989; Hoeffler et al., 1988). CREB mediates stimulus-dependent changes in the expression of genes critical

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for neuronal survival, plasticity and growth (Lee et al., 2005a; Lee et al., 2005b; Lonze and Ginty, 2002; Lonze et al., 2002). CREB is activated by phosphorylation of a particular residue, Serine 133 (Ser133). However, CREB activity can be further modulated by phosphorylation at additional sites as well as by its association with CREB co-activators (Rosenfeld and Glass, 2001). Indeed, CREB phosphorylation at Ser133 residue facilitates the binding of the transcriptional co-activator CBP (Chrivia et al., 1993; Kwok et al., 1994; Parker et al., 1996; Radhakrishnan et al., 1997). This interaction between CREB and CBP, together with the interaction with other members of the transcriptional machinery, facilitates gene expression (Ferreri et al., 1994; Parker et al., 1996). Ross and collaborators in 2001 were the first to demonstrate that CREB signaling is compromised in HD. By the expression of mutant huntingtin constructs with different lengths they observed CBP aggregation and a downregulation of CRE-mediated signaling in N2A cells (Nucifora, Jr. et al., 2001). This observation was also confirmed in PC12 cells, where inducible mutant huntingtin expression impairs the cAMP-regulated response (Wyttenbach et al., 2001). Importantly, CREB-signaling deregulation is also found in knock-in cells (Gines et al., 2003b) and R6/2 mice (Sugars et al., 2004). Moreover, it has been shown that mutant huntingtin expression down-regulates CRE-mediated transcription of numerous genes (Augood et al., 1997; Luthi-Carter et al., 2000; Zuccato et al., 2010). Overall, CREB-mediated reduced signaling became a promising target for pharmacological therapeutic intervention in HD. In this context, specific phosphodiesterase inhibitors, like rolipram and TP10, have been tested to maintain CREB activity and preserve neuronal viability (DeMarch et al., 2007; Giampa et al., 2006; Giampa et al., 2009a; Giampa et al., 2009b). The use of these drugs has been reported to decrease striatal cell loss in an excitotoxic model of HD (DeMarch et al., 2007), reduce gross brain atrophy, increase the survival of striatal neurons, increase the expression of BDNF in the striatum and cortex, decrease the number of intranuclear inclusions and improve motor deficits in HD mouse models (DeMarch et al., 2007; Giampa et al., 2006; Giampa et al., 2009a; Giampa et al., 2009b; Giampa et al., 2010).

CBP not only acts as a CREB transcriptional co-activator (Bourtchuladze et al., 1994; Chrivia et al., 1993; Ravnskjaer et al., 2007), but also as a histone

acetyltransferase (HAT) to alter chromatin structure allowing gene transcription (Kalkhoven, 2004;Ogryzko et al., 1996;Vo and Goodman, 2001). Thus, CBP exerts a dual role in controlling CREB-mediated gene transcription: 1) as a scaffolding protein to recruit CREB and 2) as a HAT protein acetylating histones to disrupt repressive chromatin structure (Chan and La Thangue, 2001). Because its functional properties, CBP has a key role in the nervous system and both mutations and deletions of CBP have been associated with the Rubinstein-Taybi syndrome. Regarding HD, Steffan and colleagues have shown that CBP directly interacts with mutant huntingtin, blocking CBP acetyltransferase activity (Steffan et al., 2001). This blockage of HAT activity causes a condensed chromatin state and a reduction of gene transcription. Importantly, hypoacetylation of histone H3 associates with downregulation of genes in R6/2 mice and knock-in cell lines (Sadri-Vakili et al., 2007), while loss of CBP function associates with striatal neurodegeneration in HD models (Jiang et al., 2006;McC Campbell et al., 2001;Nucifora, Jr. et al., 2001;Taylor et al., 2003). The reduction of striatal CBP levels has been explained by its presence and sequestration in polyglutamine aggregates (McC Campbell et al., 2000;Nucifora, Jr. et al., 2001) or by increased proteosomal degradation (Cong et al., 2005;Jiang et al., 2003;Sadri-Vakili et al., 2007). Importantly, restoration of CBP striatal function in HD by overexpression of CBP or by using HDAC inhibitors prevents cell loss and increases cell survival in HD models (Ferrante et al., 2003;Gardian et al., 2005). Overall, these studies suggest an important role of CBP loss of function in polyglutamine-dependent striatal toxicity in HD. However, deficits in synaptic plasticity and memory have been shown to precede striatal vulnerability and neurodegeneration in HD, although the precise molecular mechanisms underlying these defects remain largely unknown. Activity-induced gene transcription is required for normal hippocampal synaptic plasticity and memory consolidation (Barco et al., 2003;Kandel, 2001). Compelling evidence indicate that CREB is essential for activity-induced memory gene expression (Benito and Barco, 2010;Silva et al., 1998). Moreover, several studies have demonstrated reduced chromatin acetylation and hippocampal long-term potentiation (LTP) and long-term memory (LTM) deficits in mouse models in which CBP activity is compromised (Alarcon et al., 2004;Chen et al., 2010;Valor et al., 2011).

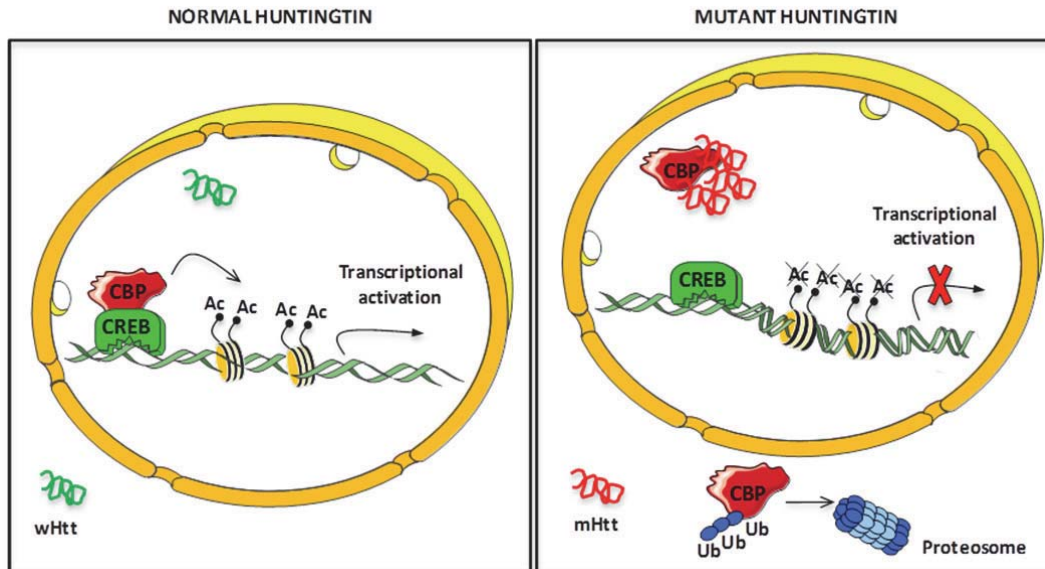


Figure 10. CREB/CBP pathway in HD. In the presence of wild-type huntingtin (wHtt), CBP binds with CREB, and by its histone acetyl transferase activity (HAT), CBP promotes histone acetylation and activates the transcription of genes involved in neuronal survival. By contrast, mutant huntingtin (mHtt) decreases CBP expression by its recruitment into intranuclear inclusions and/or by its degradation by the proteasome. Moreover, mHtt-CBP interaction blocks its HAT activity and causes a condensed chromatin state that represses gene transcription and promotes neuronal cell death.

Altogether raises the question of whether deregulation of CBP expression or activity could play a role in HD memory deficits. If so, could the modulation of CBP activity restore not only the striatal vulnerability but also the cognitive deficits in this devastating disorder? Surprisingly, the role of CBP in regulating gene expression required for memory in HD has not been investigated. Because several evidences point the restoration of gene transcription in HD as an important therapeutic strategy, in this Thesis we have evaluated the role of CBP in HD memory-related deficits and the use of HDAC inhibitors as a pharmacological treatment to restore CBP HAT activity and cognitive decline in HD.

2.2 NEUROTROPHIC DYSFUNCTION

Neurotrophins exert several functions in the CNS, from differentiation and neuronal survival to synaptogenesis and activity-dependent synaptic plasticity (Huang and Reichardt, 2001; Lewin and Barde, 1996). Four neurotrophins have been identified

in the mammalian brain, including the nerve growth factor (NGF), the brain-derived neurotrophic factor (BDNF), the neurotrophin 3 (NT3) and the neurotrophin 4 (NT4). These neurotrophins, like other secreted proteins, are initially synthesized as precursors or pro-neurotrophins (30-35kDa), which are cleaved to produce the mature proteins (12-13kDa) (Mowla et al., 2001; Seidah et al., 1996). The actions of neurotrophins is through its binding to two different transmembrane-receptors: the Tropomyosin-receptor kinase (Trk) family of receptor tyrosine kinases, which includes TrkA, TrkB and TrkC and the p75 neurotrophin receptor (p75^{NTR}), a member of the tumor necrosis factor (TNF) receptor superfamily (Dechant and Barde, 2002; Huang and Reichardt, 2001; Kaplan and Miller, 2000; Chao, 2003). Whereas p75^{NTR} receptor exhibits a similar affinity for all pro-neurotrophins and mature neurotrophins, each Trk receptor selectively show binding specificity for particular neurotrophins. Thus, NGF binds preferentially to TrkA, BDNF and NT4 to TrkB and NT3 to TrkC (Fig. 11).

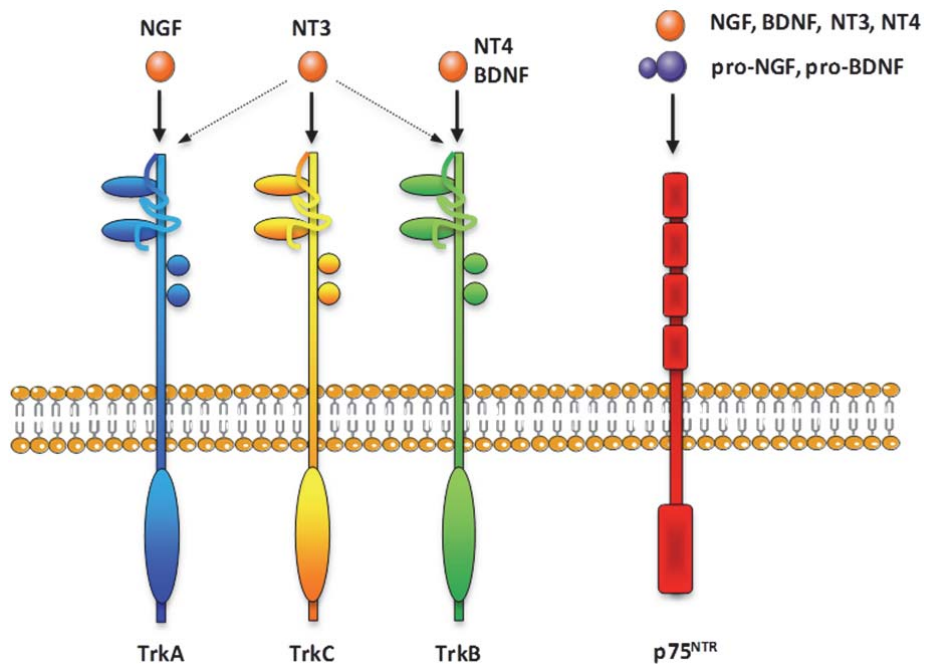


Figure 11. Neurotrophins and its receptors. Neurotrophins bind selectively to specific Trk receptors that contain extracellular immunoglobulin G domains for ligand binding and a catalytic tyrosine kinase sequence in the intracellular domain. By contrast, all neurotrophins bind to p75^{NTR} receptor, which contains an extracellular four-cystein-rich repeats, and a death domain in the intracellular part. Dashed arrows indicate low affinity of the neurotrophin for the receptor. Adapted from (Chao, 2003).

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Importantly, the affinity of a neurotrophin for its receptor can be regulated by receptor dimerization, structural modifications as well as by association with the p75^{NTR} receptor. Thus, p75^{NTR} can bind to each neurotrophin but also can act as a co-receptor for Trk receptors, leading to changes in the binding affinity for neurotrophins (Arevalo et al., 2000; Benedetti et al., 1993; Bibel et al., 1999; Esposito et al., 2001; Hempstead et al., 1991). Neurotrophins play a pivotal role in determining the balance between cell survival and cell death and neural activity modulates the expression of neurotrophins. Knock-out mice for neurotrophins die during the first weeks after birth and heterozygous mice in which the levels of neurotrophins are reduced exhibit several deficits (Table 3) (Airaksinen et al., 1996; Bartoletti et al., 2002; Bianchi et al., 1996; Carroll et al., 1998; Chen et al., 1997; Crowley et al., 1994; Dluzen et al., 2001; Elmer et al., 1997; Ernfors et al., 1994a; Ernfors et al., 1994b; Kernie et al., 2000; Korte et al., 1995; Lyons et al., 1999; Patterson et al., 1996; Rios et al., 2001; Carreton et al., 2012), indicating a pivotal role of neurotrophin expression in the brain.

<i>NGF^{+/-} mice</i>	<i>BDNF^{+/-} mice</i>	<i>NT3^{+/-} mice</i>
Decrease cholinergic innervation of the hippocampus	Hyperphagia, obesity	Cardiovascular defects
Deficiency in memory acquisition and retention	Impairment of long-term potentiation	Reduced mechanoreceptors
Loss of neurons of the peripheral nervous system	Elevated striatal dopamine levels	Loss of neurons of the peripheral nervous system
	Loss of mechanosensitivity	
	Loss of neurons of the peripheral nervous system	
	Impaired motor learning	

Table 3. Neurotrophin heterozygous mice. Heterozygous mice for neurotrophin levels are viable but exhibit important deficits.

2.2.1 Brain-derived neurotrophic factor

Among all neurotrophins, BDNF has emerged as a key regulator of neuronal survival and synaptic plasticity (Binder and Scharfman, 2004) and has been considered an excellent molecular target for drug development in neurological disorders (Binder

and Scharfman, 2004;Arancio and Chao, 2007;Liu et al., 2008). As previously reviewed, the striatum and the cerebral cortex are preferentially affected in HD (Reiner et al., 1988;Rosas et al., 2005). Interestingly, it has been demonstrated that BDNF is produced in the cerebral cortex and is anterogradely transported along the corticostriatal pathway to the MSNs (Altar et al., 1997;Baquet et al., 2004). Numerous studies have demonstrated that striatal neurons require BDNF for their survival and function. Indeed, a deficiency in BDNF-mediated signaling has been shown to cause dendritic abnormalities and neuronal loss in the striatum and cerebral cortex (Baquet et al., 2004;Gorski et al., 2003). Moreover, several studies demonstrated that BDNF shows protective effects on striatal projection neurons against the excitotoxic stimulus produced after the administration of quinolinic acid (Gratacos et al., 2001;Perez-Navarro et al., 1999;Perez-Navarro et al., 2000;Perez-Navarro et al., 2005). In non-pathological conditions BDNF is highly expressed in different brain regions that are affected in HD, such as the cerebral cortex, the striatum and the hippocampus (Altar et al., 1997;Baquet et al., 2004;Gavalda et al., 2004;Perez-Navarro et al., 2000). Importantly, a reduction in BDNF protein expression is a common phenomenon in different brain regions of HD mouse models and HD patients (Ferrer et al., 2000;Gines et al., 2003b;Zuccato et al., 2001;Zuccato et al., 2005;Zuccato and Cattaneo, 2007;Zuccato and Cattaneo, 2009), supporting the hypothesis that deficits in neurotrophic support caused by reduced BDNF levels plays a key role in HD neuropathology. In an attempt to establish whether BDNF is neuroprotective against cell death and cognitive impairments in HD, several studies have been performed. Thus, experiments in cultured cells demonstrate that BDNF protects mutant huntingtin-transfected neurons against death (Saudou et al., 1998;Zala et al., 2005). Moreover, it has also been reported that BDNF levels regulate the onset and severity of motor and cognitive symptoms, as well as synaptic plasticity deficits in several HD mouse models (Canals et al., 2004;Cho et al., 2007;Gharami et al., 2008;Giralt et al., 2009;Giralt et al., 2011a;Lynch et al., 2007;Simmons et al., 2009;Simmons et al., 2011;Xie et al., 2010). Overall, these studies indicate that treatments focused on the recovery of BDNF levels may be suitable for preventing neuronal cell death and dysfunction of neurons to improve not only motor deficits but also synaptic plasticity and cognitive impairments in HD.

2.2.2 Trk and p75^{NTR} receptors

Neurotrophins are released from neurons in an activity-dependent manner and exert their effects through binding to Trk (TrkA, TrkB, TrkC) and p75^{NTR} receptors. In the striatum, TrkB (the most abundant) and TrkC are expressed mainly in the MSN, while TrkA expression is restricted to cholinergic interneurons (Holtzman et al., 1995;Merlio et al., 1992). Accordingly with this receptor distribution, BDNF and NT-3 exert trophic effects on GABAergic projecting neurons (Ivkovic et al., 1997;Mizuno et al., 1994;Ventimiglia et al., 1995), while NGF on striatal cholinergic interneurons (Martinez et al., 1985;Mobley et al., 1985). Importantly, the activity of Trk receptors has been associated with multiple neuronal processes such as cell survival, differentiation, synapse formation and axonal growth (Ernfors et al., 1995;Huang and Reichardt, 2003;Levi-Montalcini and Booker, 1960;Snider, 1988). To mediate these functions, it is classically accepted that neurotrophin binding to Trk receptors causes receptor dimerization and autophosphorylation of their cytoplasmic kinase domains. Trk phosphorylated residues facilitate the recruitment of several adaptor proteins that in turns activate different signaling cascades, such as Ras/Rap-MAPK, PI3K-Akt or PLC γ -PKC (Arevalo and Wu, 2006;Reichardt, 2006). By contrast, the role of p75^{NTR} in the nervous system has been shown to be more complex. p75^{NTR} expression is higher during development but decreases in the adult brain (Barker, 1998;Chao, 2003;Pioro and Cuello, 1990). However, in pathological conditions p75^{NTR} expression is rapidly increased and has been associated with cell death (Chao, 2003;Kalb, 2005;Nykjaer et al., 2005). Neurotrophin binding to p75^{NTR} has been shown to cause cell death in the nervous system and in cell lines that do not expressed Trk receptors (Frade et al., 1996;Friedman, 2000), whereas apoptosis is reduced when p75^{NTR} is completely deleted (Naumann et al., 2002). However, p75^{NTR} has also been involved in survival, depending on the cellular context and the signaling pathways that are activated. In this view, it has been demonstrated that p75^{NTR} can acts as a Trk co-receptor and signal for neural survival (Fig.12) (Baker and Reddy, 1996;Culmsee et al., 2002;He and Garcia, 2004;Ip et al., 1993;Lad and Neet, 2003;Lu et al., 2005;MacPhee and Barker, 1997;Nykjaer et al., 2005;Zaccaro et al., 2001). The signal transduction pathways that are activated downstream of p75^{NTR} remain largely unknown. However, ceramide,

Ras/extracellular signal-regulated kinase (ERK), nuclear factor kappa B (NF- κ B) and Jun N-terminal kinase (JNK) have been associated with p75^{NTR} activation (Chen et al., 2009).

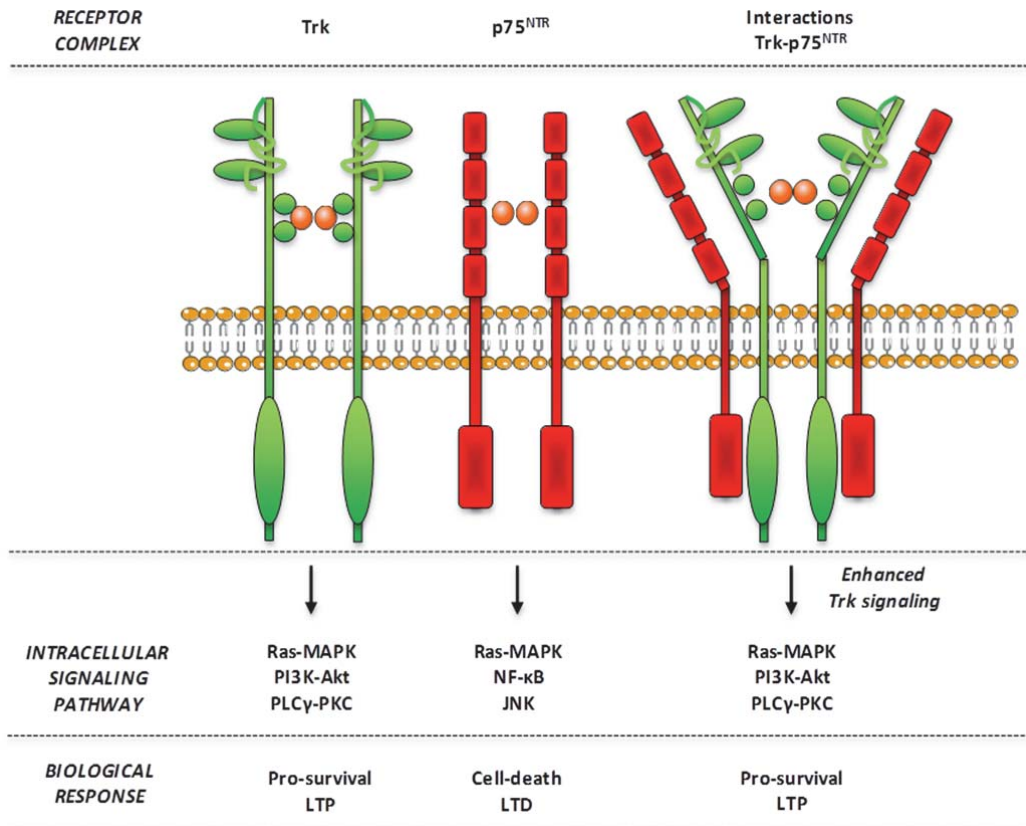


Figure 12. Trk and p75^{NTR} receptor activation in the adult brain. Neurotrophin binding causes receptor dimerization that finally results in homo- and/or heterodimers between Trk and p75^{NTR} receptors. Depending on the receptor dimerization, several signaling pathways are activated and diverse biological responses occur. Adapted from (Chao, 2003;Lu et al., 2005).

Notably, both Trk and p75^{NTR} receptors play a crucial role in activity-dependent synaptic plasticity processes, such as long-term potentiation (LTP) and long-term depression (LTD), and neurotrophins and its receptors can also cause antagonistic effects in this context. Thus, whereas BDNF induces hippocampal LTP through TrkB (Poo, 2001), p75^{NTR} has been involved in regulating LTD without affecting LTP (Rosch et al., 2005;Woo et al., 2005)(for review see (Lu et al., 2005). These changes in synaptic transmission associate with structural changes such as axonal and dendritic elongation and arborization. It has been reported that whereas neurotrophins promote these

effects through activation of Trk receptors (Cohen-Cory and Fraser, 1995;McAllister et al., 1999), p75^{NTR} blocks them by activation of RhoA, a Rho GTPase that negatively regulates neurite elongation and actin assembly (Yamashita et al., 1999). Accordingly, it has been demonstrated that p75^{NTR} negatively modulates dendritic morphology and density in adult hippocampal pyramidal neurons (Zagrebelsky et al., 2005).

Importantly, reduced TrkB expression levels have been reported in HD cellular and mouse models as well as in HD patients (Gines et al., 2006;Gines et al., 2010;Zuccato et al., 2008). Moreover, increased p75^{NTR} mRNA expression in the caudate but not in the cortex of HD patients has also been reported (Zuccato et al., 2008). Because deficits in neurotrophic support and particularly in BDNF expression are considered a key player in HD neuropathology, the administration of BDNF has been proposed as an excellent neuroprotective therapy for the treatment or delay of HD (Alberch et al., 2004;Zuccato and Cattaneo, 2009). However, although BDNF therapy has exciting results in HD animals, numerous methodological and safety issues such as dosage and the method of delivery remain to be address before being an accepted therapeutic strategy in patients (Zuccato and Cattaneo, 2009). We hypothesize that neurotrophic dysfunction could account not only for BDNF reduction, but also for an imbalance between TrkB and p75^{NTR}-mediated signaling, as suggested by altered receptor levels in HD mouse models and HD patients. Therefore, in this Thesis we have studied the role of TrkB and p75^{NTR} in HD neuropathology, as an alternative neuroprotective strategy to restore BDNF signaling in HD.

2.3 SYNAPTIC DYSFUNCTION

Neuronal dysfunction and cognitive impairments precede motor symptoms and neuronal death in HD patients and occur long before (or in the absence) of cell death in different HD mouse models, suggesting that the early deficits in cognition may be a consequence of synaptic dysfunction rather than cell loss (Marder et al., 2000;Milnerwood and Raymond, 2007;Orth et al., 2010;Schippling et al., 2009;Verny et al., 2007). Indeed, alterations in excitatory synapses have been described at early disease stages in HD animal models (Fan and Raymond, 2007;Lynch et al., 2008;Cepeda et al., 2007), and it has been suggested that these synaptic alterations could contribute

to cognitive symptoms in both HD patients and animal models (Lione et al., 1999;Montoya et al., 2006;Trueman et al., 2007;van Raamsdonk et al., 2005). Importantly, mutant huntingtin is present in presynaptic (Parker et al., 2007;Smith et al., 2005) and postsynaptic terminals of transgenic HD mice (Suopanki et al., 2006). In this cellular compartments, it has been reported that mutant huntingtin interacts with several synaptic-related proteins and impairs synaptic function (Labbadia and Morimoto, 2013;Smith et al., 2005;Zuccato et al., 2010). Some suitable candidates have been proposed to contribute to HD synaptic dysfunction, such as α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPA) subunit GluA1 (Giralt et al., 2009;Nithianantharajah et al., 2008), dopamine receptors (Augood et al., 1997;Cha et al., 1998;Jay, 2003;Luthi-Carter et al., 2000;Pavese et al., 2003;Weeks et al., 1996), post-synaptic density 95 protein (PSD95) (Nithianantharajah et al., 2008;Nithianantharajah and Hannan, 2013b;Torres-Peraza et al., 2008) and trophic factors (del et al., 2006;Gauthier et al., 2004;Zuccato et al., 2001;Zuccato and Cattaneo, 2007). Targeting synaptic dysfunction and in particular alterations in excitatory synapses as a therapeutic strategy is complex and difficult. However, since is one of the first alterations in HD neuropathology, excitatory synaptic dysfunction represents an attractive therapeutic candidate.

2.3.1 Structural changes at the synapses

Synapses are the building dynamic blocks of neuronal networks. Can be classified in two categories: type 1 (asymmetric synapses) that are located on dendritic spines and dendritic shafts and type 2 (symmetric synapses) that are located on dendritic shafts and neuronal cell bodies (GRAY, 1959). The majority of asymmetric synapses are glutamatergic whereas most symmetric synapses are GABAergic. In the mammalian brain, most of the glutamatergic excitatory synapses occur on tiny protrusions emanating along dendrites called dendritic spines.

2.3.1.1 Dendritic spine pathology

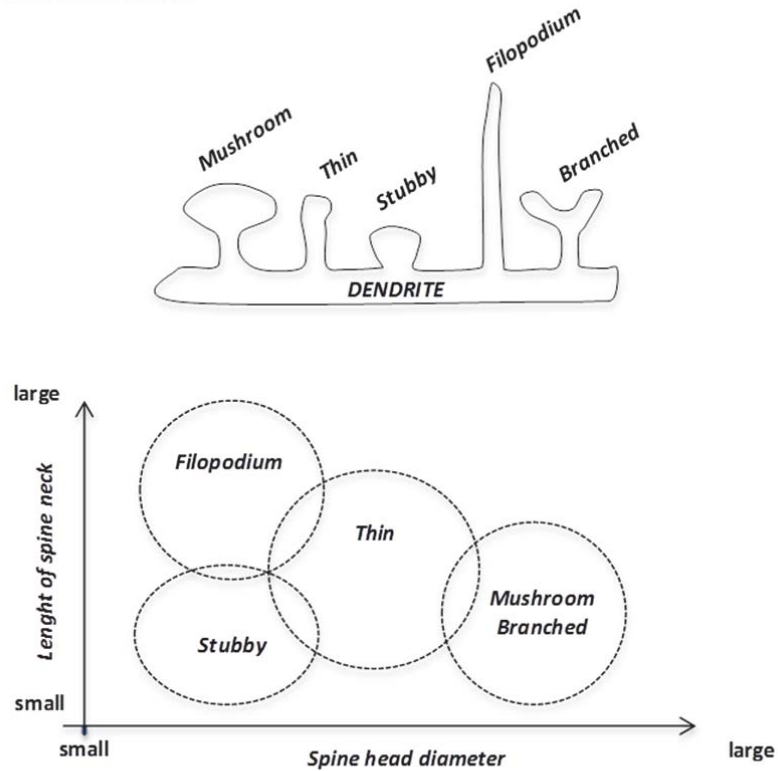
Dendritic spines play critical roles in synaptic transmission and plasticity. Changes in their morphology and density modulate the formation and maintenance of the synapse allowing the dynamics of neural circuitry (Shepherd and Erulkar, 1997;van

der Zee, 2014; von Bohlen Und, 2009). As a dynamic structure, dendritic spine density and size increases after activity-dependent changes such as LTP, and decreases after LTD (Segal, 2005). One exciting feature of dendritic spines is their morphological diversity (Fig.13). A classical dendritic spine contains a head connected to the dendritic shaft by a narrow neck, but depending on their shape and morphology can be subcategorized into:

- 1) Mushroom spines with a large head and a narrow neck
- 2) Thin spines with a smaller head and a narrow neck
- 3) Stubby spines without an obvious constriction between the head and the dendritic shaft
- 4) Filopodium, are long and thin without an obvious head
- 5) Branched spines with multiple heads that emerge from a shared origin

Dendritic spine alterations have been shown to precede the loss of neurons in HD. Several studies demonstrate synaptic alterations in striatal spiny neurons (Ferrante et al., 1991; Graveland et al., 1985) and prefrontal cortical pyramidal neurons (Sotrel et al., 1993) from HD post-mortem brain samples. Furthermore, some studies provided evidence for altered neuronal and dendritic morphology in HD animal models. Thus, it has previously reported a decrease in number and length of spines within the anterior cingulate cortex and dorsal striatum in 36 weeks old R6/1 mice (Spires et al., 2004), while other study shows an absence of dendritic spine pathology in 20 weeks old R6/1 mice, with region-specific decreases in dendritic diameter, branching, complexity and neuronal soma area (Nithianantharajah et al., 2009). Pre-symptomatic and symptomatic R6/2 mice also show a decrease in spine density in striatal spiny neurons (Heck et al., 2012; Klapstein et al., 2001), and decreased spine density and thickening of dendrites is also shown in striatal and cortical neurons from full-length *htt* cDNA mice (Guidetti et al., 2001) and in the full-length Htt YAC128 mice (Xie et al., 2010).

SPINE MORPHOLOGY



SPINE MORPHOLOGY VARIATION ON A DENDRITE

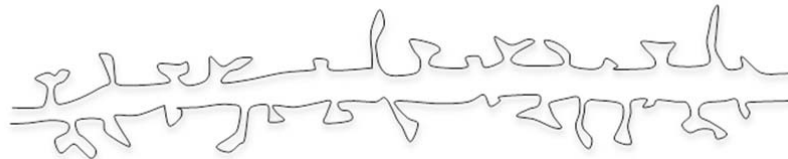


Figure 13. Spine morphology. Depending on their morphology, dendritic spines can be subdivided into different categories. Although the length of the spine neck and head diameter facilitates its classification, not all spine classification is clear (note the overlap between the dashed circles). A large variety of spine types are present in a same dendrite and due to their plastic properties their shape change over time. Adapted from (van der Zee, 2014; von Bohlen Und, 2009).

Synapses contain the postsynaptic density (PSD), an electro-dense macromolecular complex that is made up of neurotransmitter receptors, scaffolding proteins and a complex cytoskeleton network formed by actin filaments (F-actin) that properly structured the PSD to contribute to signal transduction processes (Fig. 14) (Nimchinsky et al., 2002).

Recent studies using molecular and proteomic approaches have highlighted the tremendous complexity of the PSD (Bayes et al., 2011). Interestingly, wild-type and mutant huntingtin protein, as well as huntingtin-interacting proteins are enriched in the synaptic plasma membrane and localized at postsynaptic spines (Gutekunst et al., 1999; Nithianantharajah and Hannan, 2013b; Petrasch-Parwez et al., 2007; Suopanki et al., 2006), suggesting that they may play a role at synaptic sites and in vesicle trafficking through interaction with actin cytoskeleton (Li et al., 2003a; Li et al., 2003b; Smith et al., 2005; Twelvetrees et al., 2010).

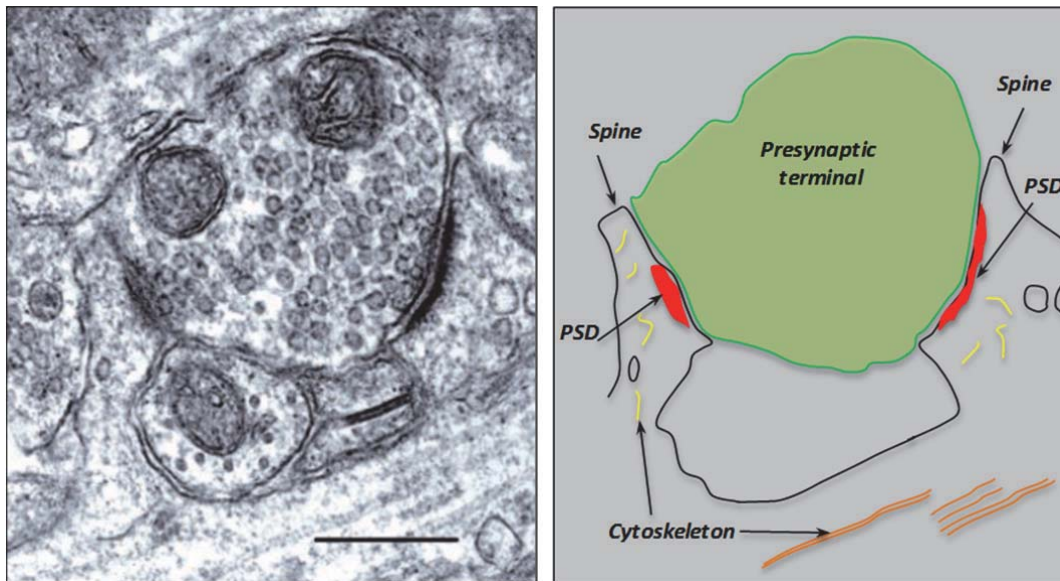


Figure 14. Glutamatergic synapses. Image showing a presynaptic terminal which forms glutamatergic synapses with two dendritic spines. Note the higher presence of synaptic vesicles containing glutamate at the site of synaptic contact (left). Tracing of the left image showing the presynaptic terminal (green), two spines that make synapses within the presynaptic terminal, PSD structure in both spines (red) and cytoskeleton structures such as actin filaments (yellow) and microtubules (orange). Scale bar in left image, 400 nm. Image from (Kennedy, 2000).

2.3.1.2 Alterations in MAGUKs proteins

Excitatory presynaptic terminals make synapses on dendritic spines and release glutamate, as excitatory neurotransmitter. The postsynaptic membrane contains distinct neurotransmitter receptors, including glutamate receptors such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-Aspartate (NMDA) receptors. Glutamate through its binding to AMPAR results in a brief membrane depolarization called excitatory postsynaptic potential (EPSP) produced by the flow of sodium and potassium ions across the membrane. By contrast, although NMDAR are

also ligand-gated ion channels, glutamate binding *per se* does not open NMDAR. Thus, to open NMDAR channels is necessary a strong depolarization of the membrane that allows the relieve blockade of the channel by extracellular magnesium. Thus, when glutamate binding and strong depolarization occurs, the NMDAR channel opens and sodium and calcium ions flow into the cell. Then, the influx of calcium ions into the cell through the NMDAR initiates a wide range of biochemical changes in the post-synaptic spine (Kennedy, 2000). The first potential targets are the proteins located in the PSD.

Membrane-associated guanylate kinases (MAGUKs) superfamily has emerged as central protein organizers of the synapses (Montgomery et al., 2004), and particularly the post-synaptic density 95 (PSD95), synaptic associated protein 102 (SAP102) and 97 (SAP97), and post-synaptic 93 (PSD93) are the most studied (Garner and Kindler, 1996;Kim and Sheng, 2004). The structural properties of these proteins allow them to act as a key scaffolding proteins in the PSD (Brenman et al., 1998;Colledge et al., 2000;Zheng et al., 2011b;Zheng et al., 2011a). It has been demonstrated that PSD95 is involved in several processes such as the stabilization and modulation of NMDA and AMPA receptors (Elias et al., 2006;Funke et al., 2005;Lin et al., 2004;Xu, 2011), the modulation of synaptic processes such as the LTP (Migaud et al., 1998) and the regulation of different NMDAR signaling pathways (Cao et al., 2005;Kim and Sheng, 2004;Sattler et al., 1999;Sheng and Sala, 2001). Importantly, PSD95, SAP102 and PSD93 protein levels are down-regulated in the caudate nucleus of HD patients, and PSD95 and SAP97 were also found to be decreased in the striatum of R6/1 mice at early disease stages (Torres-Peraza et al., 2008). Moreover, PSD95 decrease has also been reported in the hippocampus and frontal cortex of 20-weeks old R6/1 mice (Nithianantharajah et al., 2008). Interestingly, wild-type huntingtin binds to PSD95 and sequesters this scaffold protein, resulting in the inhibition of NMDA receptor activity. By contrast, when the huntingtin protein is mutated the interaction between huntingtin and PSD95 is decreased (Sun et al., 2001). Thus, the authors hypothesize that the reduced interaction between mutant huntingtin and PSD95 allows more PSD95 available to cluster to NMDA receptors, leading to overactivation or sensitization of these receptors. Moreover, while overexpression of wild-type huntingtin inhibits neuronal toxicity caused by mutant huntingtin and NMDAR-

mediated excitotoxicity by binding and sequestration of PSD95, mutant huntingtin enhances neuronal toxicity mediated by glutamate receptors. Overall, these data suggest that PSD95 is a mediator of neuronal toxicity induced by NMDAR in HD.

2.3.1.3 RhoGEF/GAP signaling

The morphology of a spine can change within few seconds and spine dynamics depends on the architecture of the actin cytoskeleton (Fifkova and Morales, 1992;Fischer et al., 1998;Matus, 2000;Nimchinsky et al., 2002). Given the actin-rich nature of spines, the Rho GTPase family plays a key role in spine dynamics. Rho-family GTPases consist of 22 members of guanine nucleotide-binding proteins that switch between an active GTP-bound form and an inactive GDP-bound form (Aspenstrom et al., 2004). In neurons and particularly at the synapses, the Rho GTPases Rac1, Cdc42 and RhoA are the best characterized for their effects on actin cytoskeleton and have been considered key regulators of spine dynamics and synaptic plasticity (Govek et al., 2011;Newey et al., 2005). Whereas Rac1 and Cdc42 promote spine formation, growth and maintenance, RhoA induces spine shrinkage and loss (Newey et al., 2005). Their activity is regulated by positive regulator guanine exchange factors (GEFs), negative regulators GTPases activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Thus, several Rho GEFs, GAPs and GDIs proteins emerged as key regulators in dendritic morphogenesis by modulating these small Rho GTPases activities (Govek et al., 2004;Irie et al., 2005;Newey et al., 2005;Saneyoshi et al., 2008;Tolias et al., 2007;Van de Ven et al., 2005;Xie et al., 2007;Zhang et al., 2005).

2.3.1.3.1 Kalirin-7

Kalirin is a brain-specific GEF protein for Rho-like small GTPases (Penzes and Jones, 2008). Promoting the exchange of GDP for GTP, kalirin stimulates the activity of Rho GTPases (Alam et al., 1997;Rossman et al., 2005). Kalirin gene (*Kalrn*) contains multiple promoters and transcriptional start sites that generate different isoforms by alternative splicing (Fig.15) (Johnson et al., 2000;McPherson et al., 2002;McPherson et al., 2004).

Kalirin isoforms have distinct subcellular localizations in neurons. Kalirin-12 is located in growth cones of immature neurons and in dendritic spines of mature

hippocampal neurons (Xin et al., 2009). Kalirin-9 is found into neurites and growth cones (Penzes et al., 2001a). Although the function of both Kalirin-9 and kalirin-12 remain largely unknown, it has been suggested that Kalirin-12 has a role in axon outgrowth, synaptic plasticity, coordination of endocytic trafficking and actin cytoskeleton modulation, whereas Kalirin-9 exogenous expression induces longer neurites and altered neuronal morphology in cultured cortical neurons (Mandela and Ma, 2012). The expression of both Kalirin-9 and -12 isoforms are higher during embryonic development but decrease in the adult brain. The decrease in these two isoforms in the adult brain associates with an increase in the expression of another isoform, Kalirin-7.

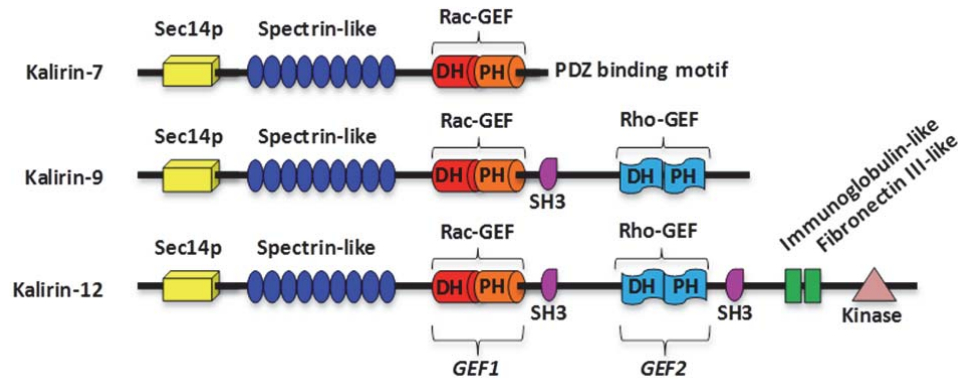


Figure 15. Major Kalirin isoforms structure. By alternative splicing different Kalirin isoforms are generated. Kalirin-7, -9 and -12 contains an N-terminal Sec14p domain, followed by nine spectrin-like repeats. The Dbl homology (DH) and pleckstrin homology (PH) domains confer Kalirin's guanine nucleotide exchange factor (GEF) activity. The first GEF domain (GEF1) is common to all isoforms and activates Rac1. Kalirin-7 also contains a unique C-terminal PDZ-binding domain. Kalirin-9 and -12 have a Src homology domain (SH3) and a second GEF domain (GEF2) that activates RhoA. Kalirin-12 has C-terminal immunoglobulin-like and fibronectin III-like domains as well as a kinase domain. Adapted from (Mandela and Ma, 2012;Remmers et al., 2014).

Kalirin-7 is the most abundant isoform in the adult brain and its expression is limited to neurons of the central nervous system. Its expression is very low at birth, but begins to increase at postnatal day 14, coinciding with synaptogenesis (Ma et al., 2003;Mandela and Ma, 2012). In neurons, Kalirin-7 is exclusively localized to the postsynaptic side of excitatory, but not inhibitory synapses (Ma et al., 2003;Ma et al., 2008;Ma et al., 2011;Penzes et al., 2001b). In the adult brain, Kalirin-7 expression is

higher in the cerebral cortex and hippocampus although it is also expressed in other brain regions. Therefore, it has been suggested that Kalirin-7 may play a role in learning and memory processes by modulating structural plasticity (Ma et al., 2003; Ma et al., 2008). Indeed, several *in vitro* studies demonstrate that Kalirin-7 plays a pivotal role in the regulation of excitatory synapse formation and signaling. Thus, the overexpression of Kalirin-7 causes an increase in dendritic spine density, spine size and synapse number, while kalirin-7 reduction promotes spine shrinkage and loss in cultured hippocampal and cortical neurons (Fig.16) (Ma et al., 2003; Ma et al., 2008; Penzes et al., 2001b).

Consistent with these *in vitro* experiments, it has been reported that kalirin-7 also plays a key role in spine and synapse formation *in vivo* as shown by the data obtained in two Kalirin knock-out (KO) mice. In Kalirin-7^{KO} mice the terminal exon unique to kalirin-7 was deleted (Ma et al., 2008), while in Kalirin^{GEF1-KO} mice the exons 27-28 in the first GEF domain were replaced by a neomycin resistance cassette leading to the elimination of both kalirin-7, -9 and -12 isoforms expression (Cahill et al., 2009). Kalirin-7^{KO} mice exhibit decreased spine density in the CA1 hippocampal pyramidal neurons, LTP deficits, decreased anxiety-like behavior in the elevated zero maze and impaired acquisition in the passive avoidance task, but normal behavior in the open field, object recognition and radial arm maze tasks (Ma et al., 2008). On the other hand, Kalirin^{GEF1-KO} mice show reduced spine density in pyramidal cortical neurons, increased locomotor activity in the open field and deficits in spatial and working memory (Cahill et al., 2009). Importantly, both Kalirin-7^{KO} and Kalirin^{GEF1-KO} mice display impaired hippocampal LTP induction, impaired contextual fear conditioning and reduced spine density in cortical neuronal cultures (Cahill et al., 2009; Ma et al., 2008; Xie et al., 2011).

Interestingly, decreased Kalirin-7 mRNA and protein levels have been reported in the hippocampus of Alzheimer's disease (Youn et al., 2007b; Youn et al., 2007a) as well as in animal models of depression (Li et al., 2010). Surprisingly, although it has been demonstrated that Huntingtin-associated protein1 interacts with Kalirin (Colomer et al., 1997), the role of Kalirin-7 in HD has not been determined.

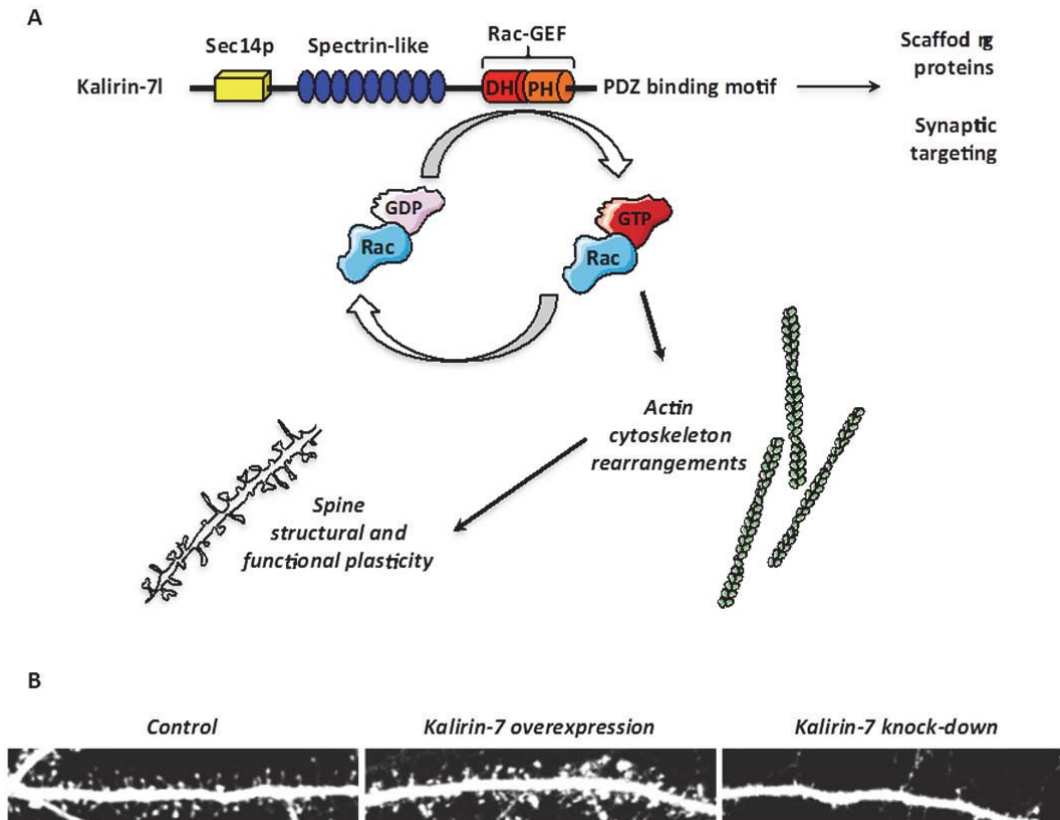


Figure 16. Kalirin-7 emerged as a key postsynaptic regulator of spine morphogenesis. **A**, DH and PH Kalirin-7 domains provide GEF activity that activates Rac1 and controls spine remodeling by modulating the actin cytoskeleton. At its C-terminus, Kalirin-7 contains a unique 20 amino acid sequence in a PDZ domain-binding motif (STYV) that allows the interaction with scaffolding proteins and confers its synaptic targeting. **B**, Kalirin-7 regulates dendritic spine morphogenesis and maintenance in pyramidal neurons. Kalirin-7 overexpression promotes spine formation and enlargement, while Kalirin-7 knock-down by an RNAi causes spine shrinkage and loss. Adapted from (Penzes and Jones, 2008).

Given that alterations in dendritic spines and synaptic plasticity deficits have been described in HD, and as previously described Kalirin-7 emerged as a major contributor in modulating spine and synapse formation *in vitro* and *in vivo*, in this Thesis we have analyzed the possible role of Kalirin-7 in excitatory synaptic dysfunction in HD.

2.3.2 From structure to function

2.3.2.1 Synaptic plasticity and transmission

One of the best characterized methods to evaluate synaptic plasticity is by electrophysiological techniques. Several studies indicate that excitatory synapses

exhibit the most important alterations in HD (Cepeda et al., 2007; Fan and Raymond, 2007; Li et al., 2003b). Several groups focused their research in the study of corticostriatal and hippocampal synaptic transmission concluding that synaptic plasticity is impaired in HD (Cepeda et al., 2007; Milnerwood and Raymond, 2007; Milnerwood and Raymond, 2010; Murphy et al., 2000; Raymond et al., 2011; Usdin et al., 1999; Milnerwood et al., 2006).

Studies in HD mouse models show severe age-related alterations in corticostriatal connectivity (Cepeda et al., 2007; Milnerwood et al., 2006; Milnerwood and Raymond, 2007), with impairments in evoked synaptic responses and spontaneous synaptic currents in different HD mice. Moreover, primary somato-sensory cortex plasticity deficits have been detected in exon-1 mouse models, as well as altered corticostriatal LTP and decreased cell capacitance of MSN (Cepeda et al., 2001; Cybulska-Klosowicz et al., 2004; Giralt et al., 2011a; Levine et al., 1999). Interestingly, other studies describe a progressive age-dependent decline in the ability to support LTD in cortical slices from HD transgenic mice (Cummings et al., 2006; Cummings et al., 2007).

The hippocampus is considered an area of the mammalian brain that is highly related to memory processes such as the formation and retrieval of some forms of memory (Bliss and Collingridge, 1993). LTP and LTD represent an excellent experimental evaluation of the synaptic properties of learning and memory in the hippocampus (Behr et al., 2009; Bliss and Collingridge, 1993; Gladding et al., 2009; Lynch, 2004). Normal basal neurotransmission (CA3-CA1 field excitatory post-synaptic potentials) but reduced LTP expression has been reported in exon-1 mice (Giralt et al., 2009; Murphy et al., 2000), YAC mice (Hodgson et al., 1999), and knock-in mice (Lynch et al., 2007; Usdin et al., 1999), indicating hippocampal synaptic dysfunction. Importantly, the alteration in hippocampal LTP expression in HD mouse models has been shown to be accompanied by impaired LTD. Thus, several studies indicate that whereas HD transgenic mice exhibit normal LTD expression at younger ages compared to controls, their synapses later regain the ability to support LTD (Hodgson et al., 1999; Milnerwood et al., 2006; Murphy et al., 2000).

Overall, these studies demonstrate a clear impairment in synaptic plasticity as demonstrated by altered LTP and LTD and suggest that synaptic deficit (including alterations in synaptic transmission, plasticity and aberrant spine density/morphology) may be the initial triggers for the cognitive deficits observed in HD.

2.4 KINASE AND PHOSPHATASE SIGNALING

Mutant huntingtin can impact on several physiological processes through inducing alterations in signaling pathways. In this scene, an appropriate balance between protein kinase and phosphatase activities is crucial for the regulation of neuronal survival and plasticity. Indeed, memory formation and synaptic plasticity processes depend on an appropriate balance between kinase and phosphatase activities (Kennedy et al., 2005; Mansuy and Shenolikar, 2006; Mayford, 2007). Changes in the expression and activity of different phosphatases (Table 4) (Ehrnhoefer et al., 2011; Fan et al., 2008; Metzler et al., 2010; Pineda et al., 2009; Runne et al., 2008; Saavedra et al., 2010; Saavedra et al., 2011; Wu et al., 2002; Xifro et al., 2008; Xifro et al., 2009) and kinases (Table 5) (Apostol et al., 2006; Fan et al., 2012; Gines et al., 2003a; Giralte et al., 2011b; Paoletti et al., 2008; Roze et al., 2008; Rue et al., 2014; Saavedra et al., 2010; Saavedra et al., 2011; Xifro et al., 2011) have been reported in HD models and human HD brain, supporting that aberrant function of these proteins are involved in the pathogenesis of HD.

Overall, these studies demonstrate kinase and phosphatase alterations in the presence of mutant huntingtin and identify them as pharmacological candidates to design therapeutic strategies to restore neuronal survival and function in HD. Among these kinases and phosphatases, Cdk5 has been emerged as one of the most versatile kinases.

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Type of phosphatase	Phosphatase	Change	HD model	Reference
<i>Ser/Thr Phosphatase</i>	Calcineurin	Increased	STHdh ^{Q7/Q111} cells and Hdh ^{Q111/Q111} mice	Xifro et al., 2008 Pineda et al., 2009
			STHdh ^{Q7/Q111} cells and Hdh ^{Q111/Q111} mice	Xifro et al., 2008 Pineda et al., 2009
	PHLPP1	Reduced	R6/1 and YAC128 mice	Xifro et al., 2009 Metzler et al., 2010
			STHdh ^{Q7/Q111} cells and Hdh ^{Q111/Q111} mice	Xifro et al., 2008 Pineda et al., 2009
			R6/1, R6/1:BDNF ^{-/-} , R6/2, Tet/HD94 Gene-On, Hdh ^{Q111/Q111} mice and STHdh ^{Q111/Q111} cells	Saavedra et al., 2010
	PP1	Unchanged Reduced	YAC128 mice	Metzler et al., 2010
			YAC128 mice	Ehrnhoefer et al., 2011
PP2A	Unchanged Reduced	YAC128, R6/1 mice	Metzler et al., 2010 Saavedra et al., 2010	
		YAC128 mice	Ehrnhoefer et al., 2011	
<i>Tyr Phosphatase</i>	STEP	Reduced	R6/1, R6/2, Tet/HD94 Gene-On, Hdh ^{Q111/Q111} mice and Primary striatal neurons Overexpressing htt171-82Q	Saavedra et al., 2011 Runne et al., 2008
	MKP1 and MKP3	Increased	PC12 cells overexpressing Exon-1 mhtt 118Q	Wu et al., 2002
	MKP2	Intracellular redistribution	HEK 293 cells Overexpressing mhtt 138Q and NR1/NR2B	Fan et al., 2008

Table 4. Phosphatases altered in HD models. Table includes information about the change in levels/activity of different phosphatases in different HD models. Pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase (PHLPP); protein phosphatase 1 (PP1) and 2A (PP2A); striatal-enriched protein tyrosine phosphatase (STEP); mitogen-activated protein kinase phosphatase 1 (MKP1), 2 (MKP2) and 3 (MKP3). Adapted from (Saavedra et al., 2012).

Type of Kinase	Kinase	Change	HD model	Reference
<i>Ser/Thr Kinase</i>	PKA	Increased	R6/1 and R6/2 mice	Giralt et al., 2011
	PKB or Akt	Increased	STHdh ^{Q111/Q111} cells and Hdh ^{Q111/Q111} , R6/1, R6/1:BDNF ^{+/-} , R6/2, Tet/HD94 Gene-On mice	Ginés et al., 2003 Saavedra et al., 2010
	GSK3β	Increased levels of inactivated protein	STHdh ^{Q111/Q111} cells and R6/1 mice	Ginés et al., 2003 Saavedra et al., 2010
	PKC	Reduced	R6/1 mice	Rué et al., 2014
	Rsk	Increased	STHdh ^{Q111/Q111} cells and Hdh ^{Q111/Q111} , R6/1 mice	Xifró et al., 2011
	Cdk5	Increased	STHdh ^{Q111/Q111} cells and Hdh ^{Q111/Q111} mice	Paoletti et al., 2008
	MAPKs			
	ERK1/2	Increased	PC12 cells overexpressing exon-1 mutant huntingtin and R6/1, R6/2 mice	Saavedra et al., 2011 Roze et al., 2008 Apostol et al., 2006
	JNK	Increased	PC12 cells overexpressing exon-1 mutant huntingtin and YAC128 mice	Apostol et al., 2006 Fan et al., 2012
	p38	Increased	YAC128 and R6/1 mice	Fan et al., 2012 Saavedra et al., 2011

Table 5. Kinases altered in HD models. Table includes information about the change in levels/activity of different kinases in different HD models. Protein kinase A (PKA); protein kinase B (PKB or Akt); glycogen synthase kinase 3 beta (GSK3β); protein kinase C (PKC); 90-KDa ribosomal S6 kinase (Rsk); cyclin dependent kinase 5 (Cdk5); mitogen-activated protein kinase (MAPKs); extracellular regulated kinase 1/2 (ERK1/2); c-Jun NH₂-terminal kinase (JNK).

2.4.1. Cyclin-dependent kinase 5

Cyclin-dependent kinases (CDKs) are a large family of serine/threonine protein kinases that associate with specific cyclin subunits in order to be activated and control the eukaryotic cell cycle. In 1992, five independent laboratories reported Cdk5 as a new member of the Cdk family (Hellmich et al., 1992; Ishiguro et al., 1992; Lew et al., 1992; Meyerson et al., 1992; Xiong et al., 1992). Although Cdk5 has >60% sequence homology to the mammalian Cdk2 and yeast cdc2, is a unique family member (Dhavan

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and Tsai, 2001;Hellmich et al., 1992;Lew et al., 1992;Meyerson et al., 1992). In contrast to other Cdks, Cdk5 does not participate in cell cycle and does not associate with cyclins (Dhavan and Tsai, 2001). However, Cdk5 needs to associate with a regulatory subunit in order to be activated, named neuron-specific activators p35 and p39 (Fig. 17) (Tang et al., 1995;Tsai et al., 1994). Importantly, p35 and p39 double-knock-out mice display an identical phenotype than Cdk5-knock-out (Cdk5 KO) mice, suggesting that no other Cdk5 activators were directly involved and that p35 and p39 are necessary and sufficient for Cdk5 activity (Ko et al., 2001).

Despite the widespread expression of Cdk5 throughout the organism, the enzymatic activity of Cdk5 is more prominent in the CNS, where its main co-activators are highest express (Hisanaga and Endo, 2010;Nguyen et al., 2002;Paglini and Caceres, 2001;Zheng et al., 1998). Both p35 and p39 are essentially localized in the cellular membrane as membrane-anchored proteins and although Cdk5 *per se* does not show a specific distribution pattern, it tends to co-localize with its activators and substrates (Delalle et al., 1997;Ko et al., 2001;Sharma et al., 1999b). However, p35 also has a dynamic localization between the nucleus and the cytoplasm due to its interaction with different importin proteins (Fu et al., 2006) indicating that Cdk5/p35 complex also exerts its function in this subcellular compartments.

Notably, Cdk5 can also be activated by its interaction with p25 and p29, the truncated forms of p35 and p39, respectively. Calpain, a calcium-activated protein, generates these proteolytic fragments (Lee et al., 2000;Nath et al., 2000;Patzke and Tsai, 2002). Importantly, whereas p35 is a protein with a short lived (a half-life of less than 20 minutes) and is rapidly degraded by the proteosome, p25 is resistant to ubiquitin-mediated proteolysis and has a fivefold longer half-life compared to p35 (Patrick et al., 1999). Moreover, whereas p35 contains in its N-terminal region a myristoylated region important for its membrane targeting, p25 lacks it (Dhavan and Tsai, 2001;Patrick et al., 1999). Overall indicate that Cdk5/p25 complex has a sustained activation over time and its subcellular location is different compared to Cdk5/p35 complex, modulating Cdk5 effects on its substrates (Fig. 17). In this context, it has been demonstrated that p25 maintains Cdk5 in hyperactive state, which is associated with neurotoxicity (Lee et al., 2000;Patrick et al., 1999). Additionally, p29 has also been

detected in the cells and it has been demonstrated that deregulates Cdk5 activity (Patzke and Tsai, 2002). Although binding to a protein partner is obligatory for Cdk5 activation, its activity can also be modulated by post-translational modifications such as phosphorylation and S-nitrosylation. Particularly, Cdk5 possesses three conserved phosphorylation sites: Tyr15, Thr14 and Ser159, common among the Cdk family. Cdk5 phosphorylation at Tyr15 by the kinases Abl, Fyn and ephrin receptor A (EphA) results in the activation of Cdk5 (Fig. 17), thereby promoting neuronal migration, neurite outgrowth and synaptogenesis (Cheng et al., 2003;Sasaki et al., 2002;Sharma et al., 1999a;Zukerberg et al., 2000). Although this phosphorylation results in physiological activities, Abl-mediated phosphorylation of Tyr15 can also hyperactivates Cdk5, resulting in neurotoxic effects (Cancino et al., 2011;Lin et al., 2007). By contrast, the kinases that phosphorylate the other Cdks at Thr14 are unable to phosphorylate Cdk5 at either position (Fattaey and Booher, 1997) and the phosphorylation of Cdk5 at Ser159 in cells remains unclear although it has been suggested that inhibits its activity (Tarricone et al., 2001). Importantly, active Cdk5 can also modulate its own activity by phosphorylation of its co-activator p35 at two different sites, Ser8 and Thr138 (Asada et al., 2012;Kamei et al., 2007;Patrick et al., 1998). Cdk5-mediated phosphorylation of p35 at Ser8 is required to retain p35 in the cytoplasm (Asada et al., 2012). By contrast, Cdk5-mediated phosphorylation of p35 at Thr138 has been shown to exert different functions: 1) prevents the intramolecular cleavage of p35 to p25, thereby protecting against neurotoxicity; 2) inhibits p35 binding to microtubules and due to its function in promoting microtubule bundling (He et al., 2008), Cdk5-mediated Thr138 phosphorylation of Cdk5 results in the loss of microtubule bundling and neurite outgrowth; 3) increases p35 degradation through proteosome. Overall these findings demonstrated that Cdk5 can autoregulates its own stability, subcellular localization and activity.

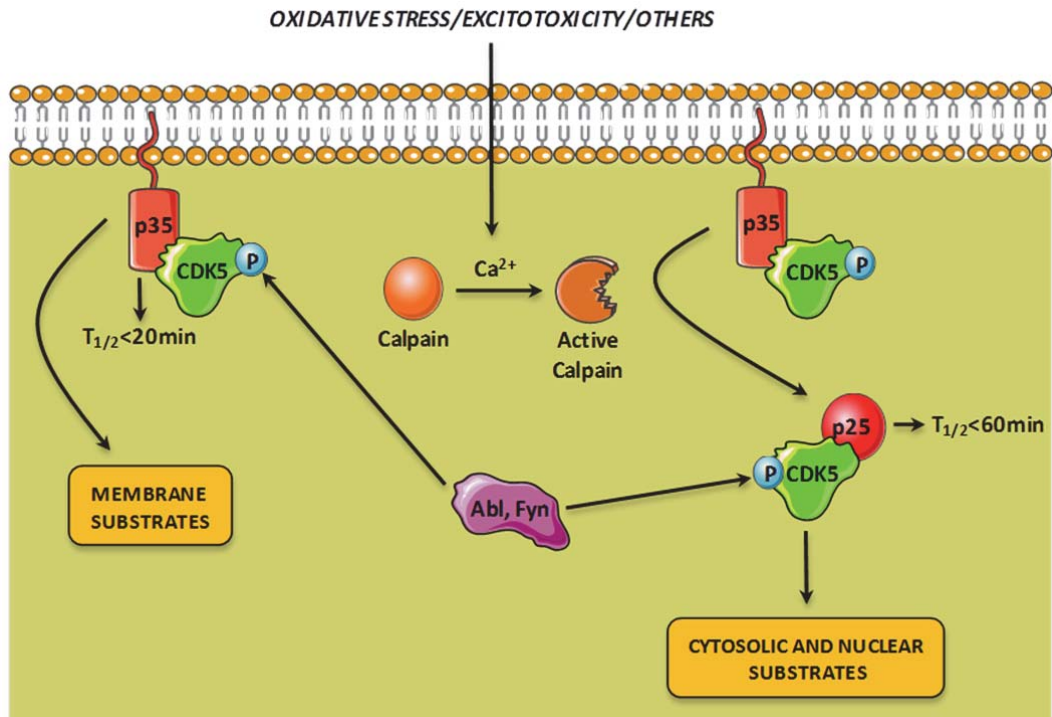


Figure 17. Schematic representation of Cdk5 regulation. Neurons exposed to oxidative stress, excitotoxicity or other insults suffer a deregulation in Ca^{2+} homeostasis, mainly caused by an influx through NMDA receptors and voltage-sensitive Ca^{2+} channels and calcium release from intracellular compartments such as endoplasmic reticulum (not shown). The increase in intracellular Ca^{2+} activates calpains, which in turn cleave the normal Cdk5 activator p35 to the pathogenic form, p25. The increase in p25 levels triggers the formation of a hyperactive Cdk5/p25 complex. p25 lacks the myristoylation signal and is mislocalized to the cytoplasm, allowing Cdk5 access to substrates away from the membrane, while p35 targets Cdk5 to the membrane, allowing its access to membrane substrates. In addition, p25 has a longer half-life ($T_{1/2}$) than p35, causing sustained activation of Cdk5. Adapted from (Dhavan and Tsai, 2001).

Cdk5 exhibit a plethora of different functions (Fig 18): is indispensable for normal brain development (Ohshima et al., 1996), regulation of cytoskeleton proteins (Hallows et al., 2003;Smith, 2003), axon guidance (Nikolic et al., 1996), membrane transport (Barclay et al., 2004;Paglini and Caceres, 2001;Shea et al., 2004) synaptic function (Cheng and Ip, 2003), neuronal survival, learning and memory formation, pain signaling, drug addiction and long-term behavioral changes (Bibb et al., 2001;Fischer et al., 2002;Pareek et al., 2006).

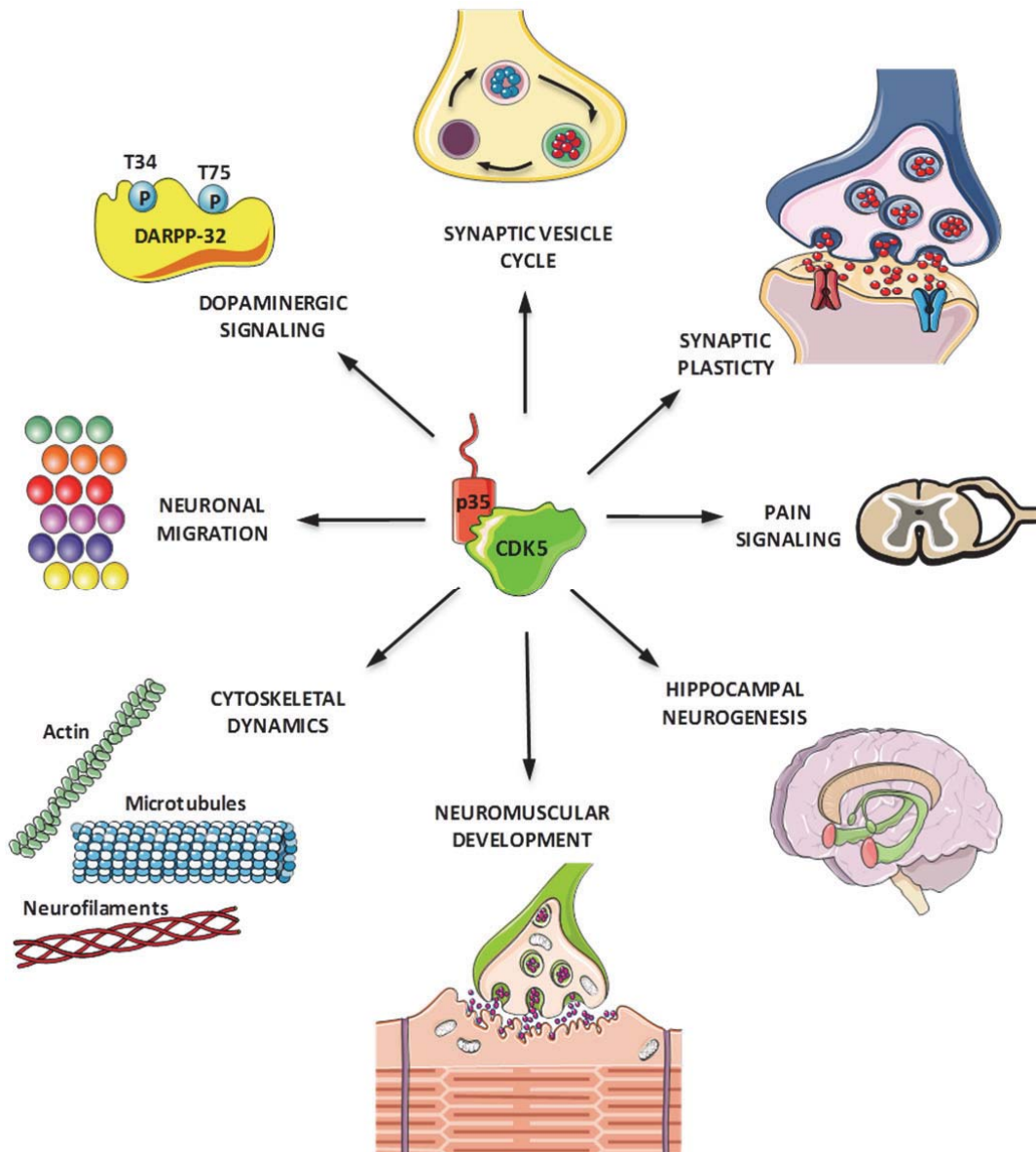


Figure 18. Schematic representation of Cdk5 impact in various cellular processes. Adapted from (Su and Tsai, 2011).

Cdk5 can exert all these functions through phosphorylation of substrates involved in 1) neuronal migration (Table 6) (Ishizuka et al., 2011;Kawauchi et al., 2006;Kesavapany et al., 2001;Keshvara et al., 2002;Kwon et al., 2000;Niethammer et al., 2000;Sasaki et al., 2000;Singh et al., 2010;Tanaka et al., 2004;Xie et al., 2003);

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NEURONAL MIGRATION

Cdk5 substrates	Functional outcome	Phosphorylation sites	References
β-Catenin	N-cadherin-mediated cell adhesion, binding	Multiple	Kwon et al., 2000 Kesavapany et al., 2001
Disabled 1	Control of neuronal positioning, reelin interaction	S491	Keshvara et al., 2002
Dixdc1	Allow DISC1-NUDEL interaction	S250	Singh et al., 2010
DISC1	Progenitor proliferation and migration switch	S710	Ishizuka et al., 2011
Doublecortin	Low affinity to microtubules, reduced effect on polymerization	S297	Tanaka et al., 2004
FAK	Nuclear translocation through microtubule fork	S732	Xie et al., 2003
NUDEL	Dynein and axonal transport regulation	S198, T219, S231	Niethammer et al., 2000 Sasaki et al., 2000
p27 (kip1)	Actin organization, cortical neuronal migration	S10, T187	Kawauchi et al., 2006

Table 6. List of major identified Cdk5 substrates in neuronal migration. Abbreviations: DISC1, Disrupted in schizophrenia1; Dixdc1, Dix-domain containing 1; FAK, focal adhesion kinase; p27(kip1), cyclin-dependent kinase inhibitor. Adapted from (Su and Tsai, 2011).

2) neurite outgrowth (Table 7) (Brown et al., 2004; Cheung et al., 2007; Humbert et al., 2000a; Kato and Maeda, 1999; Kesavapany et al., 2004; Nikolic et al., 1998; Paglini et al., 1998; Pigino et al., 1997; Zukerberg et al., 2000);

NEURITE OUTGROWTH

Cdk5 substrates	Functional outcome	Phosphorylation sites	References
Cables	Dissociation of Cdk5/Cables/c-Abl complex, Neurite outgrowth	Multiple	Zukerberg et al., 2000
CRMP2	Semaphorin 3A-induced growth cone collapse	S522	Brown et al., 2004
c-Src	Neural development, neurite outgrowth, function	S75	Kato and Maeda, 1999
MAP1B	Regulation of axonal formation and elongation	Multiple	Pigino et al., 1997 Paglini et al., 1998
p39	Regulation of actin cytoskeletal dynamics	Multiple	Humbert et al., 2000
PAK1	Actin polymerization, cytoskeletal reorganization	T212	Nikolic et al., 1998
RasGRF2	Altered RasGRF2, microtubule protein accumulation	S737	Kesavapany et al., 2004
TrkB	BDNF-stimulated dendritic outgrowth	S478	Cheung et al., 2007

Table 7. List of major identified Cdk5 substrates in neurite outgrowth. Abbreviations: Cables, Cdk5 and Abl enzyme substrate; CRMP2, Collapsin response mediator protein 2; c-Src, non-receptor tyrosine kinase; MAP1B, microtubule-associated protein 1B; p39, regulatory activator of cyclin-dependent kinase 5; PAK1, p21-Activated Kinase; RasGRF2, ras guanine nucleotide releasing factor 2. Adapted from (Su and Tsai, 2011).

3) synaptic vesicle cycle (Table 8) (Cheng et al., 2002;Fletcher et al., 1999;Floyd et al., 2001;Lee et al., 2004;Liang et al., 2007;Matsubara et al., 1996;Shuang et al., 1998;Tan et al., 2003;Taniguchi et al., 2007;Tomizawa et al., 2003);

SYNAPTIC VESICLE CYCLE

Cdk5 substrates	Functional outcome	Phosphorylation sites	References
Amphiphysin 1	Synaptic vesicle endocytosis regulation	S262, S272, S276, S285, T310	Floyd et al., 2001 Liang et al., 2007
Dynamin 1	Clathrin-mediated synaptic vesicle endocytosis	S774, S778	Tomizawa et al., 2003 Tan et al., 2003
Munc18	Modulation of synaptic vesicle exocytosis	T574	Shuang et al., 1998 Fletcher et al., 1999
Pctaire1	Synaptic vesicle exocytosis via NSF interaction	S95	Cheng et al., 2002
Septin 5	Reduction of sept5 binding to syntaxin	S17	Taniguchi et al., 2007
Synapsin 1	Impact on cytoskeletal components, actin bundling	S551, S553	Matsubara et al., 1996
Synaptojanin1	Interaction with other endocytosis components	S1144	Lee et al., 2004

Table 8. List of major identified Cdk5 substrates in synaptic vesicle cycle. Abbreviations: Munc18, mammalian uncoordinated 18; pctaire1, cyclin-dependent kinase 16 (cdk16); NSF, N-ethylmaleimide sensitive factor. Adapted from (Su and Tsai, 2011).

4) neurodegenerative diseases (Table 9) (Ackerley et al., 2003;Anne et al., 2007;Avraham et al., 2007;Baumann et al., 1993;Gong et al., 2003;Iijima et al., 2000;Kobayashi et al., 1993;Lew et al., 1994;Luo et al., 2005;Qu et al., 2007;Rashidian et al., 2009;Smith et al., 2006);

NEURODEGENERATIVE DISEASES

Cdk5 substrates	Functional outcome	Phosphorylation sites	References
APP	Potential localization of the β -amyloid protein	T668	Iijima et al., 2000
Huntingtin	Reduction in huntingtin aggregation and formation	S434, S1181, S1201	Luo et al., 2005 Anne et al., 2007
MEF2D	Inhibition of transcriptional factors and cell death	S444	Smith et al., 2006 Gong et al., 2003
NF	Regulation of axonal transport of NF	Multiple	Lew et al., 1994 Ackerley et al., 2003
Parkin	Modulation of synphilin-1/ α -synuclein inclusions	S131	Avraham et al., 2007
Prx2	Tuning sensitivity of neuron to oxidative stress	T89	Qu et al., 2007 Rashidian et al., 2009
Tau	Accumulation and disruption of axonal transport	Multiple	Baumann et al., 1993 Kobayashi et al., 1993

Table 9. List of major identified Cdk5 substrates in neurodegenerative diseases. Abbreviations: APP, amyloid precursor protein; MEF2D, myocyte enhancer factor 2D; NF, neurofilament; Prx2, peroxiredoxin 2. Adapted from (Su and Tsai, 2011).

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5) synaptic transmission and plasticity (Table 10) (Bibb et al., 1999;Kansy et al., 2004;Kim et al., 2006;Li et al., 2001;Morabito et al., 2004;Moy and Tsai, 2004;Nguyen et al., 2007;Poore et al., 2010;Samuels et al., 2007;Seeburg et al., 2008;Tomizawa et al., 2002;Wang et al., 2003;Wei et al., 2005;Xin et al., 2008;Zhang et al., 2008);

SYNAPTIC TRANSMISSION AND PLASTICITY

Cdk5 substrates	Functional outcome	Phosphorylation sites	References
δ-Catenin	Dendritic morphogenesis, synaptic activity	S300, S357	Poore et al., 2010
WAVE1	Regulation of actin polymerization and dendritic spine morphogenesis	S310, S397, S441	Kim et al., 2006
Kalirin-7	Formation, regulation of dendritic spine protrusion	T1590	Xin et al., 2008
NR2A	NMDA current, LTP, ischemia-mediated cell death	S1232	Li et al., 2001 Wang et al., 2003
NR2B	Surface expression levels of NR2B and endocytosis	T1472	Zhang et al., 2008
PP1	Signaling pathways involving PKA, calcineurin	S6, S67	Nguyen et al., 2007
PSD95	Clustering size of K ⁺ channels at dendritic spines	T19, S25	Morabito et al., 2004
SPAR	Plk2-mediated degradation during synaptic scaling	S1328	Seeburg et al., 2008
TH	Presynaptic component in dopamine synthesis	S31	Moy and Tsai 2004 Kansy et al., 2004
CASK	Promotion of synaptogenesis, presynaptic proteins	S51, S395	Samuels et al., 2007
Ca_v1.2	Decreased calcium influx upon glucose stimulation	S783	Wei et al., 2005
Ca_v2.1	Synaptic plasticity and neurotransmitter release	aa724-981	Tomizawa et al., 2002
DARPP-32	Signal transduction modulation in striatal neurons	T75	Bibb et al., 1999

Table 10. List of major identified Cdk5 substrates in synaptic transmission and plasticity. Abbreviations: WAVE 1, Wiskott-Aldrich syndrome protein-family verprolin homologous protein 1 NR2A, N-methyl D-aspartate receptor subunit 2A; NR2B, N-methyl D-aspartate receptor subunit 2B; PP-1, protein phosphatase-1; SPAR, spine-associated Rap guanosine triphosphatase activating protein; TH, tyrosine hydroxylase; CASK, calcium/calmodulin-dependent serine protein kinase; Ca_v1.2, L-type voltage-gated calcium channel; Ca_v2.1, P/Q-type voltage-gated calcium channel; DARPP-32, Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa; Plk2, polo-like kinase. Adapted from (Su and Tsai, 2011).

and 6) other cellular processes (Table 11) (Adzic et al., 2009;Cheung et al., 2008;Choi et al., 2010;Fu et al., 2001;Fu et al., 2004;Hamdane et al., 2005;Hou et al., 2007;Huang et al., 2009;Huang et al., 2010;Kino et al., 2007;Lee et al., 1997;Lee et al., 2005c;Li et al., 2002a;Li et al., 2004;Maestre et al., 2008;Miyamoto et al., 2007;Pareek et al., 2007;Patrick et al., 1998;Schubert et al., 2010;Sharma et al., 2002;Tsai et al., 1994;Zhang et al., 2002) and/or by protein-protein interactions (Hawasli and Bibb, 2007).

OTHERS

Cdk5 substrates	Functional outcome	Phosphorylation sites	References
p35	Promotion of ubiquitin-mediated proteolysis	S8, T138, S170, T197	Tsai et al., 1994 Patrick et al., 1998
p53	Increased cell cycle arrest or cell death genes	S33, S315	Zhang et al., 2002
Ape1	Involvement in regulation of DNA damage, cell death	T232	Huang et al., 2010
GR	Regulate glucocorticoid receptor transcriptional activity	S203, S211, S232, S246, others	Kino et al., 2007 Adzic et al., 2009
ATM	DNA damage, ATM activation, neuronal death	S794	Tian et al., 2009
β -2 syntrophin	Regulatory mechanisms of insulin secretion	S75	Schubert et al., 2010
Bcl-2	Neuroprotective effect, neuronal survival	S70	Cheung et al., 2008
Cdh1	Mediates cyclin B1 accumulation in excitotoxicity	S40, T121, S163	Maestre et al., 2008
Rb	Cellular growth, differentiation, apoptosis	Multiple	Lee et al., 1997 Hamdane et al., 2005
ErbB3	Nrg-induced AChR expression at the NMJ	T871, S1204	Fu et al., 2001
JNK3	Inhibition of JNK activity and reduced apoptosis	T131	Li et al., 2002
MEK1	Down-regulation of MAPK signaling pathway	T286	Sharma et al., 2002
mSds3	HDAC transcriptional co-repressor complex	S228	Li et al., 2004
Paxillin	Involvement in oligodendrocyte differentiation	S244	Miyamoto et al., 2007
PIPK γ	Blocked PI (4,5)P2 synthesis at synapses and at focal adhesions	S650	Lee et al., 2005
PPAR γ	Obesity-linked phosphorylation in fat cells	S273	Choi et al., 2010
S6K1	Regulation of S6K1 catalytic activity	S411	Hou et al., 2007
STAT3	Transcription of target genes c-fos and junB	S727	Fu et al., 2004
Talin	Regulation of Smurf1-mediated talin ubiquitination	S425	Huang et al., 2009
TRPV-1	Modulation of nociceptive signaling in DRG	T407	Pareek et al., 2007

Table 11. List of major identified Cdk5 substrates in other cellular processes. Abbreviations: p53, tumor protein 53; Ape1, apurinic/apyrimidinic endonuclease 1; GR, glucocorticoid receptor; ATM, Ataxia telangiectasia mutated; Bcl-2, B-cell CLL/lymphoma 2; Cdh1, cadherin-1; Rb, retinoblastoma protein; ErbB3, receptor tyrosine-protein kinase erbB3; JNK3, c-Jun N-terminal kinase 3; MEK1, MAP kinase kinase-1; paxillin, focal adhesion-associated adaptor protein; PIPKI, phosphatidylinositol(4) phosphate 5 kinase type I gamma; PPAR γ , Peroxisome proliferator-activated receptor gamma; S6K1, S6 Kinase 1; STAT3, signal transducer and activator of transcription 3; TRPV-1, Transient Receptor Potential Vanilloid-1; Nrg, neuregulin; AChR, acetylcholine receptor; NMJ, neuromuscular junction; DRG, dorsal root ganglion. Adapted from (Su and Tsai, 2011).

Recent advances indicate that Cdk5 is involved in synaptic plasticity in mature neurons (Cheung et al., 2006; Lai and Ip, 2003). Indeed, it is not surprising due to 1) Cdk5, p35 and p39 localization in neuronal synapses (Fu et al., 2001; Fu et al., 2005; Humbert et al., 2000b), 2) many of its substrates are isolated in the synaptosomes of adult brains (Collins et al., 2005) and 3) the induction of synaptic

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plasticity and hippocampus-dependent spatial learning are compromised in Cdk5 and p35 transgenic and KO mice (Hawasli et al., 2007; Ohshima et al., 2005; Plattner et al., 2014). Therefore, through different approaches the role of Cdk5 in plasticity has been addressed. First, by using Cdk5 pharmacological inhibitors such as roscovitine and butyrolactone I, LTP induction and context-dependent fear conditioning was impaired (Fischer et al., 2002; Li et al., 2001). Nonetheless, the use of Cdk5 inhibitors has important drawbacks as they are not specific and can inhibit other proteins. Therefore, the generation of transgenic Cdk5 mouse models serves as a valuable tool to understand how Cdk5 is involved in synaptic plasticity. Due to its relevance in CNS development, Cdk5 KO mice and p35/p39 double-KO mice are not viable after birth (Ko et al., 2001; Ohshima et al., 1996). Different studies in transgenic animal models revealed contrasting roles for Cdk5 in learning and memory. First, a positive role of Cdk5 in synaptic plasticity was identified in p35 KO mice, showing deficiencies in spatial learning and memory as well as impaired LTD and depotentiation of LTP in the Schaeffer collateral CA1 pathway (Ohshima et al., 2005). Additionally, transiently upregulation of Cdk5 in mice exposed to stress is required for memory consolidation during associative learning (Fischer et al., 2002), whereas Cdk5 ablation in the CA1 pyramidal neurons of the hippocampus induces memory and synaptic plasticity impairments (Guan et al., 2011). By contrast, a negative role of Cdk5 in learning and memory formation was also demonstrated in Cdk5 conditional KO mice as evidenced by enhanced LTP, NMDA-receptor-mediated synaptic plasticity and improved performance in hippocampal behavioral tasks (Hawasli et al., 2007). Additionally, elevated Cdk5 activity in tau-tubulin kinase-1-overexpressing mice accelerates NR2B degradation mediated by calpain, causing learning deficits (Sato et al., 2008). Overall, these findings revealed that Cdk5 acts as a crucial homeostatic regulator of synaptic plasticity and suggest that deregulations in Cdk5 activity could contribute to impair synaptic function.

Deregulation of Cdk5 activity has been associated with neurodegenerative diseases including HD, AD, prion-related encephalopathies (PRE), PD, ALS or acute neuronal injury caused by ischemia or stroke (Alvira et al., 2008; Cruz and Tsai, 2004; Lopes et al., 2007; Lopes et al., 2010; Nguyen and Julien, 2003; Paoletti et al.,

2008; Shelton and Johnson, 2004; Slevin and Krupinski, 2009; Tsai et al., 2004; Weishaupt et al., 2003). Significantly higher Cdk5 activity has been observed in brains of AD patients (Lee et al., 1999) and stroke-affected patients (Mitsios et al., 2007). Additionally, higher levels of Cdk5/p25 complexes has been detected in mouse models of AD (Lopes et al., 2010), PD (Smith et al., 2003) and HD models and patients (Paoletti et al., 2008).

2.4.1.1 Cyclin-dependent kinase 5 in HD

In the last decade, Cdk5 role in HD has been studied (Anne et al., 2007; Crespo-Biel et al., 2007; Crespo-Biel et al., 2009; Luo et al., 2005; Paoletti et al., 2008). *In vitro* immunoprecipitation experiments, have demonstrated that htt protein interacts with Cdk5, but not with its co-activator p35 (Luo et al., 2005). Cdk5 phosphorylates htt at Ser434 and this phosphorylation reduces caspase-mediated htt cleavage at residu 513, resulting in attenuated aggregate formation and toxicity in cells expressing the NH2-terminal 588 amino acids of mutant huntingtin. However, Cdk5 activity is reduced in the whole brains of N171-82Q HD transgenic mice compared to controls. The authors suggest that this reduced Cdk5 activity could be account by the presence of polyQ-expanded htt fragments that reduces the interaction between Cdk5 and its co-activator p35. Therefore, the ability of Cdk5-mediated htt phosphorylation to protect against htt cleavage by caspases, and the subsequent aggregation and toxicity is compromised in cells expressing toxic fragments of htt (Luo et al., 2005). In other study, it has been demonstrated that htt can be phophorylated by Cdk5 at Serines 1181 and 1201 (Anne et al., 2007). Importantly, whereas the absence of htt phosphorylation at these residus confers toxic properties to wild-type htt in a p53-dependent manner and accelerates neuronal death induced by DNA damage in striatal neurons, these phosphorylations protects against polyQ-induced toxicity. Additionally, the authors demonstrate that sustained DNA damage in late stages of HD associates with a decrease in Cdk5/p35 levels, causing a reduction in htt phosphorylation at Ser1181 and 1201 and accelarating p53-mediated neuronal death (Anne et al., 2007). Additionally, other studies demonstrate that intraperitoneal injection of 3-NP in rats, as an experimental model of HD, induces calpain activation that in turns increases the levels of p25 and therefore increases Cdk5 activity in the striatum (Crespo-Biel et al.,

2007). Moreover, increased Cdk5 activity associates with a reduction in the neuroprotective effect mediated by MEF2 (Crespo-Biel et al., 2007). Interestingly, lithium reduces 3-NP-mediated striatal neurodegeneration in rats by inhibiting calpain activation, thereby avoiding p25 generation and Cdk5 aberrant activity (Crespo-Biel et al., 2009). Finally, in our lab it has been demonstrated that increased vulnerability of mutant huntingtin striatal cells to glutamatergic and dopaminergic activation involves aberrant Cdk5 activity (Paoletti et al., 2008). In the same study the authors demonstrate increased Cdk5 activity in the striatum of mutant Hdh^{Q111/Q111} mice and in the putamen of HD patients, highlighting the relevance of deregulated Cdk5 pathway in HD pathology (Paoletti et al., 2008).

Although 1) Cdk5 pathway is deregulated in the striatum of HD, 2) learning and memory as well as synaptic transmission are compromised in HD, and 3) Cdk5 emerged as a crucial regulator in synaptic plasticity, the role of Cdk5 in synaptic plasticity and cognition in HD has not been addressed. Thereby, in this Thesis we have analyzed whether Cdk5 modulation could represent a new therapeutic strategy to treat cognitive dysfunction in HD.

2.5 ALTERED RECEPTOR ACTIVITY AND SIGNALING

An imbalance in neurotransmitter systems, such as glutamatergic and dopaminergic systems, has been associated with many neuropathological processes, including HD (Fan and Raymond, 2007;Mota et al., 2014;Perez-Navarro et al., 2006;Stojanovic et al., 2014).

Glutamate is the most abundant excitatory neurotransmitter in the CNS. Glutamate binds and activates NMDARs, which results in calcium influx to the cell and activation of several signaling pathways. Importantly, an excess of extracellular glutamate leads to continuous stimulation of NMDARs and neuronal death in a process termed excitotoxicity (Eidelberg and Surmeier, 2011;Raymond et al., 2011). Indeed, neuronal death via excitotoxicity has been proposed as one of the first mechanisms to explain the selective vulnerability of MSN in HD (de la Monte et al., 1988;Fan and Raymond, 2007;Martin and Gusella, 1986;Paoletti et al., 2008;Perez-Navarro et al., 2006;Raymond et al., 2011).

In addition to glutamatergic afferents from the cerebral cortex, the striatum also receives densest dopaminergic innervations from the substantia nigra pars compacta (Fig. 19) (Gerfen, 1992c).

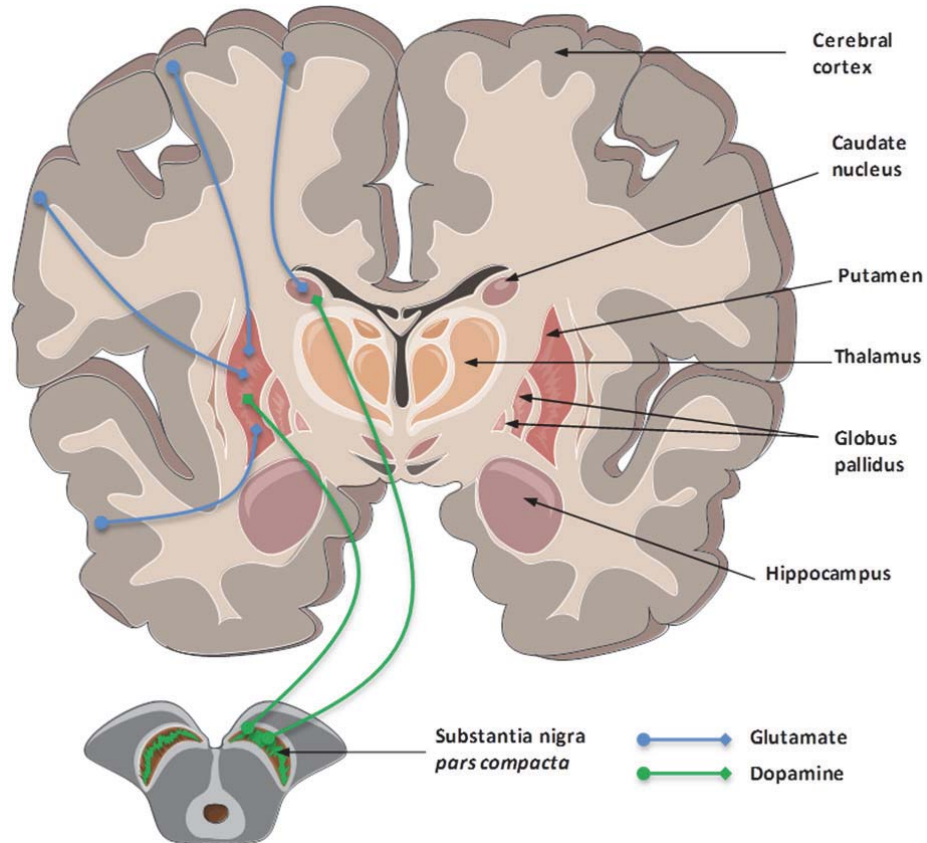


Figure 19. Anatomic organization of glutamatergic and dopaminergic afferents to the basal ganglia. Schematic representation of coronal brain section showing glutamatergic projections from cerebral cortex to caudate nucleus and putamen and dopaminergic projections from substantia nigra *pars compacta* to caudate nucleus and putamen. Image adapted from Neuroscience, 2nd edition (2001) <http://www.ncbi.nlm.nih.gov/bookshelf>.

A prevalent theory is that alterations in dopamine (DA) stimuli from the substantia nigra cause an imbalance in striatal neurotransmission, initiating cascades that induce cell death (Paoletti et al., 2008; Ross and Tabrizi, 2011).

2.5.1 Ionotropic glutamate receptors

There are two types of ionotropic glutamate receptors: the N-methyl-D-aspartate (NMDA) receptors and the non-NMDA receptors that include the α -amino-3-

hydroxy-methyl-4-isoxazolepropionic acid (AMPA) receptors and the Kainate (KA) receptors.

NMDARs are tetrameric structures (Laube et al., 1998) composed of two NR1 subunits that binds to glycine and at least two NR2 subunits (NR2A-D) that binds to glutamate or NR3 subunits (NR3A-B) that also binds to glycine, forming heteromultimers (Chatterton et al., 2002; Cull-Candy et al., 2001; Dingledine et al., 1999; Fan and Raymond, 2007; McIlhinney et al., 1998; Monyer et al., 1994; Ozawa et al., 1998). Depending on their combination different NMDARs complexes are formed with different functional characteristics (Chen et al., 1999; Cull-Candy et al., 2001; Ishii et al., 1993; Monyer et al., 1994). NMDARs exert different functions such as the regulation of gene expression (Rao and Finkbeiner, 2007) and the induction and maintenance of synaptic activity including the expression of LTP and LTD (Bliss and Collingridge, 1993). Because NMDARs are highly permeable to calcium (Ozawa et al., 1998), a deregulation in NMDARs activity can lead to excitotoxicity (Lipton and Rosenberg, 1994). Several studies at pre- and early symptomatic stages of HD in humans demonstrated a significant decrease of NMDA receptor binding (Albin et al., 1990; DiFiglia, 1990; Dure et al., 1991; London et al., 1981; Young et al., 1988). In addition, *in vitro* studies indicate that excitotoxicity in HD is produced, at least in part, by an increase in NMDARs activity (Tang et al., 2005; Zeron et al., 2004). However, *in vivo* studies regarding excitotoxicity showed controversial results. Intra-striatal injections of glutamate agonist, particularly those acting through NMDARs, causes striatal cell death in mouse similar to the one observed in human HD brains (Beal et al., 1986; Beal et al., 1991; Bruyn and Stoof, 1990; Ferrante et al., 1993; Hantraye et al., 1990; Sanberg et al., 1989; Schwarcz et al., 1984). However, whereas intra-striatal injection of quinolinic acid causes NMDAR-mediated death in YAC transgenic model of HD expressing full-length mutant huntingtin (Zeron et al., 2002), transgenic mice expressing the NH₂-terminal portion of the huntingtin protein such as R6 and N171-82Q mouse do not show neuronal death but striatal resistance to excitotoxicity (Hansson et al., 1999). This discrepancy seems to be related to the different HD mice and particularly to the size of the htt transgene that these mice express. Although at early stages of the disease no changes in total NMDAR subunit (NR1, NR2A, NR2B) levels in the striatum and hippocampus of HD mouse models are detected (Giralt et al., 2009; Jarabek et al., 2004; Torres-Peraza et al.,

2008), a significant increase in NR3A in the striatum of YAC128 mice (Marco et al., 2013) and an increase in phospho-NR1 levels in the striatum and hippocampus of R6/1 mice have been described (Giralt et al., 2011b; Torres-Peraza et al., 2008). In addition, at advanced disease stages several HD mouse models exhibit deregulated NMDARs activity (Ali and Levine, 2006; Benn et al., 2007; Cepeda et al., 2001; Fan and Raymond, 2007; Jarabek et al., 2004). Overall, these data indicate altered responsiveness of NMDA receptors in multiple HD mice, and highlight that alterations in NMDAR function may contribute to HD neuropathology.

AMPA receptors comprise four subunits termed GluA1 to GluA4, which combine to form tetramers (Palmer et al., 2005). AMPARs are ion channels that open when they bind to glutamate allowing sodium and potassium ions to flow across the membrane and producing a brief depolarization called EPSP (Kennedy, 2000). Excitotoxicity induced by calcium influx through AMPARs has also been found to cause neuronal damage (Jayakar and Dikshit, 2004). Importantly, an early decrease in GluA1 receptor has been detected in the hippocampus of HD mouse models (Giralt et al., 2009; Nithianantharajah et al., 2008; Simmons et al., 2011), suggesting its implication in the altered synaptic signaling and plasticity in HD. In addition, abnormal AMPAR function in MSN occurs at early HD stages in R6/2 mice (Cepeda et al., 2007). Importantly, by enhancing AMPAR function LTP and object recognition memory in a knock-in mice of HD can be rescued (Simmons et al., 2009) as well as locomotor dysfunction in R6/2 mice improved (Simmons et al., 2011).

Kainate receptors comprise GluR5, GluR6 and GluR7 subunits that form functional homomeric receptor-channels activated by kainite and glutamate (Bettler et al., 1990; Egebjerg et al., 1991; Lomeli et al., 1992; Schiffer et al., 1997; Sommer et al., 1992), and KA1 and KA2 subunits that show high-affinity for kainite binding but do not form functional homomeric receptor-channels (Herb et al., 1992; Werner et al., 1991). Kainate receptors have also been shown to modulate excitotoxicity (Wang et al., 2005).

Apart from glutamate receptors, also glutamate transporters such as the vesicular glutamate transporter 1 (VGLUT1) has been shown to contribute to the imbalance in glutamatergic system in HD. Whereas the expression of VGLUT1 in the pre-

synaptic terminals correlates with an appropriate synaptic proteins expression, the reduction of VGlut1 protein levels reduces synaptic density (Berry et al., 2012). In this context, a significant reduction in VGlut1 protein levels has been detected in the striatum of R6/2 mice, disrupting corticostriatal excitatory terminals in HD (Giralt et al., 2011a).

2.5.2 G-protein coupled receptors (GPCR)

GPCRs represent the largest family of integral membrane proteins (Luttrell, 2008). These membrane proteins are comprised of seven transmembrane regions (TM), an extracellular amino-terminus and an intracellular carboxyl-terminal domain and were first identified as receptor proteins that couple via heterotrimeric G-proteins (Neer, 1995). In this model, an agonist binding to GPCRs allow the adoption of an active conformation, facilitating the exchange of GDP for GTP on the G-protein $G\alpha$ -subunit, leading to the dissociation of the $G\alpha$ and $G\beta\gamma$ -subunits. The activated G-protein subunit can positively or negatively regulates different downstream effectors such as phospholipases, adenylyl cyclases (AC) and ion channels (Neer, 1995). Therefore, by positively or negatively regulation of downstream effectors, GPCRs activity regulates cAMP levels through the modulation of AC activity as well as intracellular calcium release through modulation of PLC γ /IP3/DAG pathway (Gainetdinov et al., 2004). However, it has been demonstrated that GPCRs can also signal via G-protein-independent mechanisms (Ferguson, 2001;Lefkowitz et al., 2006;Lefkowitz, 2007;Luttrell, 2008). Among other GPCRs, metabotropic glutamate receptors and dopamine receptors have been extensively studied in HD.

In addition to ligand-gated ion channels, glutamate can exert its function through G-protein-coupled metabotropic receptors (mGluR1-mGluR5). Group I mGluRs includes mGluR1 and mGluR5 and is the most studied group of mGluRs in HD due to its abundant expression in striatal MSN (Abe et al., 1992;Masu et al., 1991). Group mGluR I interact with mutant huntingtin (Anborgh et al., 2005) and mGluR-mediated signaling pathways have been shown to be altered in striatal neuronal primary cultures and striatal brain slices from presymptomatic knock-in HD mice (Ribeiro et al., 2010).

Dopamine is a catecholamine neurotransmitter that activates five types of dopamine receptors ($D_{1-5}R$). D1-like family includes D_1R and D_5R and is couple to G_s ,

whereas D2-like family includes D₂R, D₃R and D₄R and is couple to G_{i/o} type G-proteins (Sealfon and Olanow, 2000). The first study that involved the dopaminergic system in HD neuropathology was in 1970 by the observation that asymptomatic HD patients exhibited dyskinesia after L-DOPA administration (Klawans et al., 1970). Additional studies in HD patients have shown that increased DA release induces chorea (Andre et al., 2010; Spokes, 1980). In this line, a degeneration of nigrostriatal projections (Bohnen et al., 2000; Ferrante and Kowall, 1987; Ginovart et al., 1997; Suzuki et al., 2001), dopaminergic neuronal atrophy (Oyanagi et al., 1989; Yohrling et al., 2003) and significant reduction in the number of striatal dopaminergic projections (Huot et al., 2007) have been demonstrated in HD. In addition, altered dopaminergic system has been proposed as a contributing factor of striatal cell death in HD. Thus, *in vitro* studies demonstrate that dopamine-induced cell death is prevented by a D1 antagonist (Chen et al., 2003), while D1 agonist promotes cell death and cytotoxic damage (Paoletti et al., 2008).

Importantly, a decrease in D₁R and D₂R levels has been detected in asymptomatic and symptomatic HD patients (Augood et al., 1997; Ginovart et al., 1997; Weeks et al., 1996) as well as an increasing loss of D₁R binding at advanced HD stages (Glass et al., 2000). The decrease in D₁R binding has also been detected in R6/2 mice (Cha et al., 1998). Interestingly, besides a decreased in D₁R levels, mutant huntingtin may enhance the sensitivity of D₁R-mediated signals as evidenced by similar induction of immediate early genes (IEGs) transcription compared with wild-type animals (Fig.20) (Spektor et al., 2002).

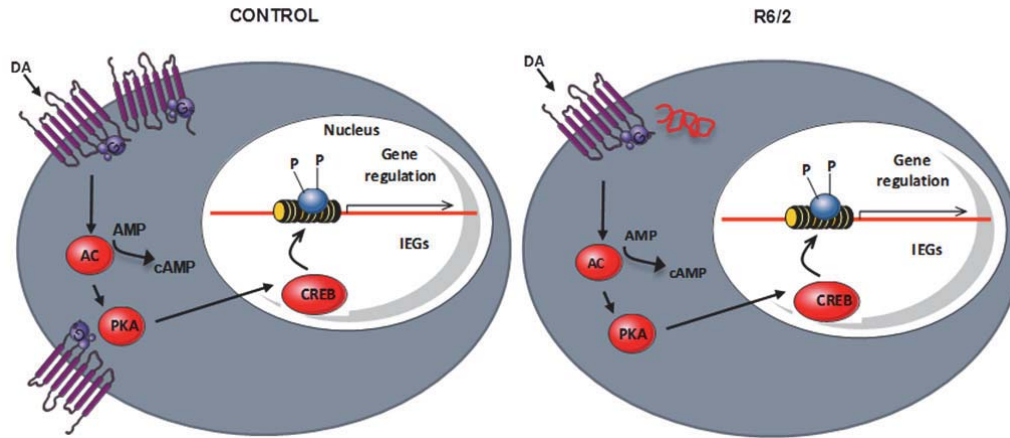


Figure 20. Dopamine receptor activity leads to the induction of IEGs in control and R6/2 mice. Dopaminergic input arising from the substantia nigra pars compacta activates D_1R activity. Stimulation of D_1R activates adenylyl cyclase (AC) that results in an increase in cAMP levels and the activation of protein kinase A (PKA). Activated PKA translocates to the nucleus where phosphorylates the cAMP response element binding (CREB) protein. Phosphorylated CREB then recruits CBP (not shown) and other transcriptional regulatory factors, resulting in the transcription of immediate early genes (IEG). In R6/2 mice, mutant huntingtin causes a significant decrease in D_1R levels. However, besides this decrease, mutant huntingtin may enhance, rather than reduce, the sensitivity of D_1R -mediated signaling. Adapted from (Spektor et al., 2002).

Importantly, although the MSNs expressing enkephalin and D_2R that form the indirect pathway are the most vulnerable in HD, the overactivation of D_1R produces an imbalance between the direct and the indirect pathway (Chen et al., 2013; Paoletti et al., 2008). Thereby, strategies that might reduce D_1R signaling have been proposed as valuable in preventing HD neuropathology. In this view, inhibitory and excitatory transmission in direct pathway neurons can be normalized by antagonizing D_1R or reducing DA signaling in HD mice (Andre et al., 2011a; Andre et al., 2011b). In addition, whereas DA receptor antagonists and agents that decrease DA reduce motor symptoms in HD patients and animal models, dopaminergic stimulation exacerbate motor symptoms (Mestre et al., 2009; Tang et al., 2007). Moreover, DA potentiates glutamate-induced cell death in cultured striatal neurons from YAC128 mice via activation of D_1R (Tang et al., 2007) and mutant huntingtin enhances DA-mediated striatal cell death via D_1R (Paoletti et al., 2008). However, D_1R and D_2R antagonist reduces spontaneous motor activity in mice exposed to a new environment (Gimenez-Llort et al., 1997), indicating that general use of D_1R antagonist could have some defects as a preventive treatment.

Most drug strategies target a single receptor and consider receptors as independent units. However, it has been shown that GPCRs cooperate and physically contact to each other forming complexes of receptors, termed heteromers, thereby conferring novel functions (Kamal and Jockers, 2011). Indeed, dopamine D₁R has been shown to form heteromers with histamine 3 receptors (H₃R), a receptor of the histaminergic system, in mammalian cells (Ferrada et al., 2008; Ferrada et al., 2009) as well as in rat brain slices (Moreno et al., 2011). In this line, dopamine D₁R signaling can be modulated via its association with H₃R (Fig. 21). Thus, upon-activation of D₁R-H₃R heteromers, H₃R ligands act as a “molecular brake” for D₁R signaling (Ferrada et al., 2008; Ferrada et al., 2009; Moreno et al., 2011).

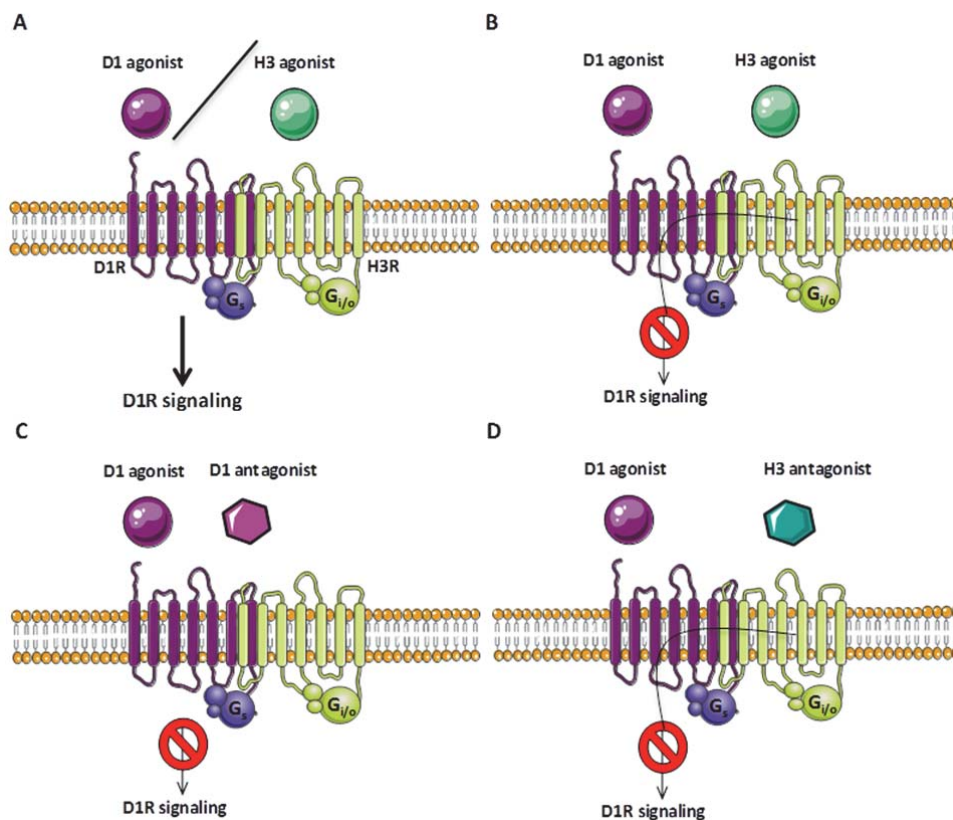


Figure 21. Dopamine D₁R signaling modulation via its association with H₃R. In D₁R-H₃R heteromer context: **A**, both D₁R agonist and/or H₃R agonist separately induces D₁R signaling. **B**, Upon co-activation with D₁R and H₃R agonist, H₃R agonist acts as a “molecular brake” for D₁R signaling. By a negative cross-talk, H₃R decreases D₁R agonist signaling. **C**, Co-treatment with D₁R agonist and D₁R antagonist induces loss of D₁R signaling. **D**, Upon co-activation with D₁R agonist and H₃R antagonist, H₃R acts as a “molecular brake” for D₁R signaling. By a cross-antagonism, H₃R antagonist decreases D₁R agonist signaling.

INTRODUCTION

Histaminergic fibers emanating from the tuberomammillary nucleus (in the posterior hypothalamus) project and arborize in the whole CNS (Haas et al., 2008). In these histaminergic neurons, histamine (HA) is formed through one-step catalytic action of histamine decarboxylase and stored in presynaptic secretory vesicles. Then, HA is released into the extracellular space where activates four subtypes of postsynaptic GPCRs designated as H₁₋₄R. Additionally, HA can also stimulates presynaptic H₃ autoreceptors inhibiting HA synthesis and/or release, while HA binding to H₃R on non-histaminergic neurons inhibits the release of a number of neurotransmitters (Fig. 22) (Blandina et al., 1996; Fox et al., 2005; Tiligada et al., 2011).

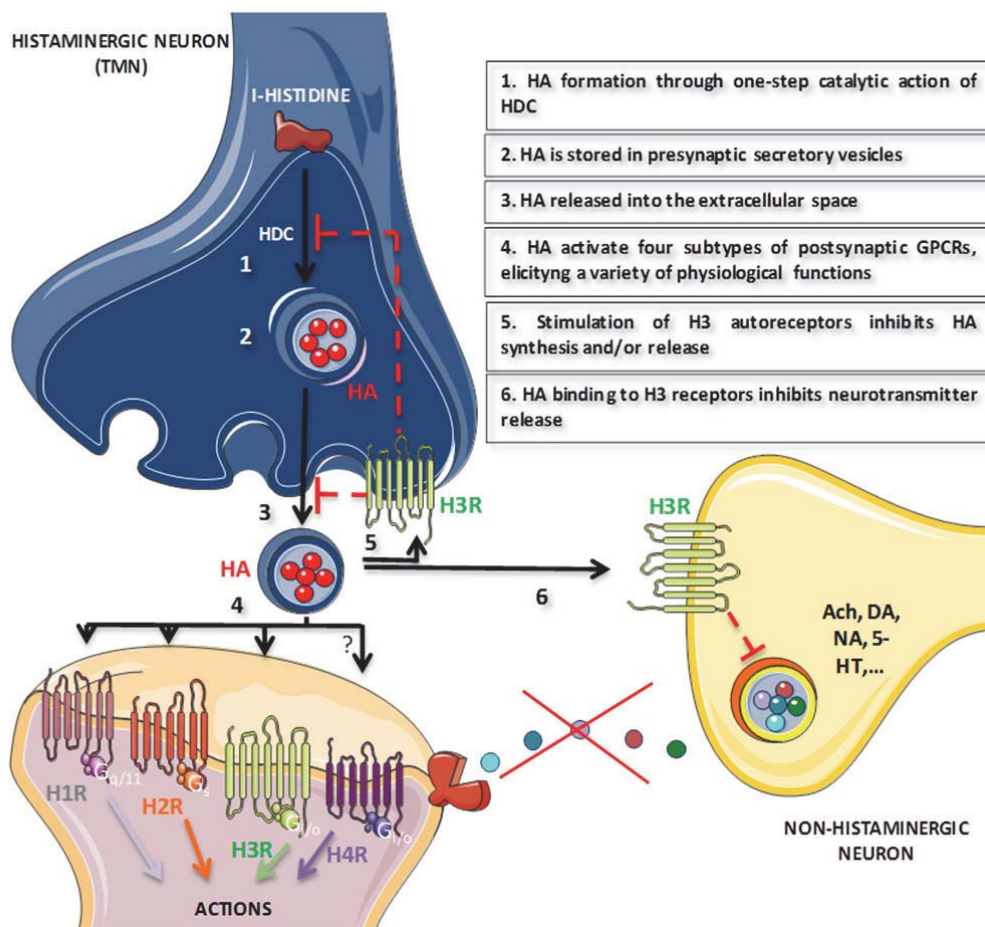


Figure 22. Schematic representation of central histaminergic components: from histamine formation to histamine binding to its receptors. Abbreviations: TMN: tuberomammillary nucleus; HA, histamine; HDC: histamine decarboxylase; Ach: acetylcholine; DA: dopamine; NA: noradrenaline; 5-HT: serotonin. Adapted from (Tiligada et al., 2011).

HA through H₁₋₄R could play different roles such as cognition, thermoregulation, Sleep/wake cycle, endocrine control, learning and memory, locomotion, circadian and feeding rhythms, pain and neuroinflammation. Whereas H₁R, H₂R and H₃R are abundantly expressed in the brain, H₄R expression is higher in peripheral tissues. From all these histamine receptors, in this Thesis we have focused in H₃R due to 1) can form heteromers with D₁R and modulates its signaling and 2) exerts relevant functions in cognition, emotion and learning and memory (Ellender et al., 2011;Haas et al., 2008;Komater et al., 2005;Mohsen et al., 2014;Orsetti et al., 2002;Pascoli et al., 2009). Importantly, H₃R expression is abundant in different brain regions, including striatum, hippocampus and cerebral cortex, whereas its expression has not been detected in the corpus callosum, spinal cord or peripheral tissues (Hamill et al., 2009;Lovenberg et al., 1999;Morisset et al., 2001;Pillot et al., 2002;Ryu et al., 1994a;Ryu et al., 1994b). H₃R can activate members of the G proteins family to modulate different cellular signaling pathways (Fig. 23).

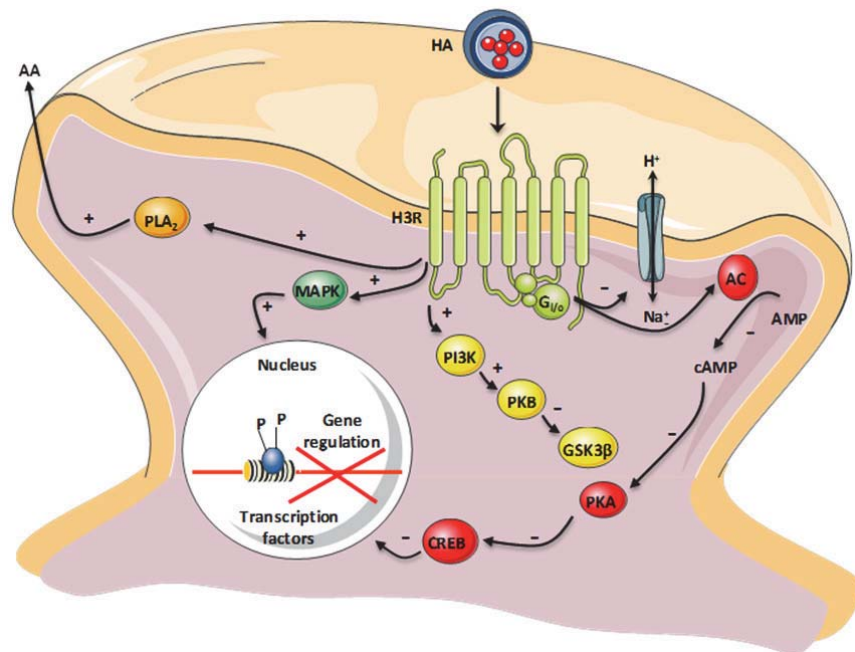


Figure 23. H₃R activation results in the modulation of several signaling pathways. Histamine through H₃R inhibits the enzyme AC and results in decreases cAMP levels, inhibition of PKA, inhibition of CREB and consequently decreased CREB-gene transcription. Other effector pathway might be activated, including MAPK and PI3K pathways. MAPK activation results in the phosphorylation of ERK that in turns could promote gene transcription. PI3K activation results in PKB or Akt phosphorylation that in turns phosphorylates and inhibits the action of GSK3β. Additionally, histamine through H₃R also leads to the inhibition of the Na⁺/H⁺ exchanger and the lowering of intracellular calcium as well as the activation of phospholipase A₂ (PLA₂), which induces the release of arachidonic acid (AA) to the extracellular space. Adapted from (Leurs et al., 2005;Bongers et al., 2007).

INTRODUCTION

Importantly, H₃R expression is still prevalent even in severe late stages of neurodegenerative diseases including HD (Haas et al., 2008; Goodchild et al., 1999), an important observation given the aim of targeting these receptors as potential therapeutic targets. Because H₃R plays a crucial role in several physiological processes, the blockage of H₃R with antagonists has been suggested as therapeutic strategy for many diseases, including cognitive disorders such as attention deficit hyperactivity disorder (ADHD), AD and schizophrenia, as well as in sleep disorders and pain (Medhurst et al., 2007; Kuhne et al., 2011).

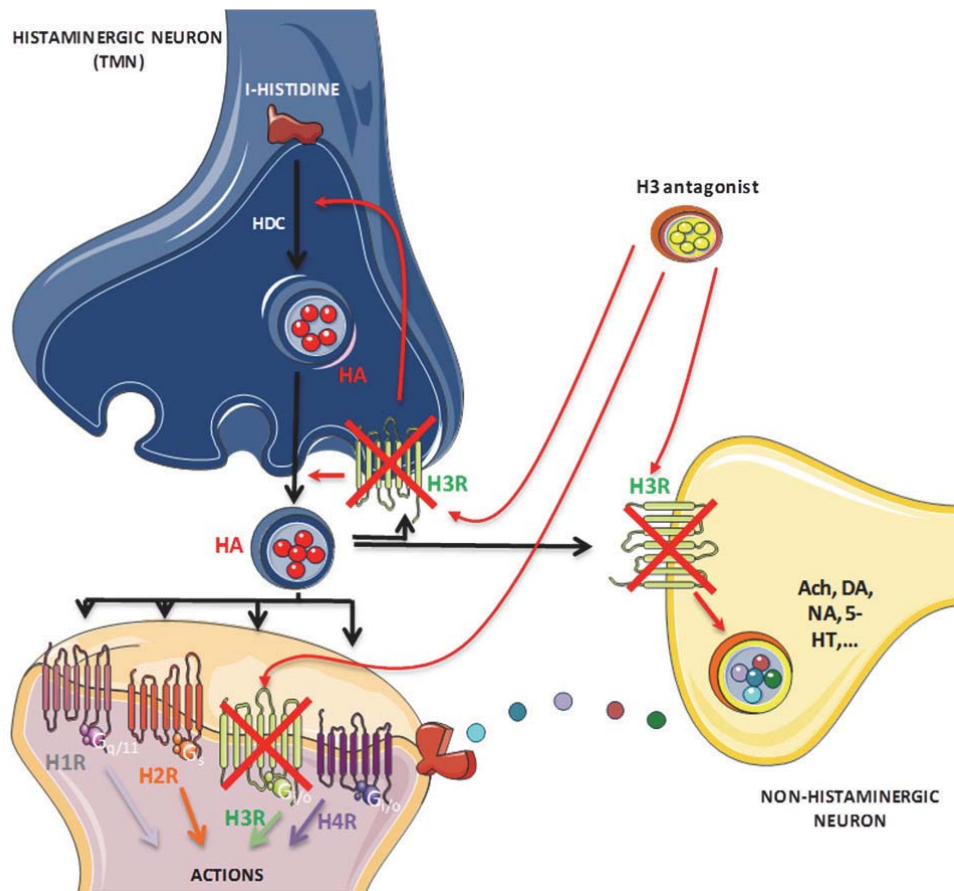


Figure 24. Schematic representation of H₃R antagonist effects on histaminergic system. By antagonizing H₃R, a blockade of presynaptic autoreceptors occurs and would therefore increase histamine formation and release. Besides controlling histamine release, H₃R antagonist blocks postsynaptic H₃R and therefore modulates neurotransmitter release and H₃R-mediated signaling pathways. Adapted from (Tiligada et al., 2011).

Because dopamine neurotransmission is affected in HD and H₃R is expressed in brain regions affected in HD and forms heteromers with dopamine receptors thereby modulating D₁R-mediated signaling, in this Thesis we have studied the role of D₁R-H₃R heteromers in dopamine-mediated cell death and cognitive deficits in HD.

II. AIMS

Understanding the molecular underpinnings of neuronal dysfunction and degeneration involved in Huntington's disease is a goal of increasing urgency for society and scientific community. In this Thesis we have studied different proteins and signaling pathways, specifically altered by the presence of mutant huntingtin, as new potential candidates to develop pharmacological strategies to treat or delay motor and cognitive deficits in Huntington's disease.

AIM 1. To study the contribution of CBP/CREB pathway in the cognitive deficits present in Huntington's disease.

AIM 2. To study the molecular mechanisms involved in neurotrophic support dysfunction in Huntington's disease.

2.1. To analyze the role of p75^{NTR}/TrkB receptors in the major striatal vulnerability in Huntington's disease.

2.2. To study the role of p75^{NTR} in cognitive deficits in Huntington's disease.

AIM 3. To study the molecular mechanisms involved in corticostriatal dysfunction in Huntington's disease.

3.1. To characterize corticostriatal deficits in HD mouse models and analyze the role of Kalirin-7 in the alteration of corticostriatal excitatory synapses in HD.

AIM 4. To study the role of Cdk5 in cognitive deficits in Huntington's disease.

AIM 5. To study the role of D₁R-H₃R heteromers in neuronal cell death and cognitive deficits in Huntington's disease.

III. RESULTS

WORK 1***“LONG-TERM MEMORY DEFICITS IN HUNTINGTON’S DISEASE ARE ASSOCIATED WITH REDUCED CBP HISTONE ACETYLASE ACTIVITY”***

This paper has been published in Human Molecular Genetics

Giralt A*, Puigdel·l·ivol M*, Carret·n·n· O, Paoletti P, Valero J, Parra-Damas A, Saura C.A., Alberch J, Gin·s· S

*These authors contributed equally to this work. Hum. Mol. Gen. 2012, 21(6):1203-16.

<http://hmg.oxfordjournals.org/content/21/6/1203.full.pdf+html>

CONTRIBUTION TO THIS WORK:

I contributed in the design of different hypothesis and in their validation. I performed the majority of genetic analysis by RT-PCR to study the specific alterations in memory-related genes that are modified in the hippocampus of HD models. I performed the immunoblots and immunoprecipitations to determine biochemical changes at protein level. Finally, I contributed in the discussion of the results as well as in writing some parts of the paper.

AIM 1. TO STUDY THE CONTRIBUTION OF CBP/CREB PATHWAY IN THE COGNITIVE DEFICITS PRESENT IN HUNTINGTON'S DISEASE

La malaltia de Huntington (MH) és una malaltia neurodegenerativa d'herència autosòmica dominant produïda per l'expansió de repeticions del triplet CAG en la regió codificant del gen de la huntingtina (*htt*). Tot i ser una malaltia clínicament caracteritzada per dèficits motors, diverses evidències posen de manifest que les alteracions cognitives precedeixen les alteracions motores en pacients afectats per la MH. En aquest treball demostrem la presència de dèficits de memòria espacial i de reconeixement en ratolins heterozigots *knock-in* $Hdh^{Q7/Q111}$ per la MH, associats a una disminució dels nivells de CBP així com d'acetilació de la histona 3 en la regió hipocampal. A més, hem demostrat una alteració selectiva en la transcripció de gens implicats en memòria, tals com *c-fos*, *Arc* i *NR4A2* dependents de l'activitat CBP/CREB. De manera significativa, l'administració d'un inhibidor general d'histones deacetilases (Trichostatin A, TSA) és capaç de rescatar els dèficits en memòria dels ratolins $Hdh^{Q7/Q111}$. Aquests resultats impliquen la disfunció de CBP en els dèficits cognitius observats en els ratolins MH i suggereixen que la inhibició de les histones deacetilases poden representar una nova estratègia terapèutica pel tractament dels dèficits de memòria presents en aquesta patologia.

Long-term memory deficits in Huntington's disease are associated with reduced CBP histone acetylase activity

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Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder caused by an expanded CAG/polyglutamine repeat in the coding region of the *huntingtin* (*htt*) gene. Although HD is classically considered a motor disorder, there is now considerable evidence that early cognitive deficits appear in patients before the onset of motor disturbances. Here we demonstrate early impairment of long-term spatial and recognition memory in heterozygous HD knock-in mutant mice (*Hdh*^{Q7/Q111}), a genetically accurate HD mouse model. Cognitive deficits are associated with reduced hippocampal expression of CREB-binding protein (CBP) and diminished levels of histone H3 acetylation. In agreement with reduced CBP, the expression of CREB/CBP target genes related to memory, such *c-fos*, *Arc* and *Nr4a2*, was significantly reduced in the hippocampus of *Hdh*^{Q7/Q111} mice compared with wild-type mice. Finally, and consistent with a role of CBP in cognitive impairment in *Hdh*^{Q7/Q111} mice, administration of the histone deacetylase inhibitor trichostatin A rescues recognition memory deficits and transcription of selective CREB/CBP target genes in *Hdh*^{Q7/Q111} mice. These findings demonstrate an important role for CBP in cognitive dysfunction in HD and suggest the use of histone deacetylase inhibitors as a novel therapeutic strategy for the treatment of memory deficits in this disease.

INTRODUCTION

Cognitive impairment is an early clinical feature of Huntington's disease (HD), a neurodegenerative disorder caused by an expanded CAG repeat in the *huntingtin* gene, that often appear before the onset of motor symptoms or neuronal loss (1–4). Deficits in synaptic plasticity and memory have also been described in different mouse models of HD (5–9), although the precise molecular mechanisms underlying these memory deficits remain largely unknown.

Activity-induced gene transcription is required for hippocampal synaptic plasticity and memory consolidation (10,11). Compelling evidence indicate that the transcription

factor c-AMP-responsive element binding protein (CREB) is essential for activity-induced gene expression mediating memory formation (12). CREB transcriptional activity depends on CREB phosphorylation and recruitment of specific co-activators (13). CREB-binding protein (CBP) is a transcriptional co-activator that regulates CREB-mediated transcription by enhancing the ability of phosphorylated CREB to activate expression of specific genes (14–16). CBP also acts as a histone acetyltransferase (HAT) to alter chromatin structure allowing gene transcription (17–19). Thus, CBP plays a dual role in CREB-mediated gene transcription as a scaffolding protein to recruit CREB and as a HAT protein acetylating histones to disrupt repressive chromatin structure (20).

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Recent studies have revealed a particular role of CBP HAT activity in memory consolidation. Mouse models in which CBP activity is compromised exhibited reduced chromatin acetylation and hippocampal long-term potentiation (LTP) and long-term memory deficits (21–23).

Loss of CBP function has been associated with striatal degeneration in HD models (24–27). Indeed, mutant huntingtin directly interacts with the acetyltransferase domain of CBP, which results in reduced acetyltransferase activity (28). Decreased striatal CBP levels either by sequestration into huntingtin nuclear aggregates or by increased protein degradation has been reported in HD cellular and mouse models and HD human brain (26,28–30). Consistent with deficient striatal CBP function, either CBP overexpression or histone deacetylase (HDAC) inhibition prevent cell loss and increase cell survival in HD models (31,32). Altogether, these data point to an important role of CBP loss of function in polyglutamine-dependent striatal toxicity in HD. Surprisingly, the role of CBP in regulating gene expression required for memory in HD has not been investigated. In this study, we address an important gap in our knowledge of cognitive deficits in HD. We demonstrate long-term spatial and recognition memory deficits in $Hdh^{Q7/Q111}$ mutant mice associated with reduced hippocampal CBP levels and selective disruption of memory-related CREB/CBP-dependent genes. Consistent with functional disruption of CBP, we found that HDAC inhibition efficiently rescued expression of specific CREB target genes and memory deficits in $Hdh^{Q7/Q111}$ mutant mice. Together, these findings implicate CBP dysfunction in HD cognitive deficits and provide support for new therapeutic approaches targeting CBP/CREB signaling pathway for treating cognitive impairment in HD.

RESULTS

Long-term object recognition memory is impaired in $Hdh^{Q7/Q111}$ knock-in mutant mice

Memory and cognitive deficits are evident in HD even before the diagnosis of motor symptoms (1,2,4,33,34). To evaluate memory function in HD, spatial and recognition memories were analyzed in heterozygous knock-in $Hdh^{Q7/Q111}$ mice, a genetically precise model of HD in which expanded HD CAG repeats are inserted into the exon 1 of the mouse's HD gene (35). To assess recognition memory, we first measured the performance of wild-type $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice at 4 and 8 months of age in the novel object recognition task (NORT) (Fig. 1). This test evaluates recognition memory by measuring the innate tendency of mice to preferentially explore a novel object. Control $Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ mutant mice were allowed to explore objects for 10 min during the training period showing no significant differences between genotypes at 4 or 8 months of age ($P > 0.05$) (Fig. 1A and B). At 15 min post-training, both $Hdh^{Q7/Q111}$ mutant and $Hdh^{Q7/Q7}$ wild-type mice spent significantly more time exploring the novel object at either 4 months ($P < 0.001$) or 8 months of age ($P < 0.05$ and $P < 0.001$), which indicates preserved short-term recognition memory in HD mice (Fig. 1A and B). In contrast, and compared with $Hdh^{Q7/Q7}$ wild-type mice, 8-month-old $Hdh^{Q7/Q111}$ mutant

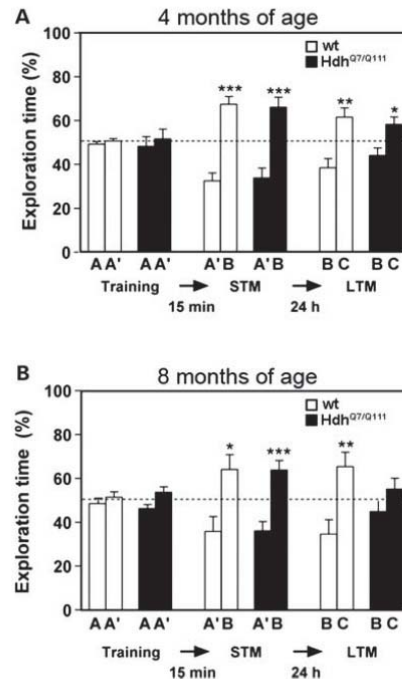


Figure 1. Impaired long-term recognition memory in mutant $Hdh^{Q7/Q111}$ knock-in mice. (A) Bar diagram illustrating the exploration time of $Hdh^{Q7/Q7}$ wild-type mice ($n = 6$) and $Hdh^{Q7/Q111}$ mutant mice ($n = 9$) at 4 months of age during the training, short-term (15 min delay, STM) and long-term (24 h delay, LTM) memory sessions in a NORT. Mice spent similar time exploring both objects during the training session. There was no significant difference in the percentage of exploration time between genotypes at either 15 min or at 24 h testing session. (B) Bar diagram illustrating the exploration time of $Hdh^{Q7/Q7}$ wild-type mice ($n = 6$) and $Hdh^{Q7/Q111}$ mutant mice ($n = 9$) at 8 months of age during the training, short-term (STM) and long-term (LTM) memory sessions in a NORT. Mice spent similar time exploring both objects during the training session. At 15 min post-training, both $Hdh^{Q7/Q111}$ mutant and $Hdh^{Q7/Q7}$ wild-type mice spent significantly more time exploring the novel object. However, during the 24 h retention test, $Hdh^{Q7/Q111}$ mutant mice displayed no significant preference for the novel object. Exploration time is shown in the bar graphs as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using a one-way ANOVA and Student *t*-test as a *post-hoc* test.

mice had a significant lower preference for the novel object when tested 24 h after training ($Hdh^{Q7/Q7}$: familiar object $37 \pm 5.6\%$ and novel object $62 \pm 5.6\%$, $P < 0.001$; $Hdh^{Q7/Q111}$: familiar object $45 \pm 4.2\%$ and novel object $53 \pm 4.4\%$, $P = 0.102$) (Fig. 1B). These results demonstrate that $Hdh^{Q7/Q111}$ mutant mice exhibit normal short-term memory but age-dependent impairment of long-term recognition memory.

Hippocampal-dependent spatial memory deficits in $Hdh^{Q7/Q111}$ knock-in mutant mice

We next examined hippocampal-dependent spatial memory in $Hdh^{Q7/Q7}$ wild-type and $Hdh^{Q7/Q111}$ mutant mice at 8 months of age in the Morris water maze (MWM) task. Mice were first tested in the visible platform version of the MWM (four

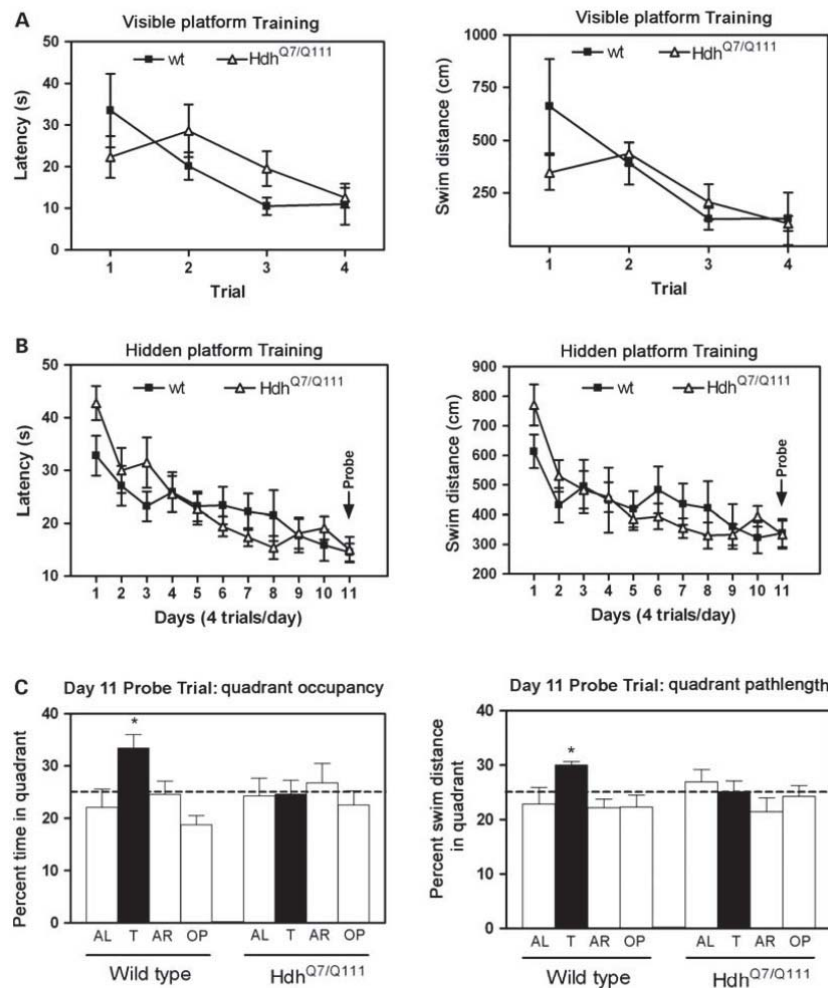


Figure 2. Impaired hippocampal-dependent spatial memory in mutant $Hdh^{Q7/Q111}$ knock-in mice. (A) In the visible platform task, mutant mice display similar escape latencies and path lengths than wild-type mice. A significant improvement across days was observed in both genotypes. (B) Eight-month-old $Hdh^{Q7/Q7}$ wild-type mice ($n = 6$) and $Hdh^{Q7/Q111}$ mutant mice ($n = 9$) were trained in the MWM task for 11 days. Both groups show similar average escape latencies and mean path-lengths with a significant improvement in their performances over training. (C) In the probe trial $Hdh^{Q7/Q111}$ mutant mice show no preference in occupancy (left) and swimming path-lengths (right) in the target quadrant compared with the rest of quadrants (T versus AL, AR, OP). AL, adjacent left; T, target quadrant; AR, adjacent right; OP, opposite quadrant. Data represent the mean \pm SEM. Statistical analysis was performed using two-way ANOVA with repeated measures (A and B) and one-way ANOVA (C) followed by Newman–Keuls as a *post-hoc* test. * $P < 0.05$, compared with the rest of quadrants.

trials per day) to control for visual ability and ensure that all mice were motivated and able to locate the platform. $Hdh^{Q7/Q111}$ mutant mice located the platform with similar escape latencies (trial effect: $F_{(3,52)} = 5.779$, $P < 0.01$; genotype effect: $F_{(1,52)} = 0.1739$, $P = 0.683$) and swimming path-lengths (trial effect: $F_{(3,52)} = 7.028$, $P < 0.001$; genotype effect: $F_{(1,52)} = 0.7512$, $P = 0.4$) than $Hdh^{Q7/Q7}$ wild-type mice, indicating that both groups improved significantly across trials (Fig. 2A).

When spatial learning was tested using the hidden platform version of the task, we found a significant improvement in their performances during training ($P < 0.001$) (Fig. 2B) with no significant differences between control $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice in escape latencies (day effect: $F_{(10,150)} = 28.03$, $P < 0.001$; genotype effect: $F_{(1, 150)} = 0.11$, $P = 0.793$) or swimming path-lengths (day effect: $F_{(10,150)} = 25.34$, $P < 0.001$; genotype effect: $F_{(1, 150)} = 0.15$, $P = 0.606$). In the probe trial, in which the platform

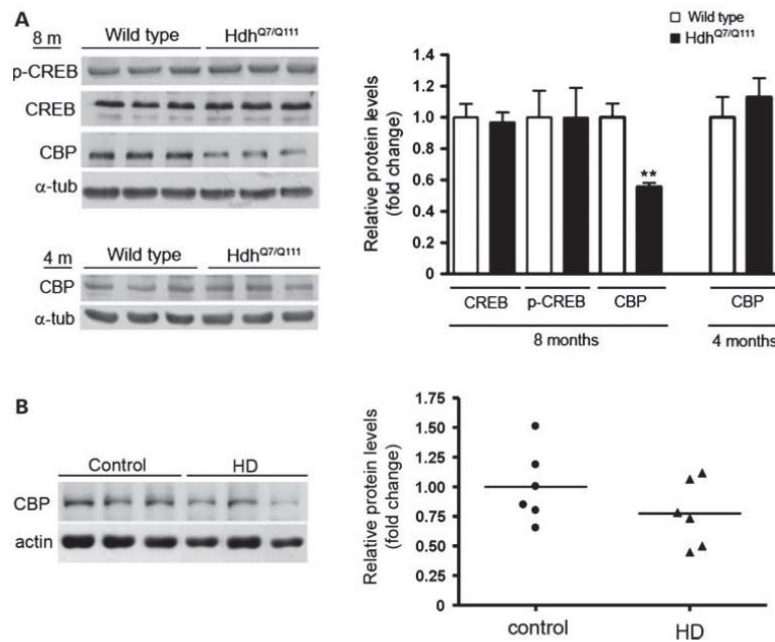


Figure 3. Reduced CBP protein levels in the hippocampus of mutant Hdh^{Q7/Q111} mice and HD human brain. (A) Western blot (left) and quantification (right) showing similar levels of CREB and phosphorylated-CREB (Ser133) in total hippocampal extracts from mutant Hdh^{Q7/Q111} mice compared with wild-type Hdh^{Q7/Q7} mice at the age of 8 months. A significant reduction in CBP levels was also observed by western blot analysis in mutant mice at the age of 8 months when compared with wild-type mice, whereas no significant changes were detected at 4 months of age. Protein levels were normalized to α -tubulin as a loading control. The histogram represents the relative protein levels expressed as fold change of wild-type mice. Values are given as mean \pm SEM of five independent samples. ** $P < 0.01$ mutant compared with wild-type mice using Student *t*-test. (B) Representative western blot showing levels of CBP and actin as a loading control from control and HD human hippocampal brain samples. Scatter plots represent the relative levels of CBP. The mean value of control samples was set as 1.

was removed, Hdh^{Q7/Q111} mutant mice showed impaired spatial memory compared with Hdh^{Q7/Q7} mice (Fig. 2C). Thus, whereas wild-type mice exhibited a preference for the target quadrant (quadrant effect: $F_{(3, 23)} = 5.406$, $P < 0.01$), evaluated as the time spent in the target quadrant versus each of the other quadrants, Hdh^{Q7/Q111} mutant mice did not show such a preference (quadrant effect: $F_{(3, 35)} = 0.301$, $P = 0.824$). Moreover, Hdh^{Q7/Q111} mutant mice displayed significantly less swimming path-lengths in the target quadrant compared with the rest of quadrants (quadrant effect: $F_{(3, 35)} = 1.080$, $P = 0.371$) than Hdh^{Q7/Q7} mice (quadrant effect: $F_{(3, 23)} = 3.409$, $P < 0.05$) (Fig. 2C). Overall, these results demonstrate that Hdh^{Q7/Q111} mutant mice exhibit normal acquisition of a MWM task but impaired hippocampal-dependent long-term spatial memory.

CBP is significantly reduced in the hippocampus of Hdh^{Q7/Q111} knock-in mutant mice and in HD human brain

CBP/CREB signaling is critical for long-lasting changes in synaptic plasticity underlying long-term memory consolidation (11). To determine the molecular mechanisms underlying the observed long-term memory deficits in Hdh^{Q7/Q111} mutant mice, we analyzed the levels of total CREB, phosphorylated

CREB (Ser133) and CBP in hippocampal extracts from Hdh^{Q7/Q7} wild-type and Hdh^{Q7/Q111} mutant mice at the age of 8 months. Western blot analysis revealed similar levels of phosphorylated and total CREB, but a significant reduction in CBP protein levels in mutant compared with wild-type mice ($\sim 45\%$, $P < 0.01$; Fig. 3A). Importantly, at 4 months of age when no cognitive deficits were observed, unchanged CBP levels were found between Hdh^{Q7/Q7} wild-type and Hdh^{Q7/Q111} mutant mice (Fig. 3A). We also investigated the levels of CBP in hippocampal samples from HD patients. Western blot analysis revealed that CBP levels were notably lower, although not significant, than that in control brains (Fig. 3B), demonstrating that altered CBP levels are also manifested in HD patients.

We next examined whether reduced CBP levels were associated with diminished CBP mRNA expression. Quantitative real-time polymerase chain reaction (PCR) analysis showed no significant differences in mRNA *CBP* transcripts between wild-type and mutant huntingtin mice (Hdh^{Q7/Q7}: 1.0 ± 0.1 and Hdh^{Q7/Q111}: 1.1 ± 0.1), indicating that decreased CBP levels are not due to altered CBP transcription.

Depletion of CBP in the striatum of HD mice models has been associated with either recruitment of CBP into mutant huntingtin aggregates (26,28,30) or enhanced CBP degradation

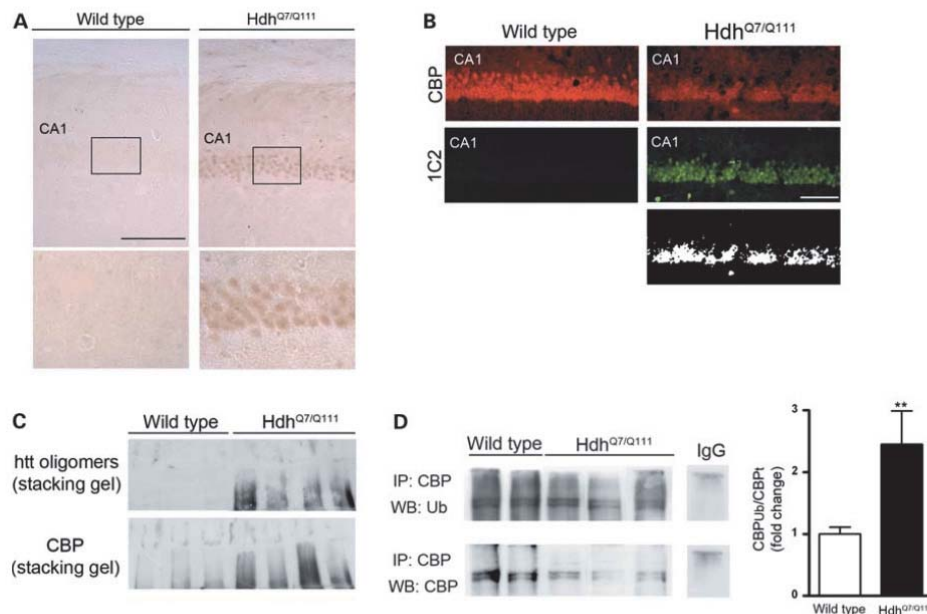


Figure 4. CBP reduction in the hippocampus of mutant $Hdh^{Q7/Q111}$ mice is associated with nuclear accumulation of mutant huntingtin and enhanced CBP ubiquitylation. (A) Intranuclear accumulation of mutant huntingtin in hippocampal neurons of the CA1 region in $Hdh^{Q7/Q111}$ mice. Immunostaining of coronal brain sections from wild-type $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice at 8 months of age using the 1C2 antibody that selectively recognizes polyglutamine repeats in mutant huntingtin. Inset shows enlarged CA1 region. Scale bar 90 μm . (B) Immunostaining of coronal brain sections from wild-type $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice at 8 months of age using CBP (red fluorescence) and 1C2 (green fluorescence) antibodies. Note the correlation between CBP and 1C2 distribution in mutant $Hdh^{Q7/Q111}$ mice (merged is shown in white). Scale bar 70 μm . (C) Western blotting of hippocampal extracts isolated from wild-type $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice at the age of 8 months. The blots were probed with 1C2 for huntingtin and CBP antibodies. In samples from HD but not from wild-type mice, CBP was detected in the stacking gel. When the blot was stripped and re-probed with the 1C2 antibody, oligomeric forms of mutant huntingtin were also detected. (D) Enhancement of endogenous CBP ubiquitylation in the hippocampus of 8-month-old mutant $Hdh^{Q7/Q111}$ mice. Hippocampal extracts isolated from wild-type $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice were subjected to immunoprecipitation with an anti-CBP antibody. Immunoprecipitates were analyzed by western blotting to detect ubiquitylated proteins and endogenous CBP. The ratio ubiquitylated CBP/total CBP was significantly increased in the hippocampus of mutant $Hdh^{Q7/Q111}$ mice. Data are presented as mean \pm SEM of five independent samples. ***P* < 0.01 using Student *t*-test.

(24,29,36). Therefore, we first tested the hypothesis that the observed reduction in soluble CBP was associated with the accumulation of nuclear mutant huntingtin in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice. Immunohistochemical analysis in hippocampal tissue of mutant and wild-type mice at 8 months of age was performed using the 1C2 antibody that selectively recognizes the expanded polyglutamine domain in mutant huntingtin (37,38). Extensive accumulation of nuclear mutant huntingtin detected as positive 1C2 immunoreactivity was found in the hippocampus of mutant $Hdh^{Q7/Q111}$ mice compared with $Hdh^{Q7/Q7}$ wild-type mice (Fig. 4A). We next determined by confocal microscopy whether 1C2 staining was associated with CBP immunoreactivity (Fig. 4B). In agreement with the biochemical analysis, an important reduction in CBP was found in the hippocampus of mutant compared with wild-type mice (Fig. 4B). Most importantly and consistent with our hypothesis, we found co-localization between CBP and 1C2 in the hippocampus of mutant $Hdh^{Q7/Q111}$ mice. Because soluble CBP was reduced in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice and CBP was found to co-localize with nuclear mutant huntingtin, we next studied the presence of aggregated forms of huntingtin

and CBP in the stacking gel of western blots (Fig. 4C). 1C2 immunostaining revealed oligomeric huntingtin forms detected as a diffuse smear in lysates from mutant but not wild-type mice. Notably, when immunoblots were re-probed with a CBP antibody, a similar large smear in the stacking gel was found in mutant mice samples. These results suggest that decreased CBP protein levels are associated with nuclear accumulation of mutant huntingtin in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice.

However, enhanced CBP degradation could also provide another mechanism involved in the observed decrease in CBP levels in mutant mice. To explore this possibility, we analyzed the levels of ubiquitinated CBP immunoprecipitated from $Hdh^{Q7/Q7}$ wild-type and $Hdh^{Q7/Q111}$ mutant hippocampal extracts (Fig. 4D). Detection of immunocomplexes with an anti-ubiquitin antibody revealed a significant increase in ubiquitylated CBP/total CBP levels (~ 2.5 -fold, *P* < 0.01) in mutant compared with wild-type mice, which is consistent with the idea that increased proteasomal-dependent ubiquitination and degradation of CBP could also be involved in the CBP reduction detected in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice.

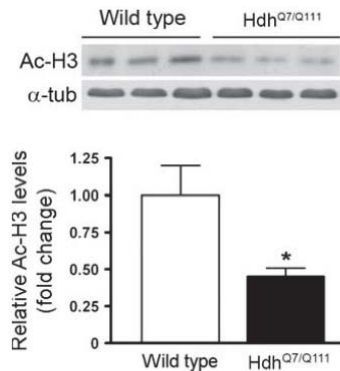


Figure 5. Decreased levels of CBP were associated with reduced acetylation of histone H3 in the hippocampus of mutant Hdh^{Q7/Q111} mice. Western blot and quantification showing a significant reduction in Ac-H3 levels in total hippocampal extracts from mutant Hdh^{Q7/Q111} mice compared with wild-type Hdh^{Q7/Q7} mice at the age of 8 months. Protein levels were normalized to α -tubulin as a loading control. The histograms represent the relative protein levels expressed as fold change of wild-type mice. Values are given as mean \pm SEM of five to seven independent samples. * $P < 0.05$ using Student t -test.

Reduced acetylation of histone H3 in the hippocampus of Hdh^{Q7/Q111} knock-in mutant mice

Since histone acetylation is significantly reduced in CBP heterozygous or conditional knock-out mice (21–23) and CBP HAT activity is essential for the conversion of short-term to long-term memory (39,40), we next tested whether reduced CBP could involve decreased histone acetylation in the hippocampus of HD mutant mice. We examined acetylation levels of histone H3 in the hippocampus of wild-type and mutant mice at 8 months of age by western blot analysis (Fig. 5). A significant decrease ($\sim 55\%$, $P < 0.05$) in acetylated H3 histone levels was detected in the hippocampus of Hdh^{Q7/Q111} mutant mice compared with Hdh^{Q7/Q7} wild-type mice. These data suggest that altered histone acetylation due to reduced CBP activity could contribute to cognitive deficits in Hdh^{Q7/Q111} mice.

Down-regulation of CREB/CBP-dependent genes in Hdh^{Q7/Q111} knock-in mutant mice

Chromatin modification via CBP-mediated histone acetylation is an important molecular pathway involved in the regulation of CREB-dependent gene transcription underlying long-term memory formation (11). To determine whether altered CREB-dependent transcription contributes to cognitive deficits in Hdh^{Q7/Q111} mutant mice, we analyzed by quantitative real-time PCR the expression of well-established CREB–CBP target genes in the hippocampus of spatial-trained wild-type Hdh^{Q7/Q7} and mutant Hdh^{Q7/Q111} mice at the age of 8 months. A significant reduction in CREB target genes related to synaptic plasticity and memory such as *c-fos* ($\sim 36\%$), *Arc* ($\sim 32\%$) or *Nr4a2* ($\sim 25\%$) but not of CREB-dependent genes associated with cell proliferation (*Cyr61*) or stress (*Fosb*) was detected in the hippocampus of trained

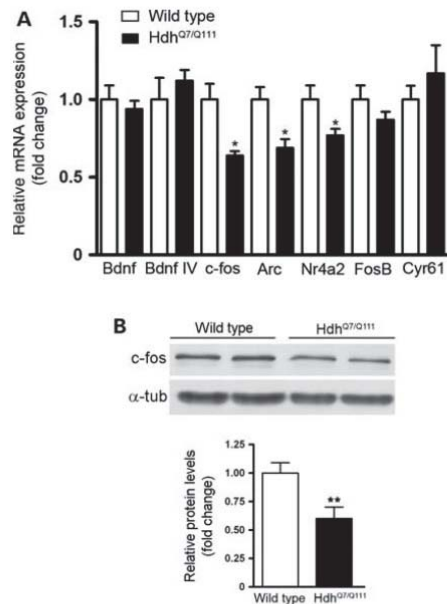


Figure 6. Reduced CREB/CBP-dependent gene transcription in Hdh^{Q7/Q111} mutant mice following MWM training. (A) Quantitative real-time RT–PCR analysis performed in hippocampal samples of 8-month-old Hdh^{Q7/Q7} wild-type ($n = 6$) and Hdh^{Q7/Q111} mutant ($n = 7$) mice following MWM training. qRT–PCR analysis reveals significant reduction in the CREB target genes, *c-fos*, *Arc* and *Nr4a2* in Hdh^{Q7/Q111} mutant mice compared with wild-type mice. mRNA expression is expressed as fold change values of wild-type mice normalized to GAPDH and 18S. * $P < 0.05$ compared with wild-type mice. (B) Western blot images (top) and quantification (bottom) showing reduced *c-fos* protein levels in the hippocampus of trained Hdh^{Q7/Q111} mutant mice ($n = 7$) compared with Hdh^{Q7/Q7} wild-type mice ($n = 6$). Protein levels were normalized to α -tubulin as a loading control. The histogram represents the relative protein levels expressed as fold change of wild-type mice. Values are given as mean \pm SEM. ** $P < 0.01$ compared with wild-type mice using Student t -test.

Hdh^{Q7/Q111} mutant mice (Fig. 6A). Notably, reduced expression of *c-fos* mRNA was associated with a significant decrease in *c-fos* protein levels ($\sim 40\%$, $P < 0.01$) (Fig. 6B). These results demonstrate selective reduction in CREB target genes in the hippocampus of memory impaired Hdh^{Q7/Q111} mutant mice, suggesting that deficient CREB/CBP-dependent gene transcription may account for the impaired hippocampal-dependent memory observed in these mice.

HDAC inhibition rescues long-term memory deficits in Hdh^{Q7/Q111} knock-in mutant mice

To further examine the possibility that CBP HAT activity was indeed involved in long-term memory deficits in mutant Hdh^{Q7/Q111} mice, we next tested whether cognitive deficits could be ameliorated by a general increase in histone acetylation. To this aim we used trichostatin A (TSA), a well-known HDAC inhibitor that increases histone acetylation in cell culture and mouse models without evident toxicity (40–42). To determine whether TSA treatment improves long-term

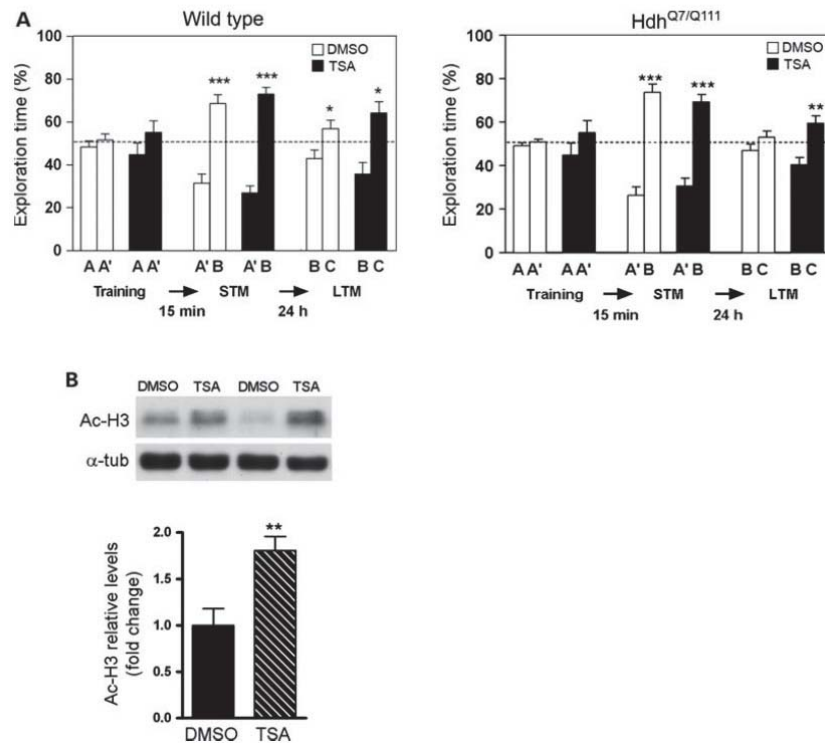


Figure 7. Administration of the histone deacetylase inhibitor TSA reverses recognition memory deficits in mutant $Hdh^{Q7/Q111}$ mice. TSA administration 2 h before training rescued recognition memory deficits in mutant $Hdh^{Q7/Q111}$ mice. (A) Bar diagram illustrating the exploration time for wild-type $Hdh^{Q7/Q7}$ ($n = 5$) (left) and mutant $Hdh^{Q7/Q111}$ ($n = 6$) (right) mice during the training, short-term (15 min delay, STM) and long-term (24 h delay, LTM) memory sessions in a NORT. TSA treatment does not affect mice performance during the training session and at 15 min post-training (short-term memory). However, TSA-treated mutant $Hdh^{Q7/Q111}$ mice exhibited significantly enhanced preference for the novel object compared with the vehicle-treated $Hdh^{Q7/Q111}$ mice 24 h after training (long-term memory). Exploration time is shown in the bar graphs as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in a one-way ANOVA with Student t -test as a *post-hoc* test. (B) Western blot and quantification showing a significant increase in Ac-H3 levels in total hippocampal extracts from TSA-treated mutant $Hdh^{Q7/Q111}$ mice compared with DMSO-treated mutant $Hdh^{Q7/Q111}$ mice following NORT task. Protein levels were normalized to α -tubulin as a loading control. The histograms represent the relative protein levels expressed as fold change of DMSO-treated mutant mice. Values are given as mean \pm SEM of six independent samples. ** $P < 0.01$ using Student t -test.

memory deficits in $Hdh^{Q7/Q111}$ mutant mice, we examined the effect of TSA injection on memory by using the NORT test (Fig. 7A). To assess short- and long-term memory, we tested mice at two different retention intervals of 15 min and 24 h. In agreement with the above results (Fig. 1), we found no significant differences in performance between groups at 15 min, indicating that TSA did not affect short-term memory acquisition in both experimental groups (Fig. 7A). Interestingly, when object recognition memory was tested 24 h after training, TSA-treated mutant $Hdh^{Q7/Q111}$ mice exhibited significantly increased preference for the novel object compared with vehicle-treated- $Hdh^{Q7/Q111}$ mice ($Hdh^{Q7/Q111}$ TSA, familiar object: $39 \pm 3.5\%$ versus novel object: $60.5 \pm 3.5\%$, $P < 0.01$; $Hdh^{Q7/Q111}$ vehicle, familiar object: $47 \pm 3.2\%$ versus novel object: $53 \pm 3\%$, $P = 0.17$) (Fig. 7A). No significant differences were found between vehicle and TSA-treated wild-type $Hdh^{Q7/Q7}$ mice. Consistent with an improvement of long-term recognition memory in $Hdh^{Q7/Q111}$ mutant

mice, a significant increase ($\sim 80\%$, $P < 0.01$) in acetylated histone H3 levels (AcH3) were found in TSA-treated $Hdh^{Q7/Q111}$ mutant mice compared with those treated with dimethyl sulfoxide (DMSO) (Fig. 7B). Altogether, these results suggest that reduced CBP HAT activity likely contributes to long-term cognitive deficits in $Hdh^{Q7/Q111}$ mutant mice and that TSA efficiently reverses histone acetylation and long-term object recognition memory deficits in HD mutant mice.

Selective increase in CREB target genes in $Hdh^{Q7/Q111}$ knock-in mutant mice by HDAC inhibition

We next tested whether rescue of memory deficits in $Hdh^{Q7/Q111}$ mutant mice by TSA treatment was associated with enhanced expression of CREB/CBP-dependent genes. Quantitative real-time PCR analysis revealed that TSA-treated mutant mice exhibited a slight, but not statistically significant increase in *Arc* expression that was not observed in wild-type mice.

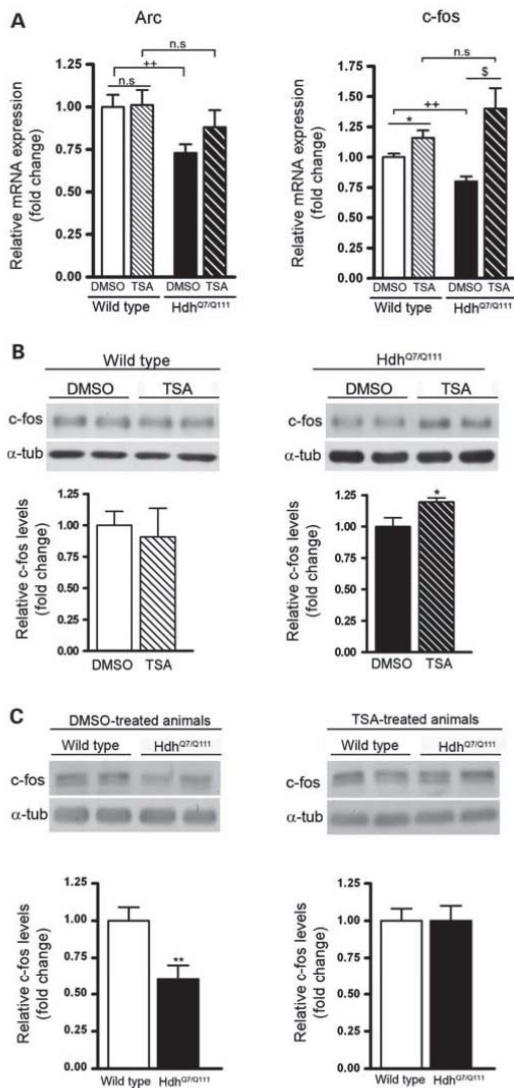


Figure 8. Increase in selective CREB target genes by TSA administration in Hdh^{Q7/Q111} knock-in mutant mice. (A) Quantitative real-time RT-PCR analysis performed in hippocampus of TSA-treated and DMSO-treated wild-type Hdh^{Q7/Q7} and mutant Hdh^{Q7/Q111} mice following NORT task. mRNA expression is expressed as the fold change values of the wild-type vehicle-treated mice. * $P < 0.05$, TSA-treated wild-type mice compared with DMSO-treated wild-type mice. ** $P < 0.01$ DMSO-treated wild-type mice compared with DMSO-treated mutant mice, ^s $P < 0.05$ TSA-treated mutant mice compared with DMSO-treated mutant mice. (B) Western blot and quantification showing hippocampal c-fos protein levels from DMSO or TSA-treated wild-type Hdh^{Q7/Q7} (left) and mutant Hdh^{Q7/Q111} mice (right) following NORT task. Protein levels were normalized to α -tubulin as a loading control. The histograms represent the relative protein levels expressed as fold change of DMSO-treated mice. Values are given as mean \pm SEM of five independent samples. * $P < 0.05$ compared with DMSO-treated mutant mice using

Student *t*-test. (C) Western blot images showing hippocampal c-fos protein levels from vehicle (left) and TSA (right)-treated wild-type Hdh^{Q7/Q7} and mutant Hdh^{Q7/Q111} mice following NORT task. Protein levels were normalized to α -tubulin as a loading control. The histograms represent the relative protein levels expressed as fold change of wild-type mice. Values are given as mean \pm SEM of five to eight independent samples. ** $P < 0.01$ mutant compared with wild-type mice using Student *t*-test.

However, it is important to notice that when TSA-treated mice were compared no significant differences in *Arc* expression were detected, while a significant reduction was still found in DMSO-treated mice ($\sim 30\%$, $P < 0.01$, Fig. 8A). Notably, while TSA treatment induced a drastic increase in *c-fos* expression in Hdh^{Q7/Q111} mutant mice ($\sim 50\%$, $P < 0.05$), the increase was much moderate in Hdh^{Q7/Q7} wild-type mice ($\sim 20\%$, $P < 0.05$). In agreement with these data, western blot analysis revealed that c-fos protein levels were significantly increased ($\sim 20\%$, $P < 0.05$) in TSA-treated mutant mice compared with those treated with DMSO, although no differences were found in wild-type mice (Fig. 8B). Consistently, when DMSO-treated mice were compared, a significant decrease in c-fos protein levels was found in mutant compared to wild-type mice ($\sim 44\%$, $P < 0.05$, Fig. 8C), while no significant differences were found between TSA-treated mice. Unfortunately, we failed to detect any specific band for *Arc* in hippocampal samples.

DISCUSSION

Memory and cognitive deficits are clinical features of HD that are present at early disease stages, when motor symptoms are not yet evident (1,3,43–47). Image analysis indicates anatomic and functional brain atrophy in regions involved in cognitive function in pre-symptomatic HD patients (34,48–51), although the pathological and molecular mechanisms underlying cognitive deficits in this motor disorder are largely unknown. In this study, we provide evidence for the first time that diminished CBP levels and altered CREB/CBP-dependent transcription are associated with long-term memory deficits in heterozygous Hdh^{Q7/Q111} mutant mice, a genetic model of HD that accurately express the *HD* CAG mutation.

Our behavioral data demonstrate that Hdh^{Q7/Q111} mutant mice exhibit age-dependent long-term object recognition and spatial memory deficits but preserved short-term memory, which is consistent with previous reports showing impaired hippocampal LTP and spatial learning in HD mouse models (5–7,9,52–54). Moreover, we found that Hdh^{Q7/Q111} mutant mice display normal acquisition but impaired consolidation of recognition and spatial memories in contrast with previous studies showing learning impairments in exon-1 HD mice (6,7,53). A more severe phenotype of exon-1 over-expressing transgenic HD mice compared with Hdh^{Q7/Q111} knock-in mice may explain this apparent discrepancy.

Student *t*-test. (C) Western blot images showing hippocampal c-fos protein levels from vehicle (left) and TSA (right)-treated wild-type Hdh^{Q7/Q7} and mutant Hdh^{Q7/Q111} mice following NORT task. Protein levels were normalized to α -tubulin as a loading control. The histograms represent the relative protein levels expressed as fold change of wild-type mice. Values are given as mean \pm SEM of five to eight independent samples. ** $P < 0.01$ mutant compared with wild-type mice using Student *t*-test.

To elucidate the specific molecular mechanism(s) underlying these memory deficits, we focused on CREB signaling, a pathway critical for hippocampal-dependent synaptic plasticity and long-term memory (14,55). We found reduced levels of CBP but unchanged levels of total and phosphorylated CREB in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice at 8 months of age when memory deficits are present. Importantly, CBP levels remain unaltered in $Hdh^{Q7/Q111}$ mutant mice at 4 months, an age in which cognitive impairments are not observed. Consistent with these findings, we also observed reduced CBP levels in the hippocampus of HD patients, which support the idea that CBP dysfunction plays a relevant role in HD memory deficits.

Several possible mechanisms may account for the CBP reduction in the hippocampus of $Hdh^{Q7/Q111}$ mice. Our data indicate that mutant huntingtin does not regulate CBP expression at the transcriptional level but at the protein level. Thus, our findings showing intranuclear accumulation of mutant huntingtin in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice as well as an association of CBP with oligomeric forms of mutant huntingtin suggest that similarly to that observed in the striatum (26,28,30), mutant huntingtin may interfere with hippocampal CBP function by reducing the levels of soluble CBP. However, in addition to depletion of soluble CBP, increased CBP-mediated proteasomal degradation may also contribute to reducing CBP. Indeed, it has been reported in hippocampal cell lines that mutant huntingtin selectively enhances CBP processing by the ubiquitin–proteasome pathway (24,36). Consistent with these studies, we found increased CBP ubiquitylation in the hippocampus of 8-month-old $Hdh^{Q7/Q111}$ mutant mice. Thus, collectively our data suggest that mutant huntingtin may alter hippocampal CBP levels via at least two different mechanisms: (i) reduction of soluble CBP and (ii) increased CBP degradation.

Loss of CBP function in the hippocampus has been extensively associated with defects in long-lasting synaptic plasticity and long-term memory in different experimental mouse models of psychiatric and cognitive disorders (21,22,40,42,56). The activity of CBP as transcriptional co-activator is essential for regulation of genes underlying memory formation (57,58) and its complete or partial inactivation in mutant mice causes impaired long-term memory without changes in learning and short-term memory (21,40,59,60). Consistent with the above results, we found that reduced CBP levels in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice were associated with selective deregulation of CREB/CBP-target genes related to synaptic plasticity and memory (*c-fos*, *Nr4a2* and *Arc*) but not to cell proliferation (*Cyr61*) or stress (*Fosb*). Interestingly, decreased CREB-dependent gene expression as a consequence of diminished CBP levels or function is associated with synaptic plasticity and memory deficits in Alzheimer's disease mouse models (56,61,62) and inactivation or reduction of *Arc* or *c-fos* in the hippocampus results in deficits in spatial memory (63,64).

Besides genetic activation, epigenetic modifications and chromatin remodeling are essential mechanisms for proper cognitive functions (65–68). Histone modifications are especially relevant for transcriptional regulation during memory consolidation (69,70). In this view, CBP HAT activity has emerged as a critical component for synaptic plasticity and

long-term memory (21,40,60,71). Thus, in several mouse models of cognitive dysfunction, diminished H3 acetylation has been associated with reduced CBP expression and/or activity (22,40,72). Consistent with these studies, we found a significant decrease in histone H3 acetylation in the hippocampus of $Hdh^{Q7/Q111}$ mutant mice providing the first demonstration of reduced H3 acetylation associated with memory deficits in a HD mouse model. If decreased histone acetylation contributes to memory dysfunction in $Hdh^{Q7/Q111}$ mutant mice, we would expect that HDAC inhibitors improve these cognitive deficits. Consistent with this hypothesis, we found that HDAC inhibition by TSA treatment efficiently reversed long-term memory impairments in $Hdh^{Q7/Q111}$ mutant mice. This result agrees with recent reports showing that HDAC inhibitors enhance synaptic plasticity and improve memory deficits in different mouse models of cognitive dysfunction (21,22,73–77). Importantly, HDAC inhibition rather than having a general effect in gene expression positively affects the transcription of specific genes involved in memory consolidation (42,75,78). Interestingly, we found that TSA treatment increases the expression of *c-fos* and *Arc* in mutant $Hdh^{Q7/Q111}$ mice resulting in similar *c-fos* and *Arc* expression between genotypes. The critical involvement of *c-fos* and *Arc* in spatial and recognition memory consolidation has been largely demonstrated (63,64,79–81). Thus, impaired long-term memory and reduced LTP have been found in mice or rats lacking *c-fos* or *Arc* expression (63,64) and increased *c-fos* levels following systemic administration of HDAC inhibitors was associated with improvement of cognitive deficits in mouse models of neurodegenerative and cognitive disorders (82,83). Altogether, our results suggest that HDAC inhibition by TSA rescue memory deficits in $Hdh^{Q7/Q111}$ mutant mice by reducing histone acetylation deficits and enhancing transcription of specific CRE-target genes related to memory. However, we cannot rule out that non-histone substrates might also be affected by TSA treatment and therefore contribute to memory improvement in HD mice. Interestingly, it has been recently reported that TSA treatment compensates for the reduction in tubulin acetylation observed in HD neuronal models leading to improvement of axonal transport deficits and the subsequent release of brain-derived neurotrophic factor (BDNF) (84).

In summary, we show evidence that reduced hippocampal CBP levels may contribute to cognitive deficits in $Hdh^{Q7/Q111}$ mice by deregulating CREB-dependent transcription of specific genes involved in synaptic plasticity and memory. Likely, both reduced CBP transcriptional and HAT activities play a critical role in long-term memory impairments in $Hdh^{Q7/Q111}$ mice. Finally, the recovery of memory deficits in $Hdh^{Q7/Q111}$ mice by a HDAC inhibitor may have important therapeutic implications for treatment of cognitive deficits in HD.

MATERIALS AND METHODS

Reagents and antibodies

HDAC inhibitor TSA was obtained from Sigma-Aldrich. CBP (A22 and C-1) and *c-fos* (A4) antibodies were purchased from Santa Cruz Biotechnology. Phospho-CREB (Ser133), CREB

and acetyl-H3 (C5B11) antibodies were from Cell Signaling Technologies. 1C2 antibody and 2166 antibody were obtained from Millipore. Ubiquitin antibody was from Abcam. Anti-tubulin and anti-actin were from Sigma-Aldrich.

Huntington's disease mouse model

Hdh^{Q111} knock-in mice, with targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues, were maintained on a C57BL/6 genetic background (35). Hdh^{Q7/Q111} heterozygous males and females were intercrossed to generate age-matched Hdh^{Q7/Q111} heterozygous and Hdh^{Q7/Q7} wild-type littermates. Only males were used for all experiments. All procedures were carried out in accordance with the National Institute of Health and were approved by the local animal care committee of the Universitat de Barcelona (99/01) and the Generalitat de Catalunya (00/1094).

Postmortem brain tissue

Hippocampal brain tissues (six controls and six HD patients) were obtained from Banc de Teixits Neurològics (Servei Científico-Tècnic, Universitat de Barcelona, Barcelona, Spain) following the guidelines of the local ethics committees. Controls (mean \pm SEM; age 53.5 ± 6.8 years; post-mortem intervals of 4–18 h), HD brain grades 3 and 4 (mean \pm SEM; age 54.5 ± 6.5 years; post-mortem intervals of 4–17 h). Hippocampal brain tissue was homogenized in cold lysis buffer [20 mM Tris base (pH 8.0), 150 mM NaCl, 50 mM NaF, 1% NP-40, 10% glycerol and supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)], cleared by centrifugation at 16 000 g for 20 min and the supernatants collected and resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). CBP protein levels were finally determined by western blot analysis as described below. All the ethical guidelines contained within the latest Declaration of Helsinki were taken into consideration and informed consent was obtained from all subjects under study.

Behavioral learning tests

Novel object recognition test (NORT). The NORT has been adapted from a previously described protocol for rats (85). For the NORT task, mice were tested in a circular open field (40 cm diameter) located in a room with dim lighting. In the object recognition protocol, two different objects were placed in the circular field during the training phase. The objects varied in color, shape and size. To avoid olfactory cues, chamber and objects were thoroughly cleansed between trials. Briefly, 4- and 8-month-old male mice were habituated to the open field in the absence of the objects for 15 min each day over 3 days. During the training period, mice were placed in the open field with two identical objects for 10 min. Retention tests were performed either 15 min (short-term memory test) or 24 h (long-term memory test) after training by placing the mice back to the open field for a 5 min session and by randomly exchanging one of the familiar objects with a novel one. Training and testing trials

were recorded and the time that mice spent exploring the novel and familiar objects was measured. Contact with a given object was defined as the mouse approaching the object nose first with the nose being within 1 cm of the object boarder. The relative exploration time was recorded and calculated using the formula $ET = 100 \times (\text{new object inspection time} / \text{total inspection time})$. Experimenters were blind to the genotypes and treatment condition of the mice.

Morris water maze. Spatial learning was assessed in a MWM task modified for use in mice (7). Briefly, 8-month-old male mice were trained in a circular pool (diameter 100 cm; height 40 cm, water depth of 25 cm) four trials per day over 11 days. Four positions around the edge of the tank were arbitrarily designated as north (N), south (S), east (E), and west (W) to provide four alternative starting positions and to divide the tank into four quadrants: NE, SE, SW and NW. A circular white escape platform (10 cm in diameter) was submerged 1 cm below the water surface and placed at the midpoint of one of the four quadrants. The mouse was allowed to swim until it reached the platform or otherwise guided onto the platform after 60 s. Mice were left on the platform for 15 s before returned to the home cage during the inter-trial interval. On day 11, the mice performed a single 1 min probe trial without the platform 4 h after the last trial. The swim path of each mouse was recorded over 60 s, while it searched for the missing platform. Thirty minutes after the probe trial, mice were sacrificed by cervical dislocation and the hippocampus was rapidly dissected and frozen at -80°C . In the visible platform test, extramaze distal cues were removed, and the platform was marked with a high-contrast striped flag. The movement of the mice was monitored using an automated tracking system and data analyzed by the SMART junior software (Panlab, Spain). All experiments were performed by operators who were blind to the mice genotypes.

Real-time quantitative reverse transcriptase-PCR

Total RNA was isolated from hippocampus of heterozygous mutant and wild-type mice using the Total RNA Isolation Nucleospin[®] RNA II Kit (Macherey-Nagel, Düren, Germany). Purified RNA (500 ng) was reverse transcribed using the StrataScript[®] First Strand cDNA Synthesis System (Stratagene, Santa Clara, CA, USA). The cDNA synthesis was performed at 42°C for 60 min in a final volume of 20 μl according to the manufacturer's instructions. The cDNA was then analyzed by quantitative reverse transcriptase (RT)-PCR using the following TaqMan[®] Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): 18S (Hs99999901_s1), Arc (Mn00479619_g1), c-Fos (Mn00487425_m1), BDNF (Mn00432069), Fosb (Mn_008036), Nr4a2 (Mn_013613.1) and Cyr61 (Mn_010516). RT-PCR was performed in 25 μl of final volume on 96-well plates, in a reaction buffer containing 12.5 μl TaqMan Gene Expression Assays and 20 ng of cDNA. Reactions included 40 cycles of a two-step PCR: 95°C for 30 s and 60°C for 1 min, after initial denaturation at 95°C for 10 min. All Q-PCR assays were performed in duplicate and repeated in at least three independent experiments. To provide negative controls and exclude contamination by genomic DNA, the RT was omitted in the cDNA synthesis step, and

the samples were subjected to the PCR reaction in the same manner with each TaqMan Gene Expression Assay. The Q-PCR data were analyzed using the MxPro™ Q-PCR analysis software version 3.0 (Stratagene). Quantification was performed with the Comparative Quantitation Analysis program of the mentioned software and using the 18S or glyceraldehyde 3-phosphatase dehydrogenase gene expression as internal controls.

Immunoprecipitation and western blot analysis

Heterozygous mutant $Hdh^{Q7/Q111}$ and wild-type $Hdh^{Q7/Q7}$ mice were killed by cervical dislocation at the age of 4 or 8 months. The brain was quickly removed, dissected, frozen in dry ice and stored at -80°C until use. Brain tissue was homogenized in cold lysis buffer [20 mM Tris base (pH 8.0), 150 mM NaCl, 50 mM NaF, 1% NP-40, 10% glycerol and supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)], cleared by centrifugation at 16 000 g for 20 min and the supernatants collected. Following determination of the protein contents by Detergent-Compatible Protein Assay (Bio-Rad, Hercules, CA, USA), protein extracts (20–60 μg) were mixed with 5 \times SDS sample buffer, boiled for 5 min, resolved on 6–10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell, Keene, NH, USA). After incubation (30 min) in blocking buffer containing 10% non-fat powdered milk in Tris buffered saline-Tween (TBS-T) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween 20), membranes were blotted overnight at 4°C with primary antibodies: CBP (1:1000), CREB (1:1000), phospho-CREB (1:1000), acetyl-H3 (1:1000), c-fos (1:1000), IC2 (1:1000), ubiquitin (1/50), anti-htt 2166 (1:1000), anti-actin (1:1000) or α -tubulin (1:50000). The membranes were then rinsed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing for 30 min with TBS-T, the membranes were developed using the enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology). The Gel-Pro densitometry program (Gel-Pro Analyzer for Windows, version 4.0.00.001) was used to quantify the different immunoreactive bands relative to the intensity of the α -tubulin band in the same membranes within a linear range of detection for the ECL reagent (86). Data are expressed as the mean \pm SEM of band density.

Immunoprecipitation was performed by incubation of protein extracts (400 μg) obtained from hippocampus of 8-month-old wild-type $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice with 3 μg of anti-CBP antibody (A-22) overnight at 4°C followed by a 2 h incubation with 30 μl of protein A-Sepharose Cl-4B (Sigma). The beads were washed by centrifugation three times, then resuspended in ice-cold phosphate buffered saline (PBS) and then boiled for 5 min for reducing SDS loading buffer. The immunocomplexes were resolved by SDS-PAGE on 8% polyacrylamide gel and transferred to nitrocellulose membranes. Immunoblot analysis was carried out as described above. Briefly, the blots were incubated with anti-CBP (C-1) and anti-ubiquitin and detected using ECL chemiluminescent reagents.

Trichostatin A administration

TSA was dissolved in 100% DMSO at a concentration of 2 $\mu\text{g}/\mu\text{l}$. For behavioral experiments, TSA or DMSO was administered by i.p. injection (1 $\mu\text{l}/\text{g}$ body weight) 2 h before training on the NORT task. Training on the NORT task was followed by memory tests at 15 min and 24 h delays. To analyze c-fos levels, hippocampus from trained wild-type and mutant mice treated with vehicle or TSA were isolated and homogenized in cold lysis buffer as previously described, cleared by centrifugation at 16 000 g and the supernatants collected. Hippocampal extracts were resolved on 10% SDS-PAGE, transferred onto nitrocellulose membranes and immunoblot analysis carried out by incubation with c-fos antibody and detection using ECL chemiluminescent reagents.

Immunohistochemistry

For immunohistochemical analysis, heterozygous mutant $Hdh^{Q7/Q111}$ and wild-type $Hdh^{Q7/Q7}$ mice at 8 months of age ($n = 3$ for each condition) were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. Brains were removed and postfixed for 1–2 h in the same solution, cryoprotected by immersion in 30% sucrose and then frozen in dry ice-cooled methylbutane. Serial coronal cryostat sections (30 μm) through the whole brain were collected in PBS as free-floating sections. Sections were rinsed three times in PBS and permeabilized and blocked in PBS containing 0.3% Triton X-100 and 3% normal goat serum (Pierce Biotechnology) for 15 min at room temperature. The sections were then washed in PBS and incubated overnight at 4°C with anti-IC2 (1:500) and anti-CBP (1:100) antibodies and detected with Cy3 anti-rabbit and Cy2 anti-mouse (1:200) secondary antibodies (Jackson ImmunoResearch). Following secondary antibody incubation, slices were rinsed in PBS and incubated with Hoechst solution (Invitrogen, 1:5000) for 10 min. As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected. Immunofluorescence was analyzed by confocal microscopy using a TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg, Mannheim, Germany).

For detection of huntingtin, sections were pre-incubated with PBS containing 3% H_2O_2 for 45 min and blocked in 5% normal goat serum for 1 h. Sections were then incubated overnight at 4°C with IC2 monoclonal antibody (1:500), washed three times in PBS and incubated with biotinylated secondary antibody (1:200; Pierce) at room temperature for 2 h and with avidin-biotin-peroxidase complex (ABC kit; Pierce). Reactions were visualized with diaminobenzidine as a chromagen. No signal was detected in controls in which the primary antibodies have been omitted. Light micrographs were obtained with an Olympus microscope BX51 (Olympus Danmark A/S).

Statistical analysis

Statistical analysis was performed using either Student's *t*-test or one-way analysis of variance (ANOVA) followed by Student's *t*-test. The MWM behavioral data were analyzed

using two-way ANOVA with repeated measures or one-way ANOVA followed by the Newman–Keuls test for *post hoc* comparisons. Data were shown as the mean \pm SEM. Differences with $P < 0.05$ were considered significant.

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Conflict of Interest statement. None declared.

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WORK 2***“IMBALANCE OF p75^{NTR}/TrkB PROTEIN EXPRESSION IN HUNTINGTON’S DISEASE: IMPLICATION FOR NEUROPROTECTIVE THERAPIES”***

This paper has been published in Cell Death and Disease

Brito V, Puigdemívol M, Giralt A, del Toro D, Alberch J, Ginés S

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CONTRIBUTION TO THIS WORK:

I contributed in the building of some hypothesis and in their validation. I helped the first author of this work in the performance of treatments and/or transfections in striatal cell lines and the performance of some immunoblots in HD mice and human samples to determine biochemical changes. After transfection and treatments in striatal cell lines, I analyzed striatal cell death by counting positive DAPI staining in some experiments. I contributed in the discussion of the results.

AIM 2. TO STUDY THE MOLECULAR MECHANISMS INVOLVED IN NEUROTROPHIC SUPPORT DYSFUNCTION IN HUNTINGTON'S DISEASE**2.1. To analyze the role of p75^{NTR}/TrkB receptors in the major striatal vulnerability in Huntington's disease**

Les teràpies basades en l'administració de BDNF han estat proposades pel tractament de la MH. No obstant, el nostre grup ha descrit prèviament que els nivells del receptor TrkB es troben disminuïts en models murins de la MH així com en mostres de cervells post-mortem d'individus afectats per la MH, suggerint així que a part de la reducció en l'expressió de BDNF, una disminució en els nivells del seu receptor TrkB pot estar contribuint en els dèficits de suport neurotròfic observats en la MH. BDNF pot unir-se també al receptor p75 (p75^{NTR}), conegut per la seva capacitat de modular la supervivència neuronal mediada per TrkB. En aquest treball hem analitzat els nivells del receptor p75^{NTR} en diversos models murins de la MH i en mostres post-mortem d'individus afectats per la malaltia. Els nostres resultats mostren un desequilibri en els nivells de p75^{NTR}/TrkB en l'estriat de ratolins knock-in homozigots per la MH (Hdh^{Q111/Q111}) i en ratolins R6/1, així com en mostres de putamen d'individus afectats per la MH. El desequilibri entre els nivells de p75^{NTR}/TrkB no afecta l'activació de TrkB mediada per BDNF de vies de pro-supervivència com ara les vies d' Akt o Erk, però induïx l'activació de cascades apoptòtiques tals com JNK. A més, els nostres resultats mostren que en les cèl·lules mutades (mhtt), els alts nivells de p75^{NTR} i els nivells disminuïts de TrkB impliquen la pèrdua de protecció per BDNF davant l'excitotoxicitat mediada per NMDA. En aquest treball hem vist que la manca de neuroprotecció per BDNF està associada a una disminució de fosforilació d' Akt que correlaciona amb l'increment dels nivells proteics de la fosfatasa PP1. Tots aquests resultats demostren que en les cèl·lules estriatals, el desequilibri entre els nivells de p75^{NTR}/TrkB produït per la presència de la mhtt altera la neuroprotecció per BDNF i contribueix a la major vulnerabilitat de les cèl·lules estriatals en la MH. Basant-nos en aquestes dades, hipotetitzem que una possible estratègia terapèutica que moduli l'expressió d'ambdós receptors (TrkB i p75^{NTR}) o de la seva senyalització podria significar una millora en les teràpies neuroprotectores en la MH.

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Imbalance of p75^{NTR}/TrkB protein expression in Huntington's disease: implication for neuroprotective therapies

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Neuroprotective therapies based on brain-derived neurotrophic factor (BDNF) administration have been proposed for Huntington's disease (HD) treatment. However, our group has recently reported reduced levels of TrkB in HD mouse models and HD human brain suggesting that besides a decrease on BDNF levels a reduction of TrkB expression could also contribute to diminished neurotrophic support in HD. BDNF can also bind to p75 neurotrophin receptor (p75^{NTR}) modulating TrkB signaling. Therefore, in this study we have analyzed the levels of p75^{NTR} in several HD models, as well as in HD human brain. Our data demonstrates a p75^{NTR}/TrkB imbalance in the striatum of two different HD mouse models, *Hdh*^{Q111/111} homozygous knockin mice and R6/1 mice that was also manifested in the putamen of HD patients. The imbalance between TrkB and p75^{NTR} levels in a HD cellular model did not affect BDNF-mediated TrkB activation of prosurvival pathways but induced activation of apoptotic cascades as demonstrated by increased JNK phosphorylation. Moreover, BDNF failed to protect mutant huntingtin striatal cells transfected with p75^{NTR} against NMDA-mediated excitotoxicity, which was associated with decreased Akt phosphorylation. Interestingly, lack of Akt activation following BDNF and NMDA treatment correlated with increased PP1 levels. Accordingly, pharmacological inhibition of PP1 by okadaic acid (OA) prevented mutant huntingtin striatal cell death induced by NMDA and BDNF. Altogether, our findings demonstrate that the p75^{NTR}/TrkB imbalance induced by mutant huntingtin in striatal cells associated with the aberrant activity of PP1 disturbs BDNF neuroprotection likely contributing to increasing striatal vulnerability in HD. On the basis of this data we hypothesize that normalization of p75^{NTR} and/or TrkB expression or their signaling will improve BDNF neuroprotective therapies in HD.

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Deficits of neurotrophic support caused by reduced levels of brain-derived neurotrophic factor (BDNF) have been implicated in the selective vulnerability of striatal neurons in Huntington's disease (HD).¹ Neuroprotective therapies based on BDNF administration have been proposed to slow or prevent the HD disease progression. However, the effectiveness of BDNF may depend on the proper expression of its neuronal receptor TrkB. Indeed, reduced striatal TrkB expression has been reported in knockin HD cellular and mouse models, exon-1 HD transgenic mice and HD human brain,^{2–4} which suggests that mutant huntingtin could lead to reduced neurotrophic support not only by altering BDNF levels but also by affecting TrkB expression. This scenario can still be more complex as BDNF also binds to p75^{NTR} a member of the tumor necrosis factor receptor superfamily.⁵ In contrast to Trk receptors, which have a well-defined trophic role, p75^{NTR} may promote a variety of complex and sometimes opposing functions ranging from trophism to apoptosis depending on

the cellular context and coexpression with Trk receptors.^{6,7} Thus, p75^{NTR} can either potentiate or reduce neurotrophic Trk receptor function or act independently to induce apoptotic signaling cascades.^{5,8,9} In the adult brain, p75^{NTR} expression is downregulated in most brain areas.^{10,11} However, in damaged or diseased conditions p75^{NTR} expression is rapidly induced, which has been associated with neuronal cell death.^{7,12} Thus, in neonatal mice brain, NMDA-induced injury is associated with upregulation of p75^{NTR}^{13,14} while in rats excitotoxicity induced by kainate administration causes an increase on p75^{NTR} levels accompanied by a significant cell death.¹⁵ Importantly, in HD, striatal neurons are selectively vulnerable to glutamate-induced neurotoxicity suggesting that excitotoxicity has an important role in HD striatal neurodegeneration.¹⁶ In this view, Zuccato *et al.* have demonstrated increased p75^{NTR} but reduced TrkB mRNA expression in the caudate but not in the cortex of HD patients⁴ while impaired TrkB-mediated ERK1/2 activation was recently reported by

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Keywords: BDNF receptors; excitotoxicity; huntingtin; neurodegeneration; neuroprotection

Abbreviation: BDNF, Brain-derived neurotrophic factor; DAPI, 4',6-Diamidino-2-Phenylindole; DARPP32, Dopamine- and cAMP-regulated protein with molecular weight of 32 kDa; ERK, Extracellular Signal-Regulated Kinase; GFAP, Glial Fibrillary Acidic Protein; GFP, green fluorescent protein; HD, Huntington's Disease; JNK, Jun N-terminal Kinase; NeuN, Neuronal nuclear antigen; NMDA, N-methyl-D-aspartate; PHLPP1, PH domain and Leucine rich repeat Protein Phosphatases; PP1, protein phosphatase 1; TrkB, Tyrosine-related kinase B receptor

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our group in HD striatal cell lines.³ Altogether, this data suggest that neurotrophic deficits in HD could account not only for BDNF reduction but also for an imbalance between TrkB and p75^{NTR}-mediated cell signaling. To test this hypothesis we have analyzed the levels of p75^{NTR} and TrkB in the striatum of two distinct HD mouse models at different HD pathological stages, as well as in the putamen of HD patients. We found an imbalance between p75^{NTR} and TrkB levels that was associated with a reduction of BDNF-mediated neuroprotection against NMDA excitotoxicity. Altogether, our findings support the idea that normalization of TrkB and p75^{NTR} protein levels or their functional signaling cross-talk would be a major step to improve neuroprotective therapies with BDNF in HD.

Results

p75^{NTR} and TrkB protein levels are altered in the striatum of R6/1 mice. Reduced BDNF neuroprotection contributes to striatal vulnerability in HD.¹⁷ Given that BDNF effects can be mediated by TrkB and p75^{NTR} we analyzed the expression of these receptors in the striatum of wild-type and mutant huntingtin R6/1 mice at different HD pathological

stages (Figure 1a). p75^{NTR} protein levels were significantly increased in the striatum of R6/1 mice at 12 weeks of age (~50%) an increase that was even more evident at 30 weeks (~120%) revealing an effect of age and genotype on changes in p75^{NTR} ($F_{(3,18)} = 4.133$; $P < 0.05$). By contrast, a significant reduction of TrkB was found in R6/1 only at late disease stages (~40%). As we described that reduced TrkB was associated with decreased TrkB transcription² we examined whether increased p75^{NTR} mRNA expression could account for upregulation of p75^{NTR} protein levels. A significant increase in p75^{NTR} mRNA was found in the striatum of R6/1 mice at 30 weeks of age (WT: 1.0 ± 0.3 and R6/1: 2.3 ± 0.4 , $P < 0.05$) indicating that both p75^{NTR} mRNA and protein are increased in R6/1 mice. To further evaluate the expression and distribution of p75^{NTR} immunohistochemistry analysis was performed in striatal slices from wild-type and R6/1 mice at 30 weeks of age (Figure 1b). According to the biochemical data a significant increase (~30%) on p75^{NTR} staining was revealed in mutant compared with wild-type mice. As striatal neuronal loss has a characteristic rostro-caudal gradient of pathological damage,¹⁸ p75^{NTR} levels were analyzed on coronal sections across the rostro-caudal extension of the striatum (Figure 1c). Significant

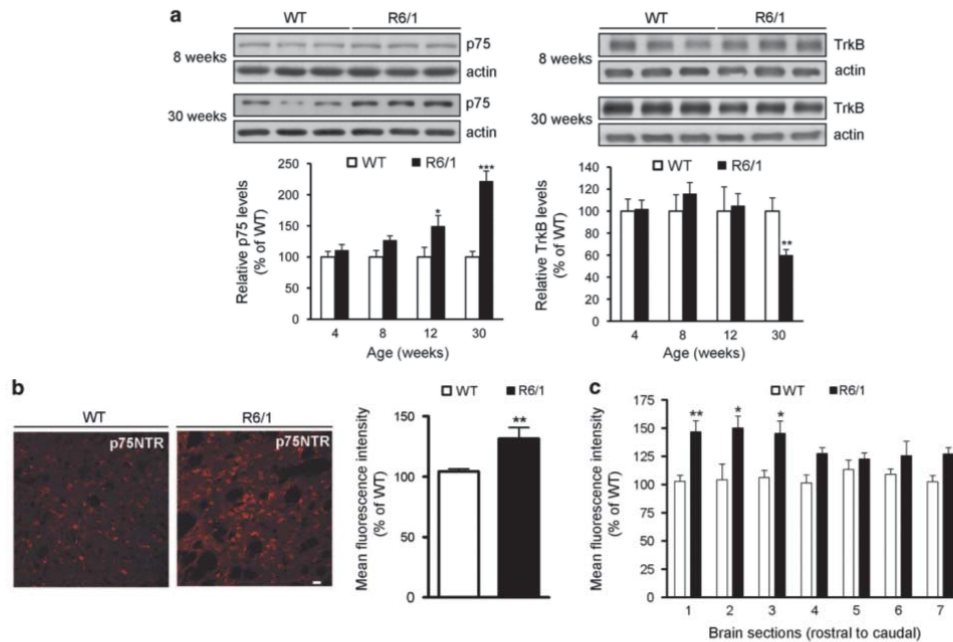


Figure 1 Imbalance between p75^{NTR} and TrkB expression in the striatum of R6/1 mice. (a) Representative immunoblots showing the levels of p75^{NTR}, TrkB and β -actin as a loading control in striatal extracts obtained from WT and R6/1 mice at 8 and 30 weeks of age. The histograms represent the relative levels of p75^{NTR} and TrkB expressed as percentage of wild-type values. Values are given as mean \pm S.E.M. of 5–6 independent samples. Data was analyzed by two-way ANOVA followed by Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ respect to wild-type mice. (b) Representative immunohistochemical images depicting levels of p75^{NTR} in the striatum of WT and R6/1 mice at 30 weeks of age. The histogram represents the mean fluorescence intensity of p75^{NTR} (mean \pm S.E.M., $n = 3$) expressed as percentage of wild-type levels. Data was analyzed by Student's *t*-test ** $P < 0.01$ respect to wild-type mice. Scale bar, 10 μ m. (c) The histogram represents the mean fluorescence intensity of p75^{NTR} staining along rostral to caudal striatal sections from WT and R6/1 mice at 30 weeks of age expressed as percentage of wild-type values (mean \pm S.E.M., $n = 3$). Data was analyzed by Student's *t*-test * $P < 0.05$; ** $P < 0.01$ respect to wild-type mice

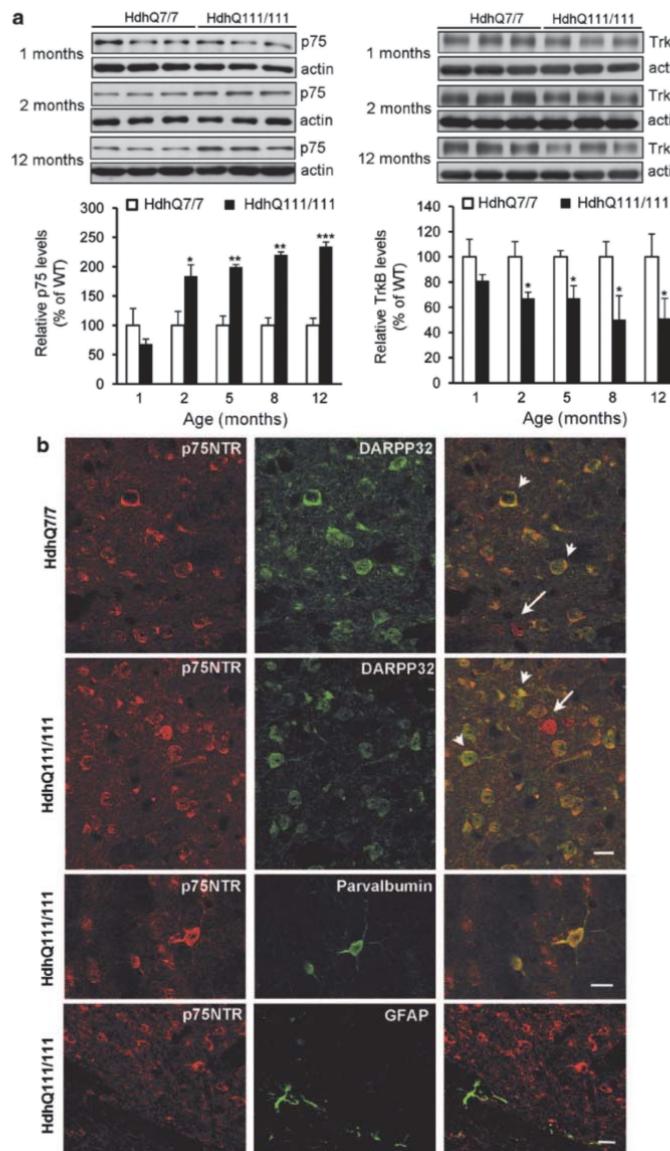


Figure 2 p75^{NTR} and TrkB protein levels are also modified in the striatum of full-length *Hdh*^{Q111/111} mutant mice. **(a)** Representative immunoblots showing the levels of p75^{NTR}, TrkB and β -actin as a loading control in striatal extracts obtained from wild-type *Hdh*^{Q7/7} and mutant *Hdh*^{Q111/111} mice at 1, 2 and 12 months of age. The histograms represent the relative levels of p75^{NTR} and TrkB expressed as percentage of wild-type values. Values are given as mean \pm S.E.M. of 5–6 independent samples. Data was analyzed by two-way ANOVA followed by Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ respect to wild-type mice. **(b)** (Upper panel) double immunostaining showing p75^{NTR} and DARPP32 in the striatum of wild-type (*Hdh*^{Q7/7}) and mutant (*Hdh*^{Q111/111}) mice at 12 months of age. Some cells are single-labeled for p75^{NTR} (arrows), whereas a high proportion of cells are double-labeled for p75^{NTR} and DARPP32 in both wild-type and HD mutant mice (arrowheads). (Middle panel) double immunostaining showing p75^{NTR} and parvalbumine staining in the striatum of mutant *Hdh*^{Q111/111} mice at 12 months of age. Most of the parvalbumine-positive neurons are also positive for p75^{NTR}. (Bottom panel) Double immunostaining showing p75^{NTR} and GFAP in the striatum of mutant (*Hdh*^{Q111/111}) mice at 12 months of age. p75^{NTR} and GFAP immunoreactivity demonstrates lack of colocalization. Scale Bar, 25 μ m



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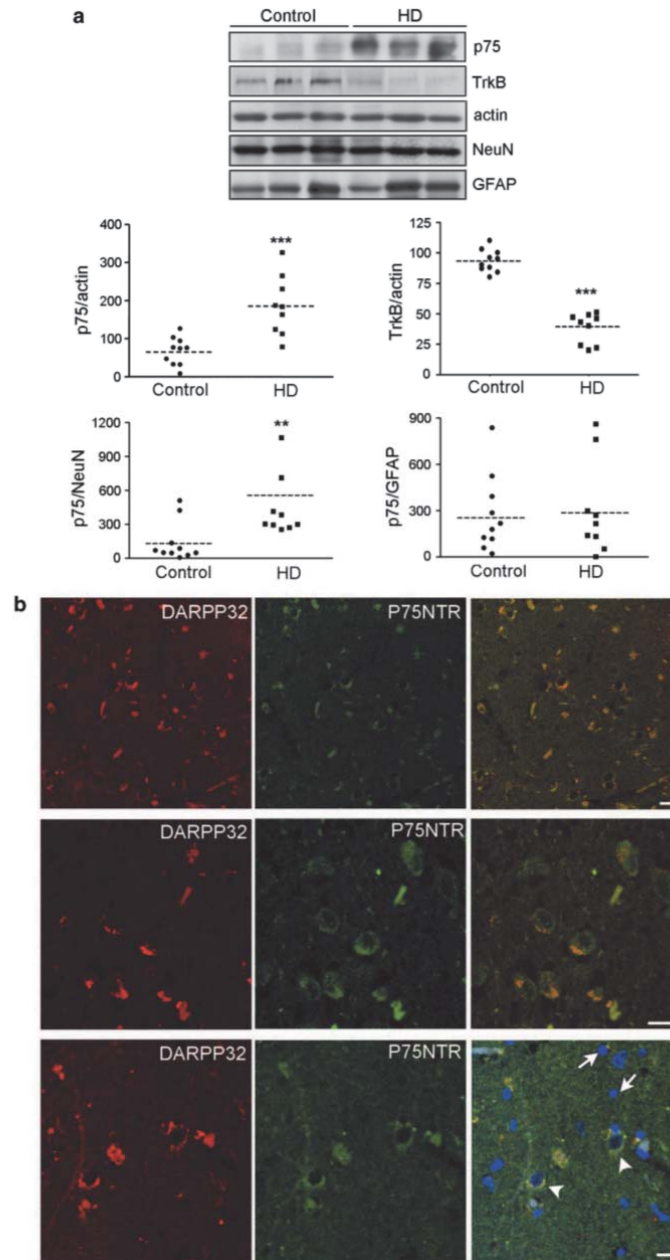


Figure 3 The putamen of HD patients shows altered p75^{NTR} and TrkB protein levels. **(a)** Human brain tissue from 10 control and 9 HD cases were analyzed by western blot. Immunoblots were probed with p75^{NTR}, TrkB, β -actin as loading control and NeuN and GFAP as neuronal and astroglial markers, respectively. Scatter plots display the relative levels of p75^{NTR} and TrkB normalized to actin, NeuN or GFAP. The horizontal line represents the normalized mean value in control and HD subjects. Data was analyzed using Student's *t*-test. ***P* < 0.01, ****P* < 0.001 compared with control. **(b)** Immunohistochemistry in caudate-putamen of HD brain (Vonsattel grade 1) showing extensive colocalization between DARPP32 (striatal neuronal marker) and p75^{NTR}. Nuclear staining was revealed by DAPI labeling to show that glia cells (arrows) are not positive for p75^{NTR} in contrast to neuronal cells (arrowheads). Scale bar, 10 μ m

differences were detected along the rostral-caudal striatum with the rostral sections having significantly more p75^{NTR} staining, which suggest a correlation between p75^{NTR} levels and striatal atrophy.

Knockin *Hdh*^{Q111/111} mutant mice also display p75^{NTR}/TrkB imbalance in the striatum. We next analyzed whether in a different HD mouse model expressing endogenous levels of full-length mutant huntingtin (*Hdh*^{Q111/111}) the expression of both receptors was also altered. No significant differences on p75^{NTR} levels between wild-type and mutant mice were found at 1 month of age while a significant increase was evident at 2 months (~80%), 5 and 8 months (~90%) and 12 months of age

(~135%) (Figure 2a). Similar to that observed in R6/1 mice the increase on p75^{NTR} was (1) progressive, starting at 2 months of age and worsening thereafter ($F_{(4,32)} = 7,415$; $P < 0.001$) and (2) associated with upregulation of p75^{NTR} mRNA expression (*Hdh*^{Q77/77}: 1.0 ± 0.1 and *Hdh*^{Q111/111}: 1.8 ± 0.3 , $P < 0.05$). When TrkB levels were examined we found a significant decrease (~40%) at early HD stages that was sustained along the disease progression (Figure 2a). Given that p75^{NTR} can be upregulated by neurons or glia¹⁹ double immunostaining for p75^{NTR}, DARPP32 and parvalbumin as GABAergic striatal neuronal markers and GFAP as astroglial marker was performed (Figure 2b). Confocal analysis revealed that the majority of DARPP32-positive cells were also positive for p75^{NTR} indicating a neuronal distribution of p75^{NTR}. We also observed p75^{NTR}-positive cells that were negative for DARPP32 that could correspond to parvalbumin interneurons. Double immunolabeling confirm colocalization between p75^{NTR} and parvalbumin indicating that p75^{NTR} was also localized in these GABAergic interneurons. Importantly, p75^{NTR} immunoreactivity failed to colocalize with GFAP demonstrating that p75^{NTR} was not detectable in astrocytes.

Changes on p75^{NTR} and TrkB protein levels are manifested in the putamen of HD patients. Next we analyzed whether aberrant p75^{NTR} and TrkB protein levels were recapitulated in HD human brain. Upon normalization to actin we found in HD samples a significant increase in p75^{NTR} (~100%) together with a decrease in TrkB (~60%) levels compared with control samples (Figure 3a). The levels of p75^{NTR} were also normalized to NeuN and GFAP. Normalization to GFAP yielded no significant changes compared with control samples while a significant increase was found when normalized to NeuN (~300%). To further

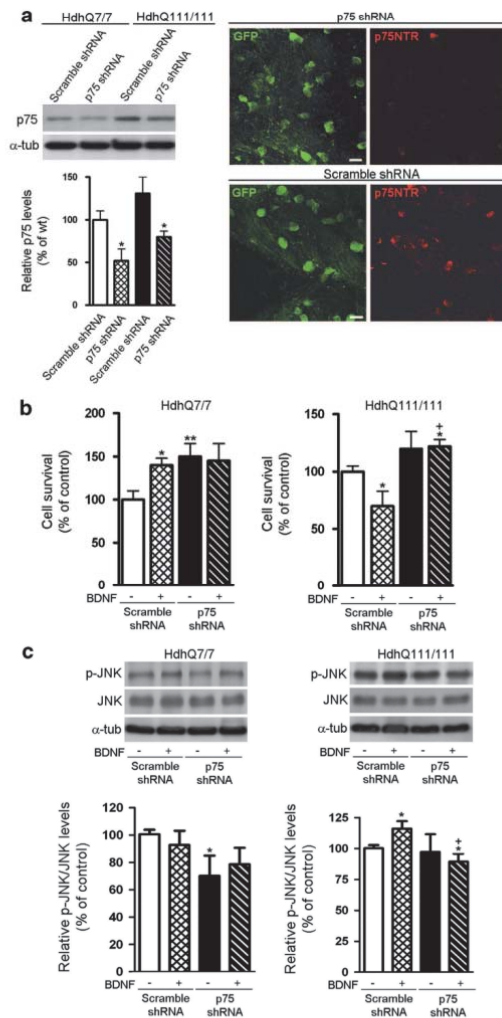


Figure 4 BDNF induces proapoptotic signaling cascades in corticostriatal slices from mutant *Hdh*^{Q111/Q111} mice. Seven-month-old wild-type (*Hdh*^{Q77/77}) and mutant (*Hdh*^{Q111/111}) mice were injected with GFP-AAV scramble shRNA (scramble shRNA) or GFP-AAV-p75shRNA (p75shRNA) and corticostriatal slices obtained 1 month later. Slices were incubated with BDNF (200 ng/ml) and cell survival determined by a CKKB assay 24 h later. (a) (Left) Representative immunoblots showing the levels of p75^{NTR} and α -tubulin as a loading control in extracts obtained from corticostriatal slices obtained from wild-type *Hdh*^{Q77/77} and mutant *Hdh*^{Q111/111} mice injected with AAV expressing scramble shRNA or p75 shRNA. The histogram represents the relative levels of p75^{NTR} expressed as percentage of wild-type values. Values are given as mean \pm S.E.M. of four slices/condition/experiment ($n = 3$). Data was analyzed by Student's *t*-test * $P < 0.05$ respect to wild-type mice. (Right) Confocal microscopy of corticostriatal slices transduced with AAV expressing p75shRNA or scramble shRNA and labeled for p75^{NTR} expression (red). Transduction of p75shRNA results in lack of p75^{NTR} expression as indicated by the absence of red p75^{NTR} labeling in GFP-positive cells. (b) Histograms showing the quantification of cell survival expressed as the percentage of scramble shRNA transduced cells (control). Values are given as mean \pm S.E.M. of four slices/condition/experiment ($n = 3$). Data was analyzed by one-way ANOVA followed by Turkey's Multiple comparison test or Student's *t*-test * $P < 0.05$; ** $P < 0.01$ respect to wild-type mice, + $P < 0.05$ respect to scramble shRNA + BDNF treatment. (c) Representative immunoblots showing the levels of JNK, p-JNK and α -tubulin as a loading control in extracts obtained from corticostriatal slices obtained from wild-type *Hdh*^{Q77/77} and mutant *Hdh*^{Q111/111} mice and incubated with BDNF for 24 h. The histograms represent the relative p-JNK/JNK ratios considering 100% the ratio obtained in scramble shRNA conditions (control). Values are given as mean \pm S.E.M. of three independent experiments. Data was analyzed by Student's *t*-test * $P < 0.05$ respect to wild-type mice, + $P < 0.05$ respect to scramble shRNA + BDNF treatment



confirm the neuronal distribution of p75^{NTR} in HD human brain double immunolabeling for p75^{NTR} and DARPP32 was performed in HD caudate-putamen brain sections. Confocal analysis demonstrated colocalization of p75^{NTR} and DARPP32 revealing that p75^{NTR} is primary neuronal in HD human brain, which agrees with our immunohistochemistry data in knockin HD mutant mice. Consistently, glia cells identified by DAPI staining as cells with bright and condensed nuclei were negative for p75^{NTR} immunostaining.

BDNF treatment reduces the cell survival of corticostriatal slices from knockin *Hdh*^{Q111/111} mutant mice. Given the importance of TrkB to mediate BDNF neuroprotection we analyzed whether the p75^{NTR}/TrkB imbalance in HD might influence BDNF-induced cell survival. To this aim adeno-associated viruses (AAV) expressing p75 shRNA or scramble shRNA were bilaterally injected into the striatum of wild-type and HD mice, corticostriatal slices obtained and cell survival analyzed following BDNF treatment (Figure 4). First, we examined viability of tissue slices by fluorescence microscopy using calcein as a vital dye and propidium iodide as a marker for cell death. An optimal preservation of slices as judged from the cell viability achieved was found (Supplementary Figure S1a). Then we analyzed the efficiency of AAV-p75shRNA to knockdown p75^{NTR}. Western blot analysis demonstrated a significant reduction of p75^{NTR} in wild-type and mutant corticostriatal slices (~50% and 40%, respectively; $P < 0.05$). Histological analysis by confocal microscopy further confirmed lack of p75^{NTR} expression in neurons transduced with p75shRNA (Figure 4a). Finally, cell survival was evaluated in BDNF-treated slices (Figure 4b). BDNF incubation increased the cell survival of wild-type corticostriatal slices (~40%) while a significant reduction was found in HD slices (~30%) suggesting that p75^{NTR}/TrkB imbalance negatively modulates BDNF neuroprotection. Interestingly, reduction of p75^{NTR} levels in the striatum of wild-type mice increased the survival of corticostriatal slices (~50%) while in HD mice not only increased the cell survival but also prevented the cell death induced by BDNF (Figure 4b). Because JNK activation mediates the proapoptotic actions of neurotrophins^{20,21} we next determined whether the cell death induced by BDNF was associated with increased JNK phosphorylation. A significant increase (~20%) in p-JNK levels was found in extracts obtained from mutant corticostriatal slices treated with BDNF suggesting a correlation between levels of p-JNK and cell death (Figure 4c). Consistent with this idea reduction of p75^{NTR} in wild-type and HD mice significantly decreased JNK phosphorylation (~25% and ~15%, respectively, Figure 4c).

Reduced cell survival of GFP-p75-transfected *STHdh*^{111/111Q} mutant cells after BDNF treatment is associated with activation of JNK pathway. To further analyze the role of p75^{NTR}/TrkB imbalance in BDNF striatal neuroprotection immortalized striatal cell lines that express wild-type (*STHdh*^{7/7Q}) or mutant full-length huntingtin (*STHdh*^{111/111Q}) were used. These mutant cells exhibit reduced levels of TrkB³ but lack p75^{NTR} expression (Supplementary Figure S2b). Therefore, to mimic the p75^{NTR}/TrkB imbalance

observed in HD mutant mice, *STHdh*^{7/7Q} and *STHdh*^{111/111Q} cells were transfected with GFP or GFP-p75^{NTR} (Supplementary Figure S2b) and then incubated with BDNF. BDNF treatment induced a reduction of cell survival (~20%) in GFP-p75 *STHdh*^{111/111Q} cells but not in GFP-p75 *STHdh*^{7/7Q} cells (Figure 5a) consistent with our data in HD corticostriatal slices. Next, we analyzed whether the reduction on cell survival was also related with increased JNK phosphorylation. A significant increase in p-JNK levels (~20%) without changes in total JNK were found in *STHdh*^{111/111Q} cells expressing GFP-p75 but not GFP (Figure 5b). Then we analyzed whether BDNF-mediated activation of Akt and ERK1/2 pathways was preserved in *STHdh*^{7/7Q} and *STHdh*^{111/111Q} cells transfected with GFP-p75. It is important to mention that *STHdh*^{111/111Q} cells exhibit increased p-Akt and decreased p-ERK levels at basal conditions,^{3,22} levels that were not altered by overexpression of p75^{NTR} (Supplementary Figure S2c). Western blot analysis revealed that BDNF treatment induced a significant increase in Akt phosphorylation in both *STHdh*^{7/7Q} and *STHdh*^{111/111Q} cells transfected with GFP (~100%) and GFP-p75 (~200%) (Figure 5c). Interestingly, the increase on Akt phosphorylation was significantly higher in GFP-p75-transfected cells than in GFP cells suggesting that overexpression of p75^{NTR} facilitates TrkB-mediated Akt activation. When ERK1/2 phosphorylation was analyzed we found that BDNF treatment induced a robust and similar activation (~140%) of p-ERK1/2 in GFP and GFP-p75 *STHdh*^{7/7Q} cells (Figure 5c). In contrast, ERK1/2 phosphorylation was not increased in BDNF-treated GFP or GFP-p75 *STHdh*^{111/111Q} cells, which agrees with our own published data showing impaired TrkB-mediated ERK1/2 activation in mutant cells.³ These findings indicate that altered p75^{NTR}/TrkB expression modifies BDNF-mediated neuroprotection by increasing the activation of the proapoptotic pathway JNK.

BDNF fails to protect GFP-p75-transfected *STHdh*^{111/111Q} mutant cells against NMDA-induced excitotoxicity. BDNF-mediated TrkB signaling is required to protect striatal neurons against NMDA-induced excitotoxicity,²³ which is well known to contribute to the striatal cell loss in HD.¹⁶ Therefore, we investigated whether the p75^{NTR}/TrkB imbalance could affect BDNF neuroprotection against NMDA. GFP or GFP-p75 transfected *STHdh*^{7/7Q} and *STHdh*^{111/111Q} cells were treated with BDNF before NMDA exposure and cell survival was analyzed 24 h later (Figure 6). NMDA treatment induced a significant reduction of cell survival in both *STHdh*^{7/7Q} (~30%) and *STHdh*^{111/111Q} cells (~50%) (Figure 6a). Addition of BDNF before NMDA completely prevented NMDA-induced cell death in *STHdh*^{7/7Q} cells independently of p75^{NTR} expression (Figure 6a). Surprisingly, in GFP-p75 *STHdh*^{111/111Q} cells BDNF treatment not only failed to protect striatal cells against NMDA-induced cell death but also potentiated it (~20%). As activation of caspase-3 following NMDA treatment was described in this mutant huntingtin cell line²⁴ we evaluated whether caspase-3 activation was involved in the lack of BDNF neuroprotection against NMDA (Figure 6b). Similar caspase-3 activation was found in GFP or GFP-p75 mutant cells following NMDA incubation (~40%). However, we found that BDNF

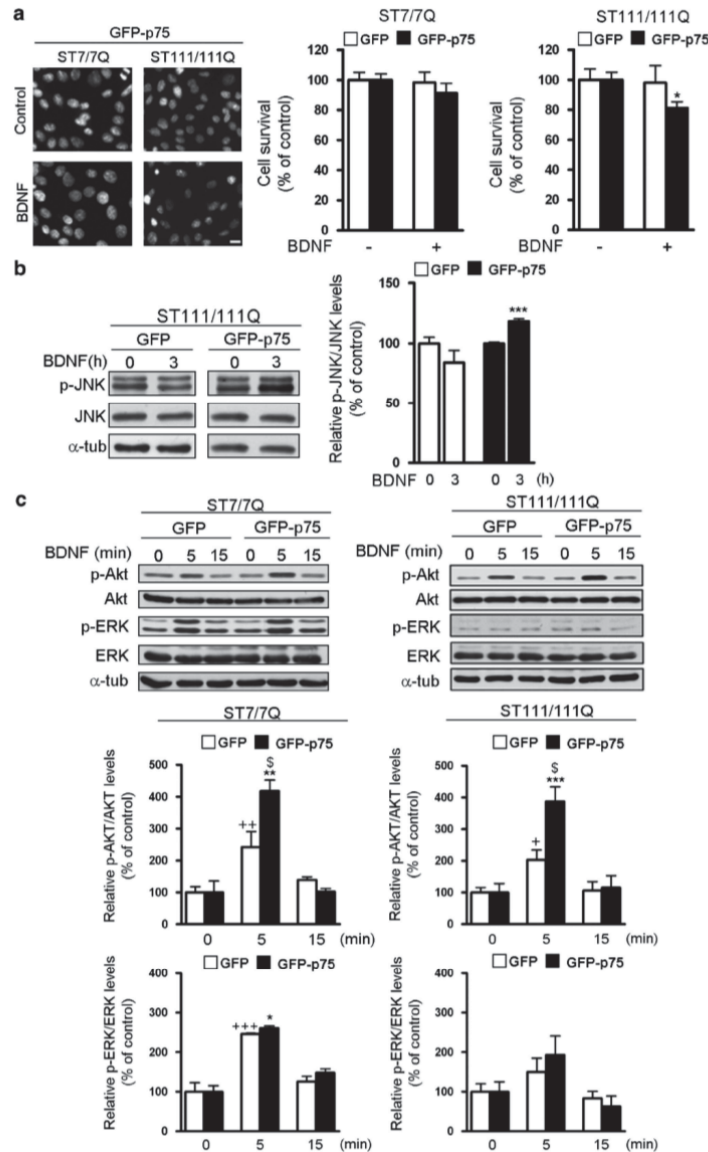


Figure 5 BDNF-mediated reduction of the survival of GFP-p75 *ST7/7Q* and *ST111/111Q* mutant cells is associated with activation of apoptotic pathways. Wild-type (*ST7/7Q*) and mutant (*ST111/111Q*) huntingtin striatal cells transfected with GFP or GFP-p75 were treated with BDNF (50 ng/ml, 30 min) and cell survival was evaluated 24 h later by scoring the percentage of Hoechst-stained nuclei. (a) Photomicrographs of *ST7/7Q* and *ST111/111Q* cells stained with Hoechst showing a significant reduction of cell survival in BDNF-treated GFP-p75 *ST111/111Q* cells. Scale bar, 10 μ m. Quantification of surviving cells is shown as the percentage of total cells in control conditions. The results are representative of seven independent experiments performed in triplicate and are expressed as the mean \pm S.E.M. Data was analyzed by one-way ANOVA followed by Student's *t*-test **P* < 0.05 versus vehicle-treated GFP-p75 mutant cells. (b) Representative immunoblot showing the levels of JNK, p-JNK and α -tubulin as a loading control in striatal extracts obtained from mutant *ST111/111Q* cells incubated with BDNF (50 ng/ml, 3 h). The histogram represents the relative p-JNK/JNK ratio considering 100% the ratio obtained in control conditions (vehicle). Values are given as mean \pm S.E.M. Data was analyzed by Student's *t*-test. ****P* < 0.001 versus vehicle-treated GFP-p75 cells. (c) Representative immunoblots showing the levels of Ser-473-Akt (p-Akt), Akt, phosphoERK1/2 (p-ERK1/2), ERK1/2 and α -tubulin as a loading control in striatal extracts obtained from wild-type (*ST7/7Q*) and mutant (*ST111/111Q*) cells transfected with GFP or GFP-p75 and treated with BDNF (50 ng/ml) for the indicated time points. Blots are representative of four independent experiments. The histograms represent the relative p-Akt/Akt and p-ERK/ERK ratios considering 100% the ratio obtained in control condition (vehicle). Values are given as mean \pm S.E.M. Data was analyzed by Student's *t*-test. † *P* < 0.05, †† *P* < 0.01 versus vehicle-treated GFP cells, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus vehicle-treated GFP-p75 cells, \$*P* < 0.05 versus BDNF-treated GFP cells

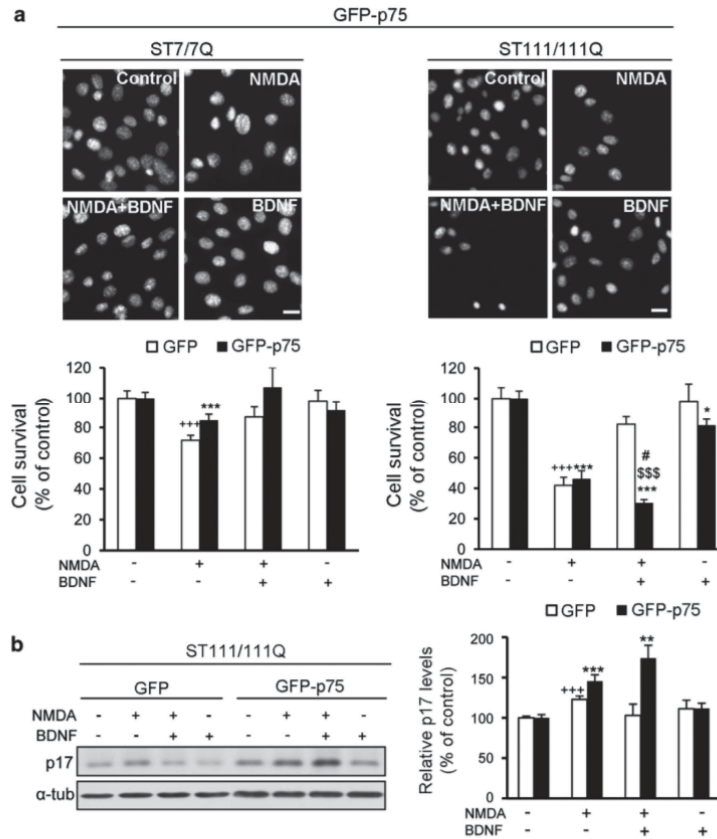


Figure 6 BDNF fails to protect GFP-p75 *STHdh*^{111/111Q} mutant cells against NMDA excitotoxicity. Wild-type (ST7/7Q) and mutant (ST111/111Q) huntingtin striatal cells transfected with GFP or GFP-p75 were treated with BDNF (50 ng/ml, 30 min), NMDA (500 μ M, 30 min) or BDNF before addition of NMDA (NMDA + BDNF) and cell survival was evaluated 24 h later by scoring the percentage of Hoechst-stained nuclei. (a) Representative photomicrographs of wild-type and mutant huntingtin striatal cells stained with Hoechst showing the number of surviving cells in the different conditions (Control, NMDA, NMDA + BDNF and BDNF) Scale bar, 10 μ m. Quantification of surviving cells is shown as the percentage of total cells in control conditions (vehicle). The results are representative of seven independent experiments performed in triplicate and are expressed as the mean \pm S.E.M. Data was analyzed by one-way ANOVA followed by Student's *t*-test. +++*P* < 0.001 versus vehicle-treated GFP cells, **P* < 0.05, ****P* < 0.001 versus vehicle-treated GFP-p75 cells, [#]*P* < 0.05 NMDA-treated GFP-p75 cells versus NMDA + BDNF-treated GFP-p75 cells, \$\$\$*P* < 0.001 NMDA + BDNF-treated GFP mutant cells versus NMDA + BDNF-treated GFP-p75 mutant cells. (b) Mutant huntingtin cells (ST111/111Q) transfected with GFP or GFP-p75 were treated with BDNF, NMDA or BDNF before NMDA and cell extracts obtained 3 h later. Immunoblots were performed to detect cleaved caspase-3 (p-17 fragment) and α -tubulin as loading control. The blot is representative of four independent experiments. The histogram represents the relative p-17 levels considering 100% the value obtained in control conditions (vehicle). Values are given as mean \pm S.E.M. Data was analyzed by one-way ANOVA followed by Student's *t*-test. +++*P* < 0.001 versus vehicle-treated GFP mutant cells and ***P* < 0.01, ****P* < 0.001 versus vehicle-treated GFP-p75 mutant cells

completely blocked NMDA-induced activation of caspase-3 in GFP mutant cells while a significant increase (~75%) was still observed in GFP-p75 mutant cells consistent with the lack of BDNF neuroprotection against NMDA.

Lack of BDNF neuroprotection against NMDA correlates with decreased Akt phosphorylation in GFP-p75 *STHdh*^{111/111Q} mutant cells. As BDNF did not protect GFP-p75 *STHdh*^{111/111Q} cells against NMDA we analyzed activation of Akt and ERK1/2 following BDNF and NMDA treatment. GFP and GFP-p75 transfected *STHdh*^{7/7Q} and

STHdh^{111/111Q} cells were incubated with NMDA alone or with BDNF before NMDA and Akt and ERK1/2 phosphorylation analyzed by western blot (Figure 7a). NMDA treatment induced a significant increase in p-Akt levels in both *STHdh*^{7/7Q} (GFP: ~80% and GFP-p75: ~150%) and *STHdh*^{111/111Q} cells (~100%) independently of p75^{NTR} transfection. We then analyzed whether BDNF treatment could modify this NMDA-induced Akt phosphorylation. We found that BDNF prevented NMDA-mediated Akt phosphorylation in *STHdh*^{7/7Q} cells (GFP or GFP-p75) suggesting that BDNF desensitize wild-type cells to further Akt activation by

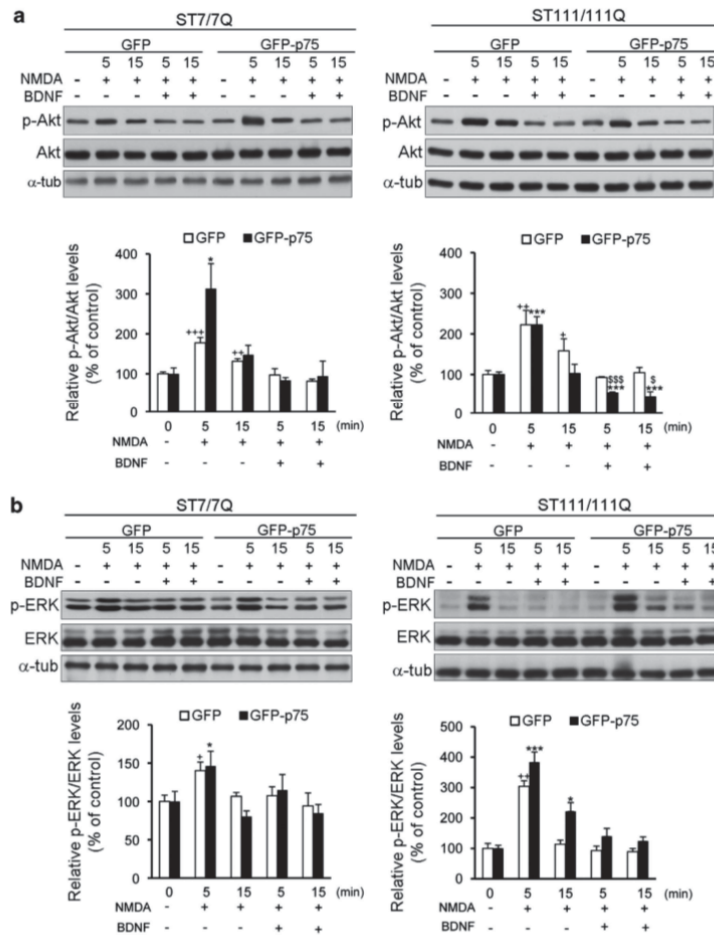


Figure 7 BDNF prevents NMDA-induced phosphorylation of Akt in GFP-p75 *STHdh*^{111/111Q} mutant cells. Wild-type (ST7/7Q) and mutant (ST111/111Q) huntingtin striatal cells transfected with GFP or GFP-p75 were treated with BDNF (50 ng/ml, 30 min), NMDA (500 μ M, 30 min) or BDNF before addition of NMDA (NMDA + BDNF) for the indicated time periods. Cells were lysed and immunoblots performed to detect Ser-473-Akt (p-Akt) and Akt (a) or phosphoERK1/2 (p-ERK1/2) and ERK1/2 (b). α -tubulin was used as a loading control. The blots are representative of four independent experiments. The histograms represent the relative p-Akt/Akt and p-ERK/ERK ratios considering 100% the ratio obtained in control conditions (vehicle). Values are given as mean \pm S.E.M. Data was analyzed by Student's *t*-test. ⁺ $P < 0.05$, ⁺⁺ $P < 0.01$, ⁺⁺⁺ $P < 0.001$ versus vehicle-treated GFP cells, ^{*} $P < 0.05$ and ^{***} $P < 0.001$ versus NMDA + BDNF-treated GFP-p75 cells, ² $P < 0.05$, ³³³ $P < 0.001$ NMDA + BDNF-treated GFP mutant cells versus NMDA + BDNF-treated GFP-p75 mutant cells

NMDA. Similar results were found when GFP- *STHdh*^{111/111Q} cells were analyzed. However, in GFP-p75 *STHdh*^{111/111Q} cells BDNF preincubation not only prevented NMDA-induced phosphorylation of Akt but also decreased to levels below those in control mutant cells (~50%, Figure 7a). NMDA treatment induced a significant increase in p-ERK1/2 levels in both *STHdh*^{7/7Q} (~50%) and *STHdh*^{111/111Q} cells (~250%). Similar to that observed for Akt, BDNF pretreatment prevented subsequent NMDA-mediated ERK1/2 phosphorylation independently of p75^{NTR} transfection. These results indicate that p75^{NTR}/TrkB imbalance disturbs

BDNF-mediated neuroprotection against NMDA by reducing Akt phosphorylation.

Reduced Akt phosphorylation in GFP-p75-transfected *STHdh*^{111/111Q} mutant cells correlates with increased PP1 levels. Akt phosphorylation at Ser-473 can be regulated by the activity of PP1 and PHLPP1 phosphatases.^{25–27} As we observed decreased Akt Ser-473-phosphorylation in GFP-p75 *STHdh*^{111/111Q} cells after BDNF and NMDA treatment, PP1 and PHLPP1 levels were evaluated. A significant increase in PP1 levels was found in GFP

RESULTS

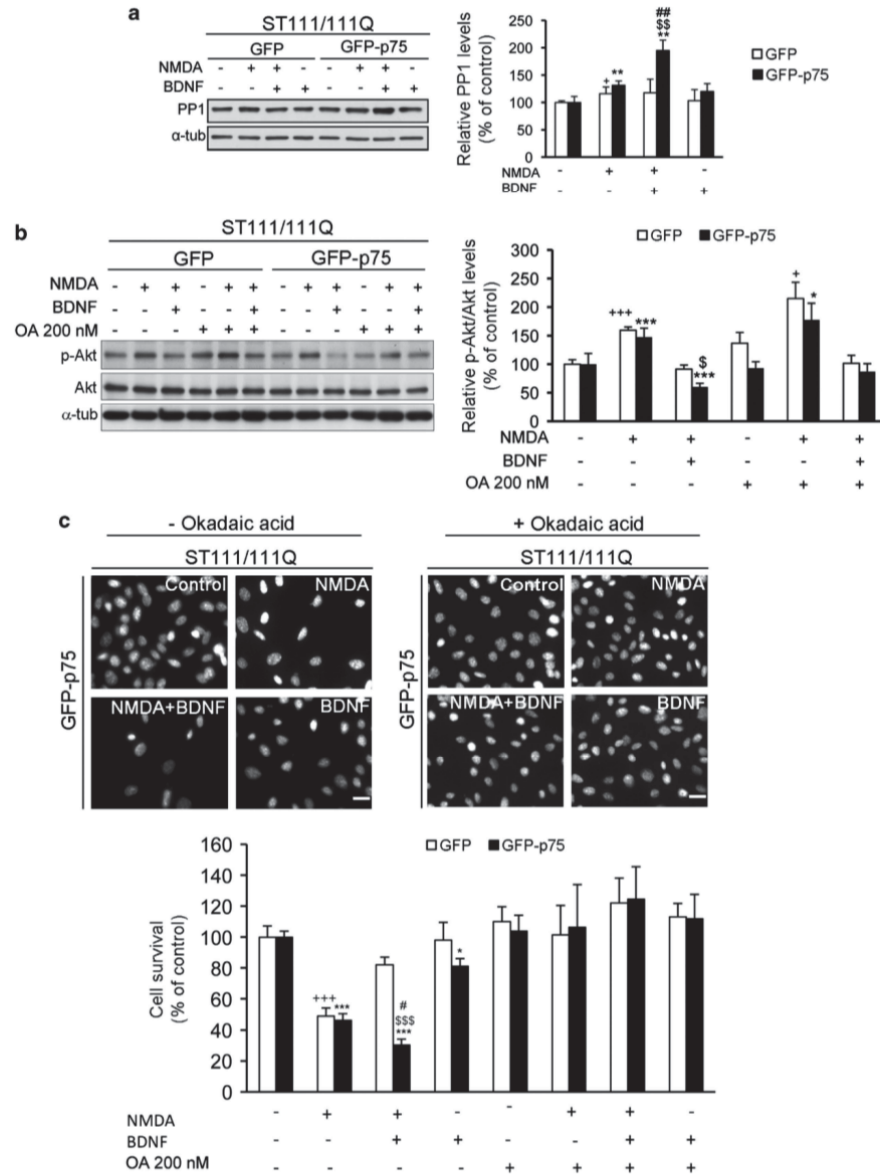


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(~20%) and GFP-p75 (~30%) *STHdh*^{111/111Q} cells following NMDA treatment (Figure 8a). Importantly, in GFP-p75 but not in GFP mutant cells incubation with BDNF before NMDA significantly increased PP1 levels (~80% compared with untreated cells and ~50% compared with NMDA-treated cells). No differences on PHLPP1 levels were found between

GFP and GFP-p75 mutant cells at any analyzed condition (Supplementary Table 2).

Inhibition of PP1 restores Akt phosphorylation and BDNF neuroprotection in GFP-p75-transfected *STHdh*^{111/111Q} mutant cells. To confirm a functional role



for PP1 in the lack of BDNF neuroprotection GFP and GFP-p75-transfected *STHdh*^{111/111Q} cells were treated with OA to inhibit PP1 activity and p-Akt levels analyzed by western blot (Figure 8b). Inhibition of PP1 in GFP-p75 *STHdh*^{111/111Q} cells blocked the reduction of Akt phosphorylation induced by BDNF treatment. Moreover and consistent with a detrimental role of PP1 on BDNF neuroprotection the cell death of GFP-p75 *STHdh*^{111/111Q} cells induced by BDNF and/or NMDA was completely prevented by pretreatment with OA (Figure 8c). Altogether, these results support the involvement of PP1 in the increased p75-mediated vulnerability of mutant huntingtin striatal cells to NMDA excitotoxicity.

Discussion

Several evidences implicate reduced levels of BDNF in HD pathology, which has emphasized the study of therapeutic strategies based on BDNF administration for HD treatment.^{1,28,29} BDNF-mediated neuronal survival is primarily associated with activation of TrkB receptors.^{30,31} However, BDNF can also binds to p75^{NTR}, which could either potentiate Trk function^{9,32–34} or signal independently to activate cell death cascades.^{20,35,36} In this study, we found an imbalance between p75^{NTR} and TrkB expression in two distinct HD mouse models: *Hdh*^{Q111/111} mutant knockin mice and R6/1 transgenic mice that was also manifested in the putamen of HD patients supporting the idea that p75^{NTR}/TrkB imbalance is relevant to HD pathology. p75^{NTR} upregulation was associated with increased p75^{NTR} mRNA expression, which agrees with previous studies showing increased p75^{NTR} and reduced TrkB mRNA levels in the caudate of HD patients.⁴ Deregulation of gene transcription has been implicated in HD pathology. In particular upregulation of the transcription factor Sp1 has been described in cellular and transgenic models of HD suggesting that increased Sp1-mediated transcription could contribute to HD pathology.³⁷ Interestingly, p75^{NTR} expression is dependent on binding of Sp1 to the p75^{NTR} proximal promoter,^{38,39} which allows us to speculate that aberrant Sp1 activity could underlie the upregulation of p75^{NTR} mRNA in HD. Our data also demonstrate that upregulation of p75^{NTR} protein levels in HD brain follows a rostro-caudal gradient with higher p75^{NTR} expression at the rostral striatum. Notably, brain tomography studies

demonstrate severe atrophy of the head caudate early in the disease process along the rostro-caudal axis⁴⁰ suggesting a correlation between increased p75^{NTR} levels and striatal atrophy and dysfunction. Consistent with this hypothesis we found a good colocalization between p75^{NTR} and DARPP32, a well known marker for striatal medium spiny neurons, the most vulnerable neurons in HD. In fact, this is the first time showing p75^{NTR} expression in DARPP32 neurons as most of the previous studies reported p75^{NTR} immunoreactivity in large cholinergic interneurons.¹¹ In this new scenario, it is important to determine whether the imbalance between p75^{NTR} and TrkB expression could affect BDNF neuroprotection. Our studies demonstrate reduced cell survival of *STHdh*^{111/111Q} mutant cells and corticostriatal slices from *Hdh*^{Q111/111} mutant mice after BDNF treatment supporting the idea that altered expression of BDNF receptors could contribute to disturbing BDNF neuronal protection. This result agrees with previous studies showing BDNF-mediated cell death of hippocampal neurons expressing high levels of p75^{NTR} but lacking TrkB or TrkC receptors.^{20,36} Moreover, we demonstrate that BDNF-induced cell death was associated with a significant activation of the JNK pathway that was reported to have an important role in p75^{NTR}-mediated cell death.^{41,42} These findings together with our published data showing lack of BDNF-mediated activation of ERK1/2 in mutant huntingtin cells (Figure 6 and Gines *et al.*³) suggest that mutant huntingtin induces neuronal cell death not only by altering p75^{NTR} and TrkB expression but also their downstream signaling. In view of this data, we propose that aberrant BDNF signaling could participate in the increased susceptibility of mutant huntingtin striatal cells to NMDA excitotoxicity. In support of this hypothesis we found that BDNF incubation before NMDA treatment not only fails to reduce GFP-p75 mutant huntingtin cell death but potentiates it. In association with increased cell death we found higher caspase-3 activation, which is known to be crucial for cell death triggered by over-activation of p75^{NTR}.^{36,43} Similarly, reduced p75^{NTR}/TrkB ratio as a consequence of seizure induction in rats also promotes neuronal cell death that was associated with increased binding of BDNF to p75^{NTR}.⁴⁴ Besides increased JNK activation lack of BDNF neuroprotection against NMDA was also related with reduced Akt activation. Dephosphorylation of Akt depends on several

Figure 8 Lack of BDNF neuroprotection against NMDA excitotoxicity is mediated by PP1-induced dephosphorylation of Akt in GFP-p75 *STHdh*^{111/111Q} mutant cells. Mutant huntingtin (ST111/111Q) striatal cells transfected with GFP or GFP-p75 were incubated with BDNF (50 ng/ml, 30 min), NMDA (500 μ M, 30 min) or BDNF before NMDA treatment and cell extracts obtained 15 min later. (a) Representative immunoblot showing the levels of PP1 and α -tubulin as loading control. The blot is representative of five independent experiments. The histogram represents the relative PP1 levels considering 100% the value obtained in control conditions (vehicle). Values are given as mean \pm S.E.M. Data was analyzed by Student's *t*-test. [†]*P* < 0.05 versus vehicle-treated GFP cells, ^{**}*P* < 0.01 versus vehicle-treated GFP-p75 cells, ^{##}*P* < 0.01 NMDA + BDNF-treated GFP-p75 cells versus NMDA-treated GFP-p75 cells, ^{§§}*P* < 0.01 NMDA + BDNF-treated GFP cells versus NMDA + BDNF-treated GFP-p75 cells. (b) Mutant huntingtin striatal cells (ST111/111Q) transfected with GFP or GFP-p75 were treated with the PP1 inhibitor OA (200 nM, 30 min) before incubation with BDNF (50 ng/ml, 30 min), NMDA (500 μ M, 30 min) or BDNF and NMDA (NMDA + BDNF). Cell extracts were obtained 15 min later and immunoblots performed to detect Ser 473-Akt (p-Akt), Akt and α -tubulin as a loading control. The blots are representative of four independent experiments. The histograms represent the relative p-Akt/Akt ratio considering 100% the ratio obtained in control conditions (vehicle). Values are given as mean \pm S.E.M. Data was analyzed by Student's *t*-test. [†]*P* < 0.05, ^{†††}*P* < 0.001 versus vehicle-treated GFP cells, ^{*}*P* < 0.05, ^{***}*P* < 0.001 versus vehicle-treated GFP-p75 cells, [§]*P* < 0.05 NMDA + BDNF-treated GFP cells versus NMDA + BDNF-treated GFP-p75 cells. (c) Mutant huntingtin striatal cells (ST111/111Q) transfected with GFP or GFP-p75 were treated with or without OA (200 nM, 30 min) before incubation with BDNF (50 ng/ml, 30 min) and then exposed to NMDA (500 μ M, 30 min). Cell survival was evaluated 24 h later by scoring the percentage of Hoechst-stained nuclei. Representative photomicrographs of wild-type (ST7/7Q) and mutant (ST111/111Q) huntingtin cells stained with Hoechst showing the number of surviving cells in the different conditions (Control, NMDA, NMDA + BDNF and BDNF). Scale bar, 10 μ m. Quantification of surviving cells is shown as the percentage of total cells in control conditions (vehicle). The results are representative of seven independent experiments performed in triplicate and are expressed as the mean \pm S.E.M.



Ser/Thr phosphatases. PP2-A dephosphorylates Akt at Thr-308,⁴⁵ whereas PP1 and PHLPP1 at Ser-473.^{25,27} No changes on PHLPP1 levels were found in BDNF/NMDA-treated GFP-p75 mutant huntingtin cells revealing that this phosphatase unlikely contributes to Akt inactivation. By contrast, PP1 levels were significantly increased following BDNF and NMDA treatment suggesting a mechanistic link between PP1 and Akt inactivation. Supporting this view, BDNF/NMDA-induced dephosphorylation of Akt was blocked by inhibition of PP1 by OA treatment. Moreover, we also demonstrate that PP1-mediated dephosphorylation of Akt contributes to mutant huntingtin cell death as inhibition of PP1 prevented BDNF/NMDA-induced cell death of GFP-p75 mutant cells. Interestingly deregulation of PP1 activity in HD mice has been associated with NMDA-mediated striatal cell death⁴⁶ and NMDA-induced cell death of retinal ganglion cells has been related with PP1-mediated Akt dephosphorylation.²⁶ Altogether, this data suggest that a dysfunctional signaling cross-talk between TrkB, p75^{NTR} and NMDAR could contribute to increase striatal cell vulnerability in HD (Supplementary Figure 3). These results may have important consequences for the potential use of BDNF as a therapeutic agent in HD. Thus, exogenous BDNF administration or pharmacological treatments that raise BDNF levels only ameliorate or partially improve morphological phenotypes or motor and cognitive behavior in different HD mouse models,^{47–51} which could be related with the dysfunctional signaling of TrkB and p75^{NTR} demonstrated in the present study. In view of these results, a better understanding of the physiological interactions between p75^{NTR} and TrkB in HD would improve the design of new neuroprotective therapies for HD treatment

Materials and Methods

Chemicals and antibodies. BDNF was obtained from Peprotech EC Ltd (London UK), Okadaic Acid was obtained from Tocris (Minneapolis, MN, USA), NMDA (N-Methyl-D-aspartic acid) from Sigma-Aldrich (St. Louis, MO, USA) and Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA). Phospho p44/42 ERK1/2 (Thr202/Tyr204), Phospho-Akt (Ser-473), Phospho-SAPK/JNK (Thr183/Tyr185), total ERK1/2, total Akt and total SAPK/JNK were obtained from Cell Signaling Technology (Beverly, MA, USA). TrkB polyclonal antibody that recognizes the full-length TrkB isoform (sc-7268; C-tal epitope) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TrkB monoclonal antibody that recognizes full-length and truncated TrkB isoforms and anti-DARPP32 was purchased from BD Bioscience (San Jose, CA, USA). Anti-p75^{NTR} was obtained from Promega (Madison, WI, USA), anti-PP1 (Protein phosphatase 1); anti-parvalbumine and anti- α -tubulin were purchased from Sigma-Aldrich. Anti-green fluorescent protein (GFP) was obtained from Abcam (Cambridge, UK). β -actin (clone C4) was obtained from MP Biomedicals (Irvine, CA, USA), anti-NeuN was purchased from Millipore (Billerica, Bedford, MA, USA) and anti-GFAP was from Dako A/S (Glostrup, Denmark). IgG HRP-conjugated anti-mouse or anti-rabbit antibodies were purchased from Promega and Cy3 anti-rabbit, Cy2 anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Alexa Fluor 488 Goat anti-Rabbit IgG conjugate was from Invitrogen.

Cell cultures. Conditionally immortalized wild-type *STHdh*^{77Q} and mutant *STHdh*^{111/111Q} striatal neuronal progenitor cell lines expressing endogenous levels of normal and mutant full-length huntingtin with 7 and 111 glutamines, respectively, were generated from wild-type *Hdh*^{277T} and homozygous *Hdh*^{311/111} littermate embryos.²² The knockin models represent faithfully the HD mutation carried by patients as elongated polyglutamine tracts are placed within the correct context of the murine *Hdh* gene. Thus, immortalized striatal cells accurately express normal and mutant huntingtin and do not exhibit amino terminal inclusions, which allow us to study changes involved in early HD pathogenesis.

Striatal cells were grown at 33 °C in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin-penicillin, 2 mM L-glutamine, 1 mM sodium pyruvate and 400 μ g/ml G418 (Geneticin; Gibco-BRL, Gaithersburg, MD, USA). Mouse septal neuron \times neuroblastoma hybrid SN56 cells (cell line with negligible TrkB expression) and T48 cells (SN56 cells transfected done expressing TrkB) were created and kindly provided by Dr. KYe (Department of Pathology, Emory University School of Medicine, Atlanta). SN56 cells were maintained at 37 °C in DMEM medium containing 1 mM pyruvate and 10% FBS and T48 cells stably transfected with rat TrkB were cultured in the same medium but containing 300 μ g/ml G418.

Genetic Huntington's disease mouse models. *Hdh*^{Q111} knockin mice expressing mutant huntingtin with 111 glutamine residues were maintained on a C57BL/6 genetic background.⁵² *Hdh*^{Q77/111} heterozygous males and females were intercrossed to generate age-matched *Hdh*^{Q111/111} homozygous and *Hdh*^{Q77/111} wild-type littermates. R6/1 transgenic mice (B6CBA background) expressing exon-1 mutant huntingtin with 145 glutamines under the *HD* human promoter and their wild-type littermates were obtained from Jackson Laboratory (Bar Harbor, ME, USA).⁵³ The animals were housed with access to food and water *ad libitum* in a colony room kept at 19–22 °C and 40–60% humidity, under a 12:12 h light/dark cycle. All procedures were performed in compliance with the European Community guidelines for the care and use of the laboratory animals (86/609/EEC), and approved by the local animal care committee of Universitat de Barcelona (404/12) and Generalitat de Catalunya (DAAM5712).

Postmortem brain tissues. Samples of the putamen nucleus from one patient with HD grade 1 (73 years, postmortem intervals of 7 h), nine patients with HD grade 3 and 4 (71, 63, 65, 60, 47, 44, 40, 39, 28 years, postmortem intervals of 4–15 h) and 10 control cases (77, 74, 71, 68, 65, 64, 60, 59, 56, 39 years, postmortem intervals of 4–23 h) were supplied by the Banc de Teixits Neurològics (Servei Científic-Tècnics, Universitat de Barcelona, Barcelona, Spain). The Banc de Teixits Neurològics was established to provide human postmortem brains to researchers in Spain taking into due consideration all the ethical guidelines of the latest Declaration of Helsinki. Informed consent was obtained from all subjects under study.

Cell transfection. All DNA constructs were transfected using Lipofectamine 2000 as instructed by the manufacturer. Both wild-type *STHdh*^{77Q} and mutant *STHdh*^{111/111Q} huntingtin striatal cells were transfected at 50% of confluence with human p75 neurotrophin receptor tagged with GFP at the C-terminus (generously provided by Dr. Formaggio, Department of Medicine and Public Health, Section Pharmacology, University of Verona, Italy). As a control condition cells were transfected with GFP vector from Clontech (Palo Alto, CA, USA). Transfection efficiencies were estimated by flow cytometry and are shown in Supplementary Table 1.

Quantitative RT-PCR. Total RNA was isolated from striatum of HD mutant and wild-type mice using the Total RNA Isolation Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany). Purified RNA (500 ng) was reverse transcribed using the StrataScript First Strand cDNA Synthesis System (Stratagene, Santa Clara, CA, USA). The cDNA synthesis was performed at 42 °C for 60 min in a final volume of 20 μ l according to the manufacturer's instructions. The cDNA was then analyzed by quantitative RT-PCR using the following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): 18S (Hs99999901_s1) and p75 (Mm00446294_m1). RT-PCR was performed in 25 μ l of final volume on 96-well plates, in a reaction buffer containing 12.5 μ l TaqMan Gene Expression Assays and 20 ng of cDNA. Reactions included 40 cycles of a two-step PCR: 95 °C for 30 s and 60 °C for 1 min, after initial denaturation at 95 °C for 10 min. All quantitative-PCR assays were performed in duplicate and repeated in at least three independent experiments. To provide negative controls and exclude contamination by genomic DNA, the RT was omitted in the cDNA synthesis step, and the samples were subjected to the PCR reaction in the same manner with each TaqMan Gene Expression Assay. The quantitative-PCR data was quantified using the comparative quantitation analysis program of MxPro Q-PCR analysis software version 3.0 (Stratagene). The C_T value for each reaction, and the relative level of gene expression for each sample were calculated using the $2^{-\Delta\Delta C_T}$ method.⁵⁴ To correct for loading differences, the values were normalized according to the level of expression of the housekeeping gene, 18S, within each sample. Its C_T value

was subtracted from that of the specific genes to obtain a ΔC_T value. Differences ($\Delta\Delta C_T$) between the ΔC_T values obtained for the control mice (calibrators) and the ΔC_T values for the R6/1 or the $Hdh^{Q111/111}$ mice were determined. The relative quantitative value was then expressed as $2^{-\Delta\Delta C_T}$, representing the fold change in gene expression normalized to the endogenous control and relative to the calibrators.

Drug treatments of striatal cell lines. All drug treatments were performed 48 h after transfection. To induce NMDA excitotoxicity transfected wild-type $STHdh^{77Q}$ and mutant $STHdh^{111/111Q}$ huntingtin striatal cells were exposed to 500 μM NMDA for 30 min in Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 3.6 mM NaHCO₃, 5 mM HEPES, 5.6 mM glucose and 10 μM glycine). After NMDA treatment, medium was replaced by fresh DMEM medium containing 2.5% FBS and cell survival was analyzed 24 h later. To analyze the neuroprotective role of BDNF against NMDA-mediated excitotoxicity transfected wild-type and mutant huntingtin striatal cells were treated for 30 min in serum-free DMEM medium containing 50 ng/ml BDNF before NMDA exposure (500 μM , 30 min). After NMDA treatment medium was replaced by fresh DMEM medium containing 2.5% FBS and cell survival was analyzed 24 h post-treatment. To analyze basal BDNF signaling, transfected wild-type and mutant huntingtin striatal cells were placed in DMEM serum-free medium for 3 h and then exposed to BDNF (50 ng/ml) for different time periods, (0, 5 and 15 min). To study BDNF signaling in NMDA-treated conditions transfected wild-type and mutant huntingtin striatal cells were treated with BDNF (50 ng/ml) for 30 min before NMDA incubation (500 μM , 30 min) and total cell extracts were collected at different time periods (0, 5 and 15 min). To analyze apoptotic cascades (cleaved caspase-3 and phospho-JNK levels), cells were treated with BDNF (50 ng/ml, 30 min) or with BDNF before NMDA exposure (500 μM , 30 min) and cell extracts collected 3 h later. Finally, to test the role of protein phosphatase 1 (PP1) in HD pathology, mutant striatal cells were exposed to 200 nM of the PP1 inhibitor, OA (30 min) before BDNF treatment (50 ng/ml, 30 min). After BDNF incubation cells were treated with NMDA (500 μM , 30 min), medium replaced by fresh DMEM medium and PP1 levels analyzed by western blot in total extracts obtained 15 min post-treatment. In parallel experiment cells were fixed with 4% paraformaldehyde and cell survival analyzed 24 h later.

Cell survival. Cell survival of striatal cells was assessed by nuclear DNA staining with Hoechst 33258. Cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, washed twice in PBS, and stained with Hoechst 33258 (1 $\mu g/ml$) for 5 min. Stained cells were then washed twice with PBS and mounted under glass coverslips with Mowiol. Cell survival is represented as the proportion of Hoechst-stained nuclei counted in treated cells compared with the number of control cells (vehicle-treated). Forty fields were counted per condition and experiment, comprising at least 30–40 cells. Data are given as mean \pm S.E.M. of values obtained in seven independent experiments performed in triplicate.

AAV-mediated shp75 expression vectors. For knockdown p75^{NTR} expression we designed a siRNA oligomer targeting the mouse p75^{NTR}: Ngfr-1 (5' GACGCATCCAGAGAGCTGA 3'). This siRNA was then used to obtain the corresponding shRNA to clone into a rAAV2/8-GFP adenoviral vector (BamHI site at 5' and AgeI at the 3'). The rAAV2/8 plasmids and infectious AAV viral particles containing GFP expression cassette with scrambled shRNA (AAV-shRNAGFP) or p75shRNA (AAV-shp75GFP) were generated by the Unitat de Producció de Vectors from the Center of Animal Biotechnology and Gene Therapy at the Universitat Autònoma de Barcelona.

Intrastriatal injection of adenoviral vectors. Seven-months-old wild-type Hdh^{277T} and mutant $Hdh^{Q111/111}$ mice ($n=4-5$) were deeply anesthetized with pentobarbital (40–60 mg/Kg) and placed in a stereotaxic apparatus for bilateral intrastriatal injections of rAAV2/8 expressing shp75 or control shRNA (3 μl ; 1.53×10^9 GC). A small incision was made in the scalp, and the striatum was marked using the following stereotaxic coordinates to Bergman: anteroposterior (AP), +0.6 mm; mediolateral (ML), +2 mm; and 2.7 mm below the dural surface with the incisor bar at 3 mm above the interaural line. Viral vector was injected using a 10 μl -Hamilton microliter syringe at an infusion rate of 0, 5 $\mu l/min$; the needle was left in place for 5 min to ensure complete diffusion of the viruses and then slowly retracted from the brain. Four weeks after injections, the mice were killed to obtain corticostriatal organotypic cultures as mentioned below.

Corticostriatal organotypic cultures. Corticostriatal slice cultures were prepared by the interface culture method⁵⁵ slightly modified. In brief, 8-months-old wild-type Hdh^{277T} and mutant $Hdh^{Q111/111}$ mice were killed by instant decapitation and their brains were quickly removed and placed in a petri dish containing dissecting salt medium (MEMp: (50% (vol/vol) MEM, 25 mM HEPES and 2 mM glutamine without antibiotics) under sterile conditions. After removal of the meninges the brains were sectioned coronally (400 μm) by using a McIlwain tissue chopper (Mickle Laboratory, Cambridge, UK). The resulting brain slices were placed in incubation culture medium (MEMi: 50% (vol/vol) MEM, 25 mM HEPES, 25% (vol/vol) HBSS, 25% (vol/vol) heat-inactivated horse serum, 2 mM glutamine, 1 ml of penicillin/streptomycin solution and 0.044% (vol/vol) NaHCO₃). These slices were then trimmed to include the dorsal one half of the striatum with the overlying frontoparietal neocortex. From each donor mice an average of seven corticostriatal cultures were obtained using both hemispheres. The slices were stored for 1 h at 4 °C before being transferred to 24-well plates contained inserts of humidified porous membranes (0.4 μm Millicell, 12 mm diameter Millipore) in 250 μl of culture medium. The cultures were maintained at 37 °C in a 5% CO₂/95% air atmosphere at a relative humidity of 95%. Culture viability was first determined by subjective judgment of the transparency, color and border morphology. To validate the method, the viability of our corticostriatal organotypic slice cultures was analyzed using a standardized protocol for PI uptake. Briefly, 2 h after transferring the slices to the transwell, 20 μl of 0,1 mM PI was added to the organotypic culture. This concentration is kept in the medium during the 24 h period incubation. After 24 h, slices were then incubated (30 min at 37 °C) with 0.01% Calcein AM, a cell-permeant dye used to determine cell viability (Molecular Probes Inc., Eugene, OR, USA) and images were analyzed by confocal microscopy.

CCK-8 cytotoxicity assay. Cell viability of corticostriatal slices was assessed by WST-8 assay using the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, St. Louis, MO, USA), in which colored formazan was formed in viable cells in response to cellular dehydrogenase activity. Briefly, after BDNF treatment (200 ng/ml), 25 μl of Cell Counting Assay Kit-8 solution was added to each corticostriatal slice. After 3 h or 24 h of incubation, 100 μl of medium from each slice culture was dispensed in a 96-well plate and absorbance at 450 nm was measured using a microplate reader. The cell viability was calculated as the ratio between the absorbance measurements at 3 h and 24 h and the results expressed as the percentage of viable cells relative to GFP-vehicle-treated slices (control). Each experiment contained four readings for each experimental condition.

Immunofluorescence. For immunohistochemical analysis, homozygous mutant $Hdh^{Q111/111}$ and wild-type Hdh^{277T} mice at 8 months of age or wild-type and R6/1 mice at 30 weeks of age ($n=3$ for each condition) were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. Brains were removed and postfixed overnight in the same solution, cryoprotected by immersion in 30% sucrose and then frozen in dry ice-cooled methylbutane. Serial coronal cryostat sections (30 μm) through the whole brain were collected in PBS as free-floating sections. Sections were rinsed three times in PBS, incubated with NH₄Cl 50 mM, and permeabilized with PBS containing 0.5% Triton X-100. Blocking was performed with PBS containing 0, 2% BSA and 5% normal goat serum (Pierce Biotechnology, Rockford, IL, USA) for 2 h at room temperature. The sections were then washed in PBS and incubated overnight at 4 °C with anti-p75 (1:100), anti-GFAP (1:100), anti-DARPP32 (1:1000) or anti-parvalbumin (1:1250) antibodies and detected with Cy3 anti-rabbit and Cy2 anti-mouse (1:200) secondary antibodies. Following secondary antibody incubation, slices were rinsed in PBS. As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected. Immunofluorescence was analyzed by confocal microscopy using a TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). For human brain immunofluorescence analysis, paraffin sections of the putamen nucleus from one patient with HD grade 1 (73 years, 7 h postmortem) was supplied by the Banc de Teixits Neurològics (Servei Científico-Tècnics, Universitat de Barcelona, Barcelona, Spain). Paraffin sections were dewaxed and rehydrated using a xylene/ethanol series followed by rinsing with PBS. Heat-induced epitope retrieval was performed in citrate buffer, pH 6.0 (DAKO) for 20 min. Sections were rinsed three times in PBS, incubated with NH₄Cl 50 mM, and permeabilized with PBS containing 0.2% Triton X-100 for 45 min. Blocking was performed with PBS containing 1% BSA and 10% normal horse serum (Pierce) for 1 h at room



temperature. After washing sections were incubated overnight at 4 °C with anti-p75 (1 : 100), and anti-DARPP32 (1 : 1000) diluted in blocking buffer and detected with Alexa Fluor 488 Goat anti-Rabbit IgG Conjugate (green) and Cy2 anti-mouse (1 : 200) secondary antibodies. Following secondary antibody incubation, slices were rinsed in PBS. As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected. Immunofluorescence was analyzed by confocal microscopy using a Leica TCS SP5 laser scanning spectral confocal microscope (Leica) with Argon and HeNe lasers attached to a Leica DM16000 inverted microscope.

Image analysis of striatal coronal sections. Coronal sections spaced 240 μ m apart comprising rostral, middle and caudal levels of the striatum (1.54 mm to -0.7 mm from Bergman) from wild-type and R6/1 mice ($n=3$) were examined with Leica TCS SP5 laser scanning spectral confocal microscope (Leica) with Argon and HeNe lasers attached to a Leica DM16000 inverted microscope. Confocal images were taken using a $\times 40$ numerical aperture objective with a $\times 1$ digital zoom and standard (one Airy disk) pinhole. For each coronal section, the entire three-dimensional stack of images from the ventral surface to the top of the section was obtained by using the Z drive in the Leica TCS SP5 microscope. The size of the optical image was 0.5 μ m. For each sample, 54 sections (6 \times 9) were captured and subsequently stitched using the tile scan feature of the Leica LAS AF 2.1.1 software to allow complete visualization of the striatum. Quantitative analyses were performed using the freeware ImageJ v1.33 by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA). Briefly, for each stitched image maximum projections was generated and mean pixel intensity was measured from the selected delineated area.

Protein extraction and western blot analysis. Wild-type and HD mice were killed by cervical dislocation and brains quickly removed, dissected, frozen in dry ice and stored at -80 °C until use. Protein extracts were prepared from striatal brain samples and striatal cell cultures by sonication on ice for 10 s in lysis buffer containing 50 mM Tris base (pH 7.4), 150 mM NaCl, 0.1 mM phenylmethylsulphonyl fluoride, 1% NP-40 and supplemented with 1 mM sodium orthovanadate and protease inhibitor mixture (Sigma-Aldrich). Samples were centrifuged at 10000g for 10 min and the protein contents determined by (Detergent-Compatible) Protein Assay (Bicinchoninic acid, BCA; Bio-Rad, Hercules, CA, USA). Protein extracts (30 μ g) were mixed with 4X SDS sample buffer, boiled for 5 min, resolved by 8–12% sSDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Blots were blocked in 10% non-fat powdered milk in Tris-buffered saline Tween-20 (50 mM Tris-HCl, 150 mM NaCl, pH7.4, 0.05% Tween-20) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies: TrkB polyclonal antibody (1 : 500; cellular extracts), TrkB monoclonal antibody (1 : 1000, striatal extracts), p75 antibody (1 : 1000), anti-NeuN (1 : 1000), anti-GFAP (1 : 1000), anti-GFP rabbit antibody (1 : 500), Phospho p44/42 ERK1/2 (Thr202/Tyr204) (1 : 1000), Phospho-Akt (Ser-473) (1 : 1000), Phospho-SAPK/JNK (Thr183/Tyr185) (1 : 1000), total ERK1/2 (1 : 2500), total Akt (1 : 1000), SAPK/JNK (1 : 1000), α -tubulin (1 : 50 000) or β -actin (1 : 20 000). The membranes were then rinsed three times with Tris-buffered saline Tween-20 (TBS-T) and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing for 30 min with TBS-T, the membranes were developed using the enhanced chemiluminescence substrate kit (Santa Cruz Biotechnology). The Gel-Pro densitometry program (Gel-Pro Analyzer for Windows- version 4.0.0.001) was used to quantify the different immunoreactive bands relative to the intensity of the α -tubulin or actin band in the same membranes. Data are expressed as the mean \pm S.E.M. of band density obtained in independent experiments or samples.

Statistical analysis. All of the data were analyzed with the program GraphPad Prism version 4.0 (GraphPad Software, La Jolla, CA, USA). Results are expressed as mean \pm S.E.M. Experimental data were analyzed either by a one- or two-way ANOVA followed by the *post hoc* Bonferroni's multiple comparison test or by Student's *t* test. A value of $P < 0.05$ was accepted as denoting statistical significant differences.

Conflict of Interest

The author declares no conflict of interest.

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WORK 3***“NEUROTROPHIN RECEPTOR p75^{NTR} MEDIATES HUNTINGTON’S DISEASE-ASSOCIATED SYNAPTIC AND MEMORY DYSFUNCTION”***

This paper has been accepted for publication in the Journal of Clinical Investigation

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CONTRIBUTION TO THIS WORK:

I participated in the building of several hypothesis and in their validation. I have obtained some RNA samples to perform the genetic analysis of BDNF receptors by RT-PCR technique and performed perfusion of mice infected with adenovirus. I contributed in the performance of several immunoblot analyses to determine biochemical changes in the hippocampus of HD and p75 transgenic mice. I performed hippocampal primary cultures, their infection with adenovirus, and the treatments with RhoA inhibitors to determine changes in spine population. I contributed in the discussion of the work.

AIM 2. TO STUDY THE MOLECULAR MECHANISMS INVOLVED IN NEUROTROPHIC SUPPORT DYSFUNCTION IN HUNTINGTON'S DISEASE**2.2. To study the role of p75^{NTR} in cognitive deficits in Huntington's disease**

Els dèficits en aprenentatge i memòria són característiques clíniques primerenques en la malaltia de Huntington (MH). Tot i que aquests dèficits estan associats a la patologia fronto-estriatal, diverses evidències indiquen que la regió hipocampal juga un paper crucial en la disfunció sinàptica i cognitiva en la MH. Degut a que el receptor p75^{NTR} juga un paper important com a regulador negatiu de la densitat d'espines dendrítiques, aprenentatge i memòria, en aquest treball ens hem plantejat estudiar si una funció aberrant del receptor p75^{NTR} pot estar contribuint a la presència de dèficits cognitius en la MH. En aquest treball demostrem que els nivells del receptor p75^{NTR} es troben significativament incrementats en l'hipocamp, però no en l'escorça, de diversos models murins de la MH així com en mostres d'individus afectats per la malaltia. La normalització genètica dels nivells del receptor p75^{NTR} en el context de la MH mitjançant la generació d'un nou model muri que expressa la huntingtina mutada i uns nivells normalitzats del receptor p75^{NTR} (Hdh^{Q7/Q111}:p75^{+/-}), restaura els dèficits cognitius i els dèficits en plasticitat sinàptica observats en el model murí de la MH. A més, la normalització dels nivells del receptor p75^{NTR} millora significativament les alteracions en espines dendrítiques observades en el model murí de la MH mitjançant la normalització de l'activitat de la RhoA. A més, en aquest treball demostrem que la sobre-expressió de p75^{NTR} mitjançant la infecció amb adenovirus en la regió hipocampal dels animals Hdh^{Q7/Q7}, mimetitzava les dèficits en aprenentatge i memòria observats en animals de la MH, mentre que la disminució dels nivells de p75^{NTR} específicament en la regió hipocampal de ratolins de la MH preveu l'aparició dels dèficits cognitius. En conjunt, tots aquests resultats estableixen p75^{NTR} com a un excel·lent candidat per modular els dèficits sinàptics, d'aprenentatge i memòria en la MH.

Neurotrophin receptor p75^{NTR} mediates Huntington's disease-associated synaptic and memory dysfunction

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Learning and memory deficits are early clinical manifestations of Huntington's disease (HD). These cognitive impairments have been mainly associated with frontostriatal HD pathology; however, compelling evidence provided by several HD murine models suggests that the hippocampus may contribute to synaptic deficits and memory dysfunction in HD. The neurotrophin receptor p75^{NTR} negatively regulates spine density, which is associated with learning and memory; therefore, we explored whether disturbed p75^{NTR} function in the hippocampus could contribute to synaptic dysfunction and memory deficits in HD. Here, we determined that levels of p75^{NTR} are markedly increased in the hippocampus of 2 distinct mouse models of HD and in HD patients. Normalization of p75^{NTR} levels in HD mutant mice heterozygous for p75^{NTR} prevented memory and synaptic plasticity deficits and ameliorated dendritic spine abnormalities, likely through normalization of the activity of the GTPase RhoA. Moreover, viral-mediated overexpression of p75^{NTR} in the hippocampus of WT mice reproduced HD learning and memory deficits, while knockdown of p75^{NTR} in the hippocampus of HD mice prevented cognitive decline. Together, these findings provide evidence of hippocampus-associated memory deficits in HD and demonstrate that p75^{NTR} mediates synaptic, learning, and memory dysfunction in HD.

Introduction

Evidence of cognitive deficits including altered acquisition of new motor skills, paired attention, planning, and memory has been demonstrated in Huntington's disease (HD) patients before the onset of motor symptoms (1–3). These clinical signs have been mainly attributed to corticostriatal dysfunction (4, 5). However, in recent years the idea has emerged that memory decline in HD is likely a reflection of a widespread brain circuitry defect and not exclusively a dysfunction of the basal ganglia (6–8). Indeed, besides the caudate and putamen, the volume of the hippocampus is reduced in premanifest HD individuals, while no changes in the amygdala, thalamus, or pallidum are observed (9). Interestingly, spatial and recognition memories have been reported to be altered in HD patients. Thus, in moderately advanced HD patients there is a simultaneous impairment of allocentric (hippocampal-dependent) and egocentric (striatal-dependent) spatial navigation, supporting the idea that beyond the atrophy of the striatum and cortex, a more general neurodegenerative process that involves the hippocampus could contribute to HD memory impairment (2, 10, 11). Actually, cytoplasmic and nuclear huntingtin aggregates within the hippocampus have been described in HD individuals (12). Importantly, such behavioral deficits, together with hippocampal

long-term potentiation (LTP) disturbances, have been replicated in different HD mouse models (2, 13–15). Although altered synaptic plasticity and aberrant dendritic spine density and morphology have been proposed as underlying mechanisms (14, 16, 17), little is known about the precise molecular pathways involved in HD synaptic and memory disturbances.

In the adult brain, neurotrophins play a critical role in synaptic plasticity regulation. Among the different neurotrophins, brain-derived neurotrophic factor (BDNF) is the best characterized for its role in regulating LTP and long-term depression (LTD) through binding to TrkB and p75^{NTR} receptors (18). It is generally accepted that BDNF via interaction with TrkB receptors modulates synaptic transmission and plasticity in adult synapses by regulating transcription, translation, and trafficking of distinct synaptic proteins (19, 20). Less is known about the role of p75^{NTR} in synaptic plasticity. Null p75^{NTR} mice show improved spatial learning and enhanced LTP (21, 22) while impaired NMDA-dependent LTD (23), which points to an antagonistic role of p75^{NTR} in synaptic plasticity. Moreover, it has been demonstrated that p75^{NTR} is a negative modulator of spine-dendrite morphology and complexity (24), likely by regulation of RhoA activity (25). Consistently, while p75^{NTR}^{-/-} mice exhibit increased hippocampal dendritic spine density, overexpression of p75^{NTR} in hippocampal neurons decreases spine number and branching (24). Interestingly, upregulation of p75^{NTR} levels has been reported in the cortex and hippocampus of Alzheimer's disease (AD) patients (26), while small-molecule p75^{NTR} ligands prevent both cognitive

Authorship note: Verónica Brito and Albert Giralt contributed equally to this work.

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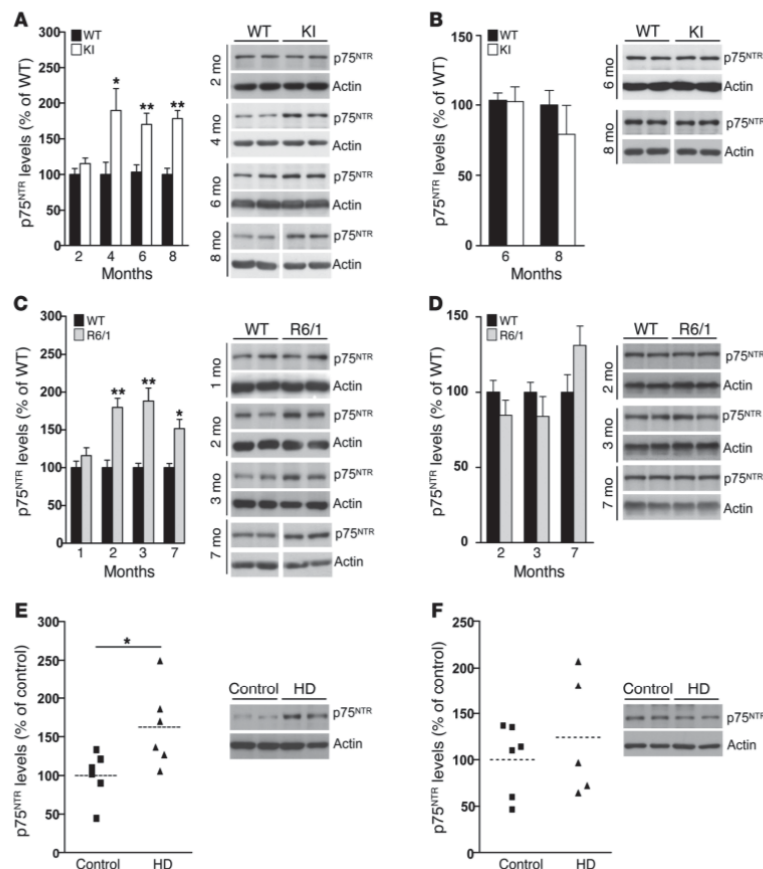


Figure 1. p75^{NTR} expression is increased in the hippocampus but not in the cortex of 2 different HD mouse models and in HD human brain. (A–D) Western blot for p75^{NTR} and actin as loading control in total hippocampus and cortex extracts from WT and KI mice (A and B) or WT and R6/1 mice (C and D) at different ages ($n = 5$ –7 per genotype). Right: Representative immunoblots. At 8 and 7 months, lanes were run on the same gel but were noncontiguous (white line). (E and F) Western blot for p75^{NTR} and actin as a loading control in total hippocampus and cortex extracts from control and HD brain samples ($n = 6$ –7). Right: Representative immunoblots. All plots represent mean \pm SEM. Student's 2-tailed t test was performed. * $P < 0.05$, ** $P < 0.01$ compared with WT mice or control human samples.

decline and neuritic dystrophy in AD mouse models (27–29). In this scenario, we were interested to explore whether p75^{NTR} plays a role in different memory deficits in HD. To this aim we have examined the levels of p75^{NTR} in the hippocampus of 2 distinct HD mouse models, exon 1 and full-length, as well as in the hippocampus of HD patients. p75^{NTR} levels were increased at early HD disease stages in the hippocampus of HD mice and also in post-mortem samples from HD patients. Increased p75^{NTR} levels were accompanied by long-term memory deficits, altered LTP, reduced levels of synaptic proteins, decreased dendritic spine density, and hyperactivation of the small GTPase RhoA. Such alterations were mimicked by overexpression of p75^{NTR} in WT mice and reversed by genetic normalization of p75^{NTR} levels in HD mutant mice. Finally, specific reduction of p75^{NTR} in the hippocampus of HD mice by intracranial injections of AAV-shp75^{NTR} prevented HD memory impairments. In summary, our findings identify p75^{NTR} as a crucial mediator of synapse loss and cognitive decline in HD and provide mechanistic rationale for p75^{NTR} as a novel target to treat memory deficits in HD.

Results

Increased p75^{NTR} expression in the hippocampus of HD mouse models and HD patients. Previous data from our group demonstrated increased levels of p75^{NTR} in the striatum of HD mice as well as in the putamen of HD patients (30). To analyze the role of p75^{NTR} in HD memory dysfunction, we extended these data by evaluating hippocampal and cortical p75^{NTR} levels in 2 HD mouse models at different stages and in postmortem human HD samples. The 2 different HD mouse models, R6/1 and *Hdh*^{Q7/Q11} knock-in mutant mice (mutant KI mice), differ in the onset and progression of HD phenotypes; R6/1 mice show earlier onset and faster disease progression than mutant KI mice (31). Quantitative Western blot analysis revealed a significant increase in hippocampal p75^{NTR} levels in HD mutant mice at early disease stages (2 months and 4 months in R6/1 and KI mice, respectively), levels that remained elevated at late stages (Figure 1, A and C). Interestingly, cortical p75^{NTR} levels were similar between genotypes (Figure 1, B and D). To analyze whether deregulation of p75^{NTR} levels also occurs in humans, we examined hippocampal and cortical tissue from controls and

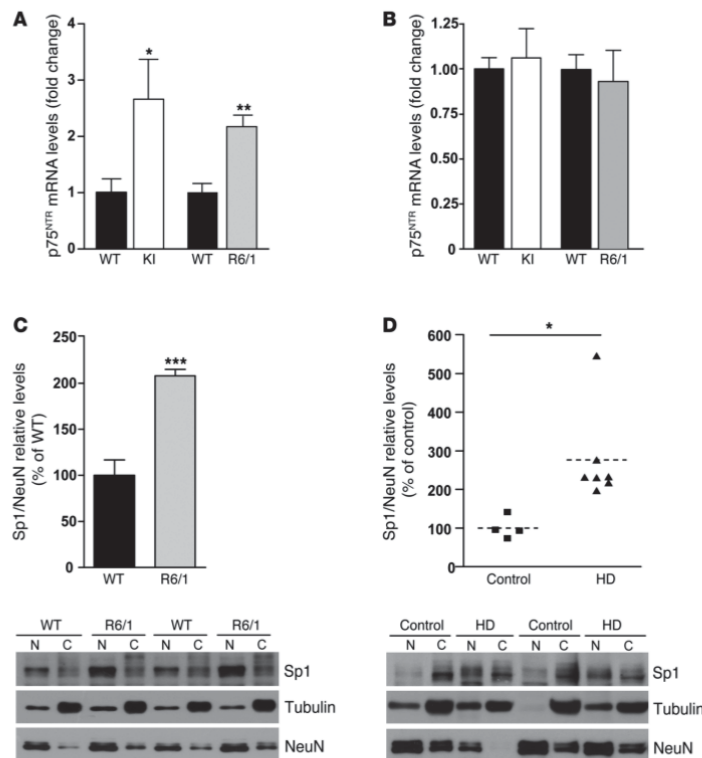


Figure 2. Increased hippocampal p75^{NTR} mRNA expression associates with higher levels of Sp1 in the mouse and human HD hippocampus. (A and B) Histograms showing p75^{NTR} mRNA expression analyzed by RT-PCR in the hippocampus (A) and cortex (B) of 8-month-old WT and KI mice and 12-week-old WT and R6/1 mice. Results were normalized to 18S gene expression. Data represent mean \pm SEM and are expressed as fold change. ($n = 5-8$ for each genotype.) (C and D) Western blot for Sp1 in nuclear and cytosolic enriched fractions from the hippocampus of WT and R6/1 mice at 14 weeks of age and from control and HD patients ($n = 4-6$). Representative immunoblots showing Sp1 protein levels in cytosolic (loading control α -tubulin) and nuclear (loading control NeuN) fractions. Data were analyzed by Student's 2-tailed t test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with WT mice or control human samples.

individuals affected by HD. Consistent with our data in HD mice, levels of p75^{NTR} were found increased in the hippocampus but not in the cortex of HD patients compared with controls (hippocampus: control, 100.1 ± 12.68 ; HD, 162.4 ± 21.69 ; cortex: control, 100.02 ± 17.14 ; HD, 124.3 ± 22.7 ; Figure 1, E and F, respectively), which supports a pathological role for p75^{NTR} in HD. The increase in hippocampal p75^{NTR} protein levels was likely due to increased p75^{NTR} transcription, since higher mRNA levels measured by RT-PCR were found in the hippocampus but not in the cortex of HD versus WT mice (Figure 2, A and B). We next aimed to determine the molecular mechanism by which mutant huntingtin induces aberrant hippocampal p75^{NTR} expression. The transcription factor Sp1 has been described to drive expression of p75^{NTR} under cellular stress conditions (32, 33), while upregulation of Sp1 has been described in cellular and mouse models of HD (34). These data prompted us to investigate whether increased p75^{NTR} expression was related to higher hippocampal Sp1 levels. Thus, Sp1 levels were determined by Western blot analysis in nuclear and cytosolic enriched fractions obtained from R6/1 and HD human hippocampus (Figure 2, C and D). Interestingly, we found a significant increase in Sp1 levels in the nuclear fraction in R6/1 and HD human hippocampus compared with WT mice and control human samples, suggesting that deregulated Sp1 expression could contribute to aberrant expression of p75^{NTR} in the HD hippocampus.

Altered p75^{NTR} synaptic location in Hdh^{Q7/Q11} knock-in mutant mice. Mutant KI and R6/1 mice develop age-dependent memory deficits (13, 15). Given the role of p75^{NTR} in synaptic plasticity (19, 24, 35), we next analyzed whether the increase in p75^{NTR} levels in the hippocampus of mutant KI mice could be involved in HD memory deficits. To address this question, levels of p75^{NTR} were normalized in KI mice by cross-mating of *Hdh^{Q7/Q11}* mice with *p75^{NTR}/ExonIII^{-/-}* mice (*p75^{-/-}* mice) to obtain double-mutant mice (*KI;p75^{-/-}* mice). Hippocampal extracts from WT, mutant KI, heterozygous *p75^{-/-}*, and double-mutant *KI;p75^{-/-}* mice were analyzed by Western blot. As expected, quantification of band intensities revealed a significant increase of p75^{NTR} in mutant KI mice, an increase that was reversed in double-mutant *KI;p75^{-/-}* mice (Figure 3A). Confocal analysis of brain sections showed p75^{NTR} immunoreactivity as a punctate staining in pyramidal cell bodies as well as in fibers concentrated in the stratum radiatum and more dispersed in the stratum oriens within the CA1 region of the hippocampus. Similar staining was observed in CA3 and the dentate gyrus (data not shown). According with our biochemical data, p75^{NTR} immunoreactivity in mutant KI mice was higher than in WT animals, whereas no significant differences were detected between double *KI;p75^{-/-}* and WT mice (Figure 3B). When subcellular localization was analyzed in the CA1 region, we found that in all analyzed genotypes p75^{NTR} immunoreactivity colocalized with MAP2, a dendritic marker,

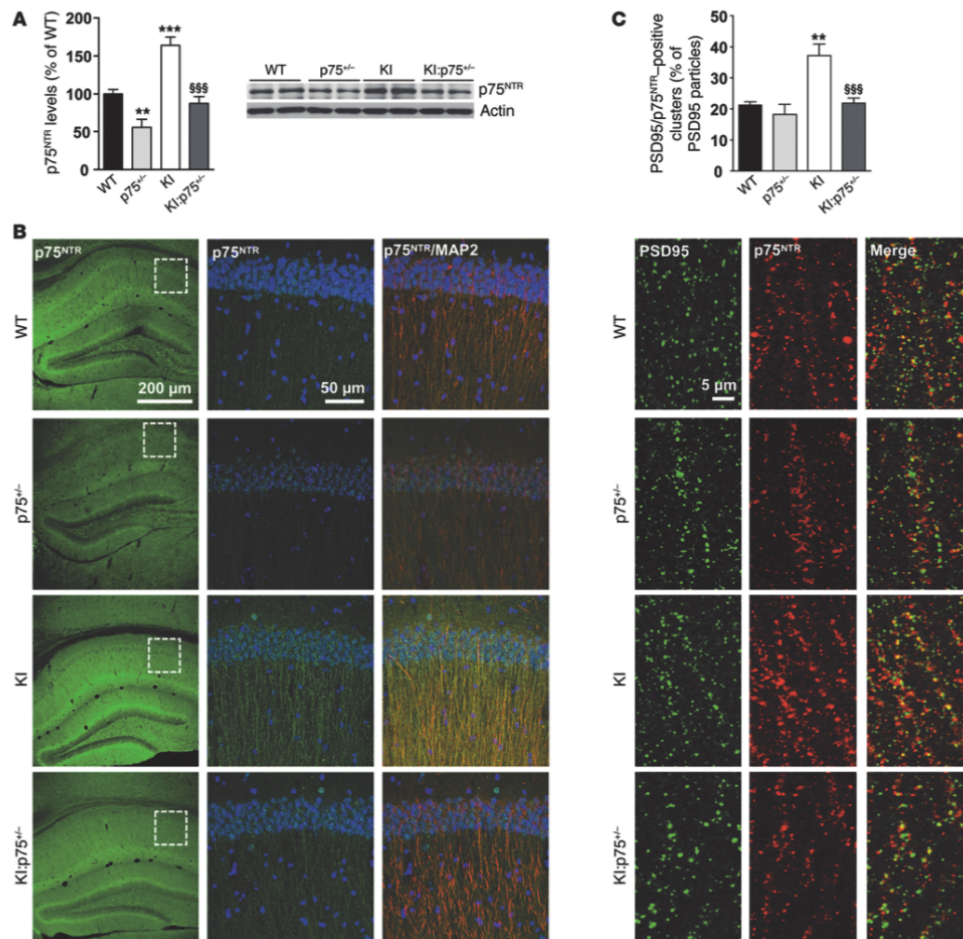


Figure 3. Increased postsynaptic localization of p75^{NTR} in mutant KI mice. (A) Western blot for p75^{NTR} and actin as loading control in total hippocampus extracts from WT, p75^{-/-}, mutant KI, and double-mutant KI;p75^{-/-} mice at 6 months of age ($n = 6-7$ per genotype) showing normalization of p75^{NTR} levels in double-mutant mice. Right: Representative immunoblots. (B) Representative confocal microscopy images (low magnification) showing the expression pattern of p75^{NTR} in 8-month-old mouse hippocampus. Magnified images (right) show colocalization between p75^{NTR} and MAP2 in the CA1 hippocampal region. (C) Representative confocal microscopy images (high magnification) showing p75^{NTR} and PSD95 colocalization in the CA1 hippocampal region. Quantitative analysis reveals a significant increase in the percentage of PSD95-positive clusters also positive for p75^{NTR} in KI mice. Data are presented as mean \pm SEM. One-way ANOVA with Tukey post hoc comparisons was performed; ** $P < 0.01$ and *** $P < 0.001$ compared with WT mice, ### $P < 0.001$ compared with KI mice.

suggesting that p75^{NTR} is expressed by hippocampal neurons. To further analyze the distribution of p75^{NTR}, double immunostaining using p75^{NTR} and PSD95 as a glutamatergic postsynaptic marker was conducted. We found that in apical dendrites of the CA1 region p75^{NTR} staining colocalized with PSD95 immunoreactivity, revealing a postsynaptic distribution of p75^{NTR} (Figure 3C). Interestingly, whereas in WT mice about 20% of PSD95-positive clusters were

positive for p75^{NTR}, in mutant KI mice this percentage rose to 40%, suggesting that the increase in p75^{NTR} in mutant KI mice is localized in spines. Importantly, this increase was completely reversed in double-mutant mice. Since p75^{NTR} is also expressed by astrocytes, especially after neuronal damage (36, 37), coimmunostaining with the astrocytic marker GFAP was performed in hippocampal slices from WT and mutant KI mice (see Supplemental Figure 6; supple-

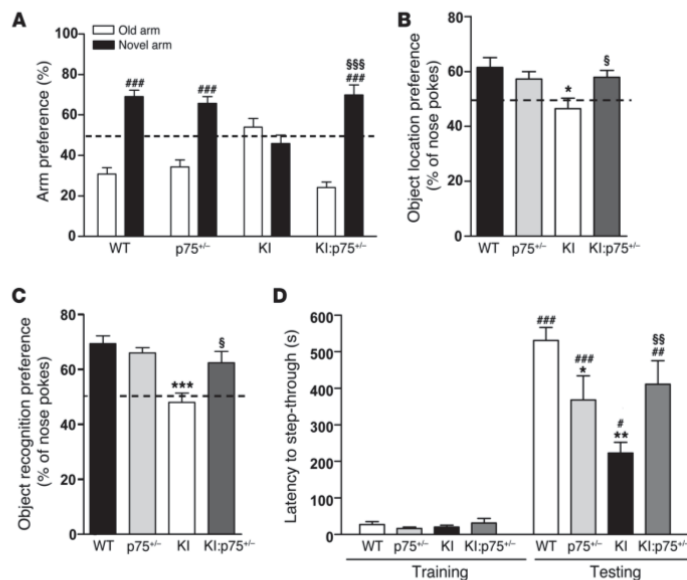


Figure 4. Normalization of p75^{NTR} levels in mutant KI mice rescues spatial and nonspatial memory deficits. (A) Percentage of time spent in arms (old versus novel) from WT, p75^{-/-}, KI, and KI:p75^{-/-} mice at 6 months of age ($n = 8-12$ per genotype). Mutant KI mice exhibit no preference for a previously unexposed (novel) arm of a T-maze. (B) Percentage of nose pokes to the displaced object from WT, p75^{-/-}, KI, and KI:p75^{-/-} mice at 6 months of age. Mutant KI mice showed significantly less preference for the novel-object location ($n = 7-8$ per genotype). (C) Percentage of nose pokes to the new object from WT, p75^{-/-}, KI, and KI:p75^{-/-} mice at 6 months of age ($n = 8-12$ per genotype) in the novel object recognition task. Mutant KI mice display no preference for the novel object. (D) Latency to step-through from light to dark compartment from WT, p75^{-/-}, KI, and KI:p75^{-/-} mice at 6 months of age ($n = 8-12$ per genotype). Mutant KI mice present worse (shorter latency to crossover) retention performance than the other genotypes. One-way ANOVA with Bonferroni post hoc comparisons; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with WT mice; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ compared with KI mice. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with old arm or latency to step-through in the training. Data are presented as mean \pm SEM.

mental material available online with this article; doi:10.1172/JCI74809DS1). Lack of colocalization between p75^{NTR} and GFAP was found, indicating that astrocytes in WT and KI mutant mice do not overexpress p75^{NTR}. These findings together with our data showing increased p75^{NTR} staining in KI hippocampus colocalizing with MAP2 suggest that neuronal p75^{NTR} upregulation underlies hippocampal HD dysfunction.

Genetic normalization of p75^{NTR} levels rescues spatial, recognition, and associative memory deficits in Hdh^{Q7/Q11} knock-in mutant mice. We next assessed whether aberrant hippocampal p75^{NTR} expression was associated with HD memory impairments. Spatial, recognition, and associative memories were evaluated in double-mutant KI:p75^{-/-} mice at 6 and 8 months of age, since previous results from our lab demonstrated lack of recognition memory deficits in mutant KI mice at 4 months of age (15). First, animals were tested in the elevated plus maze and the light-dark exploration paradigms to assess anxiety-related behaviors. These tests are based on the natural aversion of rodents to elevated open spaces and brightly illuminated spaces, respectively (38, 39).

In the elevated plus maze paradigm (Supplemental Figure 1A), all genotypes displayed similar exploratory activity measured as the time in the open arms and the distance traveled, suggesting that neither KI, p75^{-/-}, nor KI:p75^{-/-} mice exhibit higher anxiety levels compared with WT mice. As a second test for anxiety-like behaviors we performed the light-dark box test (Supplemental Figure 1B). Consistent with our previous data, we found that all mice, independently of their genotype, made similar transitions to the light compartment and spent similar time in the light. We conclude from these data that nonprominent anxiety-like behaviors are manifested by any of the genotypes studied. Then, spatial, recognition, and associative memories were analyzed (Figure 4). Spatial memory was evaluated by the T-maze spontaneous alternation task (T-SAT) and the object location task (OLT) (40, 41), both behavioral tasks sensitive to hippocampal dysfunction albeit not completely dependent (42-44). In the T-SAT test, mice have the natural tendency to alternate and enter the previously unvisited arm (novel arm) in a T-maze apparatus. At 6 months of age, mutant KI mice showed no preference for the novel versus the old arm, while a significant preference was observed in WT, p75^{-/-}, and double-mutant KI:p75^{-/-} mice (Figure 4A). In the OLT, all mice were first habituated to the open field arena

and ambient conditions and then subjected to a training session in the presence of 2 similar objects (A1 and A2). All mice similarly explored both objects, indicating no object or place preferences between genotypes (Supplemental Figure 1C). This test is based on the ability of rodents to recognize when a familiar object has been relocated. When spatial memory was assessed 24 hours after training, KI mice exhibited a significantly lower preference for the object displaced to the new location compared with WT, p75^{-/-}, or double-mutant KI:p75^{-/-} mice (Figure 4B). Overall, these results indicate that normalization of p75^{NTR} levels in KI mice rescue spatial memory deficits. Similar results were obtained at 8 months of age in the T-SAT test, suggesting that reduced p75^{NTR} expression in HD mice prevented, rather than merely delayed, spatial memory deficits (Supplemental Figure 2A). We next evaluated recognition memory by using the novel object recognition test (NORT), based on the natural tendency of mice to spend more time exploring a novel object than a familiar one and known to be dependent on hippocampal and cortical circuits (45-47). No significant differences between genotypes were found during the training period,

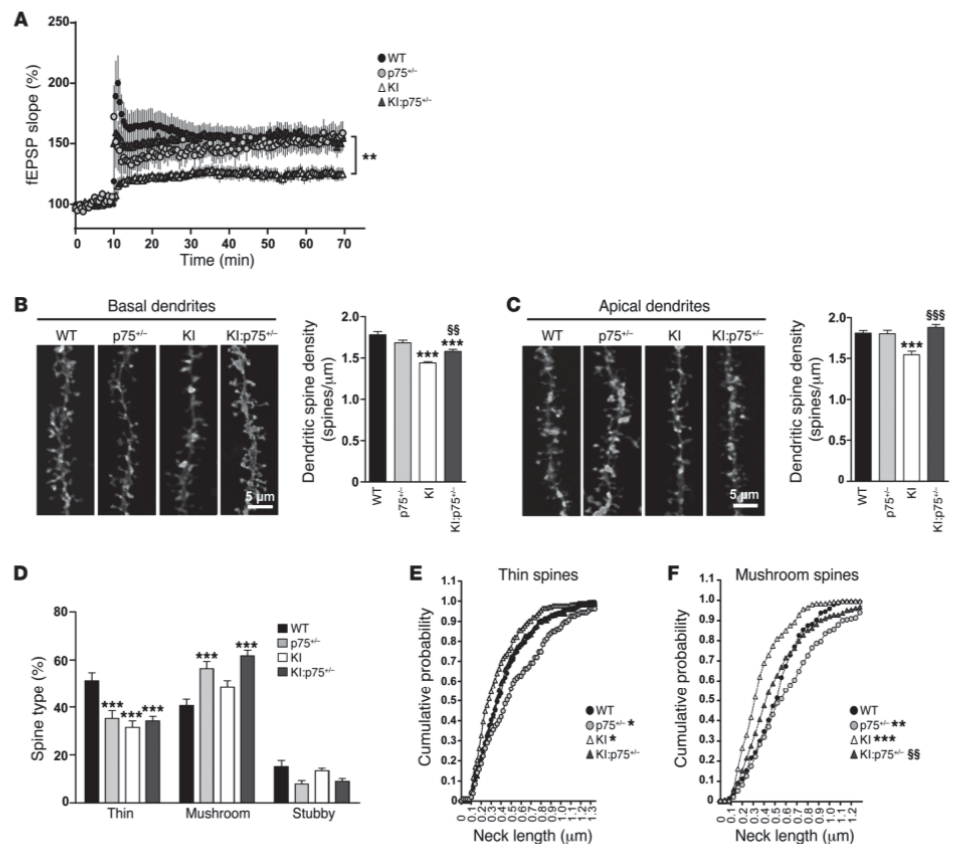


Figure 5. Normalization of p75^{NTR} levels in mutant KI mice prevents functional and structural synaptic plasticity deficits. (A) Time course of fEPSP potentiation during HSF-induced LTP in WT ($n = 3$), p75^{-/-} ($n = 4$), KI ($n = 9$), and KI:p75^{-/-} ($n = 8$) mice at 6 months of age. ** $P < 0.01$ compared with WT mice. (B and C) Representative basal (B) and apical dendrites (C) of CA1 pyramidal neurons from WT, p75^{-/-}, KI, and KI:p75^{-/-} mice at 8 months of age. Right: Quantitative analysis showing dendritic spine density per micrometer of dendritic length. Mutant KI mice exhibit a significant reduction in dendritic spines that was significantly ameliorated in double-mutant mice. One-way ANOVA with Tukey post hoc comparisons was performed (63–83 dendrites; $n = 4$ –5 animals per genotype); *** $P < 0.001$ compared with WT mice; §§ $P < 0.01$, §§§ $P < 0.001$ compared with KI mice. (D) Percentage of each morphological type of dendritic spine (see Methods and Supplemental Figure 4 for classification criteria) from WT, p75^{-/-}, KI, and KI:p75^{-/-} basal dendrites at 8 months of age. One-way ANOVA with Tukey post hoc comparisons was performed (480 spines from 100 dendrites from 4 animals per genotype were analyzed); *** $P < 0.001$ compared with WT mice. (E and F) Spine neck length distribution was examined by plotting of the cumulative frequency of neck length of all examined spines and comparison of distributions using the Kolmogorov-Smirnov test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with WT mice. §§ $P < 0.01$ compared with KI mice.

indicating no object preference (Supplemental Figure 1D). Twenty-four hours after training, mutant KI mice at either 6 months (Figure 4C) or 8 months of age (Supplemental Figure 2B) exhibited a significantly lower preference for the novel object compared with WT, p75^{-/-}, and double-mutant KI:p75^{-/-} mice, indicating preserved long-term recognition memory in double but not in mutant KI mice. Finally, we examined associative memory in the passive avoidance task, based on the association formed between an aversive stimulus (electrical foot shock) and a specific environmental context (light-dark) (Figure 4D), which relies on cortical

and hippocampal circuits (48–50). Latency to step-through during the training session was found to be similar between genotypes. However, in the testing session, although all genotypes showed a significant increase in the latencies to enter the dark compartment 24 hours after receiving an electrical shock, the time latency in mutant KI and p75^{-/-} mice was lower than in WT or double-mutant KI:p75^{-/-} mice, indicating that altered levels of p75^{NTR} contribute to associative memory impairments. Altogether, these results demonstrate memory decline in mutant KI mice that was prevented by normalization of p75^{NTR} levels.

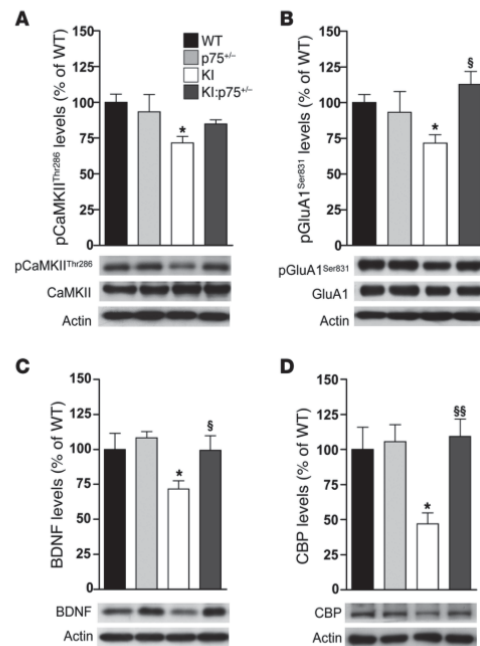


Figure 6. Normalization of p75^{NTR} levels in mutant KI mice reverses the altered expression of synaptic-related proteins. Representative Western blots showing (A) total and phospho-CaMKII^{Thr286}, (B) total and phospho-GluA1^{Ser831}, (C) BDNF, and (D) CBP and actin as loading control in total hippocampus extracts from WT, p75^{-/-}, KI, and KI:p75^{-/-} mice at 6 months of age ($n = 6-7$ per genotype). All histograms represent mean \pm SEM. One-way ANOVA with Tukey post hoc comparisons was performed. * $P < 0.05$ compared with WT mice; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with KI mice. pCaMKII^{Thr286}, phospho-Ca²⁺/calmodulin-dependent protein kinase II at threonine 286; pGluA1^{Ser831}, phospho- α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunit 1 at serine 831; BDNF, brain-derived neurotrophic factor; CBP, CREB-binding protein.

nificant decrease (~20%) in basal and apical dendritic spine density compared with WT mice (Figure 5, B and C). Normalization of p75^{NTR} levels completely (apical dendrites) or partially (basal dendrites) prevented the decay in spine density in mutant KI mice, suggesting that aberrant p75^{NTR} expression in HD could contribute to loss of dendritic spines. To elucidate whether increased p75^{NTR} levels also affect spine morphology, dendritic spine type was assessed on basal dendrites of CA1 pyramidal neurons (Figure 5D). Spines were defined as thin, stubby, or mushroom on the basis of morphology described by Harris and Stevens (ref. 52 and Supplemental Figure 4). Mutant KI mice exhibited altered spine distribution with a significant decrease (~20%) in the proportion of thin spines. Surprisingly, both p75^{-/-} and double-mutant KI:p75^{-/-} mice also presented less thin spines compared with WT mice (~20%). However, in these 2 genotypes the proportion of mushroom spines was significantly higher (~15%–20%), which may indicate a compensatory mechanism. Finally, we assessed the spine neck length, which is known to influence Ca²⁺-dependent signaling and synaptic plasticity (53). These data were plotted as cumulative distribution for each animal to examine in detail the potential differences (Figure 5, E and F). Cumulative probability indicates that either increased or decreased levels of p75^{NTR} alter spine neck morphology. Thus, while in mutant KI mice both thin and mushroom spines exhibited shorter necks, in p75^{-/-} mice the neck lengths were longer. By contrast, equal graph distributions were found between WT and double-mutant KI:p75^{-/-} mice, indicating that both genotypes display spines with similar neck lengths (Figure 5, E and F). Altogether, these data imply that HD mutant mice exhibited not only reduced dendritic spine density but also a shift in the spine distribution and morphology. Remarkably, these dendritic changes were significantly improved by reduction of aberrant p75^{NTR} levels.

Genetic normalization of p75^{NTR} levels rescues hippocampal synaptic dysfunction in Hdh^{Q2/Q11} knock-in mutant mice. Defective LTP and altered dendritic spine dynamics and morphology are involved in early cognitive and memory deficits in HD (14, 16, 51). Because normalization of p75^{NTR} levels prevented memory impairments in mutant KI mice, we next examined whether this improvement was accompanied by LTP recovery and/or amelioration of dendritic spine loss. High-frequency conditioning tetanus (HFS) to induce LTP was used to investigate synaptic plasticity in CA1 hippocampal slices at 6 months of age. Baseline responses were monitored for 10–30 minutes before conditioning and were found to be stable. Mutant KI mice showed diminished HFS-induced LTP compared with WT animals (Figure 5A). Thus, at 60 minutes after tetanus, potentiation (as mean percentage of baseline) in WT mice was $154.3\% \pm 9\%$ ($n = 8$ slices, 3 mice) versus $124.9\% \pm 5.2\%$ in mutant KI mice ($P < 0.01$, $n = 20$ slices, 9 mice). By contrast, tetanic stimulation induced in either double-mutant KI:p75^{-/-} mice or p75^{-/-} mice led to sustained LTP (KI:p75^{-/-}: $154.9\% \pm 8\%$, $P < 0.01$, $n = 14$ slices, 8 mice; p75^{-/-}: $159.02\% \pm 10\%$, $P < 0.01$, $n = 9$ slices, 4 mice), which indicates that normalization of p75^{NTR} levels in mutant KI mice restored the LTP impairment.

Next, hippocampal histology and spine density were examined. Stereological estimation of hippocampal volume and CA1 pyramidal cell density revealed no significant differences between genotypes at 8 months of age (Supplemental Figure 3), suggesting no gross anatomical deficiencies in KI or KI:p75^{-/-} mice. To compare spine density and morphology, DiOlistic labeling in fixed brain slices was used and apical and basal dendritic spines of CA1 pyramidal neurons were counted. Mutant KI mice displayed a sig-

Deregulation of synaptic-related proteins in KI mutant mice is also reversed by genetic normalization of p75^{NTR} levels. Decreased levels of synaptic-related proteins have been associated with memory impairments and aberrant synaptic plasticity in HD (15, 16, 54, 55). Thus, we next analyzed several synaptic markers and proteins involved in hippocampal synaptic plasticity in the hippocampus of mutant KI mice. Similar levels of the presynaptic marker synaptophysin and the postsynaptic markers GluN1 and GluN2B (NMDA receptor subunits) and GluA1 and GluA2/3 (AMPA receptor subunits) were found between genotypes (Supplemental Table 1). However, levels of phospho-CaMKII^{Thr286}, a key modulator of hippocampal activity through phosphorylation of NMDA and AMPA receptors (56), were significantly reduced in KI mice, a reduction

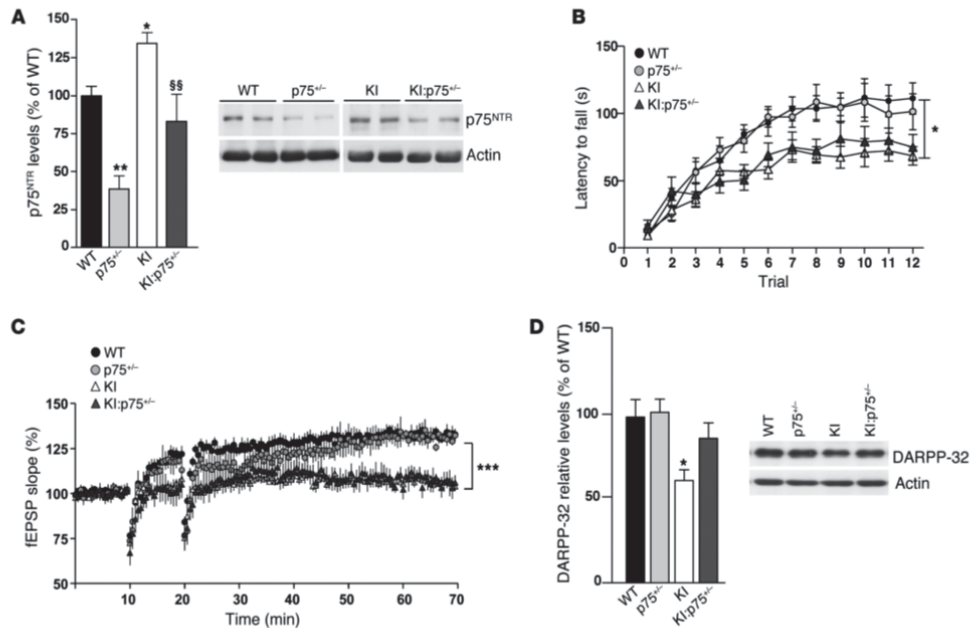


Figure 7. Normalization of p75^{NTR} levels in mutant KI mice slightly restores striatal pathology. (A) Western blot for p75^{NTR} and actin as loading control in total striatal extracts from WT, p75^{-/-}, mutant KI, and double mutant KI;p75^{-/-}. (B) Latency to fall in the accelerating rotarod task procedure in WT, p75^{-/-}, KI, and KI;p75^{-/-} mice at 6 months of age. Data represent the mean \pm SEM ($n = 9-14$ per genotype). Statistical analysis was performed using 2-way ANOVA with repeated measures. * $P < 0.05$ and ** $P < 0.01$ compared with WT; ** $P < 0.05$ compared with KI. (C) Time course of fEPSP potentiation during HSF-induced LTP in WT ($n = 7$), p75^{-/-} ($n = 8$), KI ($n = 8$), and KI;p75^{-/-} ($n = 9$) mice at 4 months of age. For each slice, data were normalized to the average slope recorded during baseline. Data represent the mean \pm SEM. Statistical differences, compared with pre-tetanus baseline amplitude values, were established using Student's 2-tailed t test. *** $P < 0.001$. (D) Western blot for DARPP-32 and actin as loading control in total striatal extracts from WT, p75^{-/-}, KI, and KI;p75^{-/-} mice at 6 months of age ($n = 6$ per genotype). Right: Representative immunoblots. Plot represents mean \pm SEM. Student's 2-tailed t test was performed; * $P < 0.05$.

that was prevented by normalization of p75^{NTR} levels in double KI;p75^{-/-} mice (Figure 6A). Consistent with these data, the levels of the CaMKII-mediated GluA1^{Ser831} phosphorylation were decreased in KI mice, while no significant differences were found in double-mutant mice (Figure 6B). Interestingly, the levels of 2 glutamatergic-scaffolding proteins such as PSD95 and SAP102 were found significantly increased in double KI;p75^{-/-} mice compared with WT mice (Supplemental Table 1), which could be related to the increase in mushroom spines previously shown in these mice (Figure 4D). Finally, levels of BDNF and CREB-binding protein (CBP), previously found to be downregulated in the HD hippocampus (15, 54), were tested. As expected, levels of BDNF and CBP were decreased in the hippocampus of mutant KI mice, whereas no significant differences were found when double KI;p75^{-/-} mice were analyzed (Figure 6, C and D). Thus, altogether these findings suggest that disruption of LTP and memory deficits in KI mutant mice involve deregulation of synaptic-related proteins and that normalization of p75^{NTR} levels prevents such alterations.

Genetic normalization of p75^{NTR} levels partially restores striatal pathology in Hdh^{Q7/111} knock-in mutant mice. Our data demonstrate that downregulation of p75^{NTR} levels in KI mutant mice fully prevented synaptic and cognitive deficits exhibited by these mice.

However, these improvements could be related to the normalization of p75^{NTR} levels in other brain regions known to be affected in HD, such as the striatum, in which p75^{NTR} levels have been demonstrated to be increased (30, 57). Therefore, we next analyzed whether striatal normalization of p75^{NTR} levels in KI mutant mice (Figure 7A) could also ameliorate striatal-dependent behavior, synaptic deficits, and striatal neuropathology. To this aim, motor learning, corticostriatal synaptic transmission, and DARPP-32 expression were analyzed (Figure 7). Compared with WT mice, KI mutant mice at 6 months of age displayed motor learning deficits as evidenced by decreased latency to fall during the accelerating rotarod test (Figure 7B). Importantly, impaired performance in the accelerating rotarod was not prevented or ameliorated by normalization of p75^{NTR} levels in KI;p75^{-/-} mice. Consistent with these findings, when corticostriatal synaptic plasticity was analyzed by induction of LTP through HFS, both KI and KI;p75^{-/-} mice displayed impaired LTP induction and maintenance (Figure 7C). Finally, the levels of the adult striatal neuronal marker DARPP-32, known to be reduced in HD mice from early stages (58), were determined by Western blot analysis in total striatal extracts obtained from WT, p75^{-/-}, KI, and KI;p75^{-/-} mice (Figure 7D). Interestingly, normalization of p75^{NTR} levels rescued reduction of striatal DARPP-32

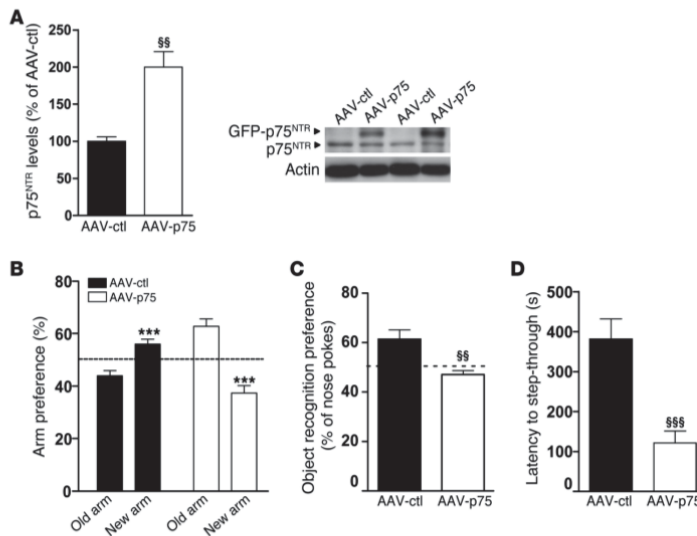


Figure 8. Intrahippocampal infusion of AAV-GFP-p75 in WT mice recapitulates the cognitive deficits observed in KI mice. (A) Western blot for p75^{NTR} and actin as loading control in total hippocampus extracts from AAV-GFP- (AAV-ctl-) and AAV-p75-transduced WT mice. A significant increase in p75^{NTR} levels was found in AAV-p75 mice. Histogram represents mean ± SEM ($n = 8$ per genotype at 6 months of age). Student's 2-tailed t test was performed. §§ $P < 0.01$ compared with AAV-ctl mice. (B) Percentage of time spent in arms (old versus novel) from AAV-ctl and AAV-p75 mice at 6 months of age ($n = 8$ –12 per genotype). AAV-p75 mice exhibit no preference for a previously unexposed (novel) arm of a T-maze. ($n = 8$ per genotype.) Student's 2-tailed t test was performed. *** $P < 0.001$ compared with old arm. (C) Percentage of nose pokes to the new object from AAV-ctl and AAV-p75 mice at 6 months of age in the novel object recognition task ($n = 8$ per genotype). AAV-p75 mice display no preference for the novel object. Student's 2-tailed t test was performed. §§ $P < 0.01$ compared with AAV-ctl mice. (D) Latency to step-through from light to dark compartment from AAV-ctl and AAV-p75 at 6 months of age ($n = 8$ per genotype). AAV-p75 mice exhibit worse (shorter latency to crossover) retention performance than AAV-ctl mice. Student's 2-tailed t test was performed. §§§ $P < 0.001$ compared with AAV-ctl mice. All histograms represent mean ± SEM.

expression in KI mice, suggesting that striatal neuronal dysfunction could be slightly ameliorated but not prevented by downregulation of aberrant p75^{NTR} expression in KI mutant mice.

Intrahippocampal infusion of AAV-GFP-p75 in *Hdh^{Q7/Q7}* WT mice reproduces HD memory deficits. To further evaluate whether the aberrant increase in hippocampal p75^{NTR} levels could contribute to memory impairments in HD mutant mice, we next tested whether overexpression of p75^{NTR} only in the hippocampus of WT mice was able to mimic HD memory deficits. First, we analyzed whether the virus serotype AAV2/8, used to overexpress p75^{NTR}, was able to infect glia and/or neuronal cells (Supplemental Figure 6B). Fluorescence microscopy analysis demonstrated that AAV2/8 efficiently transduces hippocampal neurons but not astrocytes, indicating that the 2/8 serotype is greatly specific for neurons. Then, AAV2/8 serotype specificity was tested *in vivo*. Slices from WT animals injected with AAV2/8-p75^{NTR} viruses were stained with antibodies against GFAP, and colocalization with GFP was analyzed by confocal microscopy (Supplemental Figure 6C). Consistent with our *in vitro* data, no overlap in staining between GFAP and GFP was found, indicating that AAV2/8 viruses primar-

ily infect neuronal cells within the hippocampus. First, we confirmed by Western blot analysis higher p75^{NTR} expression in the hippocampus of AAV-p75 mice compared with AAV-GFP (AAV-ctl) animals (Figure 8A). When memory function was evaluated, WT mice overexpressing p75^{NTR} showed impaired spatial and nonspatial long-term memories manifested as reduced preference for either the novel arm or the novel object in the T-SAT and NORT paradigms, respectively (Figure 8, B and C) and by a significant retention deficit in the passive avoidance task (Figure 8D). These experiments demonstrate that overexpression of p75^{NTR} in the WT hippocampus reproduces HD-like memory deficits and support an important role for p75^{NTR} in HD cognitive dysfunction.

Knockdown of p75^{NTR} expression in the hippocampus of KI mice prevents spatial, object recognition, and associative memories. Since hippocampal p75^{NTR} overexpression mirrored HD memory impairments, we next tested whether the specific reduction of p75^{NTR} in the hippocampus of KI mice was sufficient to improve such memory deficits. To this aim, adenoviruses expressing shRNAp75 (AAV-shp75) were infused bilaterally in the dorsal hippocampus of KI mice at 4 months of age, and

1 month later mice were assessed by means of spatial, recognition, and associative memories (Figure 9). First, we tested the efficiency of shRNAp75 in knocking down p75^{NTR} by testing p75^{NTR} hippocampal levels. Western blot analysis revealed a significant decrease of 30% in p75^{NTR} levels in KI mice infused with AAV-shp75 compared with those infused with AAV-ctl (Figure 9A). Then, memory function was assessed. Importantly, reduction of hippocampal p75^{NTR} levels in KI mice to levels of WT completely reversed spatial, recognition, and associative memory deficits (Figure 9, B–E), suggesting that increased expression of p75^{NTR} in the hippocampus of HD mice is a key contributor of cognitive dysfunction in HD.

Increased RhoA activity contributes to loss of dendritic spines in KI mutant mice. The small GTPase RhoA functions as a negative modulator of dendritic spine formation and maintenance (59, 60), and RhoA activity is modulated by p75^{NTR} signaling (25). Therefore, we hypothesized that aberrant p75^{NTR} expression could contribute to synaptic and memory dysfunction in HD by altering RhoA activity and therefore affecting dendritic spine density. First, we tested RhoA activity in hippocampal extracts from WT, p75^{-/-}, KI, and KI:p75^{-/-} mice by pull-down analysis. Consistent

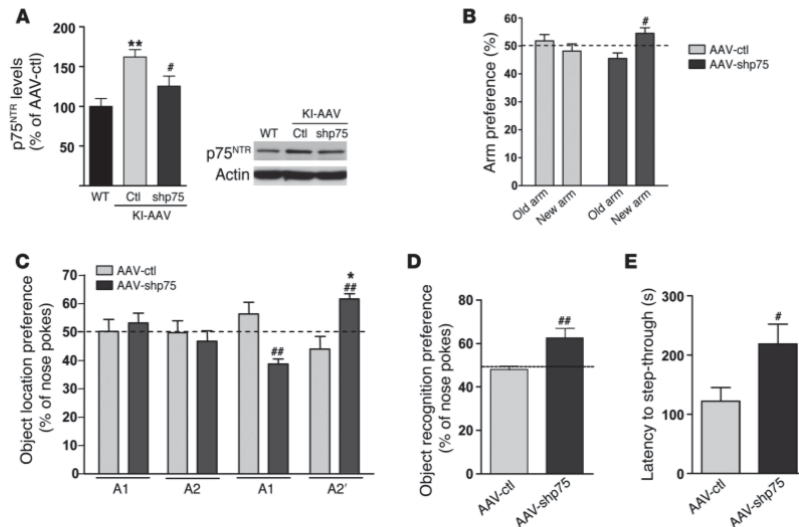


Figure 9. Intra-hippocampal infusion of AAV-shp75 in KI mice at 6 months restores spatial, recognition, and associative memories. (A) Western blot showing a significant reduction in p75^{NTR} levels in total hippocampus extracts from AAV-shp75-injected KI mice ($n = 8$ per genotype). $**P < 0.01$ compared with WT mice. $\#P < 0.05$ compared with AAV-ctrl mice. (B) Percentage of time spent in arms from AAV-ctrl and AAV-shp75 mice ($n = 8$ –12 per genotype). AAV-shp75 mice exhibit preference for a previously unexposed arm of a T-maze. ($n = 8$ per genotype.) $\#P < 0.05$ compared with AAV-ctrl mice. (C) Percentage of time exploring the new object location (A2) from AAV-ctrl and AAV-shp75 mice in the novel-object location task ($n = 7$ –8 per genotype). AAV-shp75 mice showed significantly greater preference for the novel-object location. $**P < 0.01$ compared with AAV-ctrl mice, $\#P < 0.05$ compared with familiar object location. (D) Percentage of nose pokes to the new object from AAV-ctrl and AAV-shp75 mice in the NORT ($n = 8$ per genotype). AAV-shp75 mice display preference for the novel object. $**P < 0.01$ compared with AAV-ctrl mice. (E) Latency to step-through from light to dark compartment from AAV-ctrl and AAV-shp75 ($n = 8$ per genotype). AAV-shp75 mice exhibit more latency to crossover than AAV-ctrl. $\#P < 0.05$ compared with AAV-ctrl mice. All histograms represent mean \pm SEM. Student's 2-tailed t test was performed in A, D, and E and 1-way ANOVA with Bonferroni post hoc comparisons was performed in B and C.

with our hypothesis, KI mutant mice showed a significant increase in hippocampal RhoA activity compared with either WT, $p75^{-/-}$, or $KI:p75^{-/-}$ mice (Figure 10A). Next, we tested whether p75^{NTR}-mediated RhoA activation could be responsible for hippocampal dendritic spine pathology in HD mice. To this aim, we analyzed whether exogenous overexpression of p75^{NTR} was associated with increased RhoA activity and whether this increase caused dendritic spine alterations. Notably, we found that RhoA activity in the hippocampus of WT mice overexpressing p75^{NTR} was significantly higher (twofold increase) than that in control mice (Figure 10B). Accordingly, the analysis of hippocampal GFP-labeled dendritic spines revealed a significant reduction (~50%) in dendritic spine density in AAV-p75 mice compared with AAV-GFP mice (Figure 10C). We verified that the reduction in spines was not merely due to a decrease in GFP-p75^{NTR} staining by labeling hippocampal slices with an anti-phalloidin antibody (Supplemental Figure 5). Finally, to further validate the role of RhoA in p75^{NTR}-induced dendritic abnormalities, we overexpressed p75^{NTR} in hippocampal primary cultures, and dendritic spine-like structures were analyzed in the presence of the RhoA inhibitor C3 transferase. A significant reduction (~20%) in the number of spine-like structures counted as PSD95-positive clusters (31) was found in AAV-p75 hippocampal neurons, which agrees with our previous data showing a decrease in dendritic spine density in AAV-p75-trans-

duced mice (Figure 10C). Importantly, by blocking of RhoA activity, the reduction of PSD95-positive clusters was prevented. These experiments suggest that the pathological increase of p75^{NTR} in the hippocampus of HD mice leads to aberrant RhoA activity and consequently dendritic spine alterations.

Discussion

In the present study we identify p75^{NTR} as an essential contributor to synaptic dysfunction and memory decline in HD and extend previous studies pointing to p75^{NTR} as a negative modulator of hippocampal function (23, 24, 61). We demonstrate an inverse correlation between p75^{NTR} expression in the hippocampus of mutant HD mice and synaptic function manifested as reduced dendritic spine number, altered dendritic morphology, impaired LTP, and memory deficits. In accordance, genetic normalization of p75^{NTR} in HD knock-in mutant mice rescues synaptic morphology, plasticity, and memory deficits, likely by normalization of GTPase RhoA activity. Moreover, overexpression of p75^{NTR} in the hippocampus of WT mice reproduces HD-like memory deficits, while specific hippocampal p75^{NTR} knock-down in HD mutant mice prevents such cognitive impairments.

Decades of research have long established that cognitive function declines in the premanifest and early stages of HD (62–64). Although several studies point to the contribution of other brain structures such as the hippocampus to learning and memory defi-

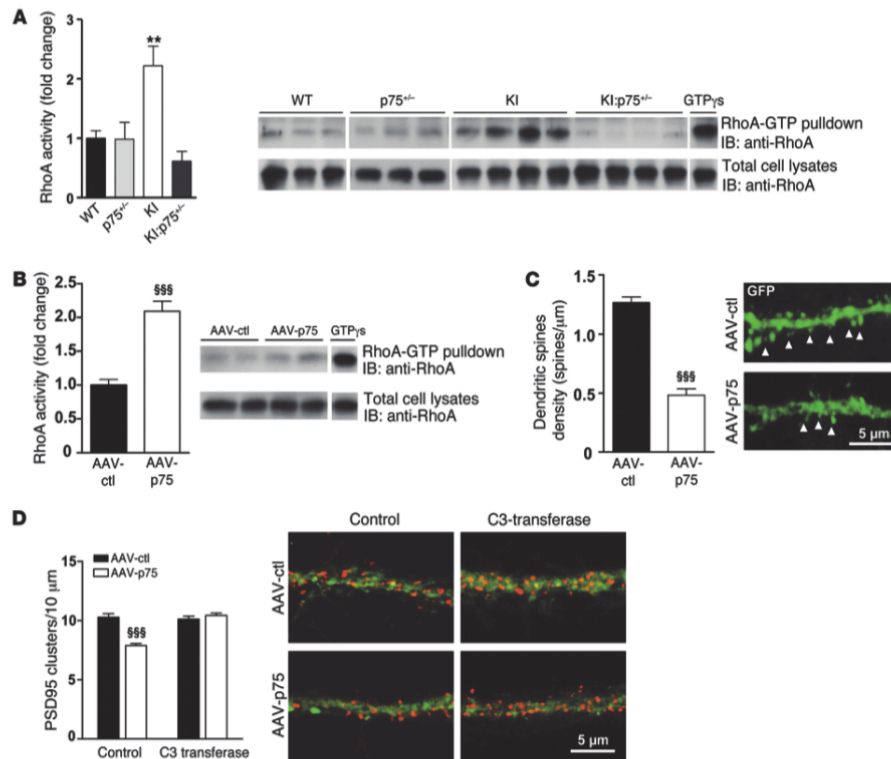


Figure 10. Increased RhoA activity contributes to p75^{NTR}-mediated dendritic spine loss in mutant KI mice. (A) Hippocampal lysates from WT, KI, p75^{-/-}, and KI:p75^{-/-} mice at 6 months of age were subjected to RhoA activity assays. Quantification of the RhoA-GTP levels adjusted to the total RhoA is shown. One-way ANOVA with Tukey post hoc comparisons was performed; $n = 5$ animals per genotype. $**P < 0.01$ compared with WT mice. (B) Hippocampal lysates from AAV-ctl- and AAV-p75-transduced WT mice were subjected to RhoA activity. Quantification of the RhoA-GTP levels adjusted to the total RhoA is shown. Student's 2-tailed t test was performed; $n = 5$ –6 per genotype. $^{***}P < 0.001$ compared with AAV-ctl mice. (C) Left: Quantitative analysis showing dendritic spine density. AAV-p75 mice exhibit a significant reduction in dendritic spines compared with AAV-ctl mice. Student's 2-tailed t test was performed; 32 dendrites (AAV-ctl) and 26 dendrites (AAV-p75) from 3 animals per condition were analyzed. $^{***}P < 0.001$ compared with AAV-ctl mice. Right: Representative confocal microscopy images of CA1 dendrites from AAV-ctl and AAV-p75 mice. Arrowheads indicate dendritic spines. (D) Hippocampal neurons were transduced at DIV14 with AAV-ctl or AAV-p75 and at DIV20 treated with the RhoA inhibitor C3 transferase. Left: Quantitative analysis showing the density of PSD95 clusters. Two-way ANOVA comparing groups \times treatment with Bonferroni post hoc comparisons was performed. $^{***}P < 0.001$ compared with control AAV-ctl-treated neurons. Right: Representative confocal microscopy images showing PSD95 clusters (red) in GFP-labeled neurons. All histograms represent mean \pm SEM.

cits in HD (7–9, 62, 63, 65), data from clinical trials are far from being conclusive on the role of the hippocampus in HD memory deficits. To shed new light on the contribution of the hippocampal circuitry to synaptic and memory decline in HD, we have evaluated spatial and nonspatial memories in a precise genetic mouse model of HD that expresses endogenous levels of mutant huntingtin, *Hdh*^{Q7/Q11} mice (30), by using a battery of different behavioral tests. We demonstrated impairments in spatial, recognition, and associative memories in *Hdh*^{Q7/Q11} mutant mice at early and mild disease stages, suggesting hippocampal dysfunction in our HD mouse models. However, we would like to emphasize that even though the contribution of the hippocampus in spatial tasks is

known to be critical (66–68), there is no consensus about which brain regions are the most important for nonspatial memory. Then, our behavioral data in HD mice do not completely rule out the contribution of other areas of the limbic system or the prefrontal cortex in such memory impairments.

To get insights into the molecular mechanisms contributing to this memory decline, we focused on p75^{NTR}. The role of p75^{NTR} in the adult brain has been mainly associated with apoptosis, whereas its involvement in synaptic plasticity and memory is poorly understood. Null p75^{NTR} mice exhibit enhanced LTP (21) and impaired LTD (23), while both null and heterozygous p75^{NTR} mice display improved spatial memory (21, 22, 69). In contrast, overexpression

of p75^{NTR} negatively modulates dendritic complexity and spine density in hippocampal neurons (24). In this scenario, we show that p75^{NTR} is upregulated in the hippocampus but not in the cortex of HD mice and HD human brain, extending our previous studies showing upregulation of p75^{NTR} in the HD striatum (30). In view of these data we wondered whether normalization of p75^{NTR} levels in KI mice would recover HD memory deficits. In agreement with our hypothesis and supporting a role of aberrant hippocampal p75^{NTR} expression in HD memory impairments, new double-mutant mice expressing mutant huntingtin but “normal” p75^{NTR} levels (*KI:p75^{-/-}* mice) showed preserved spatial, recognition, and associative memories. Moreover, and according with memory decline, our electrophysiological studies also revealed severe hippocampal LTP deficiencies in HD knock-in mutant mice, in agreement with previous studies showing significant LTP reduction in distinct HD mouse models (14, 70). Importantly, normalization of p75^{NTR} levels in *KI:p75^{-/-}* mice restored induction and maintenance of LTP, further supporting a critical role for p75^{NTR} in synaptic deficits in HD.

Our data, however, do not directly support the hypothesis that the increase of p75^{NTR} in the hippocampus of HD mice is responsible for synaptic and memory dysfunction. One could argue that downregulation of p75^{NTR} expression in other brain areas in these double-mutant mice could also contribute to the amelioration of HD memory decline. In fact, the striatal p75^{NTR} upregulation previously reported in HD mice (30, 57) raises the question of whether normalization of striatal p75^{NTR} would affect the activity of the corticostriatal circuitries by preventing or improving memory impairments. To answer this question, corticostriatal pathology was also evaluated in *KI:p75^{-/-}* mutant mice. Interestingly, normalization of striatal p75^{NTR} levels in KI mice did not prevent motor learning deficits or corticostriatal LTP abnormalities, although a recovery of DARPP-32 levels was found. These data suggest that prevention of memory deficits in *KI:p75^{-/-}* mice is associated with the normalization of p75^{NTR} expression in the hippocampus of KI mice, pointing to p75^{NTR} as a key contributor of synaptic and memory decline in our HD mice. This conclusion is further supported by our findings on overexpression of p75^{NTR} in the hippocampus of WT mice and the selective knocking down of p75^{NTR} in the HD mouse hippocampus. Notably, we found that (a) by increasing p75^{NTR} levels only in the hippocampus of WT mice, it was possible to reproduce those memory deficits observed in HD mice; and (b) the specific reduction of p75^{NTR} in the hippocampus of KI mutant mice completely prevented spatial, recognition, and associative memory deficits. Altogether, these findings suggest a negative effect of hippocampal p75^{NTR} upregulation in synaptic and memory processes in HD mice. These data are in agreement with previous findings demonstrating a critical role of p75^{NTR} in cognitive and synaptic dysfunction in AD. Increased p75^{NTR} expression has been reported in the hippocampus of AD patients (26), while inhibition of p75^{NTR} aberrant signaling by small molecules has been demonstrated to prevent memory deficits and neuritic dystrophy in AD mouse models (27, 28).

Although the precise molecular pathways by which mutant huntingtin alters p75^{NTR} expression in the hippocampus of HD mice remain unclear, our results demonstrate that increased p75^{NTR} protein levels were accompanied by increased p75^{NTR} transcripts. The mechanism of p75^{NTR} transcriptional deregulation under disease conditions is poorly understood. However, it has been proposed

that the transcription factor Sp1 drives the expression of p75^{NTR} upon neuronal injury or distinct stress inducers (32, 33). Moreover, Sp1 has been described to be increased in cellular and transgenic models of HD (34). In accordance with these findings, we found a significant increase in Sp1 protein levels in the hippocampus of HD mice and HD human brain, supporting the idea that deregulation of Sp1 activity by mutant huntingtin could underlie the increase in p75^{NTR} mRNA observed in HD hippocampus. However, we cannot rule out that other transcription factors known to regulate p75^{NTR} expression might also contribute to p75^{NTR} deregulation. For instance, it was recently shown that p75^{NTR} is a direct transcriptional target of the transcriptional activator p73 (Tap73) (71), which has also been demonstrated to be hyperactive in HD mouse models and to play a relevant role in HD pathology (72). Interestingly, hippocampal p75^{NTR} and Tap73 expression is increased in AD (73, 74), suggesting that the p73/p75^{NTR} axis may also play an important role in the pathology of neurodegenerative diseases (71).

Several mechanisms can explain how aberrant p75^{NTR} levels mediate synaptic and memory deficits in HD. First, our results indicate that p75^{NTR} directly or indirectly regulates different synaptic-related proteins previously implicated in HD synaptic and/or cognitive deficits, such as CBP, GluA1, CaMKII, or BDNF (14, 15, 54, 55). Indeed, memory improvements in double-mutant *KI:p75^{-/-}* mice correlated with a recovery of the expression and/or phosphorylation of these molecules, leading us to propose p75^{NTR} as a potential regulator of these pathways in HD synaptic pathology. In fact, altered expression of AMPA receptor subunits is observed in the hippocampus of p75^{NTR} knockout mice (61). Second, p75^{NTR} acts as a negative regulator of dendritic spine density and morphology (24, 75). Accordingly, KI mutant mice show a significant loss of dendritic spines in CA1 pyramidal neurons, a phenotype recovered by normalization of p75^{NTR} levels, suggesting that synaptic and memory deficits in HD could be related to a reduction in the number and complexity of hippocampal dendritic spines. Interestingly, spine loss in HD mice is specific to thin spines, and the remaining ones exhibit shorter neck lengths than the thin spines found in the other 3 genotypes. It has been suggested that spine necks regulate biochemical and electrical signals through compartmentalization of Ca²⁺ and diffusion of synaptic-related proteins, which could modulate synapse strength (53, 76, 77). Therefore, changes in neck lengths together with altered number and proportion of dendritic spines could mediate the reduced synaptic plasticity observed in KI mice. It is noteworthy that despite the fact that *p75^{-/-}* and *KI:p75^{-/-}* mice do not exhibit a significant reduction in the total number of dendritic spines, there was a significant decrease in the proportion of thin spines accompanied by a significant increase in the proportion of mushroom spines, thought to be highly stable with larger postsynaptic densities and to be the locus of long-term memories (78, 79). Actually, an increase in the number of mushroom spines might represent a homeostatic mechanism to compensate for the reduction of thin/learning spines (80, 81). These data suggest that adequate p75^{NTR} levels are required for normal forms of synaptic structural plasticity and cognitive processes and that any aberrant p75^{NTR} expression can be deleterious. We hypothesize that altered p75^{NTR} expression above or beyond an optimal level will be critical for hippocampal function. Thus, levels beyond this threshold will not be sufficient to activate certain transduction pathways impor-

tant for some forms of synaptic plasticity (impaired performance of $p75^{NTR}$ mice in the passive avoidance), while levels above this optimal expression, as in our HD mice, will induce overactivation of $p75^{NTR}$ signaling and deregulation of $p75^{NTR}$ -modulated molecular pathways essential for memory processes (increased RhoA activity and subsequent altered dendritic spines and cognition in KI mice).

Our results also suggest that the observed loss of spines found in mutant KI mice could be due to altered $p75^{NTR}$ synaptic localization. $p75^{NTR}$ expression in the hippocampus has been classically restricted to afferents coming from basal forebrain cholinergic neurons (82–84). However, recent evidence in vivo and in vitro also demonstrates dendritic spine localization of $p75^{NTR}$ in CA1 and dentate gyrus of hippocampal neurons (19, 23, 85, 86). In accordance with these studies, we found colocalization of $p75^{NTR}$ with MAP2 and PSD95 in hippocampal pyramidal neurons of CA1, revealing a postsynaptic localization of $p75^{NTR}$. Interestingly, $p75^{NTR}$ was increased in synapses in KI mutant mice, suggesting that postsynaptic $p75^{NTR}$ could have negative effects on synaptic function in HD. Besides $p75^{NTR}$ expression in neurons, it has been reported that astrocytes induce $p75^{NTR}$ expression after different types of injury (36, 86, 87). Increased levels of $p75^{NTR}$ have been detected in both neurons and astrocytes in the hippocampal CA3 region after seizures (37). However, our results showed no colocalization between GFAP and $p75^{NTR}$ in the hippocampus of KI mutant mice, suggesting that the increase in $p75^{NTR}$ levels in these HD mutant mice is predominantly neuronal. Accordingly, specific neuronal overexpression of $p75^{NTR}$ in the hippocampus of WT mice mimics HD-like memory deficits, further supporting the idea that the increase in $p75^{NTR}$ levels in the hippocampal neurons of KI mutant mice is responsible, at least partially, for the synaptic plasticity and memory impairments in HD mice.

How might increased $p75^{NTR}$ levels lead to dendritic spine changes? We built on work showing that $p75^{NTR}$ receptor modulates the activity of RhoA, a Rho family of small GTPases important for the maintenance of the dendritic spines (59, 88). RhoA has complex effects on spines. While sustained elevation of RhoA impairs maturation and maintenance of dendritic spines and reduces dendritic tree and neck length, brief RhoA activation after neuronal activity promotes actin reorganization needed for LTP consolidation (60, 89, 90). Given that $p75^{NTR}$ acts as a constitutive RhoA activator (25, 91), we speculate that prolonged RhoA stimulation due to high $p75^{NTR}$ levels in the hippocampus of KI mutant mice could destabilize actin spine cytoskeleton and compromise the integrity of spines. In this context, we found increased RhoA activation in the hippocampus of mutant KI but not in double-mutant $KI:p75^{NTR}$ mice compared with WT mice, suggesting a direct link between $p75^{NTR}$ -induced dendritic loss and aberrant RhoA activity. In favor of this hypothesis, we found recovery of PSD95-positive clusters in AAV- $p75^{NTR}$ -transduced hippocampal cultures treated with the RhoA inhibitor C3 transferase.

In conclusion, our findings demonstrate a detrimental role of hippocampal $p75^{NTR}$ upregulation in synaptic and memory function in HD. These results, along with previous studies in HD mouse and monkey models reporting hippocampal pathology, underline the need to address the pathogenic significance of the hippocampus in memory and synaptic decline in HD patients. By working on the hypothesis that the hippocampus can play a role in

HD memory impairments, new and exciting therapeutic alternatives can be opened. In this view, our data uncovering a new role of $p75^{NTR}$ in memory decline by regulation of structural and functional synaptic plasticity support the possibility of treating synaptic and memory impairments in HD by pharmacological or genetic modulation of $p75^{NTR}$ function.

Methods

Animals. Heterozygous $Hdh^{Q7/Q11}$ knock-in mice (KI) (92) and $p75^{NTR}$ heterozygous mice ($p75^{NTR}/ExonIII$) from the Jackson Laboratory were bred. KI and $p75^{NTR}$ were on a C57BL/6 genetic background. Only males from each genotype were used for all experiments. Male R6/1 transgenic mice (B6CBA background) expressing exon 1 of mutant huntingtin with 145 repeats were used (93).

Postmortem brain tissues. Hippocampal (6 controls and 7 HD patients) and cortical (7 controls and 6 HD patients) brain tissues from patients with HD grades 3 and 4 (mean \pm SEM; age 54.5 ± 6.5 years; post-mortem intervals of 4–17 hours) and control cases (mean \pm SEM; age 53.5 ± 6.8 years; postmortem intervals of 4–18 hours) were supplied by the Banc de Teixits Neurològics (Biobanc-HC-IDIBAPS).

Behavioral assessment. The T-maze spontaneous alternation task (T-SAT), novel object recognition task (NORT) (13), object location task (OLT) (94), and passive avoidance paradigm (38) were used to analyze hippocampal-dependent memory. Plus maze and light-dark tests (38) were used to analyze anxiety-like behaviors, while accelerating rotarod (95) was used to analyze motor learning.

T-SAT. The T-maze apparatus used was a wooden maze consisting of 3 arms, 2 of them situated at 180° from each other, and the third representing the stem arm of the T, situated at 90° with respect to the other 2. All arms were 45 cm long, 8 cm wide, and enclosed by a 20-cm wall. The maze was thoroughly painted with waterproof gray paint. Light intensity was 5 lux throughout the maze. A starting area (10 cm) was located at the end of the stem arm and closed by a wooden guillotine door. Two identical guillotine doors were placed in the entry of the arms situated at 180° . The maze was elevated 60 cm above the floor. In the training trial, 1 arm was closed (novel arm) and mice were placed in the stem arm of the T (home arm) and allowed to explore this arm and the other available arm (familiar arm) for 10 minutes, after which they were returned to the home cage. After 5 hours (long-term memory), mice were placed in the stem arm of the T-maze and allowed to freely explore all 3 arms for 5 minutes. The arm preference was determined by calculation of the time spent in each arm \times 100/time spent in both arms (familiar and novel). Animals were tracked and recorded with SMART Junior software (Panlab).

OLT. The object location memory task evaluates spatial memory and is based on the ability of mice to recognize when a familiar object has been relocated. Exploration took place in an open-top arena with quadrangular form (45 \times 45 cm). The light intensity was 40 lux throughout the arena, and the room temperature was kept at 19°C – 22°C and 40%–60% humidity. Mice were first habituated to the arena in the absence of objects (2 days, 10 min/d). On the third day during the acquisition phase mice were allowed to explore 2 duplicate objects (A1 and A2), which were placed in the far corners of the arena for 10 minutes. After a delay of 24 hours, 1 object was placed in the corner diagonally opposite. Thus, both objects in the phase were equally familiar, but 1 was in a new location. The position of the new object was counterbalanced between mice. Animals were tracked and recorded with SMART Junior software.

NORT. The device consisted of a white circular arena with 40 cm diameter and 40 cm high. The light intensity was 40 lux throughout the arena, and the room temperature was kept at 19°C–22°C and 40%–60% humidity. Mice were first habituated to the arena in the absence of objects (2 days, 10 min/d). On the third day, 2 similar objects were presented to each mouse during 10 minutes (A1 and A2 condition), after which they were returned to their home cage. Twenty-four hours later (long-term memory), the same animals were retested for 5 minutes in the arena with a familiar and a new object (A2 and B condition). The object preference was measured as the time exploring each object \times 100/time exploring both objects. The arena was rigorously cleaned between animal trials in order to avoid odors. Animals were tracked and recorded with SMART Junior software.

Passive avoidance. The passive avoidance (light-dark) paradigm was performed as described elsewhere (96, 97) with slight modifications. Experiments are conducted in a 2-compartment box, where 1 compartment is dimly lit (20 lux) and preferable to a rodent, and the other compartment is brightly lit (200 lux); both chambers are connected by a door (5 cm \times 5 cm). During training, mice were placed into the aversive brightly lit compartment, and upon the entry into the preferred dimly lit compartment (with all 4 paws inside the dark chamber), mice were exposed to a mild foot shock (2-second foot shock, 1 mA intensity). The latency of mice to enter into the dark chamber was recorded. Twenty seconds after receiving the foot shock, mice were returned to the home cage until testing. After 24 hours (long-term memory) the animal undergoes a retention test (testing). In the retention test, mice were returned to the brightly lit compartment again, and the latency to enter the shock-paired compartment (dark chamber) was measured (retention or recall latency). Ten minutes was used as a time cutoff in the retention test. The animal that learned the task would avoid the location previously paired with the aversive stimulus, and would show greater latency to enter it.

Plus maze. To analyze mouse anxiety-like behaviors, the plus maze paradigm was performed as previously described (38). Briefly, the raised plus maze was made of wood and consisted of 2 opposing open arms of 30 \times 8 cm, and 2 opposing arms of 30 \times 8 cm enclosed by 15-cm-high walls. The maze was raised 50 cm from the floor and lit by dim light. Each mouse was placed in the central square of the raised plus maze, facing an open arm, and the behavior was scored for 5 minutes. At the end of each trial, any feces were removed and the apparatus was wiped with 70% alcohol. We recorded the time spent in the open arms, which normally correlates with levels of anxiety. Animals were tracked and recorded with SMART Junior software.

Light-dark paradigm. The light-dark paradigm was performed as described elsewhere (98) with slight modifications. The light-dark box consisted of 2 compartments, which were connected by a door (5 cm \times 5 cm) located at floor level in the center of the partition. The light compartment was white and directly illuminated by a dim light (200 lux), while the dark compartment was black and completely enclosed (20 lux). The test was performed in a dimly lit room. Each animal was initially confined in the dark compartment and allowed to freely explore the apparatus for 5 minutes. The parameters recorded were time spent by each mouse in the light compartment and latency time for the first crossing to the light compartment. Between animals, the apparatus was carefully cleaned with 70% ethanol solution and allowed to dry.

Accelerating rotarod training procedure. As previously described (99), animals were placed on a motorized rod (30 mm diameter). The rotation speed gradually increased from 4 to 40 rpm over the course

of 5 minutes. The time latency was recorded when the animal was unable to keep up with the increasing speed and fell. Rotarod training and testing was performed 4 times per day during 3 consecutive days.

Viral constructs and stereotaxic injection. For p75^{NTR} overexpression the plasmid pEGFP-N3-p75 obtained from E. Formaggio (Department of Medicine and Public Health, Pharmacology Section, University of Verona, Verona, Italy) was used to clone into a rAAV2/8-GFP adenoviral vector (BamHI site at 5' and AgeI at 3'). For knockdown p75^{NTR} expression we designed a siRNA oligomer targeting the mouse p75^{NTR}:Ngfr-1 to clone into a rAAV2/8-GFP adenoviral vector as previously described. Infectious AAV viral particles containing GFP expression cassette with scrambled shRNA (AAV-ctl), shRNAp75 (AAV-shp75), GFP (AAV-GFP), or p75^{NTR} expression cassette (AAV-p75) were generated by the Unitat de Producció de Vectors from the Center of Animal Biotechnology and Gene Therapy at the Universitat Autònoma de Barcelona. Following anesthesia with pentobarbital (30 mg/kg), 5-month-old mice were injected with AAV-GFP, AAV-p75, and AAV-shp75 (viral concentration ranging from 1.53×10^9 to 3.06×10^9 GCs per injection, $n = 8$ per group) at 2 sites of the hippocampus according to the following coordinates from the bregma (millimeters); anteroposterior, -1.6 and -1.9; lateral, +2.0 and +3.0; and dorsoventral, -0.8 and -1.2. AAVs were injected over 2 minutes, leaving the cannula in place for 5 additional minutes to ensure complete diffusion of the viruses, and then slowly retracted from the brain. The animals were monitored for 2 hours after administration and then returned to the housing facility for 30 days. After this period, animals from experimental groups were subjected to behavioral assessment.

Total protein extraction and subcellular fractionation. Total protein extraction was performed as described elsewhere (30). For cytosolic and nuclear fractions, tissue was homogenized in lysis buffer (4 mM HEPES, 0.32 M sucrose, 2 mM PMSF, 10 mg/ml aprotinin, 1 mg/ml leupeptin, 2 mM Na₂VO₄, and 0.1 mg/ml benzamide) with a Teflon-glass potter and centrifuged at 800 g for 10 minutes to obtain the cytosolic (supernatant) and the nuclear (pellet) fractions. The pellet was resuspended in lysis buffer and centrifuged at 800 g for 10 minutes. The resulting pellet, containing washed nuclear fraction, was then resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM ZnCl₂, 10 mM EGTA, 2 mM PMSF, 10 mg/ml aprotinin, 1 mg/ml leupeptin, 0.1 mg/ml benzamide, 2 mM Na₂VO₄, and 100 mM NaF) and incubated for 30 minutes at 4°C in a tube rotator. Finally, after centrifugation for 20 minutes at 15,700 g, the supernatant was collected and stored at -80°C until use. Protein concentration was measured using the DC protein assay kit (Bio-Rad).

Western blotting. Western blot experiments were performed as previously described (15, 29). Antibodies used for Western blot analysis were anti-p75^{NTR} (1:1,000; Promega); anti-CaMKII, anti-phospho-CaMKII^{Thr286}, anti-BDNF, and anti-CBP (1:1,000; Santa Cruz Biotechnology); anti-GluA1 and anti-GluA2/3 (1:1,000; Upstate Biotechnology); anti-phospho-GluA1^{Ser831} (1:1,000; Millipore); anti-actin (1:10,000; MP Biochemicals); anti-PSD95 (1:2,000; Affinity Bioreagents); anti-PSD93 (1:1,000; Alomone); anti-SAP102 (1:1,000; Synaptic Systems); anti-spinophilin (1:1,000; Upstate Biotechnology); anti-GluN2B (1:1,000; Chemicon); anti-GluN1 (1:1,000; Chemicon); anti-synaptophysin (1:1,000; Abcam); anti-RhoA (1:1,000; Cell Signaling); anti-SPI (1:500; Santa Cruz Biotechnology); anti-NeuN (1:1,000; Millipore); anti-DARPP-32 (1:1,000; BD Bioscience); and anti- α -tubulin (1:20,000; Sigma-Aldrich).

RhoA pull-down assay. The Rho-GTP pull-down assay was performed using a RhoA activation assay kit according to manufacturer instructions (Cell Biolabs). Hippocampal tissue was homogenized in ice-cold 1× assay/lysis buffer. Samples were centrifuged at 14,000 g for 10 minutes at 4°C, and the supernatant (1 mg of protein) was incubated with Rhotekin RBD-agarose beads at 4°C for 1 hour on a rotator. The beads were washed 3 times with 0.5 ml of 1× assay buffer. Protein samples were eluted with 1× SDS-PAGE sample buffer and processed for Western blotting using RhoA antibody.

Real-time quantitative RT-PCR. Total RNA was isolated from the hippocampus and cortex of HD mutant and WT mice using the Total RNA Isolation Nucleospin RNA II Kit (Macherey-Nagel). Purified RNA (500 ng) was reverse transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time, Takara Biotechnology Inc.). The cDNA synthesis was performed at 37°C for 15 minutes and a final step at 85°C for 5 seconds in a final volume of 20 µl according to the manufacturer's instructions. The cDNA was then analyzed by quantitative RT-PCR using the following TaqMan Gene Expression Assays (Applied Biosystems): 18S (Hs99999901_s1) and p75^{NTR} (Mm00446294_m1). RT-PCR was performed in 12.5 µl of final volume on 96-well plates using the Premix Ex Taq. Reactions included 40 cycles of a 2-step PCR: 95°C for 5 seconds and 60°C for 20 seconds, after initial denaturation at 95°C for 30 seconds. All quantitative PCR assays were performed in duplicate. To provide negative controls and exclude contamination by genomic DNA, the RT was omitted in the cDNA synthesis step. The quantitative PCR data were quantified using the comparative quantitation analysis program of MxPro™ quantitative PCR analysis software version 3.0 (Stratagene) with the 18S gene expression as loading control.

Histology and stereology. Hippocampal volume estimations were performed following the Cavalieri method. The number of Nissl-positive neurons in the CA1 pyramidal cell layer was established using unbiased design-based stereology blinded to genotype and group and was performed using Computer-Assisted Stereology Toolbox (CAST) software (Olympus Danmark A/S). Specifically, we used the disector counting procedure in 12 hippocampal coronal sections (30 µm thick) per animal, spaced 240 µm and covering the entire rostrocaudal extent of this brain region.

Primary culture of astrocytes. Primary astrocyte cultures were obtained from P1 to P3 WT mouse pups by cortical dissection and removal of the meninges. Extracted tissue was mechanically dissociated and resuspended in MEM 1× conditioned media NM-15 (20% FBS, Gibco-BRL; 90 mM D-glucose, Sigma-Aldrich) with L-glutamine and Earle's salts (Gibco-BRL), plated in 25-cm² flasks, and cultured at 37°C with 5% CO₂. After 2 passages, cultures were purified by shaker agitation during 10 minutes at 400 rpm, and undesired floating cells were discarded. Medium was replaced and seeded astrocytes maintained 2 hours at 37°C in agitation for 16–18 hours at 250 rpm, floating cells removed, and new medium replaced. Twenty-four hours later, astrocytes were trypsinized, seeded at 1 × 10⁶ cells in P100 plates, and allowed to reach 100% confluence. Astrocytes were transduced with AAV-GFP or AAV-p75 (40,000 GCs per cell).

Primary culture of mouse hippocampal neurons and adenovirus-mediated gene transduction. Primary hippocampal neurons were prepared from E18.5 mouse embryos as previously described (100). The neuronal cell suspension was seeded (70,000 cells/cm²) on coverslips precoated with poly-D-lysine (0.1 mg/ml; Sigma-Aldrich) in 24-well plates. Neurobasal medium containing 1 ml of 1× B27 (50

supplement (Gibco-BRL) and 500 ml of GlutaMAX (100×) (Gibco-BRL) was used to grow the cells in serum-free medium conditions and maintained at 37°C in 5% CO₂. Two weeks after plating (DIV14), neurons were transduced with AAV-GFP or AAV-p75 (40,000 GCs per cell) in Neurobasal maintenance medium for up to 1 week. At DIV20, cells were treated with vehicle or C3 ADP ribosyl transferase, a cell-permeable Rho inhibitor (100 ng/ml; Cytoskeleton Inc.), for 24 hours. At DIV21, neurons were fixed for 10 minutes in 4% PFA for immunostaining and confocal analysis.

Immunocytochemistry and image analysis. Fixed cultures were permeabilized for 10 minutes at room temperature with 0.5% saponin in PBS and blocked with 15% horse serum in PBS. The cells were incubated with anti-PSD95 (1:1,000; Affinity Bioreagents) and secondary antibodies (Cy3, 1:200; Jackson ImmunoResearch). Single images were acquired digitally using Leica Confocal SP5 with a ×63 oil-immersion objective and a digital zoom of 3. Conditions such as pinhole size (1 AU) and frame averaging (4 frames per z-step) were held constant throughout the study. PSD95 clusters in dendrites from GFP-labeled neurons were quantified using the freeware NIH ImageJ version 1.33 by Wayne Rasband (NIH). At least 60 neurites (1–2 neurites per neuron) for each condition in 3 independent experiments were analyzed.

Immunofluorescence and SL confocal analysis. Immunofluorescence experiments and analysis were carried out as previously described (30, 101). Antibodies against p75^{NTR} (1:100; Promega), GFP (1:1,000; Upstate Biotechnology), PSD95 (1:1,000; Affinity Bioreagents), and MAP2 (1:500; Sigma-Aldrich) and secondary antibodies (Alexa Fluor 488 and 555, Jackson ImmunoResearch) were used. Stained coronal sections were examined blinded to genotype by confocal microscopy, using a Leica TCS SL laser scanning confocal spectral microscope with argon and helium-neon lasers. Images were taken with a 63× numerical aperture lens with 4× digital zoom and standard (1 Airy disc) pinhole. For each mouse, at least 3 slices of 30 µm containing hippocampal tissue were analyzed. Up to 3 representative images, from CA1-stratum radiatum layer, were obtained from each slice. For each image, the entire 3D stack of images was obtained by the use of the Z drive present in the Leica TCS SL microscope, and the size of the optical image was 0.5 µm, with a separation of 2 µm between each. The number of double-labeled p75^{NTR}/PSD95-positive clusters was counted by the freeware NIH ImageJ version 1.33 by Wayne Rasband (NIH).

Dendritic spine dyeing and SP5 confocal analysis. Neurons were labeled using the Helios Gene Gun System (Bio-Rad) as previously described (102, 103). Briefly, a suspension containing 3 mg of DiI (Molecular Probes, Invitrogen) dissolved in 100 µl of methylene chloride (Sigma-Aldrich) and mixed with 50 mg of tungsten particles (1.7 mm diameter; Bio-Rad) was spread on a glass slide and air-dried. The mixture was resuspended in 3.5 ml distilled water and sonicated. Subsequently, the mixture was drawn into Tefzel tubing (Bio-Rad), and then removed to allow tube drying during 5 minutes under a nitrogen flow gas. Then, the tube was cut into 13-mm pieces to be used as gene gun cartridges. Dye-coated particles were delivered in the hippocampus using the following protocol. Shooting was performed over 200-µm coronal sections at 80 psi through a membrane filter of 3 µm pore size and 8 × 10 pores/cm² (Millipore). Sections were stored at room temperature in PBS for 3 hours protected from light and then incubated with DAPI, and mounted in Mowiol to be analyzed. DiI-labeled pyramidal neurons from CA1 of the dorsal hippocampus were imaged using a Leica Confocal SP5 with a ×63 oil-immersion objective. Conditions such as pinhole size

(1 AU) and frame averaging (4 frames per z-step) were held constant throughout the study. Confocal z-stacks were taken with a digital zoom of 5, a z-step of 0.2 μm , and at 1,024 \times 1,024 pixel resolution, yielding an image with pixel dimensions of 49.25 \times 49.25 μm . Z-stacks were deconvolved using the Acoloma plug-ins from ImageJ, to improve voxel resolution and reduce optical aberration along the z-axis. Segments of basal dendrites and proximal apical dendrites were selected for the analysis of spine density and spine morphology according to the following criteria: (a) segments with no overlap with other branches that would obscure visualization of spines and (b) segments either “parallel” to or “at acute angles” relative to the coronal surface of the section to avoid ambiguous identification of spines. Only spines arising from the lateral surfaces of the dendrites were included in the study; spines located on the top or bottom of the dendrite surface were ignored. Given that spine density increases as a function of the distance from the soma, reaching a plateau 45 μm away from the soma, we selected dendritic segments of basal dendrites 45 μm away from the cell body.

For a more precise description of the dendritic shape changes, the spine head diameter was analyzed as a continuous distribution. To this end, we proceeded as follows: first all the identified spines were categorized as spines without neck (stubby spines) or with a clear neck. From the latter, blind measurements of the head diameter were performed manually using ImageJ for all the spines in control mice. Then, a distribution analysis of head diameter was performed. The Gaussian adjustment resulted in a normal distribution with a mean value of 0.5009 μm (Gaussian fit $P = 0.90$) (Supplemental Figure 4). From the estimated mean value, spine populations were divided into spines with diameters below the mean average (head diameters $<0.5009 \pm 0.006 \mu\text{m}$, thin spines), spines with head diameters above the mean value (head diameters $>0.5009 \pm 0.006 \mu\text{m}$, mushrooms), and a third category including all the non-neck spines (stubby spines). Spine neck was measured as the distance from the dendritic shaft to the head of the spine.

Electrophysiology. Coronal brain slices (400 μm thickness) were prepared from WT, $p75^{\text{NTR}}$, KI, and $KI;p75^{\text{NTR}}$ mice (6 months old) as described previously (104) and incubated for more than 1 hour at room temperature (21°C–24°C) in ACSF (in mM: NaCl 124, KCl 2.69, KH_2PO_4 1.25, MgSO_4 2, NaHCO_3 26, CaCl_2 2, and glucose 10) and were gassed with 95% O_2 and 5% CO_2 . Slices containing hippocampus or striatum and cortex were transferred to an immersion recording chamber and superfused (2.5 ml/min) with equilibrated ACSF maintained at constant temperature (34°C). To evaluate hippocampal LTP, extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode (impedances 2–3 M Ω ; filled with 1 M NaCl) positioned in stratum radiatum area CA1 of the hippocampus. Evoked fEPSPs were elicited by stimulation of the Schaeffer collateral fibers with an extracellular bipolar tungsten electrode via a 2100 isolated pulse stimulator (A-M Systems Inc.). LTP was induced by application of 4 trains (1 second at 100 Hz) spaced 20 seconds, and potentiation was measured for 1 hour after LTP induction at 0.1 Hz. Corticostriatal LTP was induced by application of tetanic stimulation twice (4 trains 1 second at 100 Hz)

spaced 10 seconds, and potentiation was measured for 1 hour after LTP induction at 0.1 Hz. For each experiment, population spike (PS) amplitude was expressed as a percentage of average pre-tetanus baseline amplitude. Data were stored on a Pentium-based PC using a PowerLab 4/26 acquisition system (AD Instruments); Scope software (AD Instruments) was used to display PS and measurements of the amplitude of PSs. Statistical differences, compared with pre-tetanus baseline amplitude values, were established using the 2-tailed Student's t test.

Statistics. All data are expressed as mean \pm SEM. Statistical analysis was performed using the unpaired Student's t test (2-tailed, 95% confidence), the Kolmogorov-Smirnov test to compare cumulative distributions, 1-way ANOVA, 2-way ANOVA, and the appropriate post hoc tests as indicated in the figure legends. Values of $P < 0.05$ were considered statistically significant.

Study approval. Human samples were provided by Banc de Teixits Neurològics (Biobanc-HC-IDIBAPS). The Banc de Teixits Neurològics was established to provide human postmortem brains to researchers in Spain, taking into consideration all the ethical guidelines of the latest Declaration of Helsinki. Informed consent was obtained from all subjects under study. Experimental animal procedures were approved by the Local Ethical Committee of the University of Barcelona (99/01) and the Generalitat de Catalunya (00/1094), following European (2010/63/UE) and Spanish (RD 1201/2005) regulations for the care and use of laboratory animals.

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WORK 4***“KALIRIN-7, A NEW MEDIATOR OF CORTICOSTRIATAL SYNAPTIC
DYSFUNCTION IN HUNTINGTON’S DISEASE”***

This manuscript is under preparation

Puigdemívol M, Cherubini M, Brito V, Giralt A, Ballesteros J.J, Zamora-Moratalla A, Martín E.D.,
Eipper B.A., Alberch J, Ginés S.

CONTRIBUTION TO THIS WORK:

I contributed to the design of the research plan and organization of the study. I performed the behavioral analysis including the ARTP, Swimming T-maze and open field. I obtained all mice brain samples for their protein analysis by immunoblot and the perfusion of HD mice and the preparation of brain slices for immunohistochemical analyses. I performed all the immunohistochemical analysis with postsynaptic markers to determine alterations in spine-like structures at several ages, as well as the biochemical analysis in different brain regions in different HD mouse models and human samples. I performed the analysis of Rac1 activity. I performed all cortical primary cultures from HD mice and their transfection with Myc and Myc-Kal7 plasmids, and analyzed the number of excitatory synapses in all these conditions. I discuss and wrote the article together with my supervisor.

AIM 3. TO STUDY THE MOLECULAR MECHANISMS INVOLVED IN CORTICOSTRIATAL DYSFUNCTION IN HUNTINGTON'S DISEASE

3.1. To characterize corticostriatal deficits in HD mouse models and analyze the role of Kalirin-7 in the alteration of corticostriatal excitatory synapses in HD.

La malaltia de Huntington (MH) és un desordre neurodegeneratiu clàssicament caracteritzat per alteracions en la coordinació motora resultants d'una selectiva degeneració de les neurones de projecció estriatal GABAèrgiques. No obstant, diverses evidències indiquen que les alteracions cognitives en la MH són d'aparició primerenca en la simptomatologia clínica, suggerint així que la disfunció neuronal i sinàptica de la via corticoestriatal precedeix la mort neuronal estriatal. En aquest estudi demostrem l'aparició de dèficits d'aprenentatge motor en dos models murins de la MH (Hdh^{Q7/Q111} i R6/1) en edats primerenques, en les quals no observem alteracions en la coordinació motora. De manera significativa, l'aparició temprana d'aquests dèficits en aprenentatge depenent de la via corticoestriatal es troba associada a alteracions en la transmissió sinàptica de la via corticoestriatal i amb una reducció significativa de partícules positives per marcadors post-sinàptics tals com PSD-95 i spinophilin en la regió cortical, però no estriatal, d'animals Hdh^{Q7/Q111}. A més, demostrem que Kalirina-7 (Kal7), una proteïna RhoGEF que en els darrers anys ha estat considerada un regulador clau en la formació i manteniment d'espines dendrítiques capaç de promoure l'activació de petites Rho GTPases tals com Rac1, es troba específicament disminuïda en l'escorça cerebral, però no en l'estriat, de models murins de la MH, a edats temperanes on prèviament hem detectat els dèficits en aprenentatge motor i transmissió sinàptica. De manera significativa, demostrem que la reducció en sinapsis excitadores en cultius primaris corticals provinents d'animals de la MH es troba completament restaurada mitjançant la sobre-expressió de Kal7. En conjunt, aquests resultats evidencien que la disfunció cortical precedeix la patologia estriatal en models murins de la MH i possiblement és la primera responsable de les alteracions sinàptiques i cognitives dependents de la via. A més, identifiquem Kal7 com a un contribuent clau en les alteracions corticals en la MH i suggerim que Kal7 pot esdevenir un important candidat molecular en les futures estratègies terapèutiques focalitzades a restaurar la funció corticoestriatal en la MH.

Kalirin-7, a new mediator of corticostriatal synaptic dysfunction in Huntington's disease

Abbreviated title: Kal7 and early corticostriatal deficits in HD

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ABSTRACT

Growing evidence demonstrates that cognitive disturbances are early clinical symptoms of Huntington's Disease that may reflect corticostriatal synaptic and neuronal dysfunction prior to striatal neuronal loss. In this study, we report corticostriatal-dependent motor learning deficits in two distinct HD mouse models at ages that precede any detectable motor coordination disturbance. Importantly, learning decline was associated with early deficits in corticostriatal long-term potentiation and with a reduction in PSD-95 and spinophilin-positive clusters in the cortex but not in the striatum of HD mice. As a potential underlying mechanism, we found that Kalirin-7 (Kal7), a guanine-nucleotide exchange factor for Rho-like small GTPases and an important regulator of dendritic spine plasticity, was specifically decreased in the cortex of HD mice at early HD stages when learning and plasticity deficits were detected. Additionally, we found that the reduction of excitatory synapses seen in cortical HD neuronal cultures was restored by over-expression of Kal7. Altogether our results provide new evidence that cortical dysfunction precedes striatal pathology and mediates early corticostriatal synaptic and cognitive deficits in HD mice. Moreover, we identify Kal7 as a key contributor of these cortical alterations, placing Kal7 as a molecular target for future therapeutic strategies to treat cognitive deficits and restore corticostriatal function in HD.

INTRODUCTION

Basal ganglia dysfunction is a clear hallmark of Huntington's disease (HD) involved in the classical motor impairments shown by HD patients. However, it is clear that HD encompass more than motor deficits with evidence of cognitive dysfunction years before chorea symptoms appear. In this view, it has been suggested that functional and morphological changes in key brain areas involved in cognitive processes such as the neocortex could precede alterations in the striatum and be the initial trigger of striatal pathology and late-stage motor symptoms (Cepeda et al., 2007;Cybulska-Klosowicz et al., 2004;Laforet et al., 2001). Thus, cognitive deficits manifested by HD patients such as impaired sensory discrimination, learning memory, planning and decision making have been related with neocortical dysfunction (Hahn-Barma et al., 1998;Ho et al., 2003;Lawrence et al., 1998;Paulsen et al., 2001;Rosenberg et al., 1995;Schneider et al., 2010) while specific thinning of the neocortex has been recently demonstrated in stage I and II of HD individuals (Rosas et al., 2010;Kassubek et al., 2004). Interestingly, deficient cortical function evidenced by decreased glutamate receptor-mediated currents (Andre et al., 2006), impaired learning-dependent cortical plasticity (Cybulska-Klosowicz et al., 2004;Mazarakis et al., 2005) and altered excitability of cortical pyramidal neurons (Cepeda et al., 2007;Cummings et al., 2009) has also been reported in different HD mouse models at early disease stages. Changes in the structure, location or number of dendritic spines, that represents the site of most glutamatergic synapses in the brain, are critically involved in the synaptic deficits in different neurodegenerative and neurodevelopmental disorders (Lai and Ip, 2013;Shepherd and Erulkar, 1997;Van Der Zee, 2014;von Bohlen Und, 2009). Thus, in symptomatic transgenic HD mice (Guidetti et al., 2001;Spires et al., 2004) and in HD post-mortem human brain (Ferrante et al., 1991;Graveland et al., 1985;Sotrel et al., 1993) it has been reported reduced dendritic spine density and altered dendritic morphology in both cortical pyramidal neurons and striatal neurons, suggesting that cognitive deficits in HD could be driven by deficiency of corticostriatal network connectivity due to dendritic spine alterations. However, the molecular mechanisms underlying these changes are poorly understood.

Dendritic spine dynamics are dependent on neuronal activity through modulation of the actin cytoskeleton by the GDP/GTP exchange factors of the Rho-family small

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GTPases (Rho-GEFS) and the corresponding GTPase-activating proteins (GAP) (Govek et al., 2004;Tolias et al., 2007;Xie et al., 2007;Zhang et al., 2005). Kalirin-7 (Kal7), is a brain-specific Rho-GEF for Rac-like GTPases localized to dendritic spines of cortical and hippocampal pyramidal neurons where plays a critical role in structural and functional plasticity of excitatory synapses (Ma et al., 2003;Ma et al., 2008a;Ma et al., 2008b;Penzes et al., 2001). Accordingly, Kal7-positive clusters were found to overlap with PSD95, NMDA and AMPA receptors staining and apposed to Vglut-1 positive clusters while no co-localization was detected with either inhibitory presynaptic (GAD65) or postsynaptic (GABA) markers (Ma et al., 2011). Moreover, it has been demonstrated that Kal7 expression is necessary for both maintenance of dendritic spines and branching (Ma et al., 2008b). Thus, a decrease in Kal7 expression in hippocampal cultured neurons results in loss of spines in apical and basal dendrites together with a significant decrease in PSD95 staining in the tips of dendritic spine-like structures while a reduction in the dendritic length and complexity was found in cortical and hippocampal neurons when Kal7 expression was genetically knockdown (Cahill et al., 2009;Cahill et al., 2012;Ma et al., 2003;Ma et al., 2008b;Penzes et al., 2001). Moreover, Kal7 knockout mice exhibit, besides a decrease in spine density, impaired hippocampal-dependent learning and deficient long-term potentiation (Ma et al., 2008a).

Interestingly, Kal7 has been reported to interact with the Huntingtin-associated protein1 (HAP1) (Colomer et al., 1997). However, no data on the role of Kal7 in HD pathology has been explored. In this scenario, we hypothesize that Kal7 may play a critical role in corticostriatal synaptic and cognitive deficits in HD by altering the structure and function of cortical and/or striatal excitatory synapses. To test our hypothesis, we have analyzed the levels of kalirin as well as the number of PSD95 positive clusters in the cortex and striatum of different HD mouse models and HD human brain and the association with deficits in corticostriatal synaptic transmission and behavior. Our results have identified Kal7 as an important player in HD corticostriatal dysfunction providing new insights for therapeutic strategies to restore early corticostriatal dependent cognitive deficits in HD.

MATERIALS AND METHODS

HD mouse models.

Hdh^{Q111} knock-in mice, with targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues, were maintained on a C57BL/6 genetic background. Male and female Hdh^{Q7/Q111} heterozygous mice were intercrossed to generate age-matched Hdh^{Q7/Q111} heterozygous and Hdh^{Q7/Q7} wild-type littermates. R6/1 mice expressing exon-1 of mutant huntingtin were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a B6CBA background. Our R6/1 colony has 145 CAG repeats (Giralt et al., 2009). Wild-type littermate animals were used as the control group. All mice used in the present study were males and were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. The animals were housed with access to food and water *ad libitum* in a colony room kept at 19-22°C and 40-60% humidity, under a 12:12h light/dark cycle. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01) and Generalitat de Catalunya (00/1094), in accordance with the Directive 2010/63/EU of the European Commission.

Postmortem brain tissue.

Samples of human cortex (four controls and six HD patients) and putamen (five controls and five HD patients) were obtained from Banc de Teixits Neurològics (Servei Científico-Tècnic, Universitat de Barcelona, Barcelona, Spain) following the guidelines of the local ethics committees. [Controls (mean \pm SEM; age 53.5 ± 6.8 years; post-mortem intervals of 4-18h), HD brain grade 3 and 4 (mean \pm SEM; age 54.5 ± 6.5 years; post-mortem intervals of 4-17h)]. All the ethical guidelines contained within the latest Declaration of Helsinki were taken into consideration and informed consent was obtained for all subjects under study.

Behavioral assessment.

Accelerating rotarod training procedure (ARTP).

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As previously described (Yang et al., 2009), separate cohorts of 1, 2 and 8-month old Hdh^{Q7/Q7} and Hdh^{Q7/Q111} animals, and 1, 2, and 3-month old wild-type (WT) and R6/1 animals were placed on a motorized rod (30 mm diameter). The rotation speed gradually increased from 4 to 40 r.p.m. over the course of 5 min. The time latency was recorded when the animal was unable to keep up with the increasing speed and fell. Rotarod training/testing was performed 4 times per day for 3 consecutive days.

Swimming T-Maze test of strategy shifting. The T-maze apparatus used was a glass maze consisting of three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° with respect to the other two. All arms were 45 cm long, 8 cm wide and enclosed by a transparent 20 cm wall. 6-months-old male mice were trained to swim from the stem arm of the T to the other arms. In one of them, there was a transparent escape platform, covered by water. During the acquisition phase of swimming T-maze of strategy shifting (two consecutive days: first day: 8 trials; second day: 4 trials), each mice was placed in the water, allowed them to swim until they reach the platform and the latency and errors (1: error, 0: non-error) to reach the platform were recorded. The mice have to be able to find the platform and learn the strategy faster across trials. After the acquisition phase, we change the location of the platform to the opposite arm. During the reversal phase of swimming T-maze of strategy shifting (two consecutive days: second day: 4 trials; third day: 8 trials) each mice was placed in the water and the latency and errors were recorded until they reach the platform. Different trials during the same day were separately by 1 hour.

Fixed Rotarod. For rotarod learning assessment, mice were trained at a fixed speed of 10 r.p.m. and subsequently tested with two trials per day spaced 1–2 h apart during 3 consecutive days. During this learning phase, mice falling from the rod were returned, and the number of falls was recorded until the addition of the latencies to fall reached a total time of 60 s per trial. The testing was performed at 5 (data not shown), 10 and 25 r.p.m. and the number of falls was recorded in a period of 60 seconds.

Open field. The device consisted of a white circular arena with 40 cm diameter and 40 cm high. The light intensity was 40 lux through-out the arena, and the room temperature was kept at 19°C-22°C and 40%-60% humidity. Mice were placed into the arena during two consecutive days (15 minutes/each day) and spontaneous locomotor activity was measured as total distance traveled. The arena was rigorously cleaned between animals in order to avoid odors. Animals were tracked and recorded with SMART Junior Software.

Electrophysiology.

Corticostriatal synaptic transmission. Transverse brain slices (450 µm thickness) were prepared from mice as described previously (Martin and Buno, 2005), and incubated for 1 h at room temperature (21-24°C) in artificial cerebrospinal fluid (aCSF). The aCSF contained (in mM) NaCl 124, KCl 2.69, KH₂PO₄ 1.25, MgSO₄ 2, NaHCO₃ 26, CaCl₂ 2 and glucose 10, and was gassed with 95 % O₂ and 5 % CO₂. Brain slices containing both striatum and cortex were transferred to an immersion recording chamber and superfused (2.5 ml/min) with gassed aCSF warmed to 32-34°C. After 1 h of equilibration, extracellular field potentials were recorded in the dorsomedial striatum by a glass microelectrode filled with 1 M NaCl on stimulation of the white matter between the cortex and the striatum with a bipolar tungsten electrode via a 2100 isolated pulse stimulator (A-M Systems, Inc.). Long-term potentiation (LTP) was induced by applying tetanic stimulation twice (four 1 s, 100 Hz trains delivered every 10 s) at an interval of 10 min, and potentiation was measured for 1 h after LTP induction at 0.1 Hz. For each experiment, population spike (PS) amplitude was expressed as a percentage of average pre-tetanus baseline amplitude. Data were stored on a Pentium-based PC using a PowerLab 4/26 acquisition system (AD Instruments, Bella Vista, Australia); Scope software (AD Instruments) was used to display PS and measurements of the amplitude of PSs. Statistical differences, compared to pre-tetanus baseline amplitude values, were established using the two-tailed Student's t-test.

Brain processing and immunohistochemistry.

RESULTS

Heterozygous mutant $Hdh^{Q7/Q111}$ and wild-type $Hdh^{Q7/Q7}$ mice at 2 (n=5-6 per genotype) and at 8 (n=6 per genotype) months of age were deeply anesthetized and immediately perfused transcardially with saline followed by 4 % paraformaldehyde (PFA)/phosphate buffer. Brains were removed and post-fixed overnight in the same solution, cryoprotected by immersion in 30 % sucrose and then frozen in dry ice-cooled methylbutane. Serial coronal cryostat sections (30 μm) through the whole brain were collected in PBS as free-floating sections. Sections were rinsed three times in PBS and permeabilized and blocked in PBS containing 0.3 % Triton X-100 and 3 % normal goat serum (Pierce Biotechnology, Rockford, IL) for 15 min at room temperature. The sections were then washed in PBS and incubated overnight at 4 $^{\circ}\text{C}$ with Spinophilin (1:250, Millipore, Temecula, CA) and PSD-95 (1:500, Thermo Scientific, MA) antibodies which were detected with Cy3 anti-rabbit and Cy2 anti-mouse secondary antibodies (1:200, Jackson ImmunoResearch, West Grove, PA). As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected.

Confocal microscopy analysis and dendritic spine-like structure counting.

PSD-95 and spinophilin positive-stained spine-like structures were examined as previously described (Rex et al., 2007). The analyses were performed by using a Leica (Mannheim, Germany) TCS SL laser scanning confocal spectral microscope with argon and helium–neon lasers attached to a Leica DMIRE2 inverted microscope. Images were taken using a 63x numerical aperture (NA) objective with 4x digital zoom and standard (one Airy disc) pinhole. Three coronal sections (30 μm thick) per animal spaced 0.24 mm apart containing the motor area M1 and dorsal-striatum were used. For each slice we obtained 3 fields/cortical layer (I, II/III and V) of the M1 area and 3 fields/dorsal-striatum region (dorso-lateral and dorso-medial). In each field, an entire Z-stack was obtained, and optical sections (3/field) of 0,5 μm were obtained separately (4 μm) in order to avoid biased counting. The number and area of positive PSD-95 and spinophilin dendritic–like spines were measured using NIH ImageJ version 1.33 by Wayne Rasband (National Institutes of Health, Bethesda, MD). Briefly, for each section, the dendritic spine area was delineated based on positive pixels for PSD-95 and

Spinophilin. Total number of positive puncta was counted and their area was registered.

Western blot analysis.

Heterozygous mutant $Hdh^{Q7/Q111}$, wild type $Hdh^{Q7/Q7}$, R6/1 and wild-type mice were killed by cervical dislocation at the age of 2 or 8 months for $Hdh^{Q7/Q7}$, $Hdh^{Q7/Q111}$ mice and 2 or 3 months for wild type and R6/1 mice. The brain was quickly removed, dissected, frozen in dry ice and stored at -80°C until use. Protein extraction from striatal and cortical tissue and Western blot analysis were performed as previously described (Giralt et al., 2012). The primary antibodies used were: GluA1 (1:1000, Upstate Lake Placid, NY); GluN1 (1:500, Chemicon, Temecula, CA); GluN2B (1:1000, Cell Signaling Technology, Beverly, MA); CamKII and Shank3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA); PSD-95 (1:1000, Thermo Scientific, MA); Spinophilin (1:1000, Millipore, Temecula, CA); Vglut1 (1:50000, Synaptic Systems, Göttingen, Germany), Synaptophysin (1:1000, Abcam, Cambridge, UK) and Rac1 (1:2000, BD Transduction Laboratories, San Diego, CA); Kalirin-7 (JH2958; 1:1000, (Penzes et al., 2000)). Loading control was performed by reprobing the membranes with an antibody to α -tubulin (1:50000, Sigma-Aldrich) or actin (1:20000, MP Biochemicals, Aurora, OH). ImageJ software was used to quantify the different immunoreactive bands relative to the intensity of the α -tubulin/actin band in the same membranes within a linear range of detection for the ECL reagent. Data are expressed as the mean \pm SEM of band density.

Rac1 activation assay.

For measurement of activated Rac, cerebral cortex from $Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ mice was rapidly homogenized in ice-cold pull-down lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl, 10 mM MgCl_2 , 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 10 % glycerol, 0.5 % β -mercaptoethanol, plus sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich). The lysates were centrifuged at $16.000g$ for 15 min at 4°C to clear insoluble material. Cleared lysates and positive control were mixed with $10\mu\text{g}$ of PAK-GST Protein Beads (Cytoskeleton INC. Denver, CO) and were incubated with shaking for 1 h at 4°C . The beads were

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washed three times with ice-cold pull-down lysis buffer. After the last wash, the supernatant was carefully removed and the pellet was re-suspended in 20 μ l of 5X SDS-PAGE sample buffer and boiled for 5 min. For positive and negative controls, 10 mM EDTA, 60 mM of $MgCl_2$ and 100 μ M of GTPYS (positive control) or 1 mM GDP (negative control) were added and incubated with lysates for 15 min at 30°C. Rac1 bound to PAK-GST was detected by Western blotting using a Rac1 antibody (1:2000, BD Transduction Laboratories).

Primary cultures of mouse cortical neurons, immunocytochemistry and transfection.

Dissociated cortical cultures prepared from E.18 wild-type and R6/1 embryos were plated at a density of 400,000 neurons onto 60-mm culture dishes (for biochemical analysis) or at a density of 50,000 neurons onto 24-well plates (for immunocytochemistry analysis) pre-coated with 0.1 mg/ml poly-D-lysine (Sigma Chemical Co., St. Louis, MO). Neurons were cultured in Neurobasal medium (Gibco-BRL, Renfrewshire, Scotland, UK), supplemented with B27 (Gibco-BRL) and GlutamaxTM (Gibco-BRL). Cultures were maintained at 37°C in a humidified atmosphere containing 5 % CO_2 . Thereafter, 50 % of the medium was replaced once a week for up to 4 weeks. For biochemical analysis, twenty-eight days after plating, neurons were homogenized in cold lysis buffer (50 mM Tris base (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 % NP-40) supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich), cleared by centrifugation at 16,000g for 15 min and the supernatants were collected. For immunocytochemical experiments, twelve days after plating, neurons were transfected with pcDNA 3.1/myc-His (+) or pEAK.Myc.His.Kalirin-7 (Penzes et al., 2000) plasmids using Fugene transfection Reagent (Promega, Madison, WI). After twenty-eight days *in vitro*, neurons were fixed with 4 % PFA/phosphate buffer for 10 min, rinsed in PBS, blocked in PBS containing 0.1 M glycine for 10 min and permeabilized in PBS containing 0.1 % saponin for 10 min and blocked in PBS containing Normal Horse Serum 15 % for 30 min at room temperature. Neurons were then washed in PBS and incubated overnight at 4°C with PSD-95 (1:250, Thermo Scientific, MA) and VGlut1 (1:500, Synaptic Systems, Göttingen, Germany) antibodies and detected with Cy3 anti-mouse and Alexa647 anti-rabbit secondary antibodies (1:200, Jackson ImmunoResearch, West Grove, PA). As negative controls, some

neurons were processed as described in the absence of primary antibody and no signal was detected. Immunofluorescence was analyzed by confocal microscopy using a TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg, Mannheim, Germany). Images were taken using a 63x numerical aperture (NA) objective. The number of positive PSD-95, Vglut1 and PSD-95/Vglut1 positive clusters was measured using NIH ImageJ version 1.33 by Wayne Rasband (National Institutes of Health, Bethesda, MD) and the NeuronJ Plugin (A Java program for Neurite Tracing and Quantification).

Statistical analysis.

All data are expressed as mean \pm SEM. Statistical analyses were performed using the unpaired Student's *t*-test, Wald's test, one-way ANOVA or two-way ANOVA with the appropriate post-hoc test as indicated in the figure legends. Differences with $p < 0.05$ were considered significant.

RESULTS

Motor learning is impaired in HD mouse models at early disease stages.

Huntington's Disease (HD) patients are impaired in various skill acquisition tasks that involve the corticostriatal circuits (Backman et al., 1997; Foroud et al., 1995; Lawrence et al., 1998; Lawrence et al., 2000; Lemièrè et al., 2004; Watkins et al., 2000). Therefore, we first evaluated motor procedural learning in two different HD mouse models, mutant Hdh^{Q7/Q111} knock-in mice and R6/1 mice at different ages by using the accelerating rotarod task (Rosas et al., 2010; Xu et al., 2009; Yang et al., 2009). These two HD models differ in the onset and progression of HD pathology, with R6/1 mice showing earlier onset and faster disease progression than Hdh^{Q7/Q111} knock-in mice (Kim et al., 2011). At 1 month of age, motor skill acquisition did not differ between genotypes (Hdh^{Q7/Q111} mice: Genotype: $F_{(1,220)}=0.8606$; $p=0.3646$, n.s; Time: $F_{(11,220)}=8.756$; $p<0.0001$; R6/1 mice: Genotype: $F_{(1,264)}=0.03423$; $p=0.8548$, n.s; Time: $F_{(11,264)}=7.391$; $p<0.0001$) (Fig. 1A and 2A). Conversely, at 2 months of age, even though wild type and HD mice showed similar latencies to fall in the last trials indicating that both genotypes learned the rotarod task (Hdh^{Q7/Q111} mice: Time: $F_{(11,231)}=59.14$;

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$p < 0.0001$; R6/1 mice: Time: $F_{(11,253)} = 11.07$; $p < 0.0001$), performance of HD mice was significantly worse than wild type mice, revealing a delay in the acquisition of new motor skills (Hdh^{Q7/Q111} mice: Genotype: $F_{(1,231)} = 13.30$; $p = 0.0015$; R6/1 mice: Genotype: $F_{(1,253)} = 7.831$; $p = 0.0102$) (Fig. 1B and 2B). Finally, at more advanced disease stages; 6 and 8 months of age in Hdh^{Q7/Q111} mice and 3 months of age in R6/1 mice, HD mice from the first trials were unable to maintain the balance on the rotarod as wild type mice that showed a significant and progressive increase in the latency to fall (Hdh^{Q7/Q111} mice: Genotype: $F_{(1,209)} = 27.77$; $p < 0.0001$; Time: $F_{(11,209)} = 27.24$; $p < 0.0001$; R6/1 mice: Genotype: $F_{(1,242)} = 11.78$; $p = 0.0024$; Time: $F_{(11,242)} = 16.84$; $p < 0.0001$) (Fig. 1C, D and Fig. 2C). Altogether these findings suggest an age-dependent impairment of motor learning in HD mice. However, poorer performance in the accelerating rotarod at advanced disease stages could be due to motor coordination problems. Indeed, R6/1 mice exhibit motor impairments in the fixed rotarod at 3 months of age while no data has been published for Hdh^{Q7/Q111} knock-in mice. Therefore, performance on the fixed rotarod was analyzed in Hdh^{Q7/Q111} knock-in mice at 2 (Fig. 1E), 6 (Fig. 1F) and 8 (Fig. 1G) months of age. While 2 and 6-month-old knock-in mice were able to appropriately perform the rotarod task, at 8 months of age motor coordination was severely impaired showing knock-in mice a significant increase in the number of falls compared to wild type mice. Similar results were found when spontaneous locomotor activity was measured in the open field showing knock-in mice no differences in the distance traveled compared to wild type mice at 2 and 6 months of age but at 8 months of age (Supplementary Fig. 1). Thus, at early and mild disease stages HD mice show motor learning deficits without motor coordination alterations while at more advanced stages cognitive deficits were accompanied by motor coordination dysfunction.

HD mice also exhibit procedural memory deficits in a swimming T-maze test.

Procedural memory deficits were further validated in HD mice by using the swimming T-maze test. The task was performed in 6-months-old (Hdh^{Q7/Q111}) and 2-months-old (R6/1) HD mice since at these ages HD mice display severe motor learning deficits without motor coordination impairments (Fig. 1 and 2). During the acquisition phase the escape platform was located in the right arm of the T-maze and the latency to

reach the platform (Fig. 3A and E) and the number of “errant” trials (turning left to reach the platform) (Fig. 3C and G) scored. After one trial, both wild-type and HD mice exhibited similar decrease in the latency to reach the platform showing by the fourth trial a constant level of performance (~ 10 sec to reach the platform). However, when the number of errant trials was analyzed, HD mice still turned left in some trials on the first two days of training. By the third day all mice regardless of the genotype turned right indicating a delay but not an impairment in the acquisition of the task in the swimming T-maze test, indicating a delay but not an impairment in the acquisition of the task.

Next, we assessed the ability of HD mice to change the strategy by switching the platform from the right arm to the left arm of the T-maze (reversal phase). Contrary to what was observed during the acquisition phase, latency to reach the platform was significantly higher in HD mice compared to wild type mice (Fig. 3B and F). This increase was likely related with the higher number of errant trials exhibited by HD mice (Fig. 3D and H). Thus, whereas by the fourth trial the error ratio in wild type mice was 0.5, meaning that ~ half turned right and half turned left, in HD mice was ~0.8 indicating that most of HD mice entered the right arm (Fig. 3D and H). Overall, these results demonstrated that HD mice had difficulty in changing strategy as evidenced by the increase in the latency to reach the platform and the error ratio in the reversal phase, but did not show any cognitive deficit in the acquisition phase of the swimming T-maze test.

Altered corticostriatal synaptic transmission in $Hdh^{Q7/Q111}$ knock-in mice.

Altered synaptic transmission and plasticity may contribute to behavioral deficits in HD (Cepeda et al., 2007; Cybulska-Klosowicz et al., 2004). Therefore, we next addressed whether impaired motor learning in HD mice was related to corticostriatal synaptic dysfunction. Since pre-symptomatic R6/1 mice are known to display deficits in cortical synaptic plasticity (Cybulska-Klosowicz et al., 2004), corticostriatal electrophysiology studies were only conducted in HD knock-in mice (Fig. 4A and B). Synaptic potentials were evoked by stimulation of corticostriatal glutamatergic fibers in slices obtained from mutant $Hdh^{Q7/Q111}$ and wild type $Hdh^{Q7/Q7}$ mice at 2-3 months of age (Fig. 4A). Baseline responses were monitored for 10-30 min before conditioning and were found

to be stable. Tetanus conditioning revealed a marked difference in the ability of mutant $Hdh^{Q7/Q111}$ mice to support long-term potentiation (LTP) with strengthening of PS significantly diminished in mutant mice (Fig. 2, $p < 0.001$). Thus, at 60 min after tetanus, potentiation in wild type mice was $133 \pm 3\%$ ($p < 0.001$; $n = 7$ slices; three mice) *versus* $104 \pm 3\%$ in mutant mice ($n = 8$ slices; three mice) (Fig. 4A), which indicates impaired induction and maintenance of corticostriatal LTP in mutant $Hdh^{Q7/Q111}$ mice.

Spinophilin-immunoreactive puncta are reduced in the cortex but not in the striatum of $Hdh^{Q7/Q111}$ knock-in mice.

Our previous data demonstrates procedural learning deficits and impaired corticostriatal LTP in 2-months-old HD mice. Therefore, we next explored whether the observed deficits in LTP and cognition could be related with structural changes in the cortex and/or striatum of HD mice. To this aim, spinophilin staining used as a marker of spines, was performed in cortical (motor cortex) and striatal (dorsal striatum) slices obtained from wild type and $Hdh^{Q7/Q111}$ mice at 2 and 8 months of age (Fig. 5). Spinophilin is detectable in the vast majority of dendritic spines where it is specifically distributed to the heads of spines, which made spinophilin immunoreactivity an excellent marker for quantitative assessment of spines (Calhoun et al., 2008; Hao et al., 2003; Radley et al., 2006; Tang et al., 2004). Confocal microscopy analysis revealed that at 2 months of age $Hdh^{Q7/Q111}$ mutant mice displayed a significant reduction ($p < 0.01$) of spinophilin-immunoreactive puncta in the layer I of the motor cortex compared to $Hdh^{Q7/Q7}$ mice wild type mice (Fig. 5A) without significant changes in the striatum (Fig. 5C). At 8 months of age, the decrease in spinophilin-immunoreactive puncta in motor cortex layer I was higher ($p < 0.05$) than that observed at 2 months of age and also involving layer II-III and layer V ($p < 0.05$) (Fig. 5B). Notice that at 8 months of age the mean size of spinophilin puncta is also reduced in motor layer I ($p < 0.01$) (Fig. 5B). Notably, spinophilin immunoreactivity was not affected in the striatum of $Hdh^{Q7/Q111}$ mutant mice at 8 months of age (Fig. 5D). Altogether these results demonstrate an early and specific decrease in spinophilin-immunoreactive spines in the cortex of HD mice suggesting that cortical structural changes may be involved in deficient corticostriatal connectivity in HD mice.

PSD-95-immunoreactive puncta are decreased in the cortex but not striatum of Hdh^{Q7/Q111} knock-in mice at early disease stages.

PSD-95 is one of the most abundant proteins present at glutamatergic excitatory postsynaptic sites in the central nervous system (Cho et al., 1992; Kistner et al., 1993) where regulates synapses stabilization and plasticity (Beique and Andrade, 2003; Chen et al., 2000; El-Husseini et al., 2000). Therefore, the number and mean size of PSD-95 - immunoreactive puncta were analyzed to evaluate cortical and striatal postsynaptic excitatory contacts in Hdh^{Q7/Q7} wild type and Hdh^{Q7/Q111} mutant mice at 2 and 8 months of age (Fig. 6). A significant decrease in the number of PSD-95-immunoreactive puncta was found in the motor cortex layer I ($p < 0.01$) and layers II-III ($p < 0.05$) of Hdh^{Q7/Q111} mutant mice compared to Hdh^{Q7/Q7} wild type mice at 2 months of age, a decrease that was accompanied by a reduction in the mean size of PSD-95 clusters in layer I (Fig. 6A). Importantly, no significant changes were found in the dorsal striatum of mutant Hdh^{Q7/Q111} mice either in the number or size of PSD-95-immunoreactive puncta (Fig. 6C). When PSD-95 immunostaining was analyzed at 8 months of age a drastic reduction ($p < 0.05$ and $p < 0.01$) in the number of PSD-95-immunoreactive puncta was detected in all analyzed cortical layers in Hdh^{Q7/Q111} mutant mice compared to Hdh^{Q7/Q7} wild type mice along with a decrease ($p < 0.01$ and $p < 0.001$) in the mean size of these PSD-95 clusters (Fig. 6B). Notably, at this age the dorsal striatum of Hdh^{Q7/Q111} mutant mice also exhibited an important decline in the number of PSD-95-immunoreactive puncta without changes in the mean size (Fig. 6D). Altogether, this data suggest a specific and early alteration of cortical excitatory synapses in HD mice worsening thereafter by involving reduction of PSD-95-immunoreactive postsynaptic puncta in the striatum.

Early structural changes in the cortex of Hdh^{Q7/Q111} knock-in mutant mice correlate with a cortical decline of the RhoGEF protein Kalirin-7.

The observed structural changes in the cortex of HD mutant mice at early disease stages evidenced by a reduction in the density of spinophilin and PSD-95 positive puncta prompted us to investigate by Western blot analysis the expression of distinct pre- and post-synaptic proteins in total cortical and striatal extracts obtained from wild type and mutant HD mice at 2 and 8 months of age (Fig. 7). First, we analyzed the

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levels of the postsynaptic brain-specific RhoGEF-protein Kalirin-7 since it has been reported to be an essential component of excitatory synapses (Ma et al., 2008b; Ma et al., 2011) and to regulate cortical spine morphogenesis and maintenance (Cahill et al., 2009; Penzes et al., 2000; Penzes et al., 2001; Xie et al., 2010). Interestingly, a significant reduction of Kalirin-7 levels was observed in the cortex (Fig. 7A) but not the striatum (Fig. 7B) of Hdh^{Q7/Q111} mutant mice compared to Hdh^{Q7/Q7} wild type mice at 2 months (~ 25 %, p<0.05) and 8 months (~ 25 %, p<0.01) of age. Notably, at 2 months of age the levels of glutamate receptors, GluA1 (AMPA receptor subunit), GluN1 and GluN2B (NMDA receptor subunits) and postsynaptic scaffolding and signaling proteins (PSD-95, Spinophilin, Shank3 and CamKII) were similar between Hdh^{Q7/Q111} mutant mice and Hdh^{Q7/Q7} wild type either in the cortex or striatum (Fig 7A, 7B). Thus, these results indicate that the specific reduction of excitatory contacts in the motor cortex of HD mice at early disease stages (2 months of age) is not accompanied by a general decrease of PSD-associated proteins but a specific decline of Kalirin-7 levels. Notably, at 8 months of age (Fig. 7B) no changes in the levels of glutamate receptors were detected whereas a significant reduction of Shank3 and CamKII (~ 25 %, p< 0.01 and p< 0.05) was observed in the cortex of HD mutant mice and a decrease in PSD-95 was found in the cortex and striatum (~ 25 %, p< 0.01) suggesting that at more advanced disease stages structural and general biochemical changes in both cortex and striatum could contribute to corticostriatal synaptic and cognitive deficits in HD. Importantly, no differences in pre-synaptic proteins (Vglut1 and Synaptophysin) were observed between genotypes and brain areas neither at 2 months nor at 8 months of age.

Kalirin-7 levels are also reduced in R6/1 mutant transgenic mice and HD human brain.

To determine whether the decrease in Kalirin-7 levels is a general hallmark of HD pathology, we analyzed by Western blot analysis the expression of Kalirin-7 in the cortex and striatum of R6/1 transgenic mice (Fig. 8A) and in HD human brain (Fig. 8B). Consistent with our previous data a significant and specific decline in Kalirin-7 protein levels were found in the cortex but not in the striatum of R6/1 mice at 2 months (~ 25 %, p<0.01) and 3 months of age (~ 50 %, p<0.001) (Fig. 8A). According with the data obtained in Hdh^{Q7/Q111} mice, the decrease in Kalirin-7 at 2 months of age in R6/1 mice

was not accompanied by a general decrease in other synaptic-related proteins (Suppl. Fig2), revealing that Kalirin-7 reduction at early disease stages is not due to a general alteration in synaptic proteins. When postmortem brain tissue from the cortex and putamen of control and HD individuals was studied, quantitative immunoblot analysis revealed a significant decrease of Kal7 levels in the cerebral cortex (Control: 99.95 ± 6.702 , HD: 52.75 ± 5.851 ; $p=0.0008$) but also in the putamen (Control: 100 ± 15.38 , HD: 29.46 ± 7.383 ; $p=0.0033$) of HD patients compared to controls (Fig. 8B), which may reflect the severe striatal cell death characteristic of HD (Reiner et al., 1988; Vonsattel and DiFiglia, 1998). These results showing reduction of Kalirin-7 levels either in HD mice and HD individuals suggests an important role of Kalirin-7 in HD pathology.

The activity of the Rho GTPase Rac1 is decreased in the cortex of Hdh^{Q7/Q111} knock-in.

Kalirin-mediated activation of Rac1 is known to be critical for the regulation of activity-dependent changes in spine morphology and plasticity (Lemtiri-Chlieh et al., 2011; Xie et al., 2007). Given the reduction of Kalirin-7 levels in the cortex of HD mice we investigated whether such decrease was associated with diminished activity of the small Rho GTPase Rac1. Pull-down assays on cortical extracts obtained from 8 month-old wild type Hdh^{Q7/Q7} and mutant Hdh^{Q7/Q111} mice revealed a significant decrease in Rac1 activation in mutant compared to wild type mice as indicated by a decrease in GTP-bound Rac1 (Hdh^{Q7/Q7}: $100\% \pm 15.5\%$, Hdh^{Q7/Q111}: $48.0\% \pm 13.0\%$, $p<0.05$). No changes in total Rac1 levels were found (Hdh^{Q7/Q7}: $100\% \pm 15.2\%$, Hdh^{Q7/Q111}: $98.4\% \pm 6.5\%$, $p=0.9462$) (Fig. 9). These results suggest that decreased Kalirin-7 levels could contribute to corticostriatal synaptic dysfunction in HD mice by reducing the activity of the GTPase Rac1.

Overexpression of exogenous Kalirin-7 restores the number of excitatory synapses in R6/1 cortical neurons.

Our previous findings showing a reduction of Kalirin-7 levels and Rac1 activity in the cortex of HD mice along with a significant decrease in cortical spinophilin and PSD-95 positive puncta suggest that deficient Kalirin-7 function could cause early cortical structural changes leading thereafter to corticostriatal dysfunction in HD mice. To further explore this hypothesis we wondered whether these deficits could also be

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reproduced *in vitro*. First, levels of Kalirin-7 were determined in 28DIV cortical primary neurons obtained from E18.5 wild type and mutant R6/1 embryos by Western blot analysis. Similar to *in vivo* data we found that Kalirin-7 levels were significantly reduced ($\sim 25\%$; $p < 0.05$) in R6/1 compared to wild type cortical neurons (Fig. 10). As we can reproduce the reduction of Kalirin-7 levels "*in vitro*" and it is essential for excitatory synapses we then analyzed whether Kalirin-7 decreased in R6/1 cortical primary neurons associates with altered number of excitatory synapses (measured as Vglut1 and PSD-95-positive co-clusters). Wild type and R6/1 cortical neurons were transfected at DIV12 with Myc or Myc-Kal7 vectors (Penzes et al., 2000), fixed sixteen days later (DIV28) and the number of excitatory synapses was evaluated by immunocytochemistry and confocal analysis. As expected, R6/1 cortical neurons transfected with the Myc vector alone exhibited a significant reduction ($\sim 30\%$, $p < 0.05$) in the intensity of Kalirin-7 immunostaining compared to Myc transfected wild type neurons (Fig. 11A). By contrast, a similar and significant increase in Kalirin-7 immunostaining was found in both wild type and R6/1 cortical neurons transfected with the Myc-Kal7 vector, indicating comparable transfection efficiencies (Fig. 11A). Then, the density of excitatory synapses as the number of Vglut1 and PSD-95 positive co-clusters was examined (Fig. 11B). Consistent with decreased Kalirin-7 levels, Myc-transfected R6/1 cortical neurons showed reduced PSD-95, Vglut1 and PSD-95/Vglut1 positive co-clusters compared to Myc-transfected wild type neurons (Fig. 11B), revealing a severe reduction of excitatory synapses in HD dissociated cortical neurons and strongly supporting a role for Kalirin-7 in HD cortical dysfunction. Importantly, after transfection with the Myc-Kal7 vector, R6/1 neurons showed comparable number of Vglut1 and PSD-95 positive clusters to wild type neurons with most of the Vglut1 clusters co-localizing with PSD-95-positive clusters (Fig. 11B). Taken together these results clearly demonstrate that loss of excitatory synapses in HD cortical cultures is related with a deficient expression of Kalirin-7 and supports the idea that altered structural plasticity in the cortex of HD mice could involve Kalirin-7 dysfunction.

DISCUSSION

Cognitive dysfunction involving psychomotor, emotional, attentional and executive functions has been reported in asymptomatic HD gene carriers years before the onset of motor symptoms (Foroud et al., 1995; Josiassen et al., 1983; Lawrence et al., 1996; Lawrence et al., 1998; Lawrence et al., 2000; Lemièrè et al., 2002; Lemièrè et al., 2004). Although there is variation in the nature and evolution of such cognitive decline most evidence indicate that these early cognitive changes derive from impairments of the corticostriatal circuits rather than as a consequence of striatal or cortical neuronal degeneration (Cepeda et al., 2004; Levine et al., 2004). However, it is not clear whether striatal dysfunction develops first involving thereafter other brain regions namely the neocortex or the limbic system, whether atrophy of pyramidal cortical neurons and consequent loss of cortical projections is the primary cause of the striatal pathology or whether both cortical and striatal changes progress in parallel inducing dysfunction of the corticostriatal pathway.

In this study, we aimed to determine the temporal sequence of brain region pathology involved in corticostriatal dependent cognitive deficits in HD mice and the molecular mechanisms underlying such impairments. We have demonstrated in two different HD mouse models; Hdh^{Q7/Q111} knock-in mice and transgenic exon-1 R6/1 mice, motor learning deficits in the accelerated rotarod evident at 2 months of age and worsening thereafter. Importantly, at early (2 months) and middle (6 months in knock-in mice and 3 months in R6/1 mice) disease stages, HD mice do not display decreased motor coordination function as evidenced by similar performance in the fixed rotarod or the open field compared to the wild type mice. Both HD mice also exhibited procedural learning deficits in the strategy-shifting task at ages in which motor coordination is not impaired. Interestingly, both motor and procedural learning disturbances appear in HD mice months before hippocampal-dependent cognitive impairments (Giralt et al., 2011; Giralt et al., 2012), indicating that alteration in corticostriatal activity precedes hippocampal dysfunction in HD mice. These results are in agreement with previous data in YAC128 mice, showing altered motor learning in the rotarod task before any other behavioral abnormality (Van Raamsdonk et al., 2005), and with data in humans in which deficits in procedural learning are among the earliest detectable cognitive symptoms (Gabrieli et al., 1997; Heindel et al., 1988; Heindel et al., 1989; Lawrence et

al., 1996;Lawrence et al., 1998;Lawrence et al., 2000;Schmidtke et al., 2002). Consistent with deficits in corticostriatal-dependent cognition, we also demonstrated impaired induction and maintenance of corticostriatal LTP in 2 months-old Hdh^{Q7/Q111} knock-in mice, an age in which motor and procedural learning start to be altered, which supports the idea that cognitive deficits in HD mice arise from changes in the corticostriatal synaptic circuit. In fact, abnormal corticostriatal activity has also been reported in YAC128 and R6/2 mice (Andre et al., 2006;Cepeda et al., 2003;Joshi et al., 2009).

Morphological and/or biochemical alterations either in pyramidal neurons from the neocortex or from the striatum may be responsible for the early cognitive and synaptic deficits observed in HD mice. Dendritic spine pathology analyzed by Golgi staining in striatal spiny neurons and pyramidal cortical neurons has been previously reported at late disease stages in different HD mouse models and in brains from HD patients (DiFiglia, 1997;Guidetti et al., 2001;Laforet et al., 2001;Sotrel et al., 1993;Spires et al., 2004). In agreement with these studies we found at 2 and 8 months of age a moderate reduction of spinophilin-positive puncta in the motor cortex of Hdh^{Q7/Q111} mice without evident changes in the striatum. However, whether this dendritic alteration could involve changes in glutamatergic synapses has not been addressed. PSD-95 is one of the most abundant proteins found in the postsynaptic density (PSD) of excitatory synapses (Cho et al., 1992;Kistner et al., 1993). Synaptic clustering of PSD-95 is critical for synapse maturation driving the remodeling of PSD through recruitment of scaffolding proteins, glutamate receptors and signaling machinery (Goda and Davis, 2003;McAllister, 2007) and controlling synaptic strength and activity-mediated synapse stabilization (Ehrlich et al., 2007;El-Husseini et al., 2000;Prange and Murphy, 2001). In this context, we found a significant reduction of PSD-95 positive postsynaptic clusters in the motor cortex layers I and II-III of Hdh^{Q7/Q111} mutant mice at 2 months of age without any major change in the striatum until 8 months, when changes in cortical positive clusters of PSD-95 are even more severe than at 2 months of age involving layer V. These findings give the idea that alterations in glutamatergic cortical activity would induce corticocortical dysfunction prior to striatal damage leading then to impaired corticostriatal function. Actually, cortical pyramidal neurons from layers II-III and V of sensorimotor cortex are the most prominent glutamatergic input to other

cortical layers and dorso-lateral striatum (Gubelini et al, 2004; McGeer et al, 1997). In accordance with our data, cortical pyramidal neurons from 3 weeks-old R6/2 transgenic mice have decreased glutamate receptor-mediated currents (Andre et al., 2006) while deficits in learning-dependent cortical plasticity have been reported in presymptomatic R6/1 mice (Cybulska-Klosowicz et al., 2004; Mazarakis et al., 2005) and abnormal motor cortex plasticity was found in HD gene carriers (Orth et al., 2010).

We next addressed the potential mechanisms that could explain the specific decrease of cortical PSD-95-positive excitatory synapses in HD mice at early disease stages. Kalirin-7 is a brain specific Rho-GEF protein enriched in the PSD of excitatory synapse where plays an essential role in synaptic structure and function through modulation of the actin cytoskeleton dynamics (Ma et al., 2003; Ma et al., 2008a; Ma et al., 2008b; Penzes and Remmers, 2012; Xie et al., 2010). Thus, Kalirin-7 knock-out (Kal7 KO) mice exhibit a 30 % reduction in the density of glutamatergic synapses in the CA1 hippocampal neurons analyzed by electron microscopy while the number of Vglut1-PSD95 positive clusters in cortical neurons from Kal7 KO mice were decreased by almost a 40 % (Ma et al., 2008a). Importantly, such changes were along with a decline on the magnitude of the LTP and impaired contextual fear learning. In this scenario, our results showing an early reduction of Kalirin-7 in the cortex but not in the striatum of HD mutant mice at 2 months of age without any change in the levels of glutamate receptors or other synaptic scaffolding proteins strongly suggest a critical role of Kalirin-7 in the structural synaptic changes found in the cortex of HD mice and underscores Kalirin-7 as a candidate to mediate HD corticostriatal synaptic transmission and behavioral deficits. Actually, reduced levels of Kalirin associated with dendritic spine disturbances have been found in schizophrenic postmortem cortices and in the hippocampus of Alzheimer's disease patients (Hill et al., 2006; Youn et al., 2007).

Our neuronal culture data further supports a crucial involvement of Kalirin-7 in cortical HD synaptic pathology. We found decreased Kalirin-7 levels in mutant cortical primary neurons from R6/1 HD mice associated with a significant reduction of excitatory synapses evidenced by a decline in the number of PSD-95/Vglut1 co-clusters, a decrease that could be completely restored by exogenous expression of Kal7.

RESULTS

The restorative effect of Kalirin-7 over-expression in excitatory synapses raises the question of how deficient Kal7 activity could be involved in synaptic alterations in HD mice. The small Rho-GTPase Rac1 regulates not only spine and synapse structure but also synaptic function. Thus, over-expression of Rac1 in hippocampal neurons enhances excitatory synaptic transmission likely by inducing remodeling of the actin cytoskeleton and the clustering of AMPAR (Wiens et al., 2005). Importantly, Kalirin-7 is a well known activator of Rac1 (Impey et al., 2010; Penzes et al., 2000; Xie et al., 2007). Thus, following neuronal activity, Kalirin-7 induces Rac1 activation that in turn causes a rapid enlargement of existing spine heads (Impey et al., 2010; Tashiro et al., 2000; Xie et al., 2007). Consistent with a role of the Kal7/Rac1 cascade in HD cortical synaptic pathology, Rac1 activity was found significantly reduced in the cortex of HD mice suggesting that decreased Rac1 function as a consequence of reduced Kalirin-7 expression could mediate the loss of excitatory PSD-95 synapses in the cortex of HD mice. We cannot however exclude, that alterations in other GEF-proteins that also regulate Rac1 activity would also contribute to HD synaptic pathology.

Overall, in the present study we demonstrate corticostriatal learning deficits in HD mice at early disease stages correlating with deficits in corticostriatal synaptic transmission and reduction of cortical excitatory synapses. This data supports the notion that corticostriatal dysfunction in HD involves early morphological and biochemical changes in the cortex long before striatal pathology becomes apparent. In the quest to elucidate the molecular pathway underlying these cortical disturbances, we have identified Kalirin-7, a critical component of excitatory synapses that control structural and functional plasticity of dendritic spines. Altogether, our data suggest that therapeutic strategies aimed to restore Kalirin-7 activity merit particular investigation to treat synaptic dysfunction in HD.

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FIGURES AND FIGURE LEGENDS

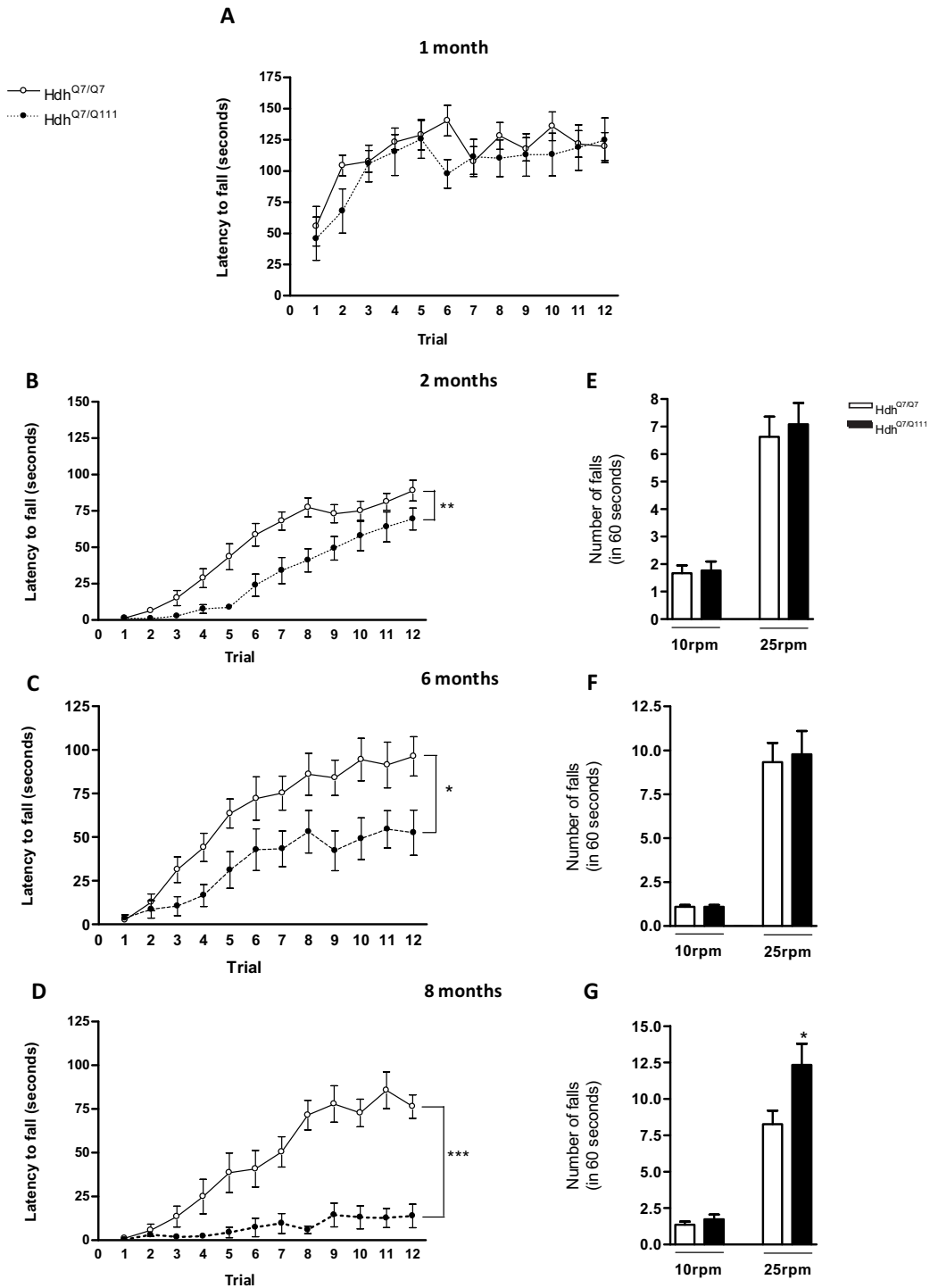


Figure 1. Hdh^{Q7/Q111} and R6/1 mice show impaired learning of new motor skills at early disease stages without motor coordination alterations. Latency to fall in the accelerating

rotarod task procedure in wild type $Hdh^{Q7/Q7}$ and knock-in mutant $Hdh^{Q7/Q111}$ mice at 1 (A), 2 (B), 6 (C) and 8 months (D) of age. An age-dependent impairment in motor learning was observed in HD mice. Data represents the mean \pm SEM (n=9-14 per genotype). Statistical analysis was performed using two-way ANOVA with repeated measures. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Number of falls in the fixed rotarod at 10 rpm and 25 rpm in wild type $Hdh^{Q7/Q7}$ and knock-in mutant $Hdh^{Q7/Q111}$ mice at 2 (E), 6 (F) and 8 months (G) of age. No motor coordination deficits were observed until 8 months of age in $Hdh^{Q7/Q111}$ mice. Data represents the mean \pm SEM (n=9-15 per genotype). Statistical analysis was performed using One-way ANOVA with Bonferroni's Multiple comparison test as a post-hoc. * $p < 0.05$.

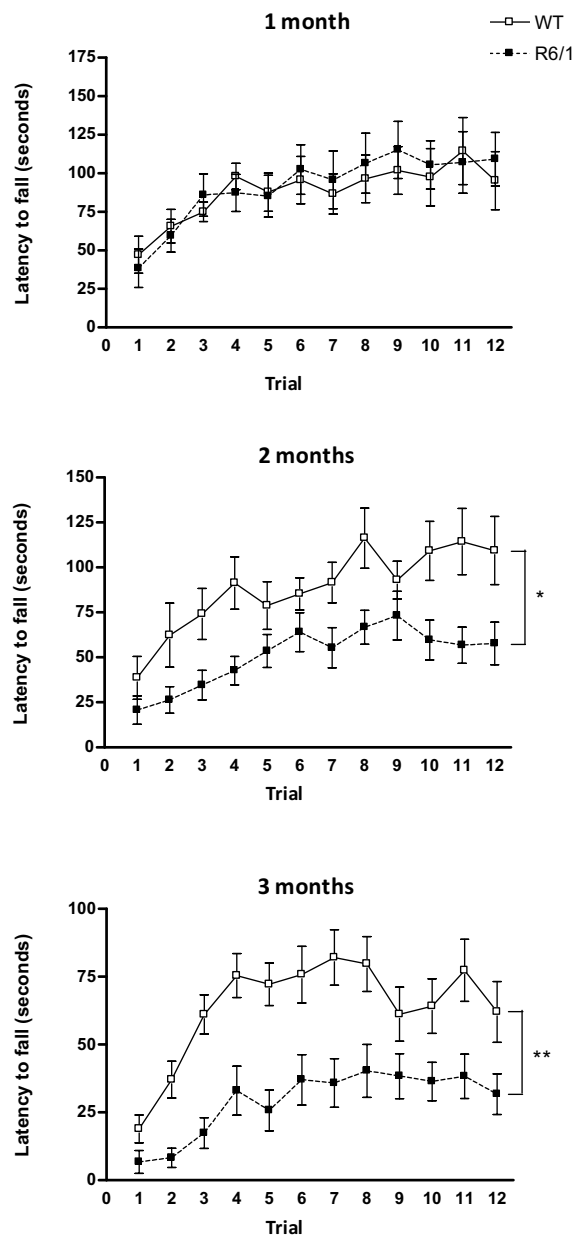


Figure 2. R6/1 mice show impaired learning of new motor skills at early disease stages. Latency to fall in the accelerating rotarod task procedure in wild type (WT) and R6/1 mice at 1 (A), 2 (B) and 3 months (C) of age. An age-dependent impairment in motor learning was observed in HD mice. Data represents the mean \pm SEM (n=9-14 per genotype). Statistical analysis was performed using two-way ANOVA with repeated measures. * $p < 0.05$; ** $p < 0.01$.

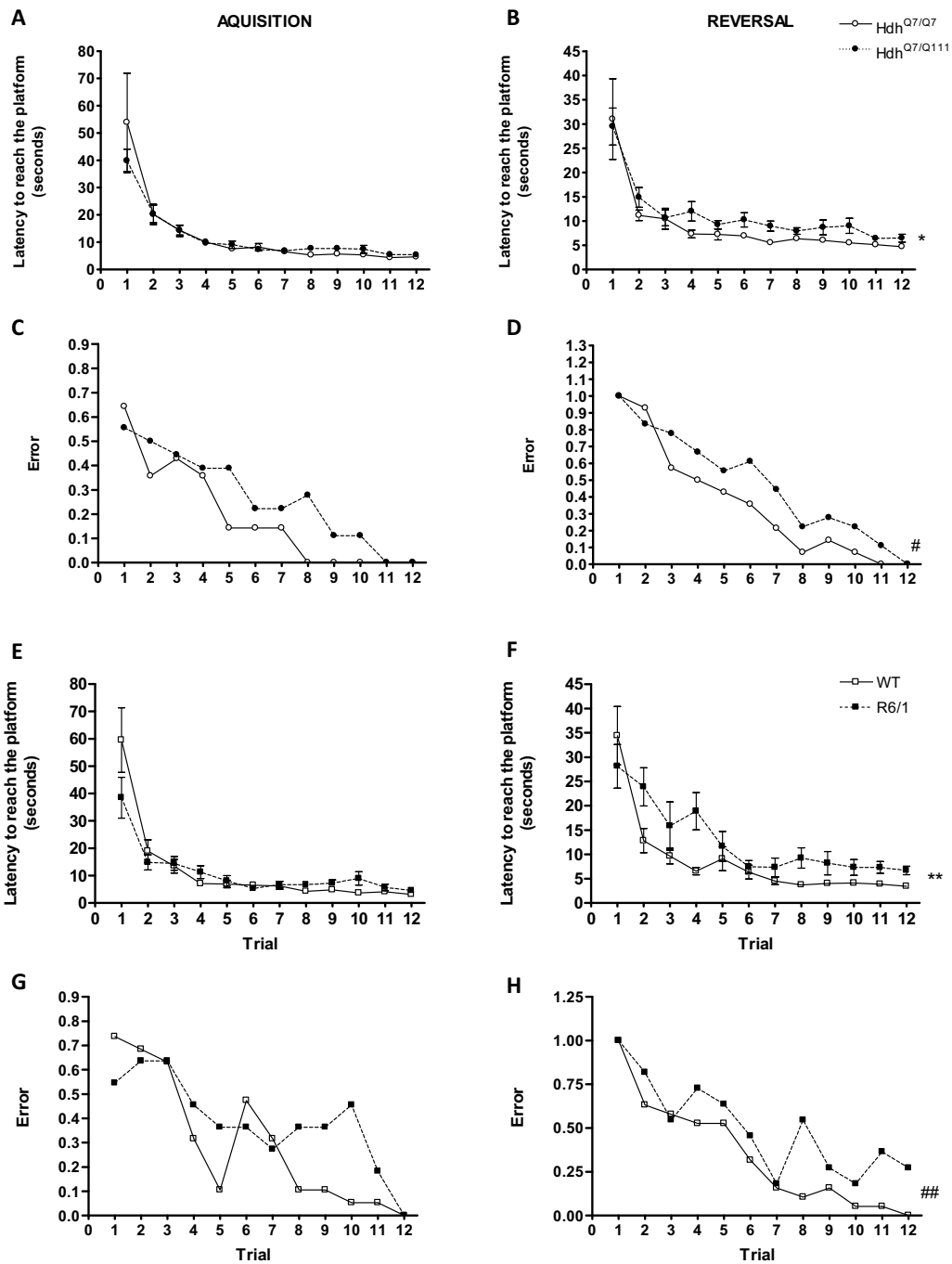


Figure 3. $Hdh^{Q7/Q111}$ and R6/1 mice show impaired procedural memory deficits. Latency to reach the platform and error trials in the swimming T-maze test in 6-months-old wild type $Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ mice and 2-months-old WT and R6/1 mice during the acquisition (A, C, E, G) and reversal (B, D, F, H) phases of the swimming T-maze test of Strategy shifting. HD mice

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exhibit procedural memory deficits in the reversal phase of the swimming T-maze test. Data represents the mean \pm SEM (n=11-19 per genotype). Statistical analysis was performed using two-way ANOVA with repeated measures to analyze the latency to reach the platform: *p<0.05; **p<0.01; and logistic regression analysis using the Wald statistical test from IBM SPSS Statistics to analyze the error probability of mice to reach the platform in the correct arm: #p<0.05; ##p<0.01.

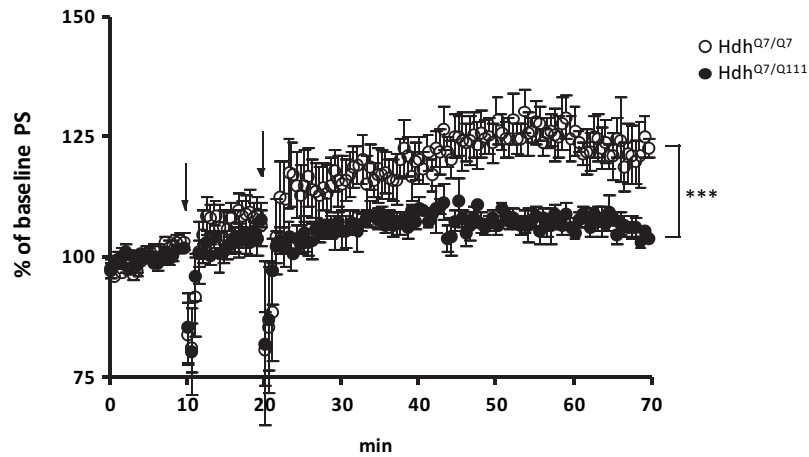


Figure 4. $Hdh^{Q7/Q111}$ mice exhibit abnormal synaptic plasticity at corticostriatal synapses.

Summary data showing the time-course of mean population spike (PS) slope in wild type $Hdh^{Q7/Q7}$ (open circle, n=7) and knock-in mutant $Hdh^{Q7/Q111}$ (filled circle, n=8) mice at 2-3 months of age in basal conditions and following LTP induction (arrows). For each slice, data were normalized to the average slope recorded during baseline. Data represent the mean \pm SEM. Statistical differences, compared to pre-tetanus baseline amplitude values, were established using Student's two-tailed *t* test. *** $p < 0.001$.

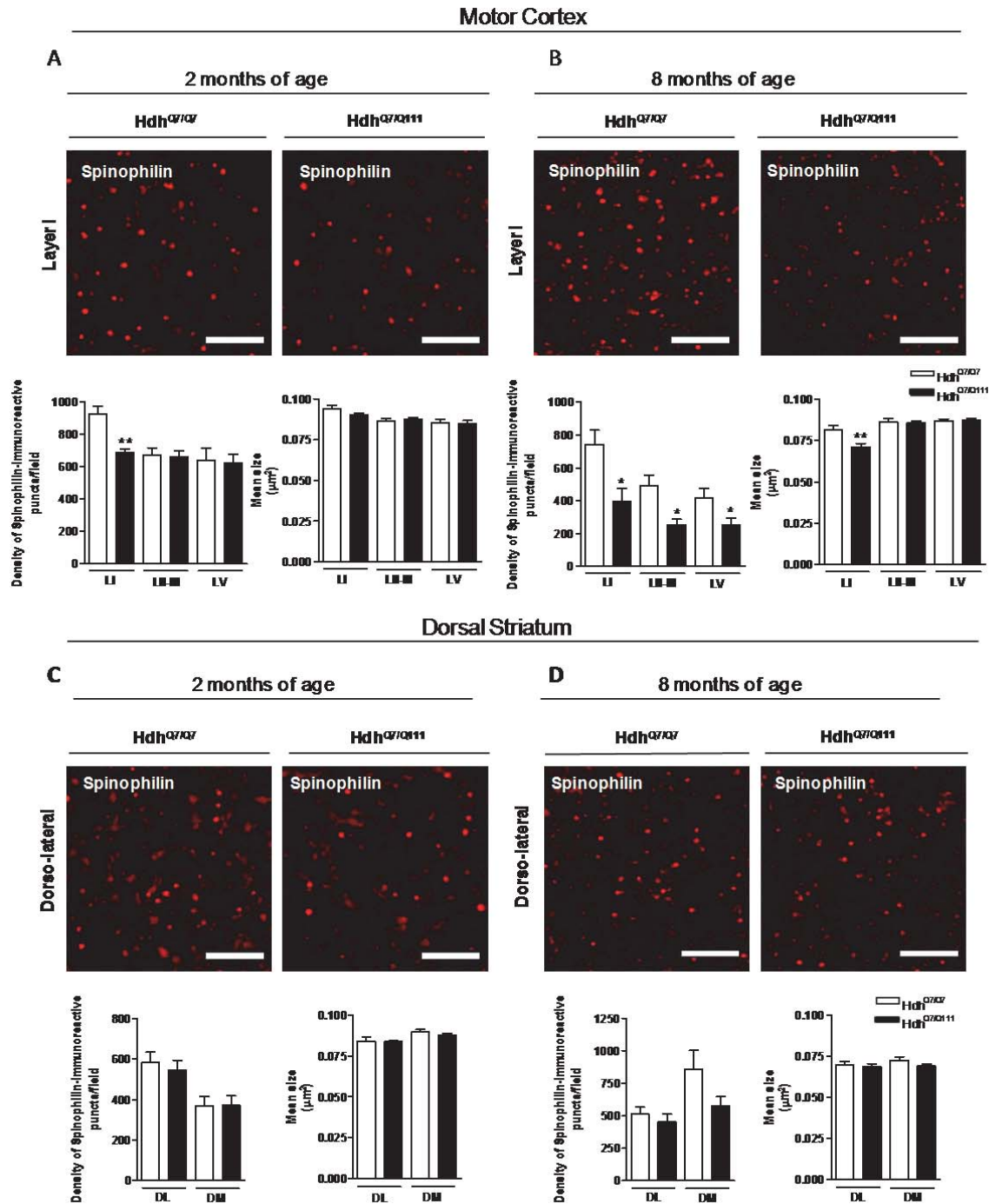


Figure 5. Specific reduction of spinophilin-immunoreactive puncta in the motor cortex, but not in the striatum, of Hdh^{Q7/Q111} mice at 2 and 8 months of age. Representative confocal images showing spinophilin (red) positive clusters in the motor cortex (**A, B**) and dorsal striatum (**C, D**) of wild type Hdh^{Q7/Q7} and mutant knock-in Hdh^{Q7/Q111} mice at 2 (**A, C**) and 8 (**B, D**) months of age. Cortical spinophilin-immunoreactive puncta were counted in layers I, II/III

and V of motor cortex area 1 (M1) and in the dorso-lateral (DL) and dorso-medial (DM) striatum and mean size was evaluated. Quantitative analysis is shown as mean \pm SEM (n=5-6 animals per group). A specific reduction in spinophilin-immunoreactive puncta was found in cortex but not striatum of Hdh^{Q7/Q111} mice at early disease stages. Statistical analysis was performed using Student's two-tailed *t* test. **p*<0.05; ***p*<0.01 compared to Hdh^{Q7/Q7} mice.

RESULTS

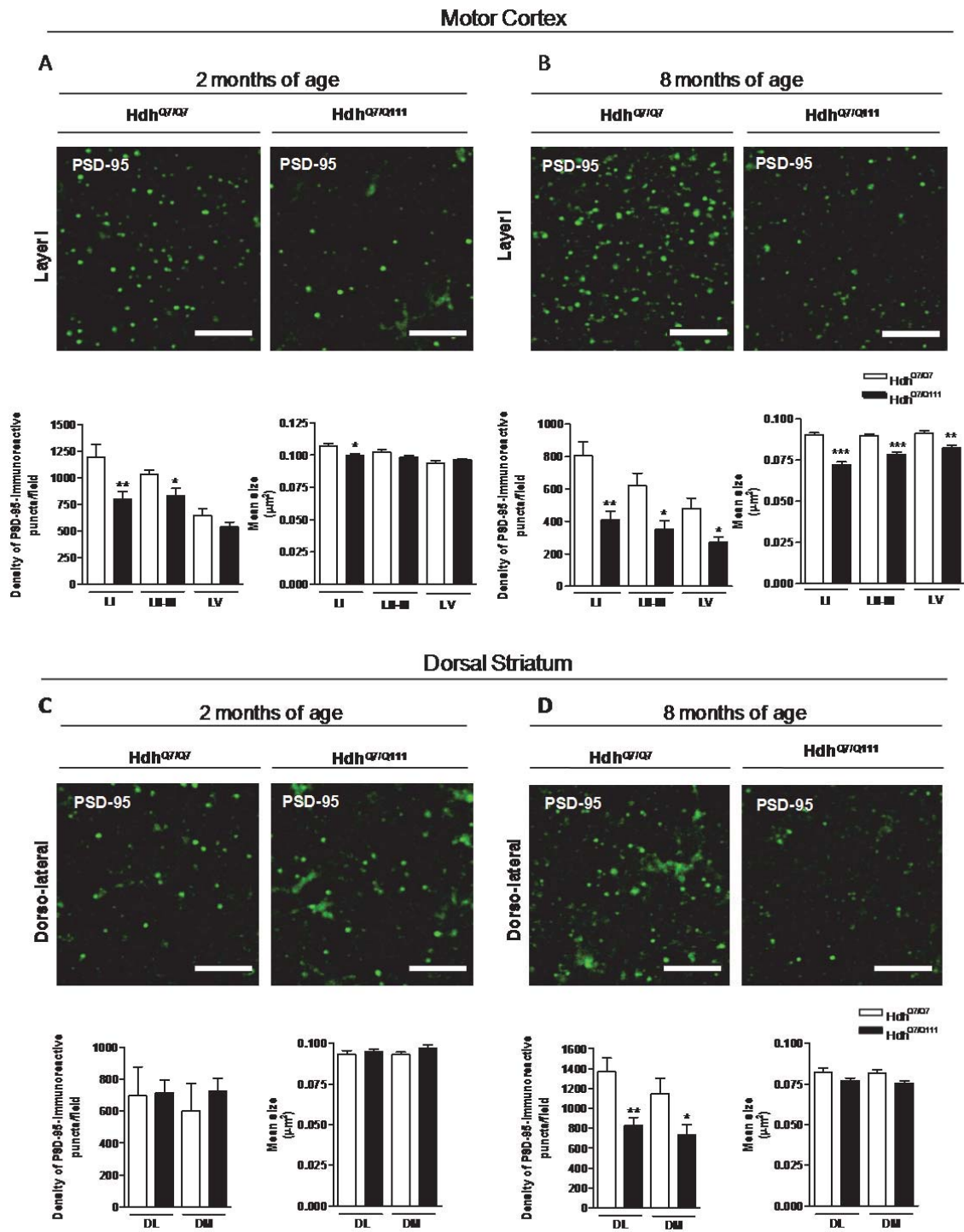


Figure 6. Specific reduction of PSD-95-immunoreactive puncta in the motor cortex of Hdh^{Q7/Q111} mice at early disease stages. Representative confocal images showing PSD-95 (green) positive clusters in the motor cortex (**A, B**) and dorsal striatum (**C, D**) of wild type Hdh^{Q7/Q7} and mutant knock-in Hdh^{Q7/Q111} mice at 2 (**A, C**) and 8 (**B, D**) months of age. Cortical

PSD95-immunoreactive puncta were counted in layers I, II/III and V of motor cortex area 1 (M1) and in the dorso-lateral (DL) and dorso-medial (DM) striatum and mean size was evaluated. Quantitative analysis is shown as mean \pm SEM (n=5-6 animals per group). A specific reduction in PSD95-immunoreactive puncta was found in cortex but not striatum of Hdh^{Q7/Q111} mice at early disease stages. At more advanced disease stages a reduction in PSD95-immunoreactive puncta was also found in the dorsal striatum. Statistical analysis was performed using Student's two-tailed *t* test. **p*<0.05; ***p*<0.01 compared to Hdh^{Q7/Q7} mice.

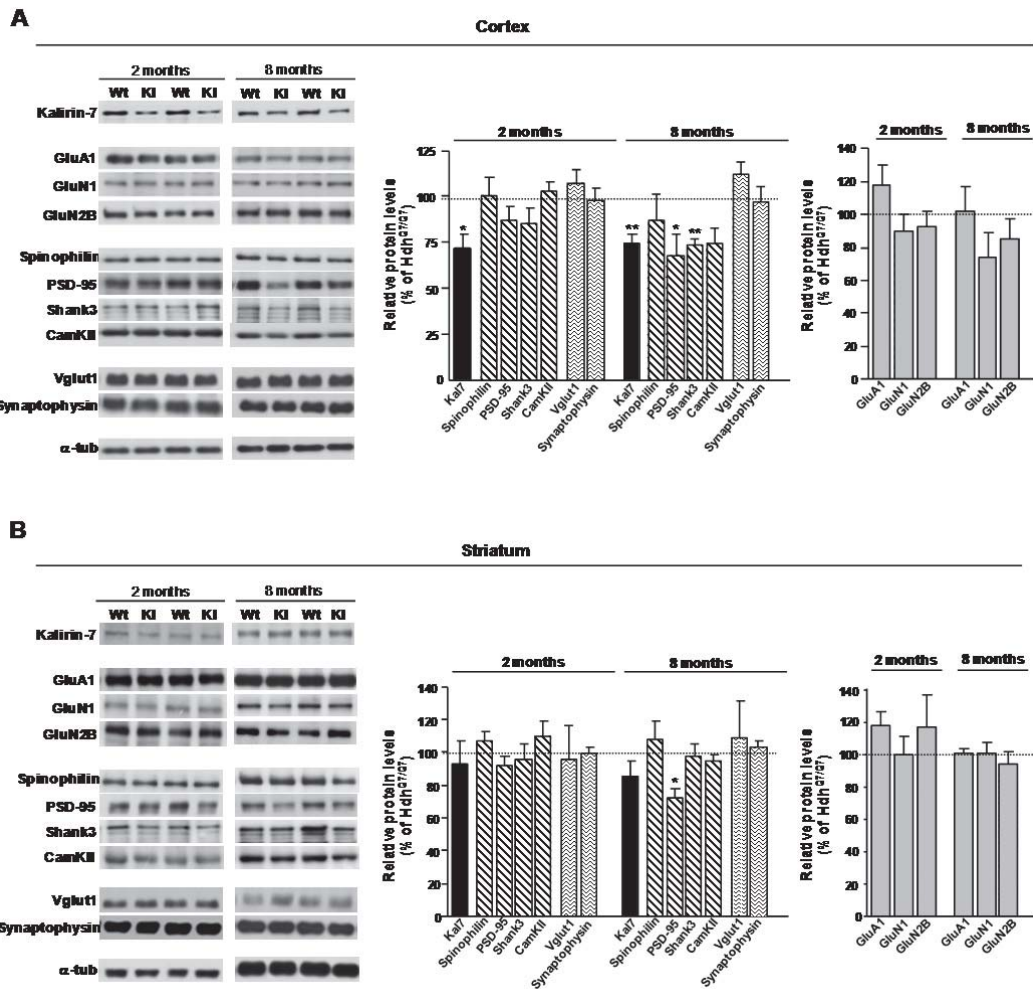


Figure 7. Levels of Kal7 are reduced in the cortex, but not in the striatum, of $Hdh^{Q7/Q111}$ mice at early disease stages. Representative Western blots showing the indicated subunits of glutamate receptors, pre-synaptic and post-synaptic scaffolding proteins and protein kinases in total cortical (**A**) and striatal (**B**) extracts from 2 and 8-month-old wild type $Hdh^{Q7/Q7}$ and knock-in mutant $Hdh^{Q7/Q111}$ mice. α -tub was used as loading control. A specific reduction in Kal7 was found in the cortex, but not in the striatum, of knock-in mutant $Hdh^{Q7/Q111}$ mice compared to wild type mice at 2 months of age. At mild disease stages a reduction on other synaptic-related proteins was found. Histograms represent the mean \pm SEM ($n=5-8$ animals per genotype). Statistical analysis was performed using Student's two-tailed t test. * $p<0.05$; ** $p<0.01$ compared to wild-type mice.

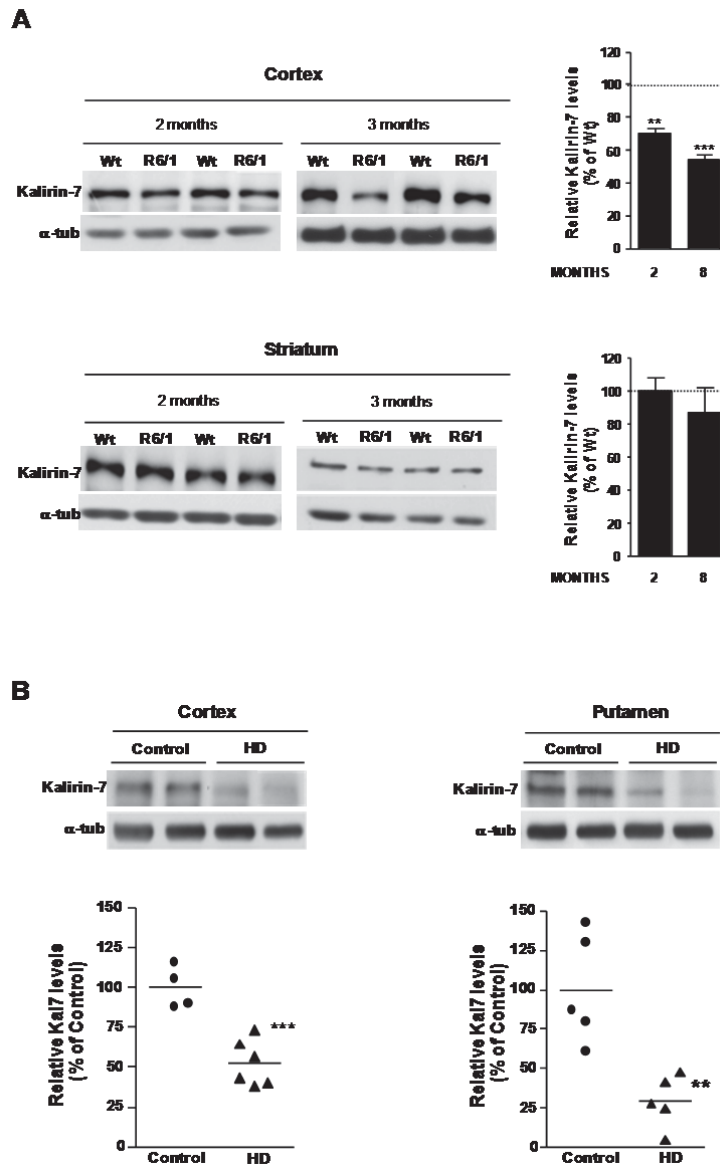


Figure 8. Kal7 levels are reduced in the cortex of R6/1 mice and in the cortex and putamen of HD brains. Representative Western blots showing Kal7 and α -tub as loading control in total cortical and striatal extracts from 2 and 3-month-old wild type and R6/1 mice (**A**). Histograms represent the mean \pm SEM ($n=5-8$ animals per genotype). Statistical analyses were performed using Student's two-tailed t test. ** $p<0.01$; *** $p<0.001$ compared to wild-type mice. Representative Western blot showing Kal7 and α -actin as a loading control in total cortical and putamen extracts from control ($n = 4-5$) and HD samples ($n = 5-6$) (**B**). Histograms represent mean \pm SEM. Student's two-tailed t test was performed. ** $p<0.01$, *** $p<0.001$; compared to control human samples.

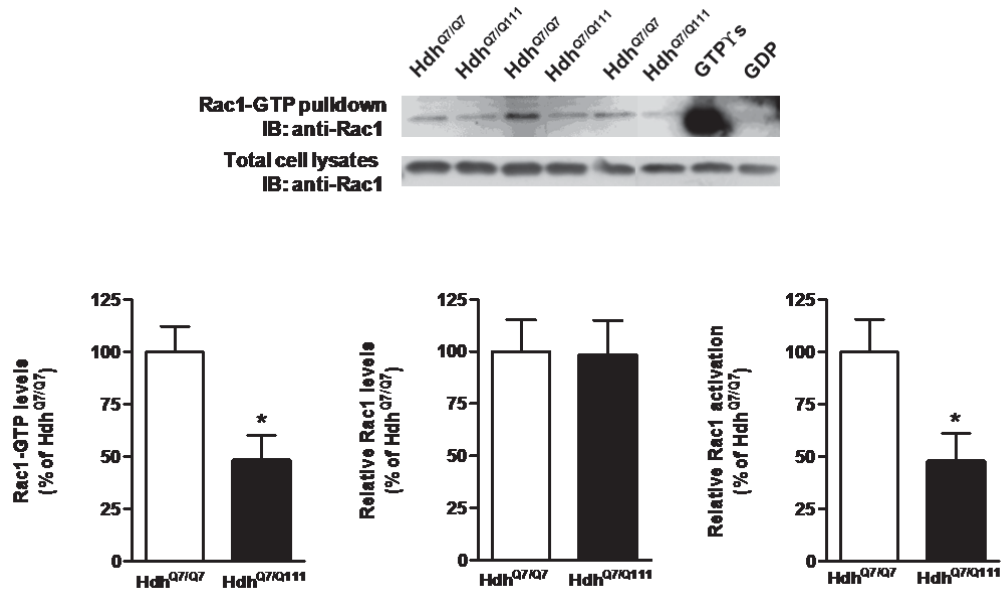


Figure 9. Rac1 activity is reduced in the cortex of Hdh^{Q7/Q111} mice. Representative Western blot of cortical extracts isolated from wild type Hdh^{Q7/Q7} and mutant Hdh^{Q7/Q111} mice at 8 months of age showing Rac1-GTP and total Rac1 levels. Activated (GTP-bound) Rac1 was detected by immunoblotting of pull-down experiments from cortical extracts. Diminished Rac1 activity was found in mutant Hdh^{Q7/Q111} compared to wild-type Hdh^{Q7/Q7} mice (n=5 animals per genotype), whereas similar total Rac1 levels were found between genotypes. Histograms represent mean \pm SEM. Student's two-tailed *t* test was performed. *p<0.05; compared to wild-type mice.

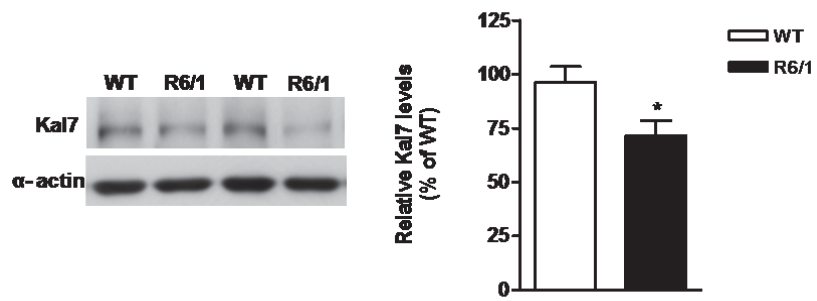


Figure 10. Reduced levels of Kal7 protein in mature cortical neurons from R6/1 mice. Representative Western blot showing Kal7 and α -actin as loading control in mature cortical neurons from wild-type (WT) and R6/1 mice (n=8-9 animals per genotype). A significant reduction of Kal7 (~ 25 %, $p < 0.05$) was found in mutant compared with wild-type mice. Statistical analysis was performed using Student's two-tailed t test. * $p < 0.05$ compared to WT cortical neurons.

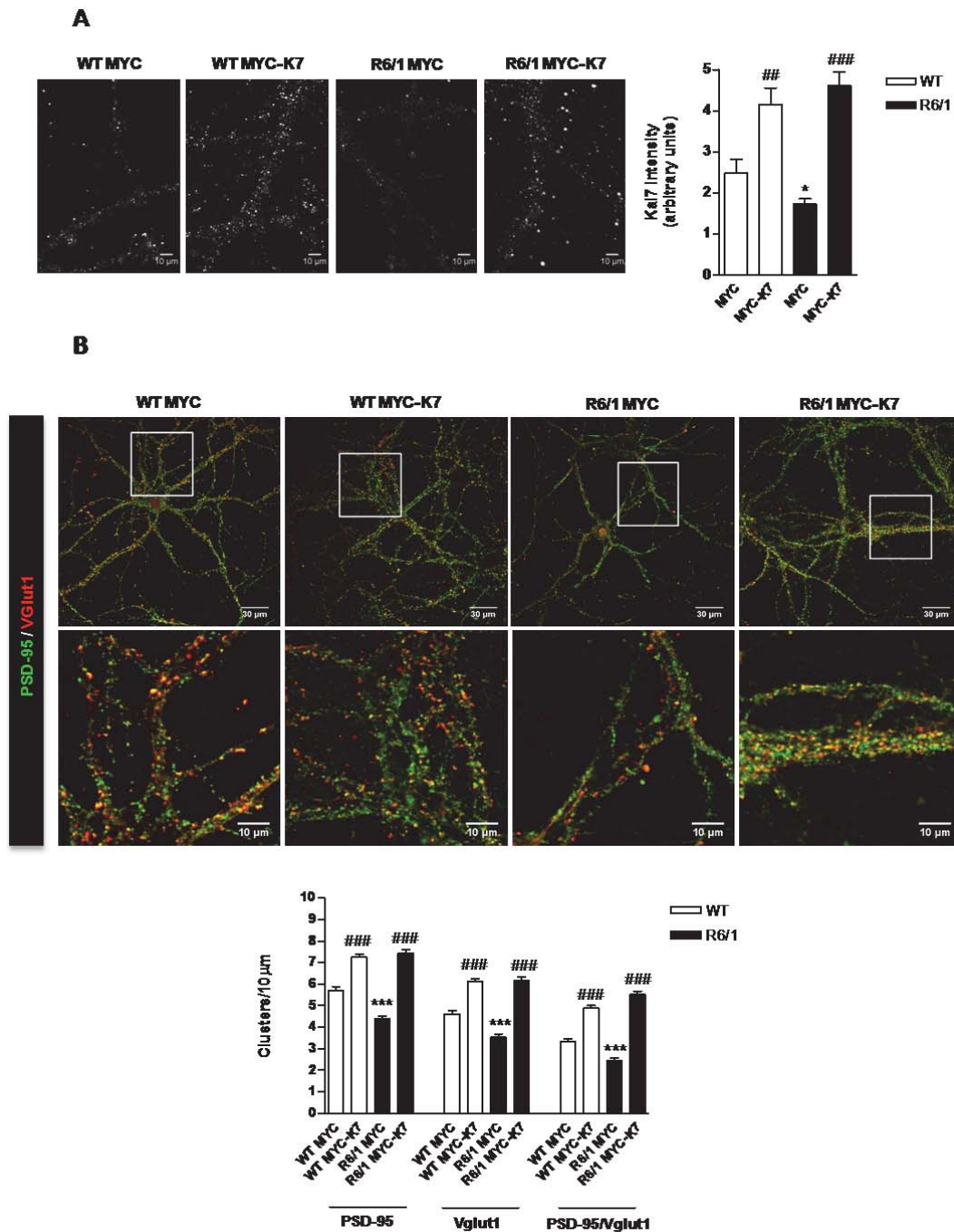
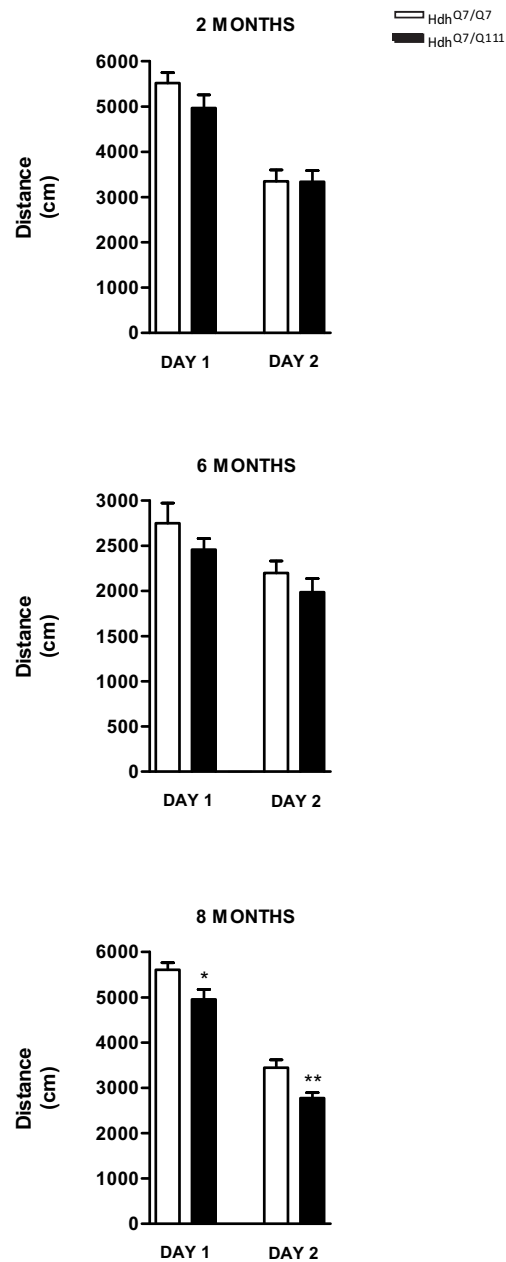


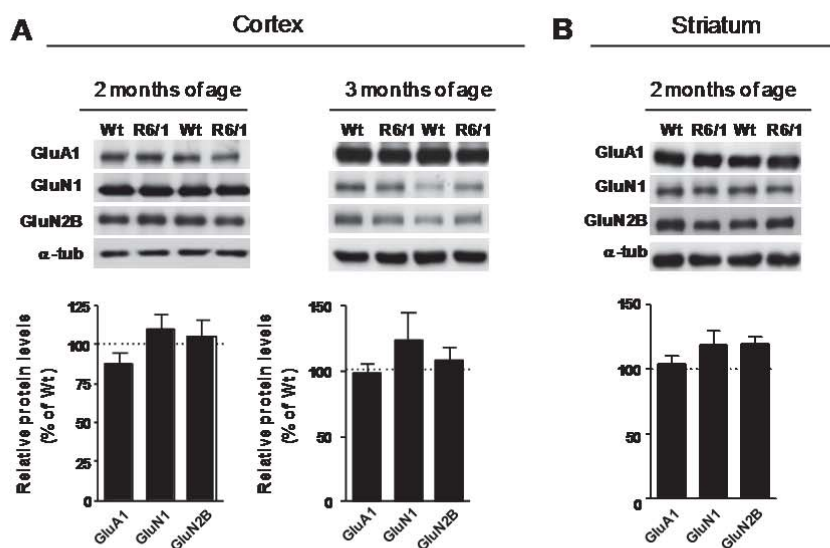
Figure 11. Exogenous expression of Kal7 restores the number of excitatory synapses in R6/1 mature cortical neurons. Representative confocal images showing Kal7 staining in mature cortical neurons from wild-type and R6/1 mice transfected with Myc or Myc-K7 vectors. Increased Kal7 staining was detected in both WT and R6/1 cortical neurons transfected with Myc-K7 compared to those transfected with Myc. Quantitative analysis of Kal7 levels is shown as mean \pm SEM (n= 21-24 fields from three replicates for WT Myc, WT Myc-K7, R6/1 Myc and

R6/1 Myc-K7) **(A)**. Statistical analysis was performed using one-way ANOVA with Student's two-tailed *t* test as a *post-hoc*. **p*<0.05, compared to WT Myc. ##*p*<0.01, ###*p*<0.001 compared to the corresponding (WT or R6/1) Myc-control. Representative confocal images showing Vglut1 (red), PSD-95 (green) and Vglut1/PSD-95 positive clusters in wild-type and R6/1 cortical neurons transfected with Myc or Myc-K7. Quantitative analysis of Vglut1, PSD-95 and Vglut1/PSD-95 positive clusters is shown as mean ± SEM (n= 65 neurons (two dendrites/each neuron) from 3 WT mice (three replicates/ animal); n=87-89 neurons (two dendrites/each neuron) from 4 R6/1 mice (three replicates/ animal). Over-expression of Myc-Kal7 restores the number of PSD-95, Vglut1 and Vglut1/PSD95-positive clusters (excitatory synapses) in R6/1 cortical neurons **(B)**. Statistical analysis was performed using one-way ANOVA with Bonferroni's Multiple comparison test as a *post-hoc*. ****p*<0.001 compared to WT Myc. ###*p*<0.001 compared to the corresponding (WT or R6/1) Myc-control.

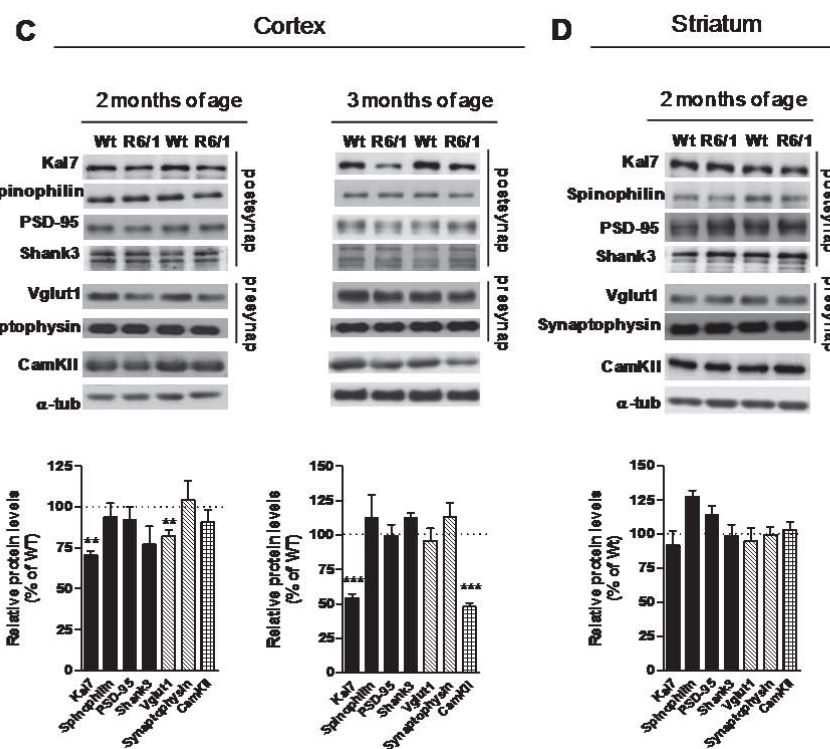


Supplementary Figure 1. Hdh^{Q7/Q111} mice do not exhibit spontaneous locomotor activity deficits until 8 months of age. Total distance traveled (cm) during 15 minutes in the open field arena in wild type Hdh^{Q7/Q7} and knock-in mutant Hdh^{Q7/Q111} mice at 2, 6 and 8 months of age. At 2 and 6 months of age no significant differences were found between genotypes, whereas at 8 months of age knock-in mutant Hdh^{Q7/Q111} exhibit decreased spontaneous locomotor activity. Data represents the mean \pm SEM (n=8-14 per genotype). Statistical analysis was performed using Student's two-tailed t test. *p<0.05; **p<0.01; compared to wild-type mice.

Glutamate receptors



Scaffolding/kinase proteins



Supplementary Figure 2. Synaptic-related proteins levels in the cortex and striatum of R6/1 mice at 2 and 3 months of age. Representative Western blots showing the indicated subunits of glutamate receptors, pre-synaptic and post-synaptic scaffolding proteins and protein kinases in total cortical (**A**) and striatal (**B**) extracts from 2 and 3-month-old wild type and R6/1 mice. α -tub was used as loading control. A specific reduction in Kal7 was found in the cortex,

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but not in the striatum, of R6/1 mice compared to wild type mice at 2 months of age. At mild disease stages a reduction on other synaptic-related proteins was found. Histograms represent the mean \pm SEM (n=5-8 animals per genotype). Statistical analysis was performed using Student's two-tailed *t* test. * $p < 0.05$; ** $p < 0.01$ compared to wild-type mice.

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WORK 5***“GENETIC REDUCTION OF CDK5 AMELIORATES COGNITIVE
DYSFUNCTION IN $Hdh^{Q7/Q111}$ KNOCK-IN HUNTINGTON’S
DISEASE MOUSE MODEL”***

This manuscript is under preparation

Puigdemívol M, Àlvarez E, Cherubini M, Alberch J, Ginés S.

CONTRIBUTION TO THIS WORK:

I contributed to the design of the research plan and organization of the study. I have participated in the design of the different mouse crossmates to obtain the final required $Hdh^{Q7/Q111};Cdk5^{+/-}$ (Double-mutant mice). I performed all the behavioral analysis, and obtained striatal, hippocampal, cortical and cerebellar samples from all mice genotypes to study biochemical changes. I performed the perfusion of animals to obtain brain slices for the immunohistochemical analysis. I build different hypothesis regarding the mechanisms by which Cdk5 modulates behavior in HD mice and contributed in the performance of biochemical analysis. I discuss and wrote the manuscript together with my supervisor.

AIM 4. TO STUDY THE ROLE OF CDK5 IN COGNITIVE DEFICITS IN HUNTINGTON'S DISEASE

Els dèficits cognitius són les primeres manifestacions clíniques en la malaltia de Huntington (MH). Desafortunadament, els mecanismes moleculars involucrats en l'aparició d'aquests símptomes resten desconeguts. Els processos d'aprenentatge i memòria es troben estretament modulats per la senyalització pre- i post-sinàptica. Cdk5, una serina/trreonina cinasa l'activitat de la qual es troba principalment restringida al sistema nerviós central ha estat considerada recentment com a un regulador clau en processos de plasticitat sinàptica, memòria i aprenentatge. A més, una aberrant activitat d'aquesta cinasa ha estat descrita en diverses malalties neurodegeneratives tals com la malaltia d'Alzheimer o la malaltia de Parkinson. Un treball recent del nostre grup, ha demostrat una aberrant activitat Cdk5 en cèl·lules estriatals que expressen la huntingtina mutada, així com en la regió estriatal d'animals $Hdh^{Q111/Q111}$ i individus afectats per la MH. Aquests estudis indiquen que la cinasa Cdk5 pot esdevenir un regulador clau en la disfunció neuronal de diverses malalties neurodegeneratives i suggereixen que la modulació de la seva activitat en condicions patològiques pot esdevenir una nova estratègia terapèutica. Per tal de determinar si una aberrant activitat Cdk5 contribueix a la patologia cognitiva de la MH, en aquest treball hem generat un nou animal transgènic que expressa la forma *full-length* de la huntingtina mutada i alhora heterozigosi per Cdk5 ($Hdh^{Q7/Q111}; Cdk5^{+/-}$). La disminució de Cdk5 en aquests nous animals transgènics es troba específicament restringida en àrees del sistema nerviós on s'expressa CamKII, tals com estriat, hipocamp i escorça cerebral. La modulació dels nivells de Cdk5 en els animals $Hdh^{Q7/Q111}$ reverteix els dèficits en aprenentatge motor i en la capacitat de canviar d'estratègia, funcions cognitives principalment atribuïdes a la via corticoestriatal. A més, la modulació dels nivells de Cdk5 en els animals $Hdh^{Q7/Q111}$ millora significativament la realització de tasques de memòria espacial i de reconeixement, suggerint així que els dèficits cognitius en la MH poden ser donats, si més no en part, a una aberrant activitat de la cinasa Cdk5. Tot i que els mecanismes pel quals la disminució de Cdk5 condueix a una millora en les funcions cognitives en la MH romanen desconeguts, els resultats exposats en aquest treball suggereixen que la disminució de Cdk5 en el context de la MH pot contribuir a la millora de les funcions cognitives en aquesta malaltia devastadora.

Genetic reduction of Cdk5 ameliorates cognitive dysfunction in Hdh^{Q7/Q111} knock-in Huntington's disease mouse model

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ABSTRACT

Cognitive deficits have been well characterized in the various stages of Huntington disease (HD) as well as in the prodrome before the motor diagnosis is given. Unfortunately, the molecular mechanisms underlying these defects remain unclear. Learning and memory formation are modulated by pre- and post-synaptic signaling events. Particularly, Cdk5 a serine/threonine kinase whose activity is primarily restricted to the nervous system has been emerged as a key regulator of synaptic plasticity and cognition. Importantly, Cdk5 has been involved in many neurodegenerative diseases such as Huntington, Alzheimer or Parkinson's Disease. Indeed, our group has previously demonstrated aberrant Cdk5 activity in the striatum of HD knock-in mouse models and in HD human brain. Overall, these studies pointed out Cdk5 as an important modulator of neuronal dysfunction in several neurodegenerative disorders and highlight the importance of therapeutic strategies aimed to inhibit Cdk5 activity to slow or prevent HD progression. To determine whether altered Cdk5 activity could also contribute to cognitive decline in HD we generated a new transgenic mice expressing mutant huntingtin but heterozygous for Cdk5 ($Hdh^{Q7/Q111}; Cdk5^{+/-}$). The genetic modulation of Cdk5 levels in $Hdh^{Q7/Q111}$ mutant mice restored corticostriatal learning deficits and improved performance in spatial and memory learning tasks, which suggests that both altered cortico-striatal and hippocampal function in HD could involve aberrant Cdk5 activity. Moreover, our data suggest that improved cognition in $Hdh^{Q7/Q111}; Cdk5^{+/-}$ mice could be mediated by the modulation of the Cdk5/Src/pTyr1472-GluN2B pathway. Altogether, these findings demonstrate that modulation of Cdk5 activity or signaling in HD may contribute to restore synaptic plasticity and learning defects in this devastating disorder.

INTRODUCTION

Huntington's disease (HD) is a genetic and progressive neurological disorder characterized by a wide range of clinical symptoms including motor disturbances (chorea) mainly associated to dysfunction and degeneration of the medium spiny neurons within the striatum and the presence of cognitive disturbances associated to an early dysfunction of the corticostriatal pathway, limbic system and even the hypothalamus (1-10).

There are several intriguing questions around how mutant huntingtin induces abnormal neuronal dysfunction in HD. One interesting possibility is that the mechanism by which mutant huntingtin causes dysfunction and ultimately degeneration resides on synaptic machinery. In fact, accumulating evidences indicate that synaptic circuitry dysfunction in HD is an early event that can become a promising therapeutic target to modulate, prevent or delay motor and cognitive disturbances (2;5;11-13). In this view, it is well established that protein kinases regulate the intracellular signaling pathways that control synaptic plasticity in the central nervous system (CNS) (14;15).

Different studies indicate that cyclin-dependent kinase 5 (Cdk5), a serine/threonine kinase whose activity is primarily restricted to the nervous system where its main activator p35/p39 is expressed (16-18), play a pivotal role in synaptic plasticity and transmission (16;19;20). Compelling evidence demonstrated the *in vivo* relevance of Cdk5 in modulating synaptic plasticity and proposed this kinase as a key regulator of higher cognitive functions (19-22). Accordingly, whereas spatial learning and memory is enhanced in brain-specific Cdk5 conditional knockout mice (23;24), prolonged Cdk5/p25 activity dramatically impairs hippocampal LTP and memory (21;25). Interestingly, our group has previously demonstrated that the increased vulnerability of mutant huntingtin striatal cells to glutamatergic and dopaminergic activation involves aberrant Cdk5 activity (26). Furthermore, sustained activation of Cdk5 has been related to other neurodegenerative disorders such as Alzheimer's or Parkinson's disease (27-30), indicating that an aberrant Cdk5 activity is a common feature in neurodegenerative disorders that leads to neuronal cell death.

In this study, we hypothesized that deregulation of Cdk5 induced by mutant huntingtin may underlie not only striatal vulnerability but also cognitive pathology in HD. To this

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aim, we have generated a new transgenic mouse model expressing full-length mutant huntingtin but heterozygous for Cdk5 (Hdh^{Q7/Q111}; Cdk5^{+/-}). We demonstrated that diminished Cdk5 levels in Hdh^{Q7/Q111} mice restored motor learning deficits assessed by the accelerating rotarod task procedure as well as the ability of the mice to replace a previously learned strategy in the reversal phase of the swimming T-maze test of the strategy shifting. Moreover, reduction of Cdk5 levels in Hdh^{Q7/Q111} mice, completely restored spatial and recognition memory deficits in the T-SAT and NORT paradigms. As an underlying mechanism by which genetic Cdk5 modulation restored cognitive function in HD, we proposed the Cdk5/Src/pTyr1472-GluN2B pathway.

Overall, our findings have identified Cdk5 as a contributing kinase to modulate cognitive dysfunction in HD and underscored Cdk5 modulation as a new therapeutic strategy to treat or delay learning and memory deficits in this devastating pathology.

MATERIALS AND METHODS

Animals. Hdh^{Q111} knock-in mice, with targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues, were maintained on a C57BL/6 genetic background. Male and female Hdh^{Q7/Q111} heterozygous mice were intercrossed to generate age-matched Hdh^{Q7/Q111} heterozygous and Hdh^{Q7/Q7} wild-type littermates. All mice used in the present study were males and were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. The animals were housed with access to food and water *ad libitum* in a colony room kept at 19-22°C and 40-60% humidity, under a 12:12h light/dark cycle. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01) and Generalitat de Catalunya (00/1094), in accordance with the Directive 2010/63/EU of the European Commission.

Generation of a new transgenic Hdh^{Q7/Q111}:Cdk5^{+/-} mice. In collaboration with Dr. James A. Bibb (Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, USA) and Dr. Paul Greengard (Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, NY, USA), we obtained the homozygous

floxed Cdk5 line that was generated as previously described (Hawasli et al., 2007). Using homologous recombination, exons encoding vital Cdk5 catalytic domain components were flanked with *loxP* elements (floxed). We crossed the homozygous floxed Cdk5 and Hdh^{Q111/Q111} mice to obtain a heterozygous floxed Cdk5 and heterozygous for full-length huntingtin mice (Cdk5^{flox/+}; Hdh^{Q7/Q111} mice). We crossed Cdk5^{flox/+}; Hdh^{Q7/Q111} mice with floxed Cdk5 mice to obtain homozygous mice for floxed Cdk5 and heterozygous for full-length huntingtin (Cdk5^{flox/flox}; Hdh^{Q7/Q111} mice). In collaboration with Dr. Carlos A. Saura (Institute of Neuroscience, Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Spain) we obtained the Cre-CamKII^{+/+} line. In these animals, Cre expression is under the control of the CamKII protein promoter. We crossed the homozygous mice for floxed Cdk5 (Cdk5^{flox/flox} mice) with the Cre-CamKII mice (Cre-CamKII^{+/+} mice) to obtain a heterozygous mice for floxed Cdk5 and heterozygous for Cre-CamKII (Cdk5^{flox/+}; Cre-CamKII^{+/-} mice). We crossed Cdk5^{flox/+}; Cre-CamKII^{+/-} mice with floxed Cdk5 mice (Cdk5^{flox/flox} mice) to obtain homozygous mice for floxed Cdk5 and heterozygous for Cre-CamKII (Cdk5^{flox/flox}; Cre-CamKII^{+/-} mice). Finally, we crossed Cdk5^{flox/flox}; Hdh^{Q7/Q111} mice with Cdk5^{flox/flox}; Cre-CamKII^{+/-} mice to obtain four different genotypes: Cdk5^{flox/flox}; Hdh^{Q7/Q7}; Cre-CamKII^{-/-} (Hdh^{Q7/Q7}; Cdk5^{+/+} or WT mice), Cdk5^{flox/flox}; Hdh^{Q7/Q7}; Cre-CamKII^{+/-} (Hdh^{Q7/Q7}; Cdk5^{+/-} or HET mice), Cdk5^{flox/flox}; Hdh^{Q7/Q111}; Cre-CamKII^{-/-} (Hdh^{Q7/Q111}; Cdk5^{+/+} or KI mice), Cdk5^{flox/flox}; Hdh^{Q7/Q111}; Cre-CamKII^{+/-} (Hdh^{Q7/Q111}; Cdk5^{+/-} or DM mice). Primers for allele genotyping had the following nucleotide sequence: 1) Cdk5: 5'-GCTGCAATGGTGACCTGGAC-3' and 5'-CCTCAGCCTTATGAGTGCTC-3', 2) Cre: 5'-GCCTGCATTACCGGTCGATGCAACGA-3' and 5'-GTGGCAGATGGCGCGCAACACCATT-3'; 3) mutant huntingtin: 5'-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC-3' and 5'-GGCGGCTGAGGAAGCTGAGGA-3'.

Western blot analysis. Heterozygous knock-in mutant Hdh^{Q7/Q111}, wild type Hdh^{Q7/Q7}, heterozygous Cdk5 (Cdk5^{+/-}) and double-mutant mice for full-length huntingtin and Cdk5 (Hdh^{Q7/Q111}; Cdk5^{+/-}) wild-type mice were killed by cervical dislocation at the age of 7 months of age. The brain was quickly removed, dissected, frozen in dry ice and stored at -80°C until use. Brain tissue was homogenized in cold lysis buffer (140 mM NaCl, 10mM HEPES, 1mM EGTA, 0.1 mM MgCl₂ (pH 7.4), 1% X-100 Triton, 0.5 % NP-40

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and supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)) by using a dounce homogenizer, 1 hour at 4°C in ice and cleared by centrifugation at 16.000g for 15 min at 4°C and the supernatants collected. Following determination of the protein contents by Detergent-Compatible Protein Assay (Bio-Rad; Hercules, CA, USA), protein extracts (20µg) were mixed with 5 X SDS sample buffer, boiled for 5 min, resolved on 6-10 % SDS-PAGE and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell; Keene, NH, USA). After incubation (30 min) in blocking buffer containing 10 % non-fat powdered milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05 % Tween 20), membranes were blotted overnight at 4 °C with primary antibodies: Cdk5 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Src (Tyr416)(1:1000; Cell Signaling Technology, Beverly, MA), phospho-GluN2B Tyr1472 (1:1000; Cell Signaling Technology) and GluN2B (1:1000; Cell Signaling Technology). Loading control was performed by reprobing the membranes with an anti-tubulin (1:50000, Sigma-Aldrich) or anti-actin (1:20000, MP Biochemicals, Aurora, OH, USA) during 30 min at room temperature. The membranes were then rinsed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (1:3000; Promega, Madison, WI, USA) for 1 h at room temperature. After washing for 30 min with TBS-T, the membranes were developed using the enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology). The ImageJ software was used to quantify the different immunoreactive bands relative to the intensity of the α -tubulin/actin band in the same membranes within a linear range of detection for the ECL reagent. Data is expressed as the mean \pm SEM of band density.

Behavioral assessment. Separate cohorts of 6-month old Hdh^{Q7/Q7};Cdk5^{+/+} (WT), Hdh^{Q7/Q7};Cdk5^{+/-} (HET), Hdh^{Q7/Q111};Cdk5^{+/+} (KI) and Hdh^{Q7/Q111};Cdk5^{+/-} (DM) mice, were used to evaluate corticostriatal function by using the accelerating rotarod training procedure (ARTP) and the swimming-simple T-Maze of strategy shifting and spatial recognition memory by using T-SAT and Novel object recognition test (NORT), respectively. To avoid stress and anxiety we performed first the less anxious and stressfull tasks as following indicated: ARTP, T-SAT, NORT, swimming T-Maze of strategy shifting.

ARTP. Animals were placed on a motorized rod (30 mm diameter). The rotation speed gradually increased from 4 to 40 r.p.m. over the course of 5 min. The time latency was recorded when the animal was unable to keep up with the increasing speed and fell. Rotarod training/testing was performed 4 times per day for 3 consecutive days.

Swimming T-Maze test of strategy shifting. The T-maze apparatus used was a glass maze consisting of three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° with respect to the other two. All arms were 45 cm long, 8 cm wide and enclosed by a transparent 20 cm wall. 6-months-old male mice were trained to swim from the stem arm of the T to the other arms. In one of them, there was a transparent escape platform, covered by water. During the acquisition phase of swimming T-maze of strategy shifting (two consecutive days: first day: 8 trials; second day: 4 trials), each mice was placed in the water, allowed them to swim until they reach the platform and the latency and errors (1: error, 0: non-error) to reach the platform were recorded. The mice have to be able to find the platform and learn the strategy faster across trials. After the acquisition phase, we change the location of the platform to the opposite arm. During the reversal phase of swimming T-maze of strategy shifting (two consecutive days: second day: 4 trials; third day: 8 trials) each mice was placed in the water and the latency and errors were recorded until they reach the platform. Different trials during the same day were separately by 1 hour.

T-SAT. The T-maze apparatus used was a wooden maze consisting of three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° with respect to the other two. All arms were 45 cm long, 8 cm wide and enclosed by a 20 cm wall. Two identical guillotine doors were placed in the entry of the arms situated at 180°. In the training trial, one arm was closed (novel arm) and mice were placed in the stem arm of the T (home arm) and allowed to explore this arm and the other available arm (familiar arm) for 10 min, after which they were returned to the home cage. After 5 h (LTM), mice were placed in the stem arm of the T-maze and allowed to freely explore all three arms for 5 min.

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NORT. The device consisted in a white circular arena with 40 cm diameter and 40 cm high. Mice were first habituated to the arena in the absence of objects (2 days, 10 min/day). On the third day, two similar objects were presented to each mouse during 10 min (A and A' condition) after which they were returned to their home cage. Twenty-four hours later, the same animals were re-tested for 5 min in the arena with a familiar (A) and a new (B) object (A, B condition; LTM). The object preference was measured as the time exploring each object \times 100/time exploring both objects.

Statistical analysis. All data are expressed as mean \pm S.E.M. Statistical analysis was performed by using the unpaired Student's t-test (95 % confidence), one-way ANOVA, two-way ANOVA, and the appropriate *post-hoc* tests as indicated in the figure legends. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Generation of a new transgenic mouse heterozygous for Cdk5 and expressing full-length mutant huntingtin.

To evaluate the functional relevance of Cdk5 in the HD context, we generated a new conditional transgenic mouse heterozygous for Cdk5 and expressing full-length mutant huntingtin (Supplemental Fig. 1 and Material and Methods). To validate our Cre-CaMKII \times Cdk5 flox/flox crossing, the levels of Cdk5 were first analyzed in heterozygous Cdk5 mice (Cdk5^{+/-}) by Western blot. A significant decrease on Cdk5 protein levels was found in the striatum, hippocampus and cortex of Cdk5^{+/-} heterozygous mice (Het) compared to wild-type Cdk5^{+/+} mice (WT) indicating that reduction of Cdk5 can be achieved by loxP elements Cre-dependent recombination (Supplemental Fig. 2). Next, the levels of Cdk5 were determined in the new conditional transgenic mice heterozygous for Cdk5 and expressing full-length mutant huntingtin. Levels of Cdk5 were found reduced in the striatum, hippocampus and cerebral cortex from both Hdh^{Q7/Q7};Cdk5^{+/-} (Het) and Hdh^{Q7/Q111};Cdk5^{+/-} (DM) mice compared to Hdh^{Q7/Q7};Cdk5^{+/+} (WT) and Hdh^{Q7/Q111};Cdk5^{+/+} (KI) mice (Fig. 1). According with the lack of CamKII expression in the cerebellum, no significant changes on Cdk5 protein expression was observed between genotypes (Fig. 1D). Overall, these data validate the reduction of

Cdk5 in both $Hdh^{Q7/Q7};Cdk5^{+/-}$ and $Hdh^{Q7/Q111};Cdk5^{+/-}$ mice, indicating that our model is useful to evaluate the functional impact of Cdk5 in HD.

Genetic reduction of Cdk5 ameliorates corticostriatal learning deficits in $Hdh^{Q7/Q111}$ knock-in mutant mice.

Given that Cdk5 has been emerged as a key modulator of synaptic plasticity, learning and memory processes, we next assessed whether normalization of Cdk5 function in our $Hdh^{Q7/Q111}$ mice by genetic reduction of Cdk5 could improve cognitive dysfunction in these mice. Alterations in corticostriatal-dependent behavioral tasks have been described in our $Hdh^{Q7/Q111}$ mice and other HD mouse models (31-36). Therefore, procedural learning and acquisition of new motor skills were evaluated in 6-months-old $Hdh^{Q7/Q7};Cdk5^{+/+}$ (WT), $Hdh^{Q7/Q7};Cdk5^{+/-}$ (Het), $Hdh^{Q7/Q111};Cdk5^{+/+}$ (KI) and $Hdh^{Q7/Q111};Cdk5^{+/-}$ (DM) mice in the accelerating rotarod paradigm and the swimming T-Maze test of strategy shifting (37-39). All genotypes learned the rotarod task (Fig 2). However, whereas KI mice were unable to maintain their balance on the rotarod with increasing speed, DM mice showed a significant and progressive increase in the latency to fall without significant differences compared to WT or Het mice (Fig. 4), indicating that genetic reduction of Cdk5 in KI mice rescues motor learning deficits. Importantly, spontaneous locomotor activity measured in the open field did not show differences between genotypes (Suppl Fig. 2), further supporting the idea that is the motor learning but not the locomotor activity what is altered in KI mice at 6 months of age. Next, we explored whether procedural memory deficits could also be reverted in DM mice. During the acquisition phase the escape platform was located in the right arm of the T-maze and the latency to reach the platform and the number of “errant” trials (turning left to reach the platform) scored. After the first trial, all genotypes exhibited similar decrease in the latency to reach the platform (Fig. 3A). However, in the last acquisition trials even though all genotypes learned to turn right to reach the platform, KI mice exhibited higher number of errant trials compared to the other genotypes (Fig. 3B), indicating a delay in learning during the acquisition phase of the strategy shifting in KI mice. Next, the ability of mice to change the strategy by switching the platform from the right arm to the left arm of the T-maze (reversal phase) was assessed. A significant increase in both the latency to reach the platform and the number of errant

trials was found when KI mice were analyzed compared to WT mice (Fig. 3C and 3D). Importantly, Cdk5 reduction in DM mice completely prevented procedural learning deficits in the reversal phase of the T-Maze evidenced by similar latency and number or error trials compared to WT mice. Overall, these data clearly demonstrate that genetic Cdk5 reduction in KI mice improves motor learning and procedural memory deficits.

Genetic reduction of Cdk5 rescues spatial and recognition memory deficits in $Hdh^{Q7/Q111}$ knock-in mutant mice.

We next explored whether genetic reduction of Cdk5 in KI mice could modulate spatial and recognition memory processes. Spatial memory was analyzed in the T-SAT paradigm that evaluates the time that a mouse spends exploring a novel versus a familiar arm in a T-Maze. During the training session, all genotypes spent similar time exploring the open arm (Suppl. Fig 2). However, when spatial long-term memory (LTM) was evaluated, KI mice exhibited no preference for the novel arm indicating spatial memory deficits (Fig. 4A). Importantly, reduction of Cdk5 in DM mice preserved spatial LTM demonstrated by a similar preference for the novel arm compared to both WT and Het mice (Fig. 4A). Next, recognition memory was evaluated by using the Novel Object Recognition task (NORT). This test is based on the natural tendency of mice to spend more time exploring a novel versus a familiar object. No differences between genotypes were found during the training period indicating no preference for object (A and A') (Fig. 4B). After 24 hours, recognition LTM was evaluated. Mutant KI mice exhibited a significant lower preference for the novel object compared to WT, Het and DM mice indicating preserved long-term recognition memory in double but not in mutant KI mice (Fig. 4B). No anxiety-like behaviors evaluated by the percentage of distance, time and entries in the periphery and the center were found during the first and second day of habituation in the open field arena (Supp Fig. 3) indicating that deficits in long-term memory deficits in KI mice are not due to anxiety behaviors. Altogether, these results demonstrate spatial and recognition memory deficits in 6-months-old KI mice, a decline that was prevented by genetic reduction of Cdk5.

Genetic reduction of Cdk5 in Hdh^{Q7/Q111} mice increases Src-mediated GluN2B phosphorylation.

Our data demonstrate that genetic reduction of Cdk5 in KI mice prevents HD cognitive deficits. To get insight into the molecular mechanism by which reduction of Cdk5 ameliorates cognitive dysfunction we investigated the Src-GluN2B pathway. It is known that Cdk5-mediated phosphorylation of Src induces ubiquitination and degradation of active Src (40;41). Interestingly, Src activity promotes surface expression of GluN2B through phosphorylation of GluN2B at residue Tyr1472 (42-45). Moreover, it has also been reported that Cdk5 inhibition increases the binding of Src to postsynaptic density-95 (PSD95) facilitating the phosphorylation of GluN2B at Tyr1472 (54). Therefore, we analyzed pTyr416-Src levels in the cortex of WT, KI, Het and DM mice as an indicator of Src activity. Our data demonstrated decreased pTyr416-Src in KI mice compared to WT mice, a decrease that was associated with a reduction in pTyr1472-GluN2B levels and a decrease in GluN2B total levels (Fig. 5A). Interestingly, genetic reduction of Cdk5 in both Het and KI mice induced a significant increase in pTyr416-Src, pTyr1472-GluN2B or total GluN2B. Altogether, these results indicate that an aberrant Cdk5 activity in KI mice correlates with Src inactivation, as indicated by reduced pTyr416-Src that results in decreased pTyr1472-GluN2B and total GluN2B levels. Importantly, reduction of Cdk5 not only reverses this phenotype but increases Src-mediated GluN2B phosphorylation and GluN2B total expression compared to WT mice.

DISCUSSION

In this study, we present Cdk5 as a mediator of learning and memory deficits in Huntington's Disease (HD), an inherited, fatal neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin gene and characterized by cognitive decline and motor disturbances (1-5;7;8;37;46;47).

A proper balance between kinase and phosphatase activities is crucial for the regulation of memory processes and synaptic plasticity (14;15;48). Cdk5 has emerged as one of the most versatile kinases playing crucial roles in the modulation of neuronal survival and synaptic plasticity through either phosphorylation of different substrates or protein-protein interaction (22;23). Although Cdk5 is required for LTP induction (25)

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and associative learning (21), conditional Cdk5 knock-out mice exhibit enhanced learning and synaptic plasticity (24) suggesting a complex role of Cdk5 in memory processes. Interestingly, we have previously reported increased Cdk5 activity in HD mice and HD human brain (26) that associates with increased vulnerability of striatal cells to glutamatergic and dopaminergic toxicity. However, the potential role of Cdk5 in HD cognitive deficits has not been addressed. To this aim knock-in Hdh^{Q7/Q111} mutant mice were crossed with conditional Cre-CamKII Cdk5 mice (Cdk5^{+/-} mice) to obtain double-mutant mice expressing mutant huntingtin but heterozygous for Cdk5. We found that genetic reduction of Cdk5 (about ~50% of protein expression compared to wild-type mice) in the striatum, hippocampus and cerebral cortex of mutant Hdh^{Q7/Q111} knock-in mice reversed spatial and recognition memories as well as motor learning and procedural memory, revealing a critical role for Cdk5 in hippocampal and cortico-striatal-dependent cognitive deficits in HD mice.

Several studies have shown that Cdk5 activity modulates structural plasticity, learning and memory through phosphorylation of distinct synaptic targets (21;24;49;50). In an attempt to elucidate the molecular mechanisms involved in the restoration of cognitive deficits in Hdh^{Q7/Q111};Cdk5^{+/-} (DM) mice, we focused on one of the Cdk5 substrates known to modulate synaptic activity, the tyrosine kinase Src (40). Src kinase activity is, in part, responsible for the up-regulation of GluN2B-containing NMDARs at the synaptic membrane through phosphorylation of GluN2B at Tyr1472 (43). Interestingly, it has been described that Cdk5 activity increases the ubiquitination and degradation of Src, reducing total Src activity (41). Moreover, Cdk5 phosphorylates the N terminus of PSD95 in a region overlapping a Src-binding site thereby modulating the binding of Src to PSD95 that in turns regulates the phosphorylation of GluN2B at residue Tyr1472 (54). We hypothesize that aberrant Cdk5 activity in KI mutant mice could lead to abnormal synaptic activity and therefore impaired memory and learning by decreasing Src activity and therefore the presence of GluN2B at the synapse. To validate this hypothesis we determined the Src activity by measuring the levels of Src autophosphorylated at residue Tyr416. This tyrosine is located, in the activation loop of the kinase domain and is known to up-regulate Src kinase activity (51-53). We found a significant reduction of pTyr416-Src levels in the cerebral cortex of Hdh^{Q7/Q111} knock-

in mutant mice compared to Hdh^{Q7/Q7} wild-type mice. Notably, genetic reduction of Cdk5 in Hdh^{Q7/Q111} mutant mice reversed such decrease suggesting that diminished Src activity in KI mutant mice is related to aberrant Cdk5 activity. Consistent with a reduction on Src activity, the levels of pTyr1472-GluN2B in KI mutant mice were significantly lower than in wild-type mice a decrease that was not detected in double mutant mice (Hdh^{Q7/Q111}, Cdk5^{+/-} mice). These results agree with previous studies showing increased levels of pTyr1472-GluN2B after pharmacological inhibition of Cdk5 by roscovitine treatment or in brain lysates from Cdk5^{-/-} mice (54). Interestingly, increased phosphorylation of GluN2B at Tyr1472 inhibits its binding to AP-2 and promotes surface expression of GluN2B (45;55), while decreased levels of pTyr1472 GluN2B correlate with increased GluN2B internalization (56). In this view, we found a significant decrease of GluN2B total levels in KI mutant mice that was not reproduced in DM mice. Therefore, we can hypothesize that the decrease in pTyr1472 GluN2B in KI mice due to Cdk5-mediated reduction in Src activity promotes GluN2B subunit internalization, thereby facilitating its degradation. Accordingly with our hypothesis, enhanced synaptic plasticity in conditional Cdk5 knock-out mice has been attributed to increased GluN2B levels in the membrane surface, and decreased GluN2B degradation by calpains (23). However, our data does not rule out that other mechanisms, namely, decreased transcription could also contribute.

Because GluN2B subunit, together with others NMDAR subunits, is a crucial mediator of NMDAR-mediated currents that in turns are closely linked to synaptic transmission and plasticity events (57), our results suggest that genetic reduction of Cdk5 in KI mutant mice could restored HD cognitive deficits through normalization of Cdk5 activity that in turns will enhance Src activity and increase pTyr1472-GluN2B maintaining GluN2B expression in the surface.

In summary, our work highlights the relevance of Cdk5/Src/pTyr1472-GluN2B pathway in HD cognitive deficits and demonstrates that genetic reduction of Cdk5 prevents cognitive dysfunction in HD mice. Altogether, we propose Cdk5 as a new promising therapeutic strategy to treat learning and memory deficits in HD.

ACKNOWLEDGMENTS

RESULTS

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

FIGURES AND FIGURE LEGENDS

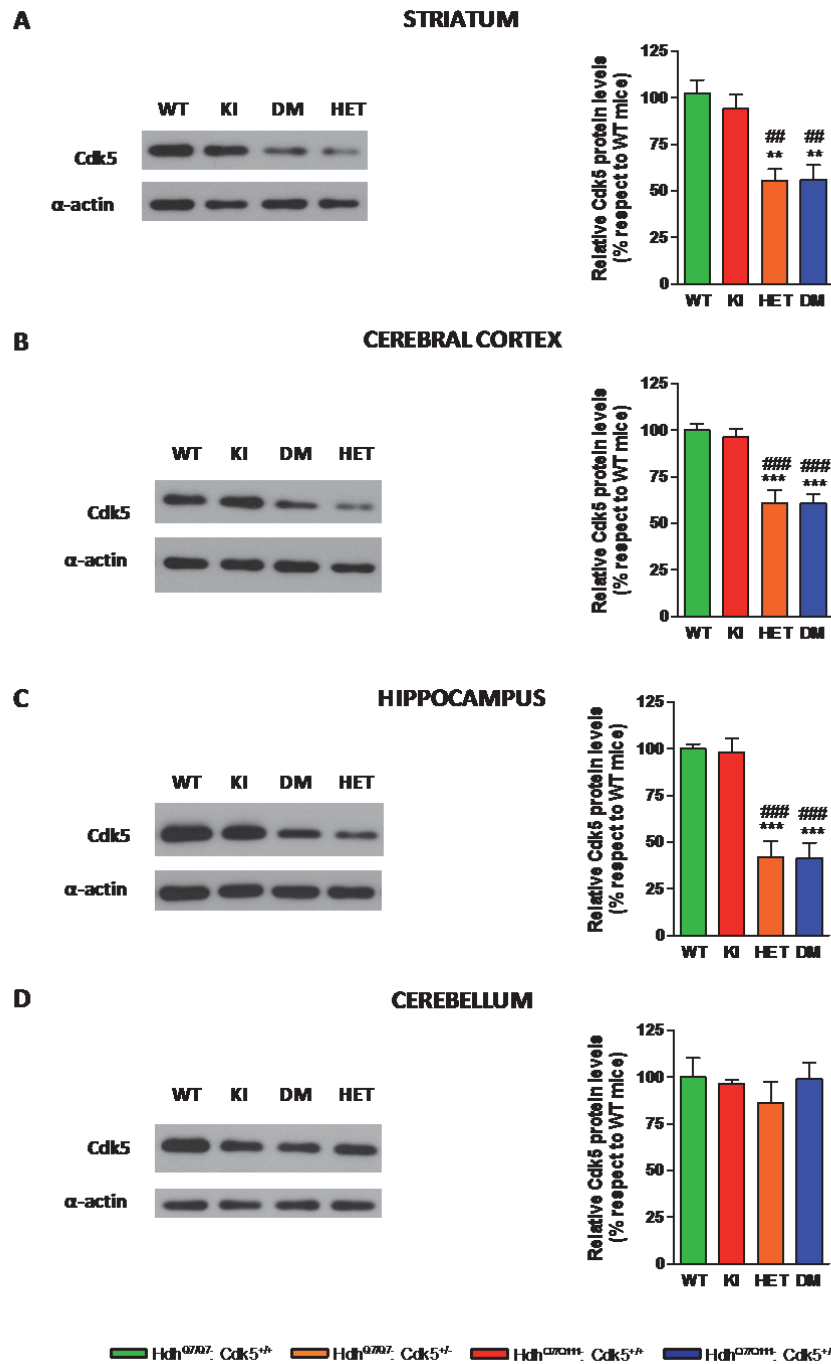


Figure 1. Decreased Cdk5 protein levels in the striatum, hippocampus and cerebral cortex, but not cerebellum, of $Hdh^{Q7/Q7};Cdk5^{+/-}$ and $Hdh^{Q7/Q111};Cdk5^{+/-}$ mice at 7 months of age. Representative Western blots for Cdk5 and actin as loading control and histograms showing a significant reduction in Cdk5 protein levels in striatal (A), hippocampal (B) and cortical (C) extracts of $Hdh^{Q7/Q7};Cdk5^{+/-}$ (Het) and $Hdh^{Q7/Q111};Cdk5^{+/-}$ (DM) mice compared to

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Hdh^{Q7/Q7}:Cdk5^{+/+} (Wt) and Hdh^{Q7/Q111}:Cdk5^{+/+} (KI) mice (n=5-10 per genotype). No significant differences were found in the cerebellum between genotypes (n=3 per genotype), accordingly with the lack of CamKII expression in this brain region. The values are expressed as mean \pm SEM. Statistical analysis was performed using Student's two tailed *t* test. ***p*<0.01, ****p*<0.001 compared to WT mice. ## *p*<0.01, ### *p*<0.001 compared to KI mice.

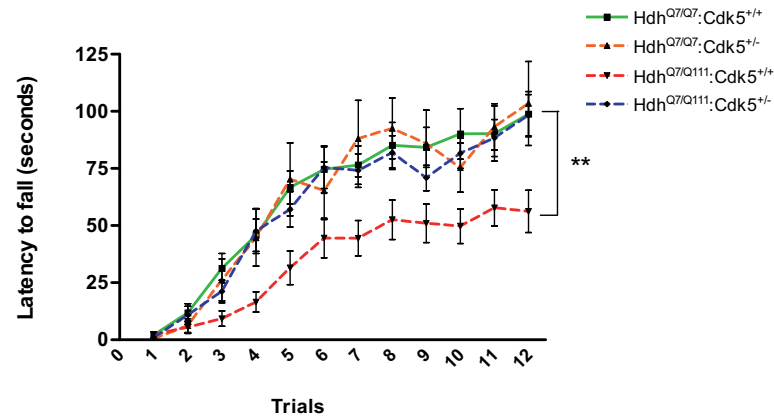


Figure 2. Impaired motor learning in Hdh^{Q7/Q111} mice is rescued in Hdh^{Q7/Q111}:Cdk5^{+/-} mice at 6 months of age. Latency to fall in the accelerating rotarod task procedure in six different cohorts of Hdh^{Q7/Q7}:Cdk5^{+/+} (Wt), Hdh^{Q7/Q7}:Cdk5^{+/-} (Het), Hdh^{Q7/Q111}:Cdk5^{+/+} (KI) mice and Hdh^{Q7/Q111}:Cdk5^{+/-} (DM) mice (n=9-26 per genotype). Whereas Hdh^{Q7/Q111}:Cdk5^{+/+} (KI) mice exhibit motor learning deficits in the ARTP compared to Hdh^{Q7/Q7}:Cdk5^{+/+} (Wt) mice, genetic reduction of Cdk5 in Hdh^{Q7/Q111}:Cdk5^{+/-} (DM) mice reverse this corticostriatal-dependent deficits. Data represents the mean \pm SEM. Statistical analysis was performed using two-way ANOVA with repeated measures. ** $p < 0.01$.

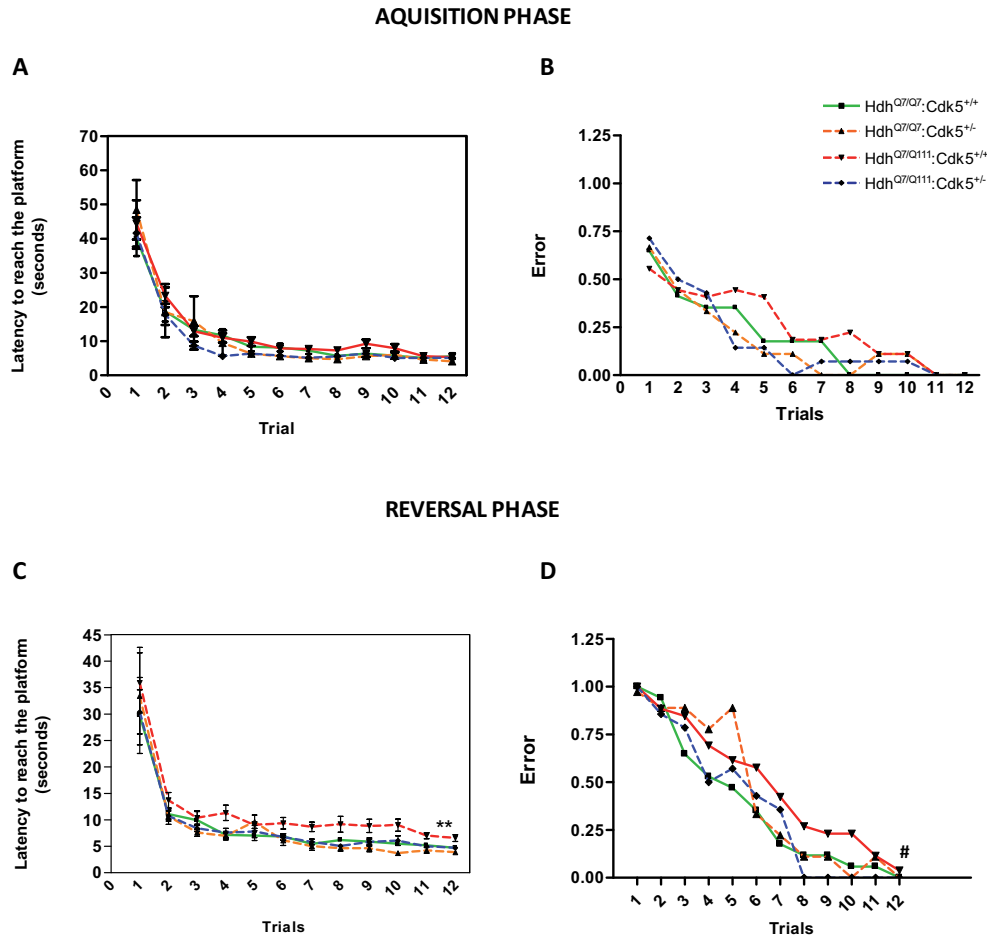


Figure 3. Genetic reduction of Cdk5 in $Hdh^{Q7/Q111};Cdk5^{+/-}$ mice rescue procedural memory deficits in the reversal phase of the swimming T-Maze test of strategy shifting. Histograms showing the Latency to reach the platform and the the number of “errant” trials to reach the platform in the correct arm during the acquisition (A, B) and reversal (C, D) phases of the swimming T-Maze test of Strategy Shifting of $Hdh^{Q7/Q7};Cdk5^{+/+}$ (WT), $Hdh^{Q7/Q7};Cdk5^{+/-}$ (HetCdk5), $Hdh^{Q7/Q111};Cdk5^{+/+}$ (KI) and $Hdh^{Q7/Q111};Cdk5^{+/-}$ (DM) mice at 6 months of age were represented. Whereas no significant changes were found between genotypes in the latency to reach the platform during the acquisition phase, in the reversal phase, $Hdh^{Q7/Q111};Cdk5^{+/-}$ (KI) mice showed a significant increase in the latency to reach the platform compared with $Hdh^{Q7/Q7};Cdk5^{+/+}$ (WT) mice. Interestingly, $Hdh^{Q7/Q111};Cdk5^{+/-}$ (DM) mice showed a significant decrease in the latency to reach the platform without differences versus $Hdh^{Q7/Q7};Cdk5^{+/+}$ (WT) mice, which indicates an improvement in this cortico-striatal-dependent task. Data represents the mean \pm SEM. (Six different cohorts of animals with a total of n=9-26 mice per genotype were evaluated). Statistical analysis was performed using two-way ANOVA with repeated measures. ****** $p < 0.01$; **#** $p < 0.05$

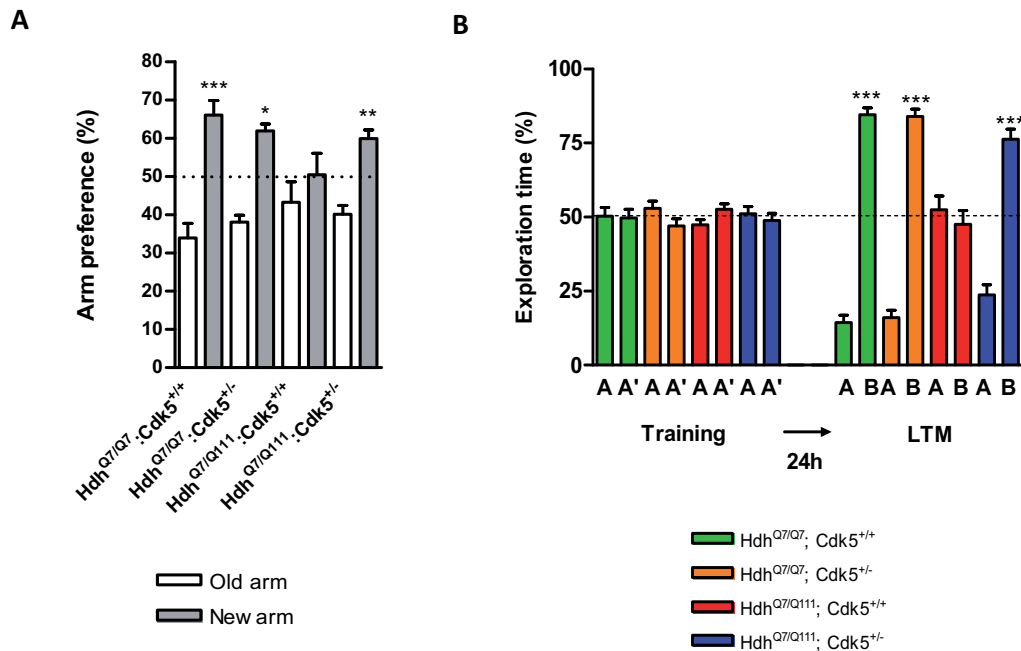


Figure 6. Hdh^{Q7/Q111};Cdk5^{-/-} exhibit restored spatial and recognition long-term memories in the T-SAT and NORT paradigms, respectively. (A) Percentage of time spent in arms (old versus novel) from Hdh^{Q7/Q7};Cdk5^{+/+} (WT), Hdh^{Q7/Q7};Cdk5^{-/-} (HetCdk5), Hdh^{Q7/Q111};Cdk5^{+/+} (KI) and Hdh^{Q7/Q111};Cdk5^{-/-} (DM) mice at 6 months of age. Hdh^{Q7/Q111};Cdk5^{+/+} (KI) exhibit no preference for the novel arm of a T-maze, while Hdh^{Q7/Q111};Cdk5^{-/-} (DM) exhibit higher preference for the previously unexposed arm of a T-maze as Hdh^{Q7/Q7};Cdk5^{+/+} (WT) and Hdh^{Q7/Q7};Cdk5^{-/-} (HetCdk5), indicating preserved spatial long-term memory in the T-SAT. **(B)** Percentage of time spent in exploring similar objects (A, A') during training session of the NORT, showing no preference for an object in all genotypes. 24 hours later, LTM was addressed by changing a previously object (A') for a new one (B). Percentage of time spent in exploring the old versus new objects during testing session of the NORT, showing that Hdh^{Q7/Q111};Cdk5^{+/+} (KI) exhibit no preference for the novel object compared to the old one, while Hdh^{Q7/Q111};Cdk5^{-/-} (DM) exhibit higher preference for the new object compared to the old one as Hdh^{Q7/Q7};Cdk5^{+/+} (WT) and Hdh^{Q7/Q7};Cdk5^{-/-} (HetCdk5), indicating preserved recognition long-term memory. Data represents the mean \pm SEM. (Four different cohorts of animals with a total of n=8-16 mice per genotype were evaluated). Statistical analysis was performed using One-way ANOVA with Bonferroni post hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the old arm (in A) and to the old object (in B).

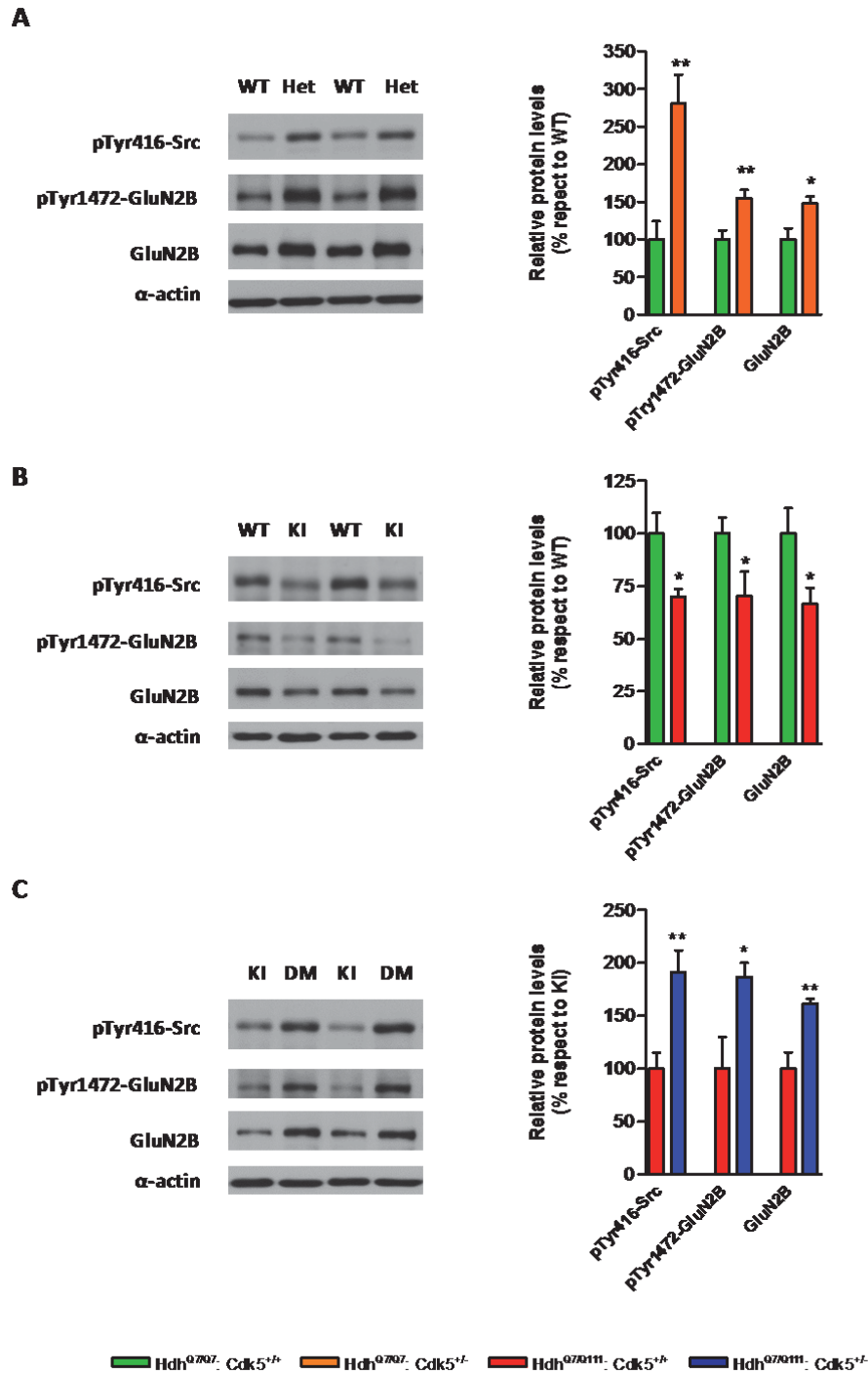
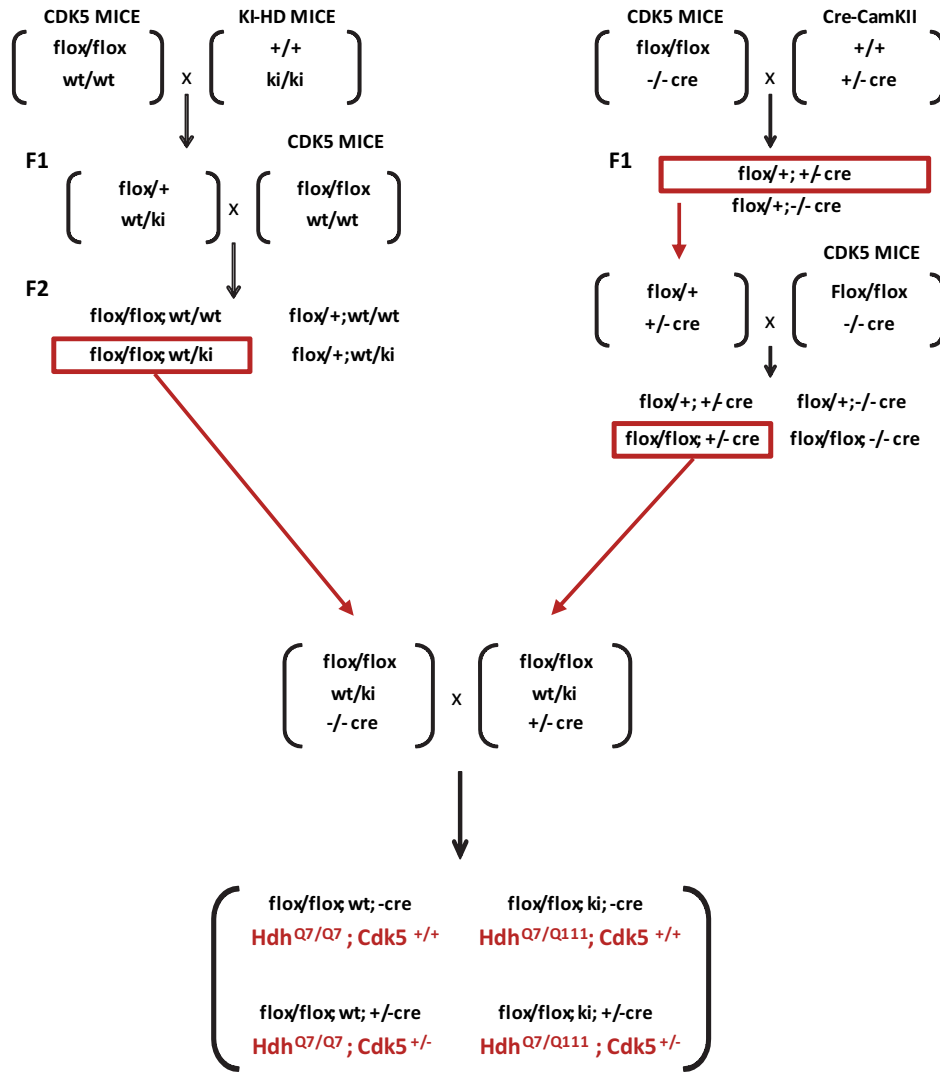


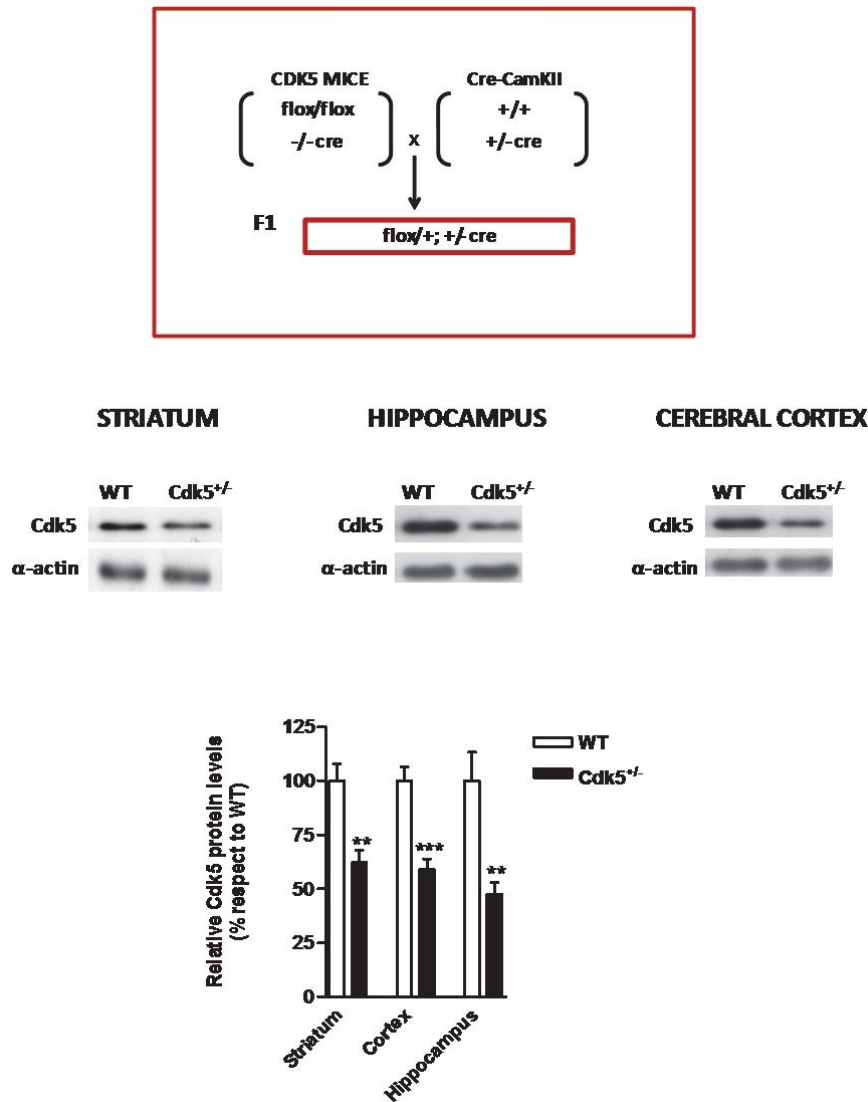
Figure 7. Altered Cdk5/Src/pTyr1472-GluN2B pathway in $Hdh^{Q7/Q111}; Cdk5^{+/+}$ mice are reverted in $Hdh^{Q7/Q111}; Cdk5^{-/-}$ mice. (A, B, C) Representative Western blots for pTyr416-Src, pTyr1472-GluN2B, GluN2B and actin as loading control in cortical extracts of 7-months-old $Hdh^{Q7/Q7}; Cdk5^{+/+}$ (Wt) and $Hdh^{Q7/Q7}; Cdk5^{-/-}$ (Het), $Hdh^{Q7/Q111}; Cdk5^{+/+}$ (KI) mice and $Hdh^{Q7/Q111}; Cdk5^{-/-}$ (DM) mice. Histogram showing significant increased in pTyr416-Src,

pTry1472-GluN2B and GluN2B protein levels in Hdh^{Q7/Q7}:Cdk5^{+/-} (Het) compared to Hdh^{Q7/Q7}:Cdk5^{+/+} (Wt) mice **(A)** and in Hdh^{Q7/Q111}:Cdk5^{+/-} (DM) mice compared to Hdh^{Q7/Q111}:Cdk5^{+/+} (KI) mice **(C)**. By contrast, histogram showing significant decreased in pTyr416-Src, pTry1472-GluN2B and GluN2B protein levels in Hdh^{Q7/Q111}:Cdk5^{+/+} (KI) mice compared to Hdh^{Q7/Q7}:Cdk5^{+/+} (Wt) mice **(B)**. The values are expressed as mean \pm SEM (n=6-7 per genotype). Statistical analysis was performed using Student's two tailed *t* test. **p*<0.05, ***p*<0.01, compared to WT or KI mice.

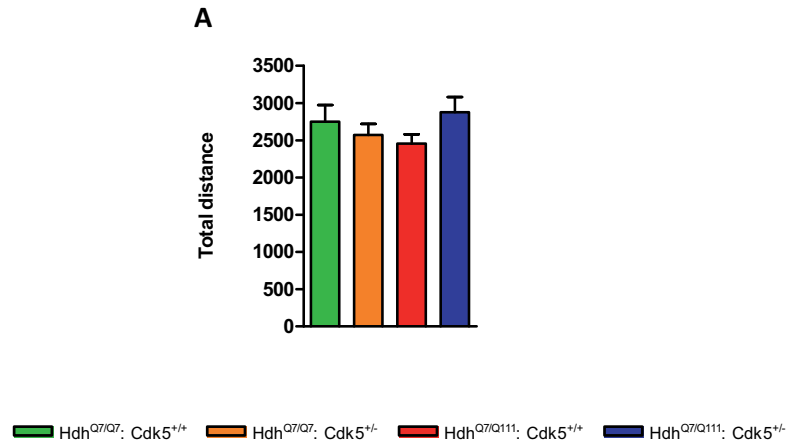
RESULTS



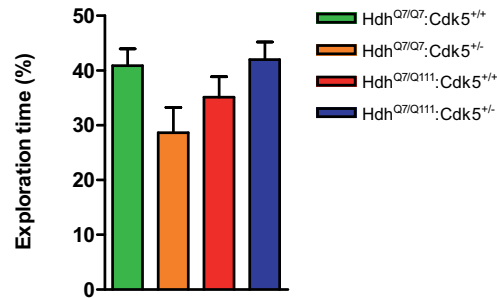
Supplementary figure 1. Generation of a new transgenic mouse heterozygous for Cdk5 and full-length mutant huntingtin. Scheme illustrating the different crossings to obtain the final genotypes (in red): $\text{Hdh}^{Q7/Q7}; \text{Cdk5}^{+/+}$ or WT mice, $\text{Hdh}^{Q7/Q7}; \text{Cdk5}^{+/-}$ or Het mice, $\text{Hdh}^{Q7/Q111}; \text{Cdk5}^{+/+}$ or KI mice and $\text{Hdh}^{Q7/Q111}; \text{Cdk5}^{+/-}$ or DM mice.



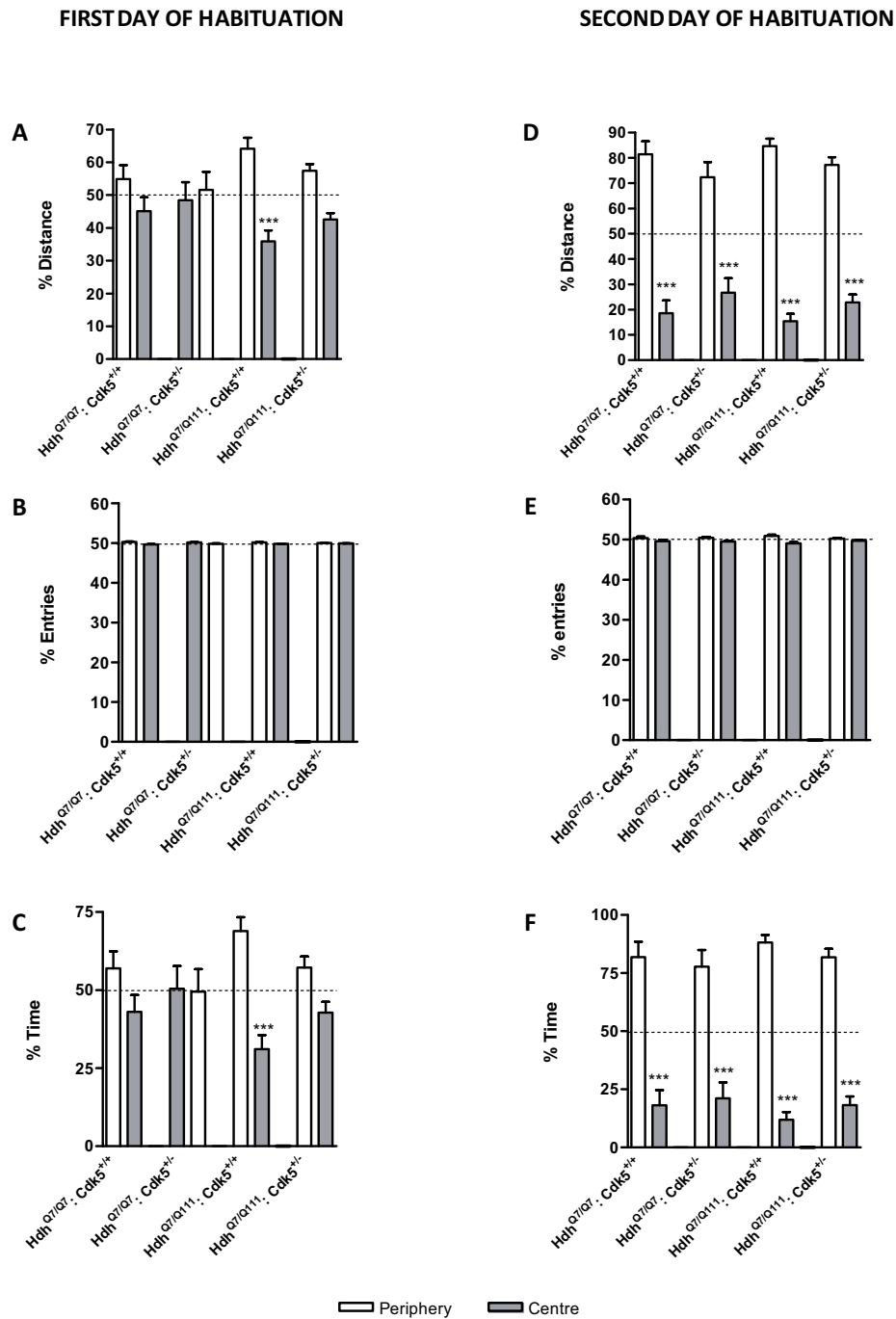
Supplementary figure 2. Cdk5 protein levels are reduced in adult Cdk5^{+/-} mice. Scheme illustrating the crossing between homozygous floxed Cdk5 mice and homozygous Cre-CamKII mice to obtain a mouse heterozygous for floxed Cdk5 and heterozygous for Cre-CamKII (Cdk5^{+/-} mice). Representative Western blots for Cdk5 and actin as loading control and quantification showing a significant reduction in Cdk5 protein levels in striatal, hippocampal and cortical extracts of Cdk5^{+/-} mice compared to WT (Cdk5^{+/+}) mice (n=6-8 per genotype). The values are expressed as mean ± SEM. Statistical analysis was performed using Student's two tailed *t* test. ***p*<0.01, ****p*<0.001 compared to WT mice.



Supplementary figure 3. No alterations in spontaneous locomotor activity are detected between Hdh^{Q7/Q7}:Cdk5^{+/+} mice, Hdh^{Q7/Q7}:Cdk5^{+/-} mice, Hdh^{Q7/Q111}:Cdk5^{+/+} mice and Hdh^{Q7/Q111}:Cdk5^{+/-} mice. Bar diagram illustrating total distance traveled in the open field arena during 15 minutes of habituation showing no significant differences between genotypes. The values are expressed as mean ± SEM (Four different cohorts of animals with a total of n=8-16 mice per genotype were evaluated). Statistical analysis was performed using One-way ANOVA.



Supplementary figure 4. Similar percentage of exploration time during the training session in the T-SAT between Hdh^{Q7/Q7}:Cdk5^{+/+} mice, Hdh^{Q7/Q7}:Cdk5^{-/-} mice, Hdh^{Q7/Q111}:Cdk5^{+/+} mice and Hdh^{Q7/Q111}:Cdk5^{-/-} mice. Bar diagram illustrating the percentage of time that mice spent in exploring the open arm of the T-Maze during the training session (10 minutes). No significant differences between genotypes were found. The values are expressed as mean \pm SEM (Four different cohorts of animals with a total of n=8-16 mice per genotype were evaluated). Statistical analysis was performed using One-way ANOVA.



Supplementary figure 5. No anxiety-like behavior neither at the first nor at the second day in the Open-field arena habituation are detected between $Hdh^{Q7/Q7};Cdk5^{+/+}$ mice, $Hdh^{Q7/Q7};Cdk5^{-/-}$ mice, $Hdh^{Q7/Q111};Cdk5^{+/+}$ mice and $Hdh^{Q7/Q111};Cdk5^{-/-}$ mice. Bar diagrams illustrating the percentage of distance (A, D), entries (B, E) and time (C, F) that mice spent between the periphery and the center during the first (A, B, C) and second (D, E, F) days of habituation in the open-field arena (15 minutes each day), showing no significant differences

between genotypes at the second day of habituation, indicating no anxiety-like behaviors genotype-dependent. The values are expressed as mean \pm SEM (Four different cohorts of animals with a total of n=8-16 mice per genotype were evaluated). Statistical analysis was performed using One-way ANOVA with Bonferroni post hoc comparisons. *** p <0.001 compared to the periphery.

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WORK 6***“TARGETING DOPAMINE D1-HISTAMINE H3 RECEPTOR HETEROMERS
REVERTS LEARNING AND LONG-TERM MEMORY DEFICITS IN A MOUSE
MODEL OF HUNTINGTON’S DISEASE”***

This manuscript is under preparation

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CONTRIBUTION TO THIS WORK:

I contributed in the building of different hypothesis and in their validation. I performed all the chronic treatments with saline and thioiperamide during several months and performed all the behavioral analysis. I obtained HD mice samples and HD brain slices at different ages and after treatment, or not, with saline or thioiperamide to give them to my colleagues who performed organotypic analysis and PLA methods. I discussed all the results and together with my supervisor wrote part of results, materials and methods and discussion.

AIM 5. TO STUDY THE ROLE OF D₁R-H₃R HETEROMERS IN NEURONAL CELL DEATH AND COGNITIVE DEFICITS IN HUNTINGTON'S DISEASE.

En edats tempranes de la malaltia de Huntington (MH), hi ha un excés de producció de dopamina i una sobre-activació del receptor de dopamina D₁ (D₁R) que produeix no tan sols un desequilibri en la senyalització dopaminèrgica sinó que també indueix l'activació de cascades de senyalització pro-apoptòtiques. En aquest treball, proposem una nova i provocativa estratègia per reduir la sobre-expressió de D₁R en la MH que consisteix en modular l'activitat dels heteròmers formats entre el D₁R i el receptor d'histamina 3 (H₃R). En aquest treball demostrem l'expressió d'heteròmers D₁R-H₃R en un model cel·lular estriatal de la MH així com en diverses regions cerebrals de models murins de la MH i individus afectats per la MH. No obstant, l'expressió d'aquests heteròmers en els models animals de la MH i individus afectats per la MH desapareix en edats més avançades de la MH. Davant la co-activació dels heteròmers D₁R-H₃R, els lligands d'H₃R actuen com a "molecular brake" de la senyalització via D₁R. Així, en aquest treball demostrem que el tractament amb lligands del H₃R rescata de la mort cel·lular mediada per D₁R en línies cel·lulars i en talls de cervell provinents d'animals Hdh^{Q7/Q111}. A més, demostrem que el tractament crònic amb un antagonista del H₃R a edats pre-simptomàtiques de la MH, rescata els déficits en aprenentatge motor i memòria espacial i de reconeixement en els animals de la MH. Aquestes millores cognitives es troben associades a la inhibició de la pèrdua dels heteròmers en els animals Hdh^{Q7/Q111}, tractats crònicament amb l'antagonista del H₃R. En conjunt, aquests resultats demostren que els heteròmers D₁R-H₃R juguen un paper crucial en el control de la senyalització dopaminèrgica i indiquen que aquests heteròmers poden esdevenir unes potents dianes terapèutiques pel tractament de la MH en estadis pre-simptomàtics de la malaltia.

Targeting dopamine D1-histamine H3 receptor heteromers reverts learning and long-term memory deficits in a mouse model of Huntington's disease

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Abstract

In the early stages of Huntington's disease (HD) there is an excess of dopamine production and an over-activation of dopamine D₁ receptors (D₁R) that can produce not only an imbalance in dopaminergic neurotransmission but can also directly lead to signaling cascades that induce cell death. Here we propose a new and provocative strategy to reduce the D₁R over-activation effects in HD by targeting the recently described receptor complexes of D₁R and the histamine receptors H₃ (H₃R). We show the expression and function of D₁R-H₃R heteromers in a HD model of striatal neuronal progenitor cells and in different brain areas of mouse models of HD in the early but not in the late stages of the illness. D₁R-H₃R heteromers are also expressed in human control subjects and in grade 2 HD patients but not in grade 3 or 4 severe HD patients. Upon co-activation of D₁R-H₃R heteromers, H₃R ligands act as a "molecular brake" for D₁R signaling. D₁R-induced cell death in cells and in brain slices and the signaling cascades responsible for this death are reduced by H₃R ligands targeting D₁R-H₃R heteromers. Treatment of presymptomatic mouse models of HD with the H₃R antagonist thioperamide restores both cognitive and motor deficits and inhibits the loss of heteromer expression in these animals. Our results demonstrate that D₁R-H₃R heteromers play a pivotal role in controlling dopaminergic neurotransmission and indicate that D₁R-H₃R heteromers can be target for treating HD in the pre-symptomatic stages of the illness.

INTRODUCTION

Huntington's disease (HD) is a dominant inherited progressive neurodegenerative disorder with severe motor, cognitive and psychiatric disturbances caused by expansion of a CAG repeat, coding a polyglutamine repeat within the N-terminal region of huntingtin protein (1993;Vonsattel and DiFiglia, 1998). Dysfunction and death of striatal medium-spiny neurons is the major feature of neuropathological changes in HD (Ferrante et al., 1991;Vonsattel et al., 1985). Initially, it was assumed that cell death was due to excitotoxicity caused by aberrant NMDA receptor activation produced by an excessive glutamate release as a cause of mutant huntingtin toxicity (de la Monte et al., 1988;Fan and Raymond, 2007;Martin and Gusella, 1986;Paoletti et al., 2008;Perez-Navarro et al., 2006). In addition to glutamatergic afferents, the striatum also receives the densest dopaminergic innervation in the brain from the ventral midbrain neurons (Gerfen, 1992) and there is increasing evidence that the dopaminergic system may contribute to HD neuropathology. A predominant theory of what leads to HD development at early stages is that alterations in dopamine (DA) stimuli from the substantia nigra produce an imbalance in striatal neurotransmission initiating signaling cascades that induce cell death (Paoletti et al., 2008;Ross and Tabrizi, 2011). Studies in HD patients show that increased DA release induces chorea (Andre et al., 2010;Spokes, 1980). The idea that aberrant DA signaling underlies behavioral abnormalities was first proposed as a predictive test and later neurochemical studies of HD patients suggested that increased DA occurs in the early stages of the disease (Garret et al., 1992). Whereas striatal medium-sized spiny neurons (MSNs) expressing enkephalin and dopamine D₂ receptors (D₂R), forming the indirect pathway, are believed to be the most vulnerable in HD, this primary neuronal loss can lead to an overactivation of the MSNs expressing substance P and dopamine D₁ receptors (D₁R), forming the direct pathway, which are believed to be relatively spared early in the progression of the disorder (Albin et al., 1990;Vonsattel et al., 1985). Thus, over-activation of D₁R produces an imbalance between the direct and indirect pathway (Chen et al., 2013a;Paoletti et al., 2008), indicating that strategies that might reduce D₁R signaling could prove successful towards preventing HD. In support of this strategy, Andre et al have shown in an animal model of HD that

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reducing DA or antagonizing D₁R normalized inhibitory and excitatory transmission in direct pathway neurons (Andre et al., 2011b;Andre et al., 2011a). This is in line with observations that DA receptor antagonists and agents that decrease DA reduce chorea and motor symptoms in patients and in animal models, while dopaminergic stimulation exacerbate symptoms (Mestre et al., 2009;Tang et al., 2007). It has been reported that DA potentiates glutamate-induced apoptosis in cultured striatal neurons from *YAC128* HD mice via activation of D₁R (Tang et al., 2007). Accordingly, it has been demonstrated that mutant huntingtin enhances DA-mediated HD striatal cell death via D₁R by a mechanism involving p25 and Cdk5 activation (Paoletti et al., 2008). Together, these data point to D₁R as an important therapeutic target for HD. However, D₁R are highly expressed in many tissues. Thus, general use of D₁R antagonists as a preventive treatment can have important drawbacks as it also reduces spontaneous locomotor activity during the exploratory period in mice exposed to a new environment (Gimenez-Llort et al., 1997).

We hypothesized that targeting D₁R through an associated modulator protein present in brain areas involved in HD is a new and provocative strategy that might be a more effective treatment for HD. One potential mechanism for achieving this could be through the recently described receptor complexes of D₁R and the histamine receptors H₃ (H₃R) (Ferrada et al., 2009;Moreno et al., 2011;Moreno et al., 2014). Histamine is an important neuromodulator and both D₁R and H₃R are co-expressed in striatal GABAergic dynorphinergic neurons (Pillot et al., 2002;Ryu et al., 1994b;Ryu et al., 1994a), where we and others have found that they establish functional negative interactions (Moreno et al., 2011;Sanchez-Lemus and Arias-Montano, 2004) by forming molecular complexes termed heteromers (Ferrada et al., 2008;Ferrada et al., 2009;Moreno et al., 2011). Here we explore whether H₃R ligands by binding to D₁R-H₃R heteromers can act as protective agents against dopamine damage in a HD model of striatal neuronal progenitor cells and in different brain areas of mouse models of HD. We have found that, in the early stages of the illness, D₁R-H₃R heteromers are expressed in HD related brain areas in different mice models and human brain samples of HD but the heteromerization is lost at symptomatic stages. D₁R-H₃R heteromers targeted by H₃R ligands reduce the overstimulation of D₁R and improves motor and

cognitive deficits in HD animals opening a new and promising pharmacological strategy to restore cognitive function and prevents neuronal death in HD.

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Functional D₁R-H₃R heteromers are expressed in wild type STHdH^{Q7} and HD STHdH^{Q111} striatal cells

To test whether D₁R-H₃R heteromers could indeed be targets for controlling D₁R signaling in HD we first utilized the previously developed and characterized conditionally immortalized striatal neuronal progenitor cells expressing normal (STHdH^{Q7}) or mutant (STHdH^{Q111}) full-length huntingtin with 7 or 111 glutamines under the endogenous promoter, respectively (Gines et al., 2010;Paoletti et al., 2008;Trettel et al., 2000). These cells do not exhibit amino-terminal inclusions allowing the study of changes involved in early HD pathogenesis (Trettel et al., 2000). By ligand binding we determined that both STHdH^{Q7} and STHdH^{Q111} cells endogenously express D₁R and H₃R (Supplemental Table 1a). By proximity ligation assays (PLA), we detected D₁R-H₃R heteromers as red spots surrounding the blue stained nuclei in both cell types (Fig. 1a and Fig. S1a, b) but not in cells depleted of H₃R by shRNA (Fig. 1b and Fig. S2) or in negative controls (Fig. S1c and d), indicating the heteromer expression in these cells. To ensure that D₁R-H₃R heteromers are functional in STHdH cells, we next performed cell signaling experiments. Our previous studies on the D₁R-H₃R heteromer have shown that upon co-activation, H₃R acts as a “molecular brake” for D₁R signaling. In fact, H₃R agonist, by a negative cross-talk, and H₃R antagonist, by a cross-antagonism, decreased the D₁R agonist signaling (Ferrada et al., 2009;Moreno et al., 2011;Moreno et al., 2014). The negative cross-talk and cross-antagonism is achieved via a protein-protein interaction between receptors in the heteromer. This is a common biochemical property of receptor heteromers by which the ligand binding to one receptor unit in the heteromer, changes positively or negatively the binding and/or the functional properties of another receptor unit in the heteromer (Ferre et al., 2009). Using both STHdH^{Q7} and STHdH^{Q111} cells and concentrations of ligands previously shown to be optimal for receptor activation of ERK 1/2 pathway (Ferrada et al., 2009;Moreno et al.,

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2011;Moreno et al., 2014), the D₁R agonist SKF 81297 and the H₃R agonist, imetit were able to increase ERK 1/2 phosphorylation whereas the co-activation with both agonists completely reverted the ERK 1/2 signaling demonstrating a clear negative cross-talk between receptors (Fig. 1c and d). Moreover, the SKF 81297 signaling was reverted not only by the D₁R antagonist SCH 23390 but also by the H₃R antagonist thioperamide (Fig. 1c and d). Neither SCH 23390 nor thioperamide had no effect on ERK 1/2 signaling on they own (results not shown). These data are evidence of the above described cross-antagonism. Similar negative cross-talk and cross-antagonism were also seen on an alternative signaling pathway activated downstream of D₁R as Ca²⁺ mobilization. To measure calcium mobilization in STHdH cells we used the recently described GCaMP6 biosensor (Chen et al., 2013b). When cells were treated with the D₁R agonist SKF 81297 a robust and rapid increase in fluorescence was seen in both STHdH^{Q7} and STHdH^{Q111} cells (Fig. 1e and f). Importantly, this calcium release could be dampened upon either co-activation with the H₃R agonist imetit showing negative cross-talk, or by H₃R antagonist thioperamide, showing cross-antagonism (Fig. 1e and f). The above signaling data strongly support the presence of functional D₁R-H₃R heteromers in STHdH cells and implicate D₁R-H₃R heteromers in controlling cell signaling in these striatal cells. To directly demonstrate that receptor-receptor interactions are indeed responsible for the observed effects we sought to design transmembrane (TM) domain mimicking peptides that might disrupt the heteromer and impede H₃R's ability to allosterically influence D₁R signaling. First, a homology model of D₁R was constructed from the crystal structure of D₃R and the same was done for H₃R using the proposed structure of H₁R. Possible interfaces were modeled considering the molecular constraint of two G-proteins most likely are bound (Moreno et al., 2014). This provided a potential interface around TMs 5 and 6. Using the HIV TAT peptide fused to synthetic peptides corresponding to D₁R TMs 5 and 7 (as negative control), we tested the ability of these peptides to disrupt the heteromer detected by the proximity ligation assay (PLA). When cells were incubated with TM5 peptide there was a near complete loss in fluorescence dots in both cell types (Fig. 1h), similar than with TM6 peptide (Fig. 1i) that was not seen using the TM7 peptide (Fig. 1j). Together, these data support the model of the D₁R-H₃R interface occurring around TM 5 and 6 being TM5 clue for heteromerization. We next checked if TM5 and TM7 influence the above observed

cross-talk and cross-antagonism in calcium mobilization (Fig. 1f and g). We found that pre-treatment with TM5 (Fig. 1f) but not TM7 (Fig. 1g) peptide could disrupt H₃R's ability to dampen D₁R calcium signaling in both STHdH^{Q7} and STHdH^{Q111} cells. Importantly, these results validate the D₁R-H₃R heteromer as being responsible for the cell signaling effects observed in both STHdH cells, and support the use of disrupting peptides as an additional tool to test for heteromer function.

H₃R ligands revert the D₁R -induced decrease in STHdH^{Q7} and STHdH^{Q111} cell viability.

Dopamine toxicity is thought to be one of the major drivers of neuronal death in HD pathogenesis and has been shown to be dependent on D₁R (Andre et al., 2010; Andre et al., 2011b). We next explored whether H₃R ligands could reduce D₁R driven cell death in these neuronal progenitor STHdH^{Q7} and STHdH^{Q111} cells and if this is mediated by D₁R-H₃R heteromers. The D₁R agonist SKF 81297 dose-responses revealed a decrease in cell viability being negligibly more sensitive with STHdH^{Q111} cells than STHdH^{Q7} cells (Fig. 2a). We found that significant cell death did not begin until ~30 μM SKF 81297 was used, results that were similar to those published earlier using a partial D₁R agonist (Paoletti et al., 2008) and was reverted by the D₁R antagonist SCH 23390 (Fig S 3a). Using the lowest SKF 81297 concentration where decreases in viability were seen, we observed that H₃R ligands diminished the D₁R-promoted decrease in cell viability. In fact, cell pre-treatment with either imetit (Fig. 2b) or thioperamide (Fig. 2c), that by they own did not modify cell viability (Fig. S 3a), led to up to a two fold increase in the number of cells surviving in the presence of SKF 81297. The effect of H₃R ligands is specific and mediated by D₁R-H₃R heteromers since there was no reversion of D₁R agonist-induced cell death in cells depleted of H₃R by shRNA (Fig. 2d and e) or in cells pre-treated with D₁R TM5 peptide (Fig. 2d and e). These results confirmed that receptor-receptor interactions in the D₁R-H₃R heteromers were responsible for the H₃R ligands induced cell protection and point out D₁R-H₃R heteromers in striatal cells as targets to protect against D₁R-mediated loss of cell viability.

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Next we sought to determine the effect of H₃R ligands on the cellular signaling pathways implicated in D₁R-mediated cell death. An important pathway known to be involved in cell death is the p38 pathway (Gianfriddo et al., 2004; Saavedra et al., 2011). We measured changes in p38 phosphorylation levels at low and high concentrations of the D₁R agonist SKF 81297. Interestingly, we found phosphorylation of p38 only occurred at the higher concentration (30 μM) that led to decrease of cell viability but not at the lower concentration (1 μM) that did not lead to decreases in viability (Fig. S 4a, b). The effect of 30 μM SKF 81297 on p-38 phosphorylation was reverted by the p38 pathway inhibitor SB 203580 in a dose dependent manner (Fig. 2f) confirming that p38 is the specific pathway under D₁R-mediated cell death. Next, we tested whether H₃R agonist or antagonist could reduce the D₁R agonist-induced p-38 signaling. Treatment with either imetit or thioperamide could reduce p-38 phosphorylation (Fig. 2h), with the effects more pronounced in the STdH^{Q111} cells. Release of calcium is an important upstream event prior to p-38 signaling. In fact, SKF 81297 at both 1 and 30 μM concentration can induce calcium release being more persistent along time at 30 μM concentration (Fig. S 4c and d). The calcium release induced by 30 μM SKF 81297 was reverted by the H₃R agonist imetit or the antagonist thioperamide (Fig. 1e and f). Together these results indicate that H₃R ligands are inhibiting the different signaling pathways under the D₁R-promoted decreases in cell viability.

We next focused on the mechanism by which H₃R ligands prevent the D₁R-mediated decreases in cell viability. It has been very well described that overstimulation of D₁R induces receptor internalization promoting a rapid intracellular signaling (Kotowski et al., 2011). We here observed that 30 μM SKF 81297, that decreased cell viability, promotes D₁R internalization in both STdH^{Q7} and STdH^{Q111} cells, detected by immunocytochemistry and confocal microscopy (Fig. 3a). Interestingly, the 30 μM SKF 81297-induced D₁R internalization correlates with a SKF 81297-induced D₁R-H₃R heteromer disruption. In fact, in cells treated with 30 μM SKF 81297 heteromers were not detected by PLA neither in STdH^{Q7} nor in STdH^{Q111} cells (Fig. 3b). Thus, D₁R overstimulation disrupts D₁R-H₃R heteromerization by internalizing D₁R. One potential way GPCRs can influence each other in a heteromer is to alter the trafficking

of the partner receptor (He et al., 2011). To test if this might be the case for D₁R-H₃R heteromers we investigated if 30 μM SKF 81297-induced heteromer disruption can be prevented by H₃R ligands. We repeated the PLA experiments with STHdH^{Q7} and STHdH^{Q111} cells pre-treated with the H₃R agonist imetit or the antagonist thioperamide prior the overstimulation with 30 μM SKF 81297. Pre-treatment with imetit or thioperamide restored the punctate PLA spots decreased after overstimulation with the D₁R agonist (Fig. 3c). These data strongly suggest that H₃R ligands, interacting with D₁R-H₃R heteromers can prevent D₁R internalization and consequently the D₁R-induced decreases in cell viability.

Functional D₁R-H₃R heteromers are expressed in wild type Hdh^{Q7/Q7} and mutant knock-in Hdh^{Q7/Q111} mice at early but not late HD stages.

To test whether D₁R-H₃R heteromers can indeed be targets for treating HD, we investigated their expression and function in a mouse model of HD, the heterozygous Hdh^{Q7/Q111} knock-in mice expressing mutant huntingtin with 111 glutamine residues and wild type mice Hdh^{Q7} expressing normal full-length huntingtin with 7 glutamine residues (Giralt et al., 2012). Among others, three regions have been implicated in the pathology of HD, including the striatum, cerebral cortex and hippocampus (Reiner et al., 1988; Rosas et al., 2003; Vonsattel and DiFiglia, 1998). Thus, we tested these three areas for the expression of the heteromer using the PLA technique. In 2 months old (Fig. S 5) or 4 months old (Fig. 4a) Hdh^{Q7/Q7} and pre-symptomatic Hdh^{Q7/Q111} mice, the heteromer was expressed in all three areas. We detected D₁R-H₃R heteromers as red spots surrounding the blue stained nuclei in brain slices (Fig. 4a) but not in negative controls in which one of the PLA primary antibodies was missing, demonstrating that not random alignment of the PLA probes can be detected (Fig. S 6). The heteromer expression was similar in all brain areas and no differences were observed between Hdh^{Q7/Q7} and Hdh^{Q7/Q111} mice (Fig. 4b). To test the role of D₁R-H₃R heteromers on D₁R-mediated cell death in these animal models, we used organotypic mouse striatal, cortical or hippocampal cultures to determine cell death by comparing DAPI and propidium iodide staining and to explore whether H₃R ligands could prevent cell death

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induced through D₁R overstimulation. As expected, D₁R overstimulation doubled cell death in all three regions compared to untreated conditions without significant changes between genotypes (Fig. 4c). Importantly, slices pre-treated with H₃R ligands imetit or thioperamide, that do not influence cell death by their own, protect cells from D₁R elicited cell death, bringing cell death back to untreated levels (Fig 4c). These results indicate that functional D₁R-H₃R heteromers are expressed in different brain areas of wild type and pre-symptomatic HD model mice, where H₃R ligands protect the D₁R-mediated cell death

To investigate if the appearance of HD phenotype in Hdh^{Q7/Q111} mice influences the role of D₁R-H₃R heteromers in cell death, we first analyzed by PLA the heteromer expression in symptomatic 8 months old Hdh^{Q111} mice (Fig. 5). To our surprise, the PLA experiments showed an almost complete loss of red spots staining D₁R-H₃R heteromers in striatum, cortex or hippocampus of symptomatic Hdh^{Q111} mice but not in 8 months old Hdh^{Q7} wild-type mice (Fig. 5a, b). By ligand binding we detected a decrease in D₁R expression in the Hdh^{Q111} compared with Hdh^{Q7} in striatum (-51%), cortex (-29%) and hippocampus (-27%) (Supplemental Table 1b). The loss of heteromer expression compared with wild type littermates was also seen in others mice models of HD as R6/1 and R6/2 mice transgenic for exon 1 of the human huntingtin gene with a greatly expanded CAG repeat (Fig. S 7), suggesting that this could be a general characteristic of HD. The dramatic change in heteromer expression in 8 months old Hdh^{Q111} mice was mirrored in the lack of H₃R ligands imetit and thioperamide protection in SKF 81297-induced cell death in organotypic striatal, cortical or hippocampal cultures (Fig. 5c). In one hand, these results corroborate that the heteromer expression is needed to detect the H₃R ligands protection of D₁R-mediated cell death and, in the other hand these results indicate that D₁R-H₃R heteromers can be target for treating HD in the pre-symptomatic states of the illness.

Treatment with thioperamide reverts cognitive and motor learning deficits and D₁R-H₃R heteromers expression in Hdh^{Q111} mice.

The results above described indicate that the H₃R antagonist thioperamide can protect the cell death induced by D₁R overstimulation and suggest that one interesting way to modulate D₁R signaling in HD can be through thioperamide binding to D₁R-H₃R heteromers in the first states of the illness. To test this hypothesis we investigated the effect of chronic thioperamide treatment on cognitive and motor learning deficits in Hdh^{Q7/Q111} mice. We choose 5 months old animals to start the thioperamide treatment (Fig. S 9), since thioperamide reverted the D₁R-mediated cell death in brain organotypic cultures indicating functional heteromers at this HD stage and because impaired recognition, associative and spatial long-term memories have been demonstrated in 6 months-old mutant Hdh^{Q111} mice without significant deficits at 4 months of age (Brito et al., 2014;Giralt et al., 2012). First, we evaluated corticostriatal function in saline and thioperamide-treated wild-type Hdh^{Q7/Q7} and mutant Hdh^{Q7/Q111} mice by using the accelerating rotarod task procedure that evaluate the acquisition of new motor skills (Marco et al., 2013). As shown in Fig 6a, saline-treated Hdh^{Q7/Q111} mice were unable to maintain their balance on the rotarod as wild-type mice revealing impaired acquisition of new motor skills. Importantly, chronic treatment with thioperamide completely rescued motor learning deficits in Hdh^{Q7/Q111} mice evidenced by a similar latency to fall in the rotarod than wild-type mice. No significant effect was observed in thioperamide-treated wild-type Hdh^{Q7/Q7} mice (Fig 6a). Next, hippocampal-dependent cognitive deficits were analyzed. To this aim, spatial and recognition long-term memory in saline and thioperamide-treated mice was evaluated by using the T-maze spontaneous alternation task (T-SAT) (Fig 6b) and the novel object recognition test (NORT) (Fig 6c). The T-SAT is based on the innate preference of animals to explore an arm that has not been previously explored and it has been used to evaluate spatial memory mainly attributed to hippocampal function (Cunningham et al., 2009;Deacon and Rawlins, 2006). During the training session in the T-maze, similar exploration time (Fig 6b) was found in all genotypes and treatments, indicating no differences in spontaneous locomotor activity or anxiogenic components. After 5 hours, testing session was performed, evaluating the time that mice spend exploring the new *versus* the old arm in the T-maze. Whereas saline-treated mutant Hdh^{Q7/Q111} mice did not show any preference for the novel arm respect to the old arm indicating spatial long-term memory (LTM) deficits, mutant Hdh^{Q7/Q111} mice treated with thioperamide spent

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more time in the novel *versus* the old arm revealing preserved LTM (Fig 6b). Then, we analyzed recognition long-term memory by using the NORT task that evaluates the ability of mice to identify a previously introduced object versus a new different one. First we habituated all mice in the open field arena and ambient conditions during two consecutive days (first day Fig S 10a, b, c and second day Fig S 10e, f, g) and the percentage of distance that mice rove in the periphery and in the center (a, e), the number of entries in the periphery and in the center (b, f) and the percentage of time (c, g) that mice spent exploring the periphery and the center of the open field arena were measured. To rule out alterations in spontaneous locomotor activity depending on genotype or treatment we also evaluated the total distance that all mice rove during the first (Fig S 10d) and second (Fig S 10h) day of habituation. In the second day of habituation, no significant differences were found between genotypes and/or treatments in any parameter, demonstrating no alterations in motivation, anxiety or spontaneous locomotor activity and therefore indicating an optimal habituation in the open field. After the habituation process, animals were subjected to a training session in the open field arena in the presence of two similar objects (A and A'). Both saline and thioperamide-treated wild-type $Hdh^{Q7/Q7}$ and mutant $Hdh^{Q7/Q111}$ mice similarly explored both objects indicating neither object nor place preferences (Fig 6c). After 24 hours, LTM was evaluated by changing one of the old objects (A') for a novel one (B). Whereas mutant $Hdh^{Q7/Q111}$ -saline mice did not show any preference for the novel object respect to the familiar one, indicating recognition LTM deficits, thioperamide treatment completely restored this LTM deficit in mutant $Hdh^{Q7/Q111}$ mice (Fig 6c). Importantly, thioperamide treatment did not affect the performance of wild-type $Hdh^{Q7/Q7}$ mice. Overall, these data demonstrate the effectiveness of thioperamide treatment in restoring motor learning and spatial and recognition long-term memory deficits in HD mice.

We next tested if the reversion of motor learning and cognitive deficits in mutant $Hdh^{Q7/Q111}$ mice induced by thioperamide treatment correlated with the restoration of heteromer expression. By PLA experiments we first analyzed the heteromer expression in 6 months old mice just after the thioperamide treatment (Fig. S 11). At 6 months of

age the heteromer expression in mutant Hdh^{Q7/Q111} mice was significantly diminished with respect to the aged matched wild-type Hdh^{Q7/Q7} mice (see Fig. 5a).

Interestingly, we observed a complete recovery of red spots staining D₁R-H₃R heteromers in striatum, cortex or hippocampus of 6 months old Hdh^{Q7/Q111} mice chronically treated with thioperamide (Fig. S 11). More importantly, this recovery was maintained in 8 months old Hdh^{Q7/Q111} mice that were treated with thioperamide from 5 months of age to 8 months of age (Fig. 5a and Fig. 6d). In fact red spots surrounding DAPI stained nuclei appeared in striatum, cortex or hippocampus of 8 months old Hdh^{Q7/Q111} mice chronically treated with thioperamide (Fig. 6d) at a similar level of age matched Hdh^{Q7/Q7} mice (Fig. 6e).

D₁R-H₃R heteromers are expressed in human brain putamen in control subjects and in grade 2 HD patients but not in grade 3-4 HD patients.

The fact that thioperamide can revert cognitive and motor deficits in an animal model of HD is suggestive that thioperamide, or future pharmacological improved H₃R antagonist specifically targeting D₁R-H₃R heteromers, can be used to treat HD in humans. To go in deep with this possibility is first needed to know if D₁R-H₃R heteromers are expressed in the human brain and if the expression is maintained in the early stages of HD and decreases in the late stages of the illness. Here we investigated the D₁R-H₃R heteromers expression in human caudate-putamen slices from age matched control subjects and grade 2, grade 3 or grade 4 HD patients by PLA technology. D₁R-H₃R heteromers were detected as red spots surrounding the blue stained nuclei in control samples (Fig. 7a). The expression of D₁R-H₃R heteromers in human caudate-putamen is high (Fig. 7e) being more than 70 % cells expressing heteromers. Interestingly, heteromers were also detected surrounding the blue stained nuclei in grade 2 HD samples (Fig. 7b). In contrast, red spots were almost absent in samples from grade 3 (Fig. 7c, e) or grade 4 (Fig. 7d, e) HD. These results indicate that, also in humans, D₁R-H₃R heteromers can be target for treating HD in the pre-symptomatic states of the illness.

DISCUSSION

The imbalance of dopamine inputs created by the onset of Huntington's Disease (HD) represent a potential "point of no return" for HD patients as this disequilibrium can eventually lead to dramatic neuronal dysfunction and death. Here we sought to dampen the D₁R driven signaling by targeting previously identified and described receptor complexes consisting of D₁R and H₃R. Our efforts provide the first demonstration of GPCR heteromers as potential targets to treat HD.

The fact that GPCRs can dimerize has long been appreciated but the challenges in how and whether to target these dimers are numerous (Ferre et al., 2009). The mechanism of action of receptor heteromers can be varied. We have previously shown that D₁R-H₃R heteromers alter ligand binding, suggesting that at least part of the mechanism involves allosteric interactions. The use of the disrupting peptides supports this hypothesis. In addition, the fact that TM5 are at the interface lends credence to previous reports of GPCR heteromers being a rhombus and not linear or square complexes. In addition, to the allosteric affects it appears that D₁RH₃R heteromers also alter the trafficking of D₁R, which may have a variety of pleiotropic effects on signaling. The signaling effects we see appear to be on a variety of timescales. It has been suggested and shown that there are multiple signaling timescales involved upon activation of GPCRs. Indeed, part of the concern of trying to target GPCR heteromers for therapeutic purposes is the uncertainty around their stability and thus indirectly whether they can impact GPCR signaling at every timescale. For the case of D₁R-H₃R heteromers, it appears that they are stable and that they can affect both rapid receptor signaling (eg. Ca²⁺ mobility) and longer cell signaling pathways like p-38, two signaling pathways involved in neuronal cell death in HD (Dau et al., 2014; Fan et al., 2012; Muller and Leavitt, 2014; Taylor et al., 2013; Wang et al., 2013) opening the potential of D₁-H₃ heteromers as a therapeutic strategy in HD. Indeed, we demonstrated *in vitro* by using striatal cellular models of HD and *ex vivo* in organotypic striatal, cortical and hippocampal cultures from HD knock-in mice that targeting D₁R in D₁-H₃ complexes by H₃ antagonists completely abrogate D₁R-induced cell death likely by inhibition of D₁R-mediated calcium influx and p38 activation. The role of D₁R in HD pathology has been previously reported by our lab and others. D₁R but not D₂R

activation induces striatal cell death being significantly higher in mutant cells derived from Q111 knock-in mice than in derived Q7 wild-type cells (Paoletti et al., 2008; Tang et al., 2007). Moreover, mice in which striatal D₁R are genetically ablated display some of the neuropathological features of HD including reduced body weight, impaired locomotor and striatal atrophy (Kim et al., 2014). However, a role for D₂R has also been demonstrated, and D₂R expressing cells of the indirect pathway are believed to be the most vulnerable. D₂R but not D₁R contribute to dopamine-mediated potentiation of huntingtin aggregates and neuronal cell death in R6/1 transgenic HD models (Charvin et al., 2005; Deyts et al., 2009) and D₂R antagonists are largely used as symptomatic treatment of chorea and psychiatric disturbances in HD patients (Ginovart et al., 1997; Joyce et al., 1988; Pavese et al., 2003; Richfield et al., 1991; van Oostrom et al., 2009). However the role and the time frame of D₂R and D₁R associated pathways can be dramatically different. From the cell viability results in striatal cells and organotypic cultures it is possible to conclude that D₁R should be the executor of the signalling cascades that lead to cell death as D₂R activation did not produce any effect on cell viability. However, D₂R related pathways could initiate the dopaminergic imbalance and even exacerbate D₁R executive action in HD. Anyway we cannot rule out that by targeting D₂R-H₃R heteromers (Ferrada et al., 2008) a blockade of D₂R signaling could be also achieved. Our group has described that H₃R can form heteromers with other GPCRs, so we cannot discard that thioperamide can display effects on other different heterodimers or even by the H₃R homodimer and future effort might be focused in a deeper characterization. Even though these data we not allow to conclusively assert that is the D₁R-H₃R heteromer the unique responsible of the effects of thioperamide on cognitive improvement in HD mice several findings can argue in favor.

First, as demonstrated in our manuscript at middle disease stages (6 and 8 months of age) mutant huntingtin expression induces loss of the D₁R-H₃R heteromer evidenced by lack of PLA in all analyzed brain regions a loss that was associated with impairments in learning and long-term memory. Interestingly, chronic treatment with thioperamide starting at 5 month of age prevented disruption of the heteromer and the subsequent cognitive decline, which stress the need of functional D₁R-H₃R heteromers to reverse behavioral deficits.

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Second, in StHdH cells we demonstrate D₁R overactivation induce in one hand activation of cell death related pathways and in the other hand D₁R internalization and D₁R-H₃R disruption. In addition, H₃R ligands pre-treatment can block D₁R-induced cell death and prevent D₁R-H₃R loss. D₁R-H₃R stability in the membrane might be influenced by several parameters such as protomers expression or trafficking. D₁R-H₃R loss of function and expression at 8 months old Hdh^{Q7/Q111} is correlated with decreases in Bmax and Kd, more important for D₁R than H₃R which could mean a decrease of the total amount of D₁R and H₃R at membrane level. A decrease of D₁R expression in the striatum has been previously described; however D₁R-H₃R heteromers have different binding properties in comparison with their homomers, so it is also possible that part of the effect observed in binding experiments could be due to D₁R-H₃R heteromer disruption.

Finally, the effect of TAT-peptides analogues of D₁R transmembrane domains in D₁R-H₃R stability and function in STHdH cells makes us confident that we are observing specific D₁R-H₃R signaling and function. It also opens the possibility of treatment with these peptides at “in vivo” level, but further knowledge of the dynamics, binding capacity and reversibility of these peptides should be performed in order to scale the use of this technology to more complex models.

Clinical diagnosis of HD relies on the manifestation of motor abnormalities classically attributed to striatal and cortical cell death, growing evidence points to neuronal dysfunction as the earliest HD disturbance responsible of the cognitive and behavioral changes that are evident at least 15 years prior to the time at which motor diagnosis is given (Lemiere et al., 2004). From last years, converging evidence involving studies in both humans and HD animal models illustrates the idea that cognitive deficits in HD not only involve basal ganglia and cortical dysfunction but also the participation of the hippocampus (Brito et al., 2014;Giralt et al., 2012;Ille et al., 2011;Paulsen et al., 2014;Majerova et al., 2012). Given this brain circuitry complexity involved in learning and memory processes not only in HD but in other neurodegenerative diseases no effective treatments are currently available to treat cognitive decline in these neurological disorders. Moreover, along with this complexity timing of intervention is also critical since atrophy and dysfunction progress with age and treatment may be different according to the stage of illness.

In this scenario and given the well known role of both dopamine and histamine in synaptic plasticity and memory (Cahill et al., 2014; Ellender et al., 2011; Haas et al., 2008; Komater et al., 2005; Lopez de and Sanchez-Pernaute, 2010; Mohsen et al., 2014; Orsetti et al., 2002; Pascoli et al., 2009; Wiescholleck and Manahan-Vaughan, 2014) we hypothesized that the demonstrated role of D₁-H₃ heteromer in cell death could also be extended to cognition. Accordingly, deficits in motor and procedural learning as well as impaired spatial and recognition memories were restored by chronic treatment with thioperamide which suggests that D₁-H₃ heteromers are also critical for behavioral changes in HD.

How the H₃R antagonism reversed cognitive impairments through the D₁R-H₃R heteromer it is not clear. Substantial data support the importance of dopamine receptors for synaptic plasticity in the cortex and hippocampus (Collins et al., 2000; Levy and Goldman-Rakic, 2000; Navakkode et al., 2004; Robbins, 2000b; Robbins, 2000a; Sajikumar and Frey, 2004). Thus, dopaminergic activation through D₁/D₅ receptors gates long-term changes in CA1 synaptic strength that are critical for acquisition of novel information (Bethus et al., 2010; Lemon and Manahan-Vaughan, 2006) while working memory processes in the cortex are regulated by modulation of GABAergic activity via the dopaminergic system (Seamans et al., 2001). Importantly, any dopamine imbalance with both suboptimal and supra-optimal dopamine activity has been reported to modify cognitive performance (Mattay et al., 2003; Vijayraghavan et al., 2007). As the early stages of HD may reflect a hyperdopaminergic stage (Chen et al., 2013a; Mochel et al., 2011), treatments reducing dopamine signalling may have therapeutical benefits. In fact, dopamine-depleting drugs such as tetrabenzine or dopamine-stabilizers as pridopidine have been prove to improve motor coordination abnormalities (de Yebenes et al., 2011; Pearson and Reynolds, 1988) while specific D₁R inhibition rescues electrophysiological changes in excitatory and inhibitory synaptic transmission in full-length HD mouse models (Andre et al., 2011a). However none of these treatments have demonstrated cognitive improvements. Our study showing that H₃R antagonists restore learning and memory deficits by blocking D₁R in D₁R-H₃R complexes along with the role of these heteromers on neuronal cell death predict a critical role of the histaminergic system as modulator of the dopamine imbalance in HD and may help to overcome the deleterious effects of directly manipulate DA-

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production and/or signalling opening new and important alternatives for HD therapeutics.

MATERIALS AND METHODS

Human brain slices

Human caudate-putamen samples were obtained from patients with Huntington's disease, or from age matched controls without neurological disease, according to the standardized procedures of the Banco de Tejidos para Investigación Neurológica (Madrid, Spain). Briefly, both patients with Huntington's disease and control subjects had signed during their life a donation protocol that was in custody of their relatives and the brain bank according to the Declaration of Helsinki. After death, the corpses were immediately stored at 4 °C until autopsy, which was performed within a time interval ranging from 2 to 12 h post mortem. After removal of the brain, the quality of the samples was checked by their pH. The brain was split in two parts by a sagittal section through the midline. The right hemibrain was used for histopathological studies and the left hemibrain for Western blot and other biochemical analyses. Both hemibrains were dissected in coronal sections (approximately 1 cm thick) to evaluate the presence of additional lesions, such as cerebral infarctions. The right hemibrain was immersed in formalin, and the slices (15 µm thick) obtained from the left hemibrain were frozen in a metal plate cooled at -80 °C. The frozen samples were stored at -80 °C. All protocols were approved by the institutional ethic committee. All samples (3 control subjects, 1 grade 2 patient, 3 grade 3 and 3 grade 4 patients) were from male 40 to 50 years old subjects

Animal models of HD

Knock-in mice, with targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues, and the corresponding littermates having 7 glutamine residues were maintained on a C57BL/6 genetic background (Lloret et al., 2006). Hdh^{Q7/Q111} heterozygous males and females were intercrossed to generate age-matched Hdh^{Q7/Q111} heterozygous (Hdh^{Q111} mice) and Hdh^{Q7/Q7} wild-type littermates (Hdh^{Q7} mice) (Giralt et al., 2012). Only males were used for all experiments. Male transgenic R6/1 mice expressing exon 1 of human huntingtin carrying 115 CAG

repeats and hemizygous male mice transgenic for exon 1 of the human huntingtin gene with a greatly expanded CAG repeat (R6/2 mice) (Mangiarini et al., 1996) and wild-type littermates were used when indicated in proximity ligation assays. Animals were housed under a 12 h light/dark cycle with food and water ad libitum. All procedures were carried out in accordance with the National Institute of Health and were approved by the local animal care committee of the Universitat de Barcelona (99/01) and the Generalitat de Catalunya (00/1094) or by Complutense University Animal Research Committee in accordance with Directive 86/609/EU of the European Commission.

Striatal Cells and cell cultures

Conditionally immortalized wild type STHdh^{Q7} and mutant STHdh^{Q111} striatal neuronal progenitor cell lines expressing endogenous levels of normal and mutant full-length huntingtin with 7 and 111 glutamines, respectively, were generated from wild type Hdh^{Q7/Q7} or homozygous Hdh^{Q111/Q111} embryos (Trettel et al., 2000). The knock-in striatal models faithfully represent the HD mutation carried by patients because elongated polyglutamine tracts are placed within the correct context of the murine Hdh gene. Thus, immortalized striatal cells accurately express normal and mutant huntingtin and do not exhibit amino-terminal inclusions, which allow us to study changes involved in early HD pathogenesis (Trettel et al., 2000). Striatal cells were grown at 33°C in DMEM(Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS), 1% streptomycinpenicillin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 400 µg/ml G418 (Geneticin; Invitrogen).

Mouse brain slices preparation

For PLA experiments, mouse brains were rapidly removed and were fixed with 4% paraformaldehyde solution for 24 h at 4°C. Mouse brains were then washed in PBS, cryo-preserved in a 30% sucrose solution for 48 h at 4°C and stored at 20°C until sectioning. Striatum, cortex and hippocampal 15 µm thick slices were cut on a freezing cryostat (Leica Jung CM-3000) and mounted on slide glass. For cell death determination, mouse brains were rapidly removed and placed in ice-cold oxygenated (O₂/CO₂: 95%/5%) Krebs-HCO₃⁻ buffer (124 mM NaCl, 4 mM KCl, 1.25 mM NaH₂PO₄, 1.5

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mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose and 26 mM NaHCO₃, pH 7.4). Cerebral hemispheres were split and sliced coronally at 4°C using a brain matrix (Zivic Instruments, Pittsburgh, PA) and McIlwain chopper (Ted Pella, Inc, California) in sterile conditions. Striatum, cortex and hippocampal slices (500 µm thick) were kept at 4°C in Krebs-HCO₃⁻ buffer during the dissection and transferred into a Millicell Insert (Millipore).

Cell death determination in striatal cells and in mouse organotypic slice cultures

Brain slices (500 µm thickness, see above) were cultured in Neurobasal medium supplemented with 20 % horse serum, 0.5% B27, 2 mM L-glutamine, 100 µg/ml sodium pyruvate, MEM Non-Essential Amino Acids Solution (1/100) and 100 units/ml penicillin/streptomycin (all supplements were from Invitrogen, Paisley, Scotland, UK). After 24 h of culture for organotypic slices or striatal cells growing at 50% density in a 24 well plates, culture medium was replaced by fresh medium. After 1 h, medium, SCH 23390, imetit or thioperamide were added at the indicated concentrations and incubated for an additional 1 h before the addition of D₁R agonist. Slices or cells were maintained 48 h more in culture. After the total 72 h of culture, 10µM propidium iodide (PI) was added to brain slices and maintained at 37°C for 1 h, then slices or cells were washed twice in cold-PBS and fixed with 4 % paraformaldehyde for 1 h at 4°C. Sample nuclei were stained with Hoechst 1:1000. The Hoechst stained cells were counted to analyze striatal cell viability and Hoechst stained and PI positive nuclei were counted to evaluate cell death in the brain slices. Quantification was performed using Leica SP2 confocal microscope (20x; UV, 561 lasers) and the quantification performed with the program Image-based Tool for Counting Nuclei for ImageJ

Lentivirus production and cell transduction

Silencing lentiviral vectors were produced by co-transfecting striatal cells with lentiviral silencing plasmids GIPZ Human histamine H₃ receptor shRNA (Thermo Scientific) with packing plasmid psPAX2 and envelope coding plasmid pMD2.G (Addgene#12260 and #12259, respectively) using the calcium phosphate method. For production of control non silencing lentiviral particles the H₃R silencing plasmid were substituted with GIPZ

Non-silencing Lentiviral shRNA Control (RHS4346, ThermoFisher). Infectious lentiviral particles were harvested at 48 h post-transfection, centrifuged 10 minutes at 3000 rpm to get rid of cell debris, and then filtered through 0.45 μ m cellulose acetate filters. The titer of recombinant lentivirus was determined by serial dilution on HEK293T cells. For lentivirus transduction, striatal cells were subcultured to 50% confluence, cells were transduced with H₃R-shRNA-expressing lentivirus or control-shRNA-expressing lentivirus (LV control) at a multiplicity of infection (MOI) of 10 in the presence of polybrene 5 μ g/ml. Virus-containing supernatant was removed after 3 h. Puromycin was added to the culturing media at the final concentration of 1 μ g/ml 2 days after infection. 5 days after puromycin selection cells were transduced with the second H₃R-shRNA-expressing lentivirus to improve the level of silencing achieved. LV control infected cells were re-infected with control-shRNA-expressing lentivirus. The second infection was carried out as the first one. Cells were tested 72 h after the second transduction was performed.

In Situ Proximity Ligation Assays (PLA)

Cells or mouse or human brain slices were mounted on slide glass and treated or not with the indicated receptor ligands or TAT-TM peptides for the indicated time. Then, cells or slices were thawed at 4°C, washed in 50 mM Tris-HCl, 0.9% NaCl pH 7.8 buffer (TBS), permeabilized with TBS containing 0.01% Triton X-100 for 10 min and successively washed with TBS. Heteromers were detected using the Duolink II in situ PLA detection Kit (OLink; Bioscience, Uppsala, Sweden) following the instructions of the supplier. A mixture of equal amounts of the primary antibodies: guinea pig anti-D₁R antibody (1/200 Frontier Institute, Ishikari, Hokkaido, Japan) and rabbit anti-H₃R antibody (1:200, Alpha diagnostic, San Antonio, Texas, USA) were used to detect D₁R-H₃R heteromers together with PLA probes detecting guinea pig or rabbit antibodies, Duolink II PLA probe anti-guinea pig minus and Duolink II PLA probe rabbit plus. Then samples were processed for ligation and amplification and were mounted using a DAPI-containing mounting medium. Samples were observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an apochromatic 63X oil-immersion objective (N.A. 1.4), and a 405 nm and a 561 nm laser

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lines. For each field of view a stack of two channels (one per staining) and 9 to 15 Z stacks with a step size of 1 μm were acquired. A quantification of cells containing one or more red spots versus total cells (blue nucleus) and, in cells containing spots, the ratio r (number of red spots/ cell) were determined, using the Fiji package (<http://pacific.mpi-cbg.de/>), considering a total 600-800 cells from 6 different fields within each brain region from 3 different mice per group or from 3 human control subjects, 3 human grade 3 or grade 4 HD patients or from 1 grade 2 HD patient. Nuclei and red spots were counted on the maximum projections of each image stack. After getting the projection each channel was processed individually. The nuclei were segmented by filtering with a median filter, subtracting the background, enhancing the contrast with the Contrast Limited Adaptive Histogram Equalization (CLAHE) plug-in and finally applying a threshold to obtain the binary image and the regions of interest (ROI) around each nucleus. Red spots images were also filtered and thresholded to obtain the binary images. Red spots were counted in each of the ROIs obtained in the nuclei images.

Membrane preparation and radioligand binding

Striatal cells or mouse striatal, cortical or hippocampal tissue were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing a protease inhibitor mixture (1/1000, Sigma). The cellular debris was removed by centrifugation at 13,000 g for 5 min at 4°C, and membranes were obtained by centrifugation at 105,000 g for 1 h at 4 °C. Membranes were washed three more times at the same conditions before use. Ligand binding was performed with membrane suspension (0.2 mg of protein/ml) in 50 mM Tris-HCl buffer, pH 7.4 containing 10 mM MgCl_2 , at 25°C. To obtain saturation curves, membranes were incubated with 2nM of [^3H]SCH 23390 (PerkinElmer, Boston, MO, USA) or 3 nM of [^3H]R- α -methyl histamine ([^3H]RAMH, Amersham, Buckinghamshire, UK) providing enough time to achieve stable equilibrium for the lower ligand concentrations. Nonspecific binding was determined in the presence of 30 μM non-labeled ligand. Free and membrane bound ligand were separated by rapid filtration of 500 μl aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated overnight with 10 ml

of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, USA) at room temperature and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer, Boston, MO, USA) with an efficiency of 62%. Protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard. Monophasic saturation curves were analyzed by non-linear regression, using the commercial Grafit software (Erithacus Software), by fitting the binding data to the equation previously deduced (equation (3) in Gracia et al., 2013)

Western blot

Striatal cell membranes (see above) were solubilized in lysis buffer containing 50nM Tris-HCl, pH 7.4, 50mM NaF, 150mM NaCl, 45mM β -glycerophosphate, 1% Triton X-100, 200 μ M phenylarsine oxide, 0.4mM NaVO₄ and protease inhibitor cocktail. Proteins were resolved by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS/PAGE) using 10 % polyacrylamide gels. Proteins were transferred to PVDF membranes using a semi-dry transfer system and immunoblotted as previously described (Navarro et al., 2010; Moreno et al., 2011) using as primary antibody rabbit anti-H₃R antibody (1:1000, Alpha diagnostic, San Antonio, Texas, USA).

Immunocytochemistry

For immunocytochemistry, cells were not treated or treated for 24 hours with 30 μ M SKF 81297. Cells were fixed in 4% paraformaldehyde for 15 min and washed with PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Then, after permeabilization with buffer A containing 0.2% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin. After 1 h at room temperature, cells were labeled with the primary guinea pig anti-D₁R antibody (1/200, Frontier Institute, Ishikari, Hokkaido, Japan) for 1 h, washed, and stained with the secondary Cy3 donkey anti-mouse antibody (1/200, Jackson Immunoresearch Laboratories, West Grove, PA, USA). Samples were rinsed and observed in a Leica SP2 confocal microscope.

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To determine ERK 1/2 phosphorylation, cells and mouse brain slices (500 μm thick) were treated or not with the indicated ligand for the indicated time and were lysed by the addition of 500 μl of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β -glycerophosphate, 1% Triton X-100, 20 μM phenyl-arsine oxide, 0.4 mM NaVO_4 and protease inhibitor cocktail). The cellular debris was removed by centrifugation at 13,000 $\times g$ for 5 min at 4°C and the protein was quantified by the bicinchoninic acid method using bovine serum albumin dilutions as standard. ERK1/2 phosphorylation were then detected as described previously (Moreno et al., 2011; Gracia et al 2013). To determine calcium release, striatal cells growing in 25 cm^2 flasks at 60% confluence were transfected with 4 μg of GCaMP6 calcium sensor (Addgene Plasmid #40753) using Lipofectamine 2000 Transfection Reagent according to the manufacturer's protocol. 48 h after transfection, cells were gently scrapped, centrifuged at 3,000 g for 5 min and adjusted in Mg-free Locke's buffer supplemented with 10 μM glycine, pH 7.4 containing 154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO_3 , 2.3 mM CaCl_2 , 5.6 mM glucose and 5 mM HEPES to 0.2 mg of protein/ml by the bicinchoninic acid method (see above). To detect GCaMP6s fluorescence changes triggered by calcium release, cells (20 μg of cell suspension protein per well in 96-well black, clear bottom microtiter plates) were pre-treated when needed for 1hour with medium or 4 μM TAT-TM5 or TAT-TM7 peptides in the absence or presence of the H_3R and D_1R ligands (see figure legends). Fluorescence emission intensity of GCaMP6s was recorded at 515 nm upon excitation at 488 nm in EnSpire® Multimode Plate Reader (PerkinElmer, Boston, MO, USA) for 330 s every 5 s and 100 flashes per well. The fluorescence gain was defined as a delta function of $\Delta F/F(t) = (F(t) - F_0)/F_0$, expressed in %, where F_0 is the average fluorescence intensity in the first six measures from the start of recording and $F(t)$ is the fluorescence intensity at a given time. Data were normalized and processed using GraphPad Prism 5 (GraphPad Software, Inc, La. Jolla, CA).

Mice thioperamide treatment

Thioperamide maleate salt (Sigma-Aldrich, St. Louis, USA) was prepared fresh daily being dissolved in sterile 0,9% saline (NaCl) in order to deliver a final dose of 10mg/kg in a final volume of 0.01 ml/g of body weight. The control treatment consisted of an

equal volume of saline solution. All injections were given via the intra-peritoneal route (*i.p.*). 5 months-old wild-type Hdh^{Q7/Q7} and mutant knock-in Hdh^{Q7/Q111} mice were treated chronically with saline or thioperamide. Three *i.p.* injections per week were administered from 5 months of age until 6 months of age (when one cohort of animals were perfused to analyze PLA after behavioral assessment) or until 8 months of age (when a second cohort of animals were perfused to analyze PLA at this more advanced disease stage). A total of 11 saline-Hdh^{Q7/Q7} mice, 10 thioperamide-Hdh^{Q7/Q7} mice, 7 saline-Hdh^{Q7/Q111} mice and 9 thioperamide-Hdh^{Q7/Q111} mice were treated. All treatments were performed in the afternoon to avoid the stress caused by the treatments during the behavioral assessment. Thus, during behavioral analysis treatments were performed after the evaluation of motor learning or cognitive tasks.

Behaviour assays

ARTP. As previously described (Brito et al., 2014;Marco et al., 2013), animals were placed on a motorized rod (30mm diameter). The rotation speed gradually increased from 4 to 40 rpm over the course of 5 minutes. The time latency was recorded when the animal was unable to keep up on the rotarod with the increasing speed and fell. Rotarod training/testing was performed as 4 trials per day during 3 consecutive days. A resting period of one hour was left between trials. The rotarod apparatus was rigorously cleaned with ethanol between animal trials in order to avoid odors.

T-SAT. The T-maze apparatus used was a wooden maze consisting of three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° with respect to the other two. All arms were 45 cm long, 8 cm wide and enclosed by a 20 cm wall. Two identical guillotine doors were placed in the entry of the arms situated at 180°. In the training trial, one arm was closed (new arm) and mice were placed in the stem arm of the T (home arm) and allowed to explore this arm and the other available arm (old arm) for 10 min, after which they were returned to the home cage. After 5 h (LTM), mice were placed in the stem arm of the T-maze and allowed to freely explore all three arms for 5 min. The arm preference was determined by calculating the time spent in each arm x 100/time spent in both arms

(old and new arm). The T-maze was rigorously cleaned with ethanol between animal trials in order to avoid odors.

NORT. The device consisted in a white circular arena with 40 cm diameter and 40 cm high. Mice were first habituated to the open field arena in the absence of objects (2 days, 15 min/day). During these two days of habitation, several parameters were measured to ensure the proper habituation of all mice in the new ambient. As a measure of anxiety or motivation behaviors, the distance that each mice rove in the periphery or in the center of the open field arena was measured as the distance rove in the periphery or in the center \times 100/the total distance. The same analysis was performed by counting the number of entries in the periphery and in the center as well as the time that each mice spent exploring the periphery or the center. The total distance that each mice rove during this two days of habituation was also recorded as a measure to evaluate spontaneous locomotor activity. On the third day, two similar objects were presented to each mouse during 10 min (A, A' condition) after which the mice were returned to their home cage. Twenty-four hours later (LTM), the same animals were re-tested for 5 min in the arena with a familiar and a new object (A, B condition). The object preference was measured as the time exploring each object \times 100/time exploring both objects. The arena was rigorously cleaned with ethanol between animal trials in order to avoid odors. Animals were tracked and recorded with SMART junior software (Panlab, Spain).

Animal perfusion and mouse brain slices preparation

For PLA experiments 2, 4, 6 and 8 months-old $Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ mice were deeply anesthetized and immediately perfused transcardially with saline (PBS) followed by 4% paraformaldehyde (PFA)/phosphate buffer. Brains were removed and post-fixed overnight in the same solution, cryoprotected by immersion in 10, 20, 30% gradient sucrose (24 hours for each sucrose gradient) at 4°C and then frozen in dry ice-cooled methylbutane. Serial coronal cryostat sections (30 μ m) through the whole brain were collected in PBS-0.025% azida as free-floating sections and stored at 4°C until PLA experiments were performed.

FIGURES AND FIGURE LEGENDS

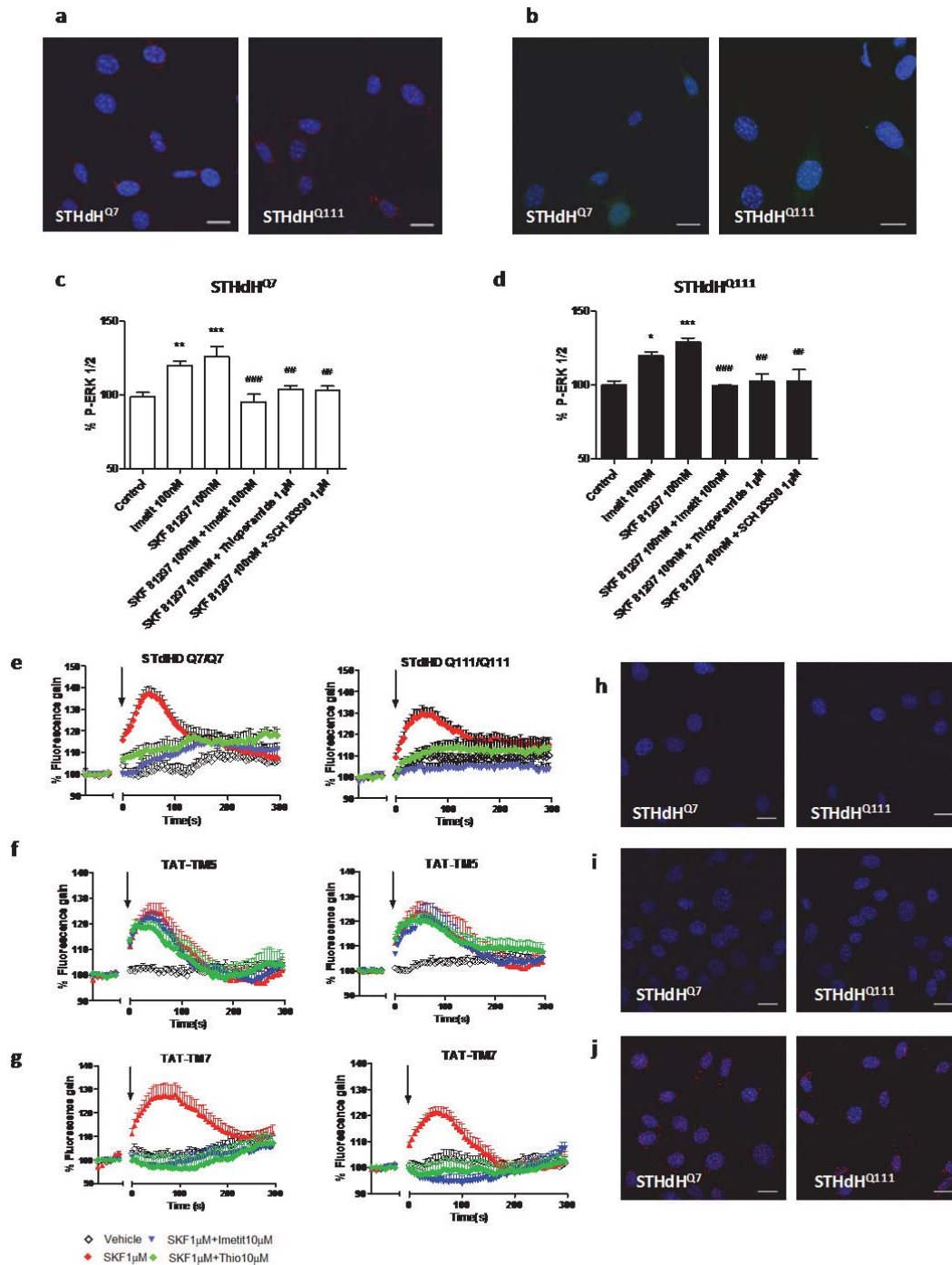


Figure 1. Functional D₁R-H₃R heteromers are expressed in STHdH^{Q7} and STHdH^{Q111} cells. In (a and b) Proximity Ligation Assays (PLA) were performed in striatal wild type STHdH^{Q7} and HD model STHdH^{Q111} cells (a) or in these cells infected with shH₃R vector to silence H₃R (b). D₁R-H₃R heteromers were visualized in (a) but not in (b) as red spots around blue colored DAPI

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stained nucleus. Scale bar: 20 μm . In (c and d) STHdH^{Q7} (c) or STHdH^{Q111} (d) cells were treated for 1 hour with the D₁R antagonist SCH 23390 (1 μM) or the H₃R agonist imetit (100 nM) or antagonist thioperamide (1 μM) before the addition of SKF 81297 (100 nM) for an additional incubation period of 24 hours and ERK 1/2 phosphorylation was determined. Values represent mean \pm SEM of percentage of phosphorylation relative to basal levels found in untreated cells (control). One-way ANOVA followed by Bonferroni post hoc tests showed a significant effect over basal (*p < 0.05, **p < 0.01, ***p < 0.001) or over SKF 81297 treatment (##p < 0.01, ###p < 0.001). In (e to g) intracellular calcium increases were measured in STHdH^{Q7} (left panels) or STHdH^{Q111} (right panels) cells treated with medium (e) or with 4 μM of TAT-TM5 (f) or 4 μM of TAT-TM7 (g) peptides in the absence or in the presence of the H₃R agonist imetit (10 μM) or antagonist thioperamide (10 μM) before the addition of vehicle or SKF 81297 (1 μM). For each curve values are expressed as a percentage of increase respected to not treated cells and are mean \pm SEM of three independent experiments. In (h to j) Proximity Ligation Assays (PLA) were performed as in (a) in STHdH^{Q7} (right panels) and STHdH^{Q111} (left panels) cells pre-treated with 4 μM TAT-TM5 and TAT-TM6 (h), TAT-TM5 (i) or TAT-TM7 (j).

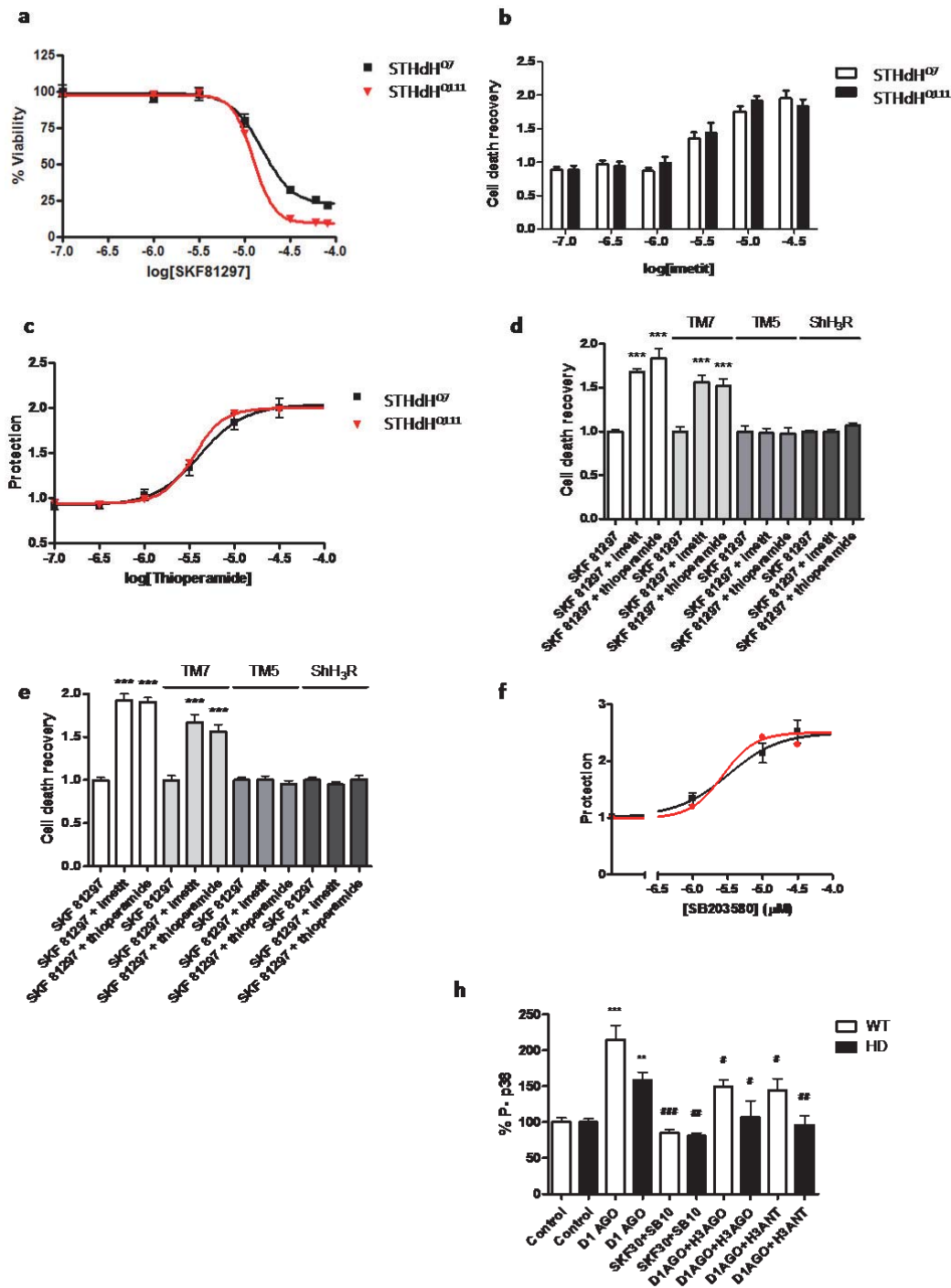


Figure 2. H₃R ligands revert the D₁R-mediated decreases in STHdH^{Q7} and STHdH^{Q111} cell viability. In (a to f) cell viability was determined in STHdH^{Q7} or STHdH^{Q111} cells treated for 1 hour with medium (a), with the H₃R agonist imetit (10 μM), b) or antagonist thioperamide (10 μM), c), with vehicle (white columns, d and e), TAT-TM7 (4μM) (pale grey columns, d and e) or TAT-TM5 (4μM) (grey columns, d and e), infected with shH₃R vector to silence H₃R (dark grey

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columns, d and e) or treated with the p38 inhibitor SB 203580 (f) prior overstimulation for 24 hours with SKF 81297 (30 μ M). Values represent mean \pm SEM of percentage of viable cells respect to vehicle-treated cells (a) or the cell viability recovery expressed as in-fold respect to SKF 81297 treated cells (b to f). Student's t test showed a significant (*p < 0.05, **p < 0.01, ***p < 0.001) effect over SKF 81297 treated cells. In (g) STHdH^{Q7} or STHdH^{Q111} cells treated for 1 hour with medium (control), with SB 203580 (10 μ M) or with the H₃R agonist imetit (10 μ M,) or antagonist thioperamide (10 μ M) were overstimulated with SKF 81297 (30 μ M; 24 hours) and p38 phosphorylation was determined. Values represent mean \pm SEM and are expressed as percentage over control. One-way ANOVA followed by Bonferroni post hoc tests showed a significant effect over control (**p < 0.01, ***p < 0.001) or over SKF 81297 treatment ([#]p < 0.05, ^{##}p < 0.01, ^{###}p < 0.001). In (h and i), STHdH^{Q7} (h) or STHdH^{Q111} (i) cells were treated with medium (red), or with the H₃R agonist imetit (10 μ M, green) or antagonist thioperamide (10 μ M, blue) and were overstimulated with SKF 81297 (30 μ M) prior intracellular calcium release determination. For each curve values are expressed as a percentage of increase respected to not treated not overstimulated cells (black) and are mean \pm SEM of three independent experiments.

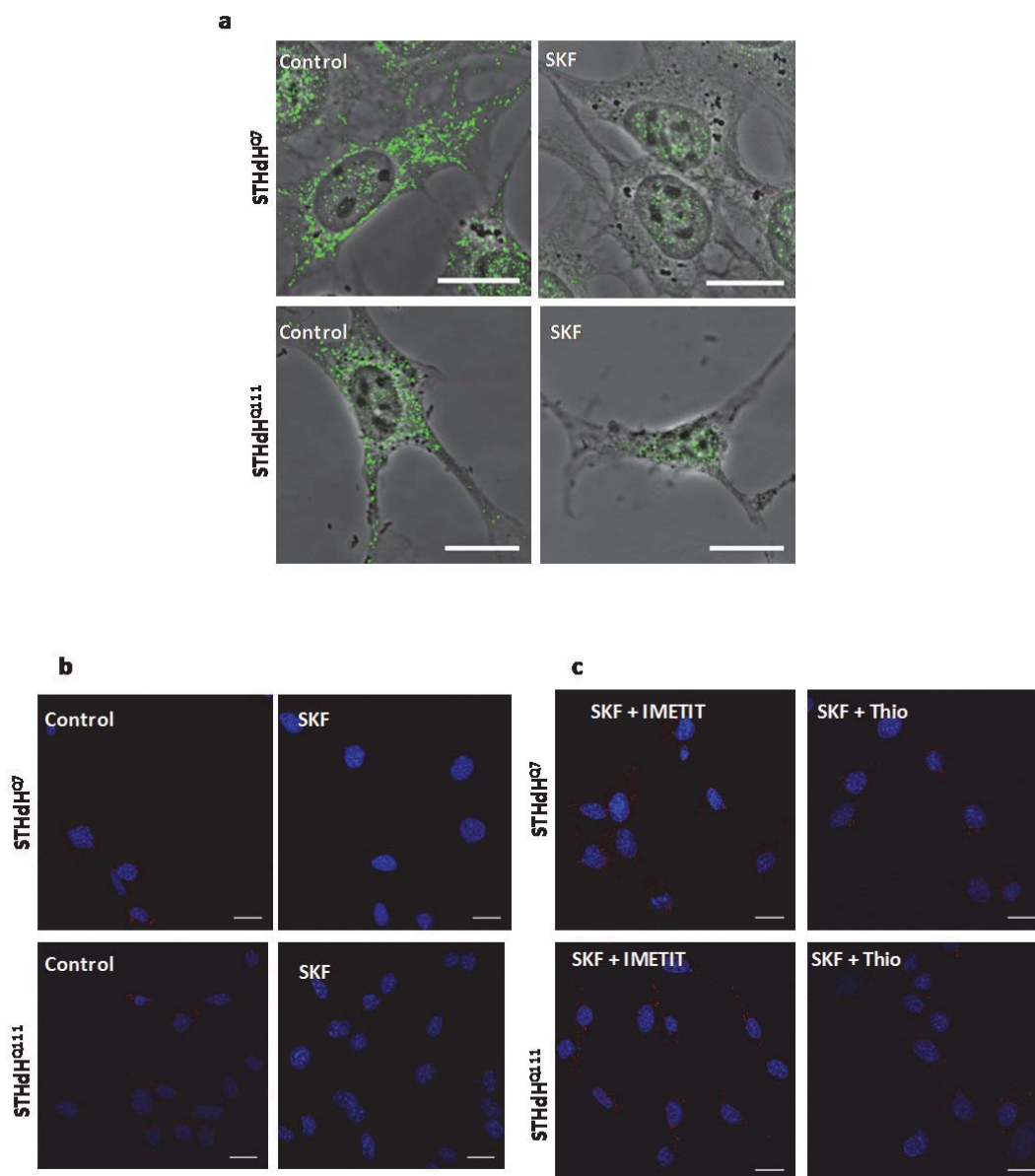


Figure 3. H₃R ligands revert the D₁R overstimulation-induced heteromer disruption in striatal cells. In (a) STHdH^{Q7} or STHdH^{Q111} cells pre-treated with medium or with sucrose were treated with medium (control) or with SKF 81297 (30 μM). Cells were processed for immunocytochemistry using a D₁R antibody (see Materials and Methods) and D₁R internalization was observed by confocal microscopy. In (b and c) Proximity Ligation Assays (PLA) were performed in STHdH^{Q7} or STHdH^{Q111} cells pretreated with vehicle (c) or with the H₃R ligands imetit (10μM, SKF+IMETIT) or thioperamide (10μM, SKF+THIO) (d) before the treatment with medium (control) or SKF 81297 (30 μM, 45 min). D₁R-H₃R heteromers were

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visualized as red spots around blue colored DAPI stained nucleus in control and H₃R ligands-treated cells, but not in SKF 81297 only treated cells. Scale bar: 20 μm.

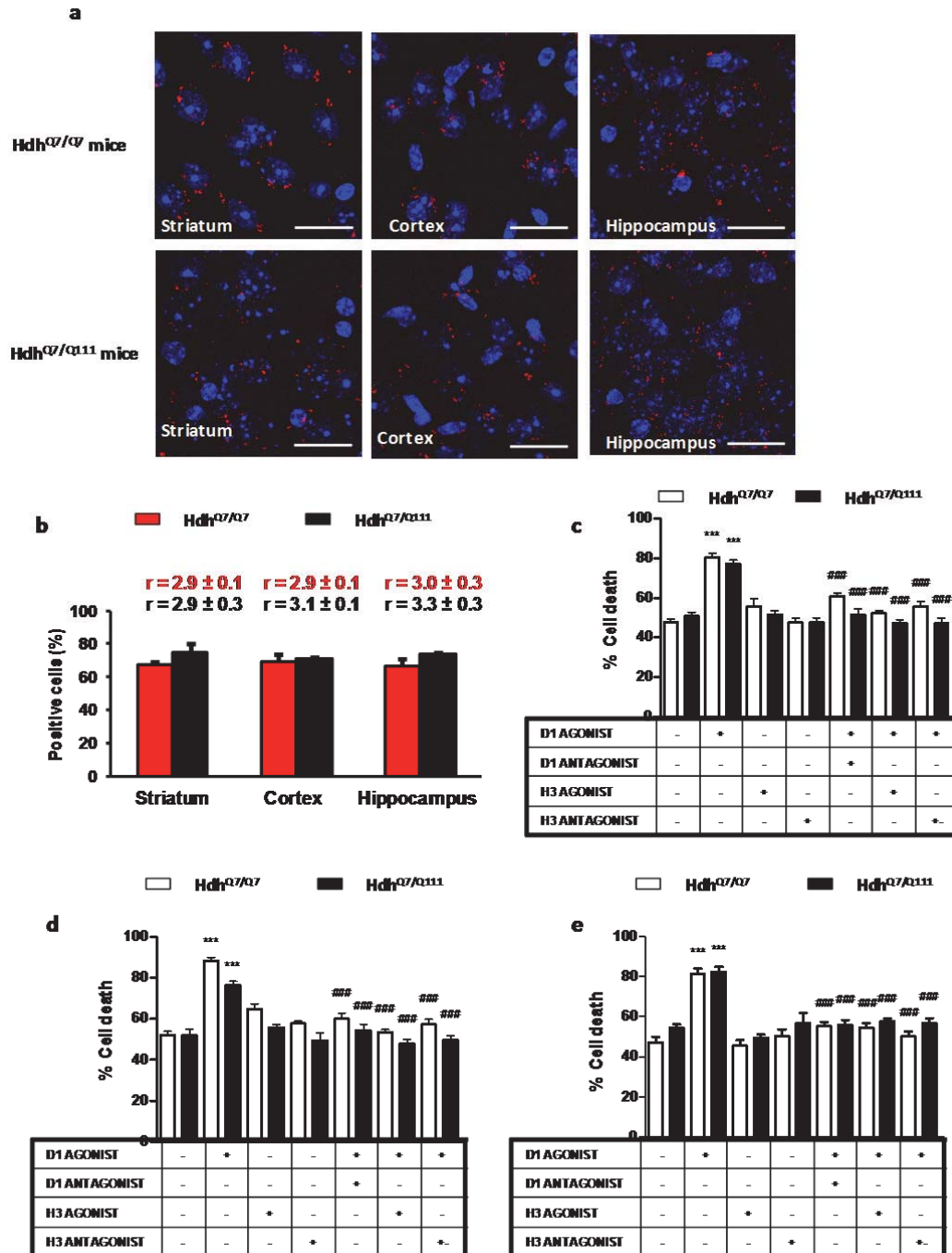


Figure 4. Functional D₁R-H₃R heteromers are expressed in wt Hdh^{Q7} and pre-symptomatic Hdh^{Q111} mice. Striatal, cortical or hippocampal slices from four months old Hdh^{Q7} and Hdh^{Q111} mice were used. In (a), by Proximity Ligation Assays (PLA) D₁R-H₃R heteromers were visualized in all slices as red spots around blue colored DAPI stained nucleus. Scale bar: 20 μ m. In (b), the number of cells containing one or more red spots is expressed as the percentage of the total number of cells (blue nucleus). *r* values (number of red spots/cell containing spots) are shown

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above each bar. Data (% of positive cells or r) are the mean \pm SEM of counts in 600-800 cells from 6 different fields from 3 different animals. In (c) organotypic cultures were treated for 1 hour with medium, the D₁R antagonist SCH 23390 (10 μ M) or the H₃R agonist imetit (10 μ M) or antagonist thioperamide (10 μ M) before the addition of SKF 81297 (50 μ M; 48 hours) and cell death was determined. Values represent mean \pm SEM of percentage of cell death. One-way ANOVA followed by Bonferroni post hoc tests showed a significant effect over non-treated organotypic cultures (**p < 0.001) or of the H₃R ligands plus SKF 81297 treatment over the SKF 81297 treatment (###p < 0.001).

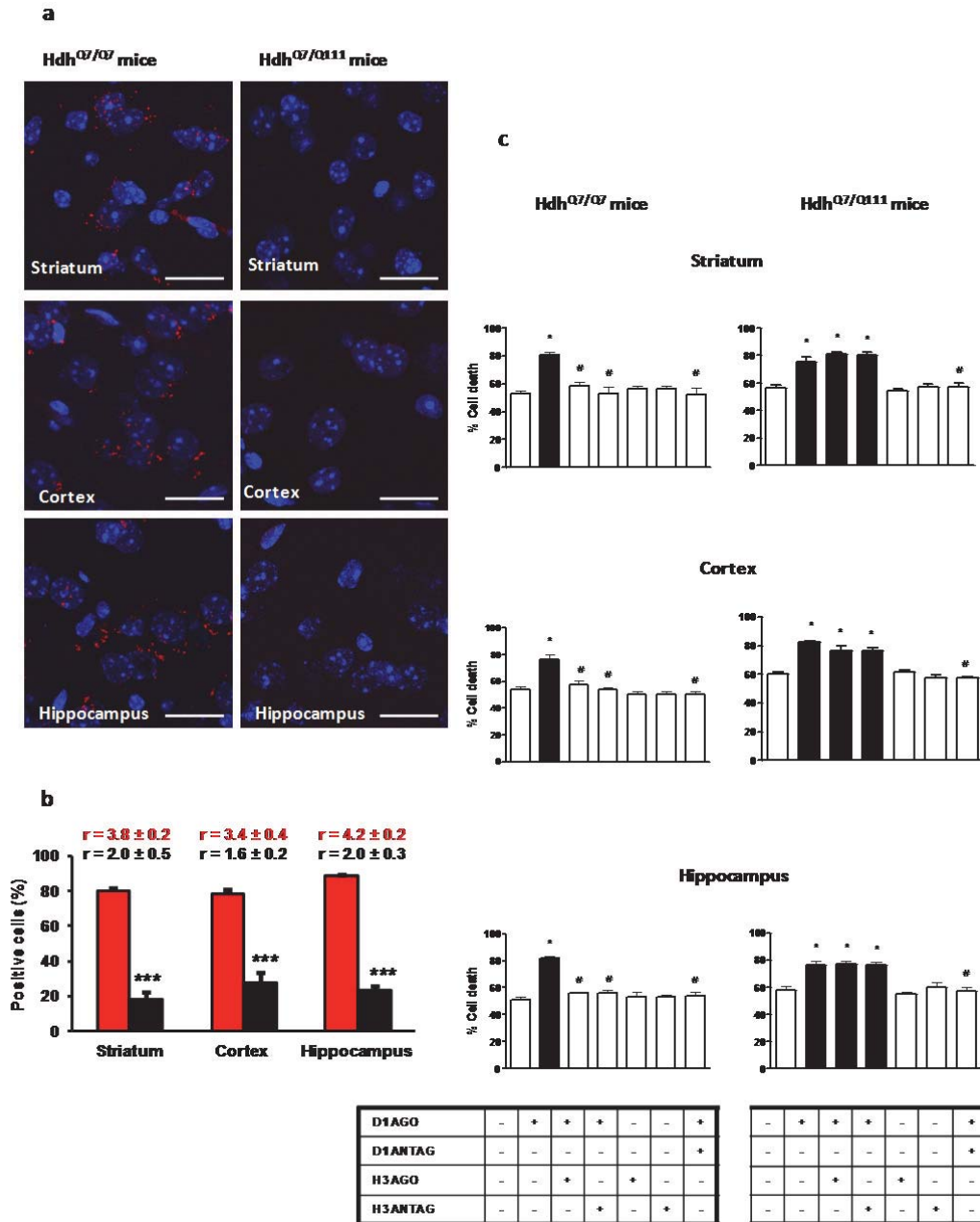


Figure 5. Functional D₁R-H₃R heteromers are expressed in wt Hdh^{Q7} mice but not in symptomatic Hdh^{Q111} mice. Striatal, cortical or hippocampal slices from 8 months old Hdh^{Q7} and Hdh^{Q111} mice were used. In (a), by Proximity Ligation Assays (PLA) D₁R-H₃R heteromers were visualized in Hdh^{Q7} mice but not in Hdh^{Q111} mice as red spots around blue colored DAPI stained nucleus. Scale bar: 20 μ m. In (b), the number of cells containing one or more red spots is expressed as the percentage of the total number of cells (blue nucleus). *r* values (number of red spots/cell containing spots) are shown above each bar. Data (% of positive cells or *r*) are the mean \pm SEM of counts in 600-800 cells from 6 different fields from 3 different animals.

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Student's t test showed a significant (***) $p < 0.05$) decrease of heteromers expression in Hdh^{Q111} mice compared to the respective Hdh^{Q7} mice. In (c) organotypic cultures were treated for 1 hour with medium, the D₁R antagonist SCH 23390 (10 μ M) or the H₃R agonist imetit (10 μ M) or antagonist thioperamide (10 μ M) before the addition of SKF 81297 (50 μ M; 48 hours) and cell death was determined. Values represent mean \pm SEM of percentage of cell death. One-way ANOVA followed by Bonferroni post hoc tests showed a significant effect over non-treated organotypic cultures (* $p < 0.05$) or of the H₃R ligands plus SKF 81297 treatment over the SKF 81297 treatment ([#] $p < 0.05$).

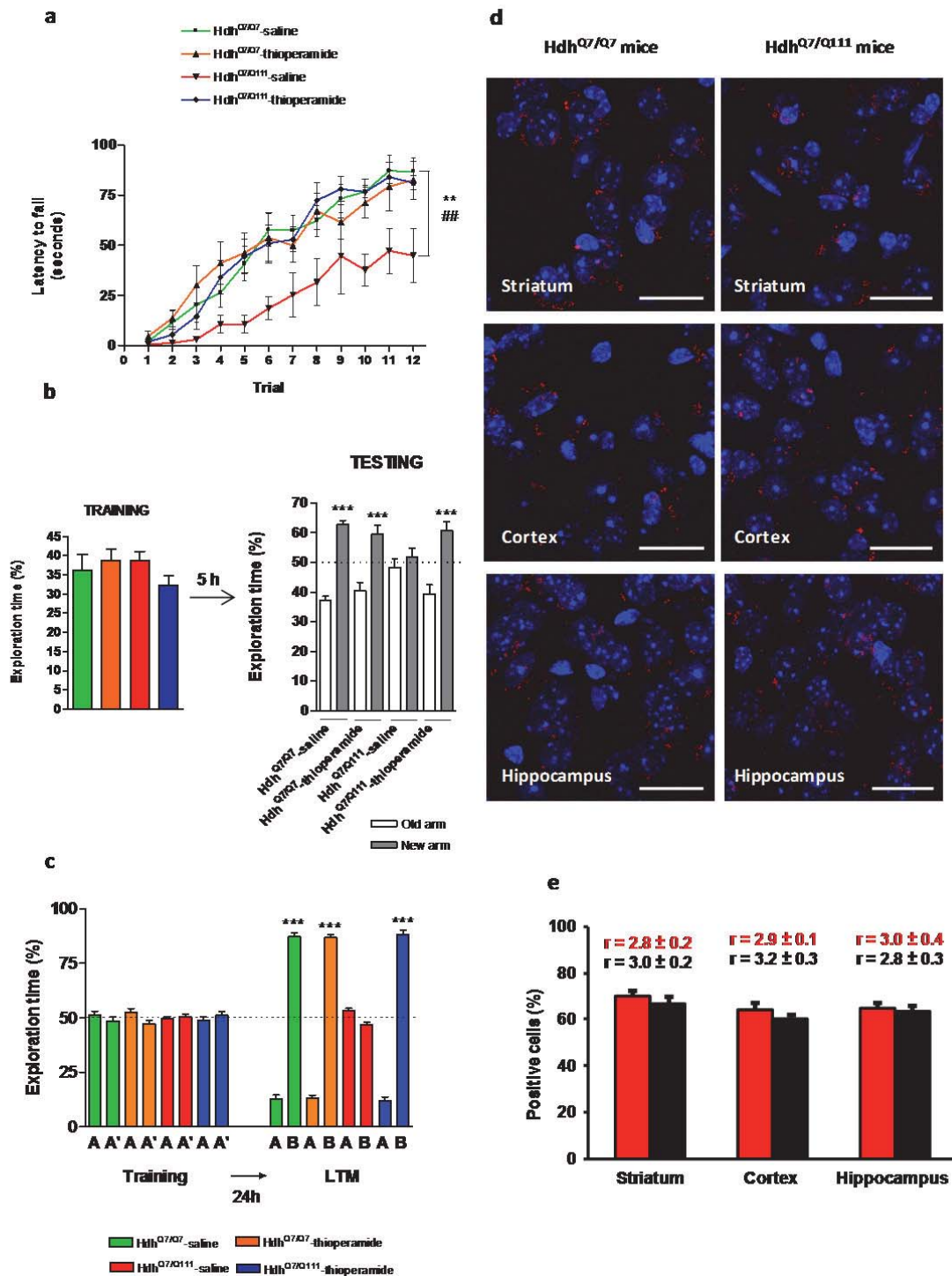


Figure 6. Thioperamide chronic treatment restores receptor heteromerization and reverses motor learning and spatial long-term memory (LTM) deficits in Hdh^{Q7/Q11} mice. In (a), histogram illustrating the latency to fall in the accelerating rotarod of 6-months-old Hdh^{Q7/Q7} wild type mice and Hdh^{Q7/Q11} mutant knock-in mice treated with saline or thioperamide from 5 months of age. Rotarod training/testing was performed 4 times per day during 3 consecutive

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days. Thioperamide treatment reverses motor learning deficits in $Hdh^{Q7/Q111}$ mice and did not affect the performance of $Hdh^{Q7/Q7}$ mice. Statistical analysis was performed using two-way ANOVA with repeated measures; ** $p < 0.01$ saline- $Hdh^{Q7/Q111}$ mice compared to saline- $Hdh^{Q7/Q7}$ mice, ## $p < 0.01$ saline- $Hdh^{Q7/Q111}$ mice compared to thioperamide- $Hdh^{Q7/Q111}$ mice. In (b), bar diagram illustrating the exploration time for saline or thioperamide-treated $Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ mice during the training and the testing period in the T-SAT. In the testing session, $Hdh^{Q7/Q111}$ -saline mice showed no preference for the novel arm indicating spatial long-term memory (LTM) deficits. By contrast, thioperamide- $Hdh^{Q7/Q111}$ mice treated spent more time in the novel *versus* the old arm revealing preserved LTM, such as $Hdh^{Q7/Q7}$ animals treated with saline or thioperamide. Statistical analysis was performed using one-way ANOVA with Bonferroni *post hoc* comparisons; *** $p < 0.001$ compared to the old arm. In (c) Bar diagram illustrating the exploration time for saline or thioperamide-treated $Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ mice during the training and the testing (24 h delay, LTM) sessions in a novel-object recognition task. Long-term recognition memory is impaired in saline- $Hdh^{Q7/Q111}$ mice at 6 months of age as showed by no preference for the novel object. Importantly, LTM memory deficits are rescued in the thioperamide- $Hdh^{Q7/Q111}$ mice. Statistical analysis was performed using one-way ANOVA with Bonferroni *post hoc* comparisons; *** $p < 0.001$ compared to the old object. In all paradigms, 11 saline- $Hdh^{Q7/Q7}$ mice, 10 thioperamide- $Hdh^{Q7/Q7}$ mice, 7 saline- $Hdh^{Q7/Q111}$ mice and 9 thioperamide- $Hdh^{Q7/Q111}$ mice were evaluated. In (d), by Proximity Ligation Assays (PLA), D_1R - H_3R heteromers were visualized as red spots around blue colored DAPI stained nucleus in both 8 months old wt Hdh^{Q7} and Hdh^{Q111} mice chronically treated with thioperamide. Scale bar: 20 μm . In (e), the number of cells containing one or more red spots is expressed as the percentage of the total number of cells (blue nucleus). *r values* (number of red spots/cell containing spots) are shown above each bar. Data (% of positive cells or *r*) are the mean \pm SEM of counts in 600-800 cells from 6 different fields from 3 different animals.

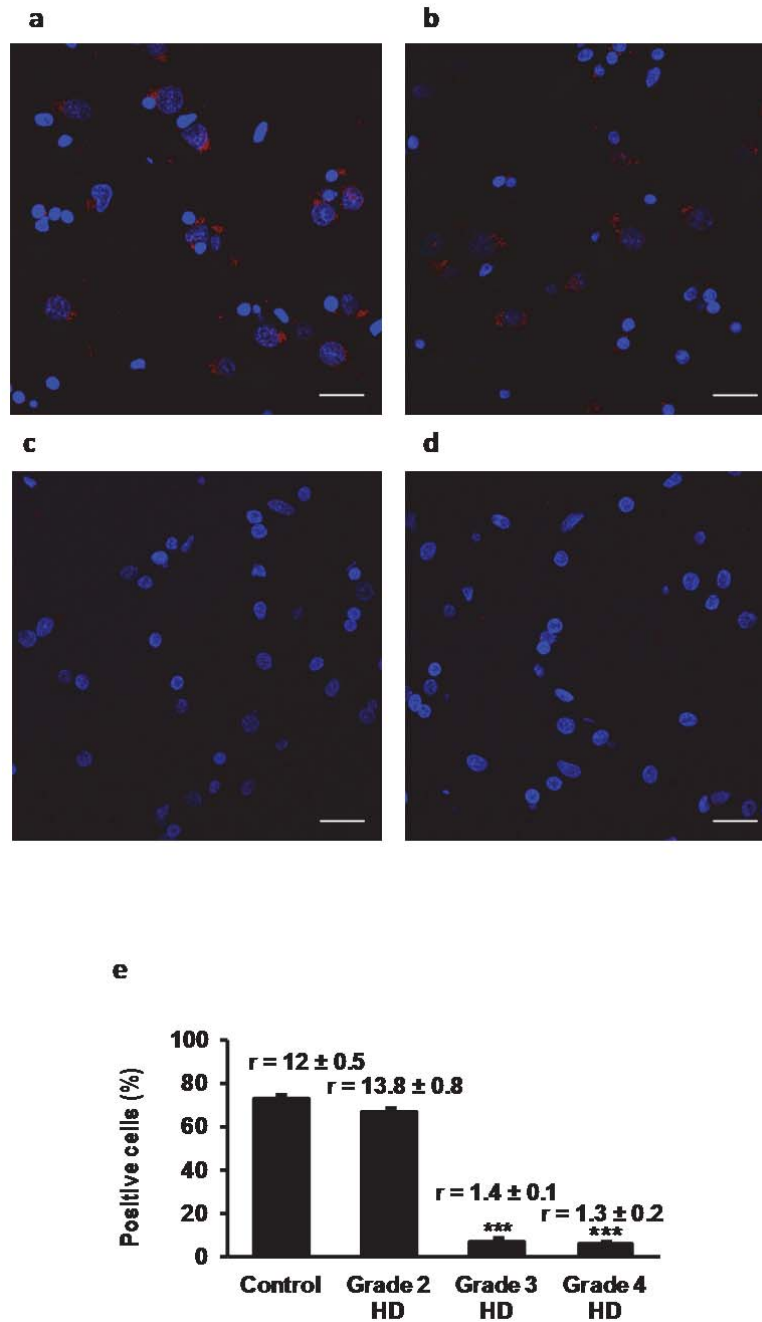


Figure 7. Striatal D₁R-H₃R heteromers are expressed in human control subjects and grade 2 HD patients but not in grade 3-4 HD patients. By Proximity Ligation Assays (PLA), D₁R-H₃R heteromers were visualized as red spots around blue colored DAPI stained nucleus in human striatal slices from age matched control subjects (a) and grade 2 HD patients (b) but not in grade 3 (c) or grade 4 (d) HD patients. Scale bar: 20 μ m. In (e), the number of cells containing one or more red spots is expressed as the percentage of the total number of cells (blue

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nucleus). *r values* (number of red spots/cell containing spots) are shown above each bar. Data are mean \pm SEM of counts in 600-800 cells from 10 different fields from subject described in Materials and Methods. Student's t test showed a significant (***) $p < 0.001$ decrease of heteromers expression in HD grade 3 and 4 patients compared to control subjects.

a

Receptor	CELLS	STHdhQ7 B _{max} (fmol/mg protein)	STHdhQ7 K _D (nM)	STHdh Q111 B _{max} (fmol/mg protein)	STHdh Q111 K _D (nM)
H ₃ R	Striatal cells	160 ± 2	1.92 ± 0.07	160 ± 34	0.71 ± 0.25
D ₁ R	Striatal cells	200 ± 20	0.3 ± 0.1	220 ± 40	0.16 ± 0.08

b

Receptor	BRAIN REGION	Hdh Q7 / Q7 B _{max} (fmol/mg protein)	WT Q7 K _D (nM)	Hdh Q7/ Q111 B _{max} (fmol/mg protein)	HD Q111 K _D (nM)
H ₃ R	Striatum	193 ± 6	0.27 ± 0.03	155 ± 6	0.26 ± 0.03
	Cortex	146 ± 4	0.22 ± 0.02	152 ± 8	0.24 ± 0.04
	Hippocampus	92 ± 1	0.20 ± 0.01	88 ± 3	0.37 ± 0.1
D ₁ R	Striatum	1088 ± 50	0.17 ± 0.02	529 ± 32	0.11 ± 0.01
	Cortex	99 ± 5	0.05 ± 0.01	73 ± 4	0.01 ± 0.01
	Hippocampus	257 ± 10	0.03 ± 0.02	187 ± 19	0.09 ± 0.07

Supplementary Table 1. Endogenous expression of D₁R and H₃R in striatal STHdh^{Q7} and STHdh^{Q111} cells and in different brain regions from Hdh^{Q7/Q7} and Hdh^{Q7/Q111} mice.

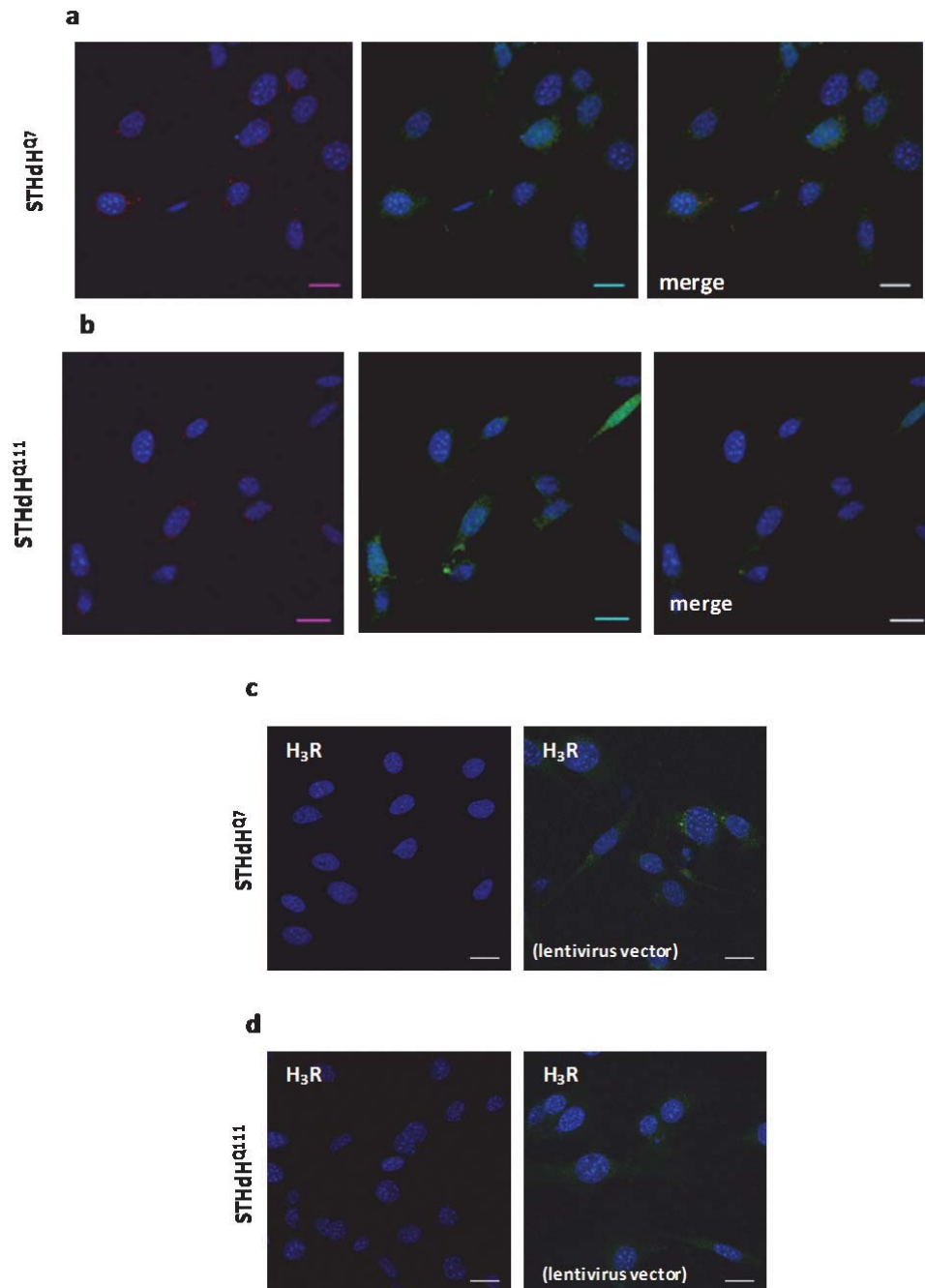


Fig. S1. Negative controls for Proximity Ligation Assays (PLA) in striatal cells. In (a and b), Proximity Ligation Assays (PLA) were performed in striatal wild type STHdH^{Q7} (a) and HD model STHdH^{Q111} (b) cells infected with GIPZ Non-silencing Lentiviral shRNA Control plasmid. D₁R-H₃R heteromers were visualized as red spots around blue colored DAPI stained nucleus (left panels), in infected cells stained in green due to the GFP expression included in the plasmid (middle panel). Merge images are given in the right panels. In (c and d) PLA were performed in

the absence of the D₁R primary antibody using STHdH^{Q7} (c) and STHdH^{Q111} (d) cells not infected (left panels) or infected (right panels) with GIPZ Non-silencing Lentiviral shRNA Control plasmid. Scale bar: 20 μm.

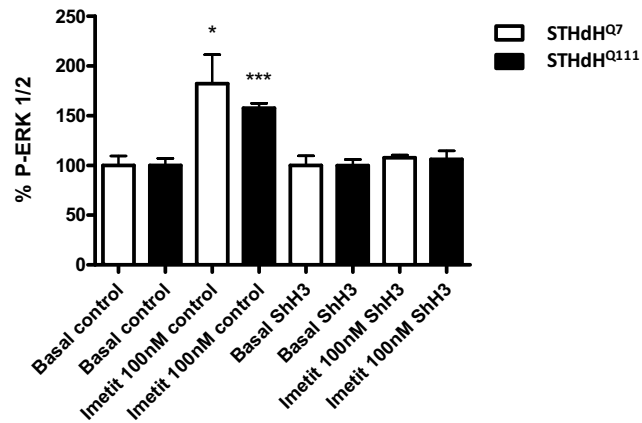


Fig. S2. H₃R are not expressed in cells depleted of H₃R by shRNA. Wild type STHdH^{Q7} and HD model STHdH^{Q111} cells were not infected (control) or infected with lentiviral silencing plasmid GIPZ Human histamine H3 receptor shRNA (ShH3). Cells were not stimulated (basal) or stimulated with the H₃R agonist imetit (100 nM) and ERK 1/2 phosphorylation was determined. Values represent mean \pm SEM of percentage of phosphorylation relative to basal levels found in untreated cells. Student's t test showed significant differences over basal conditions (*p<0.05, ***p<0.001).

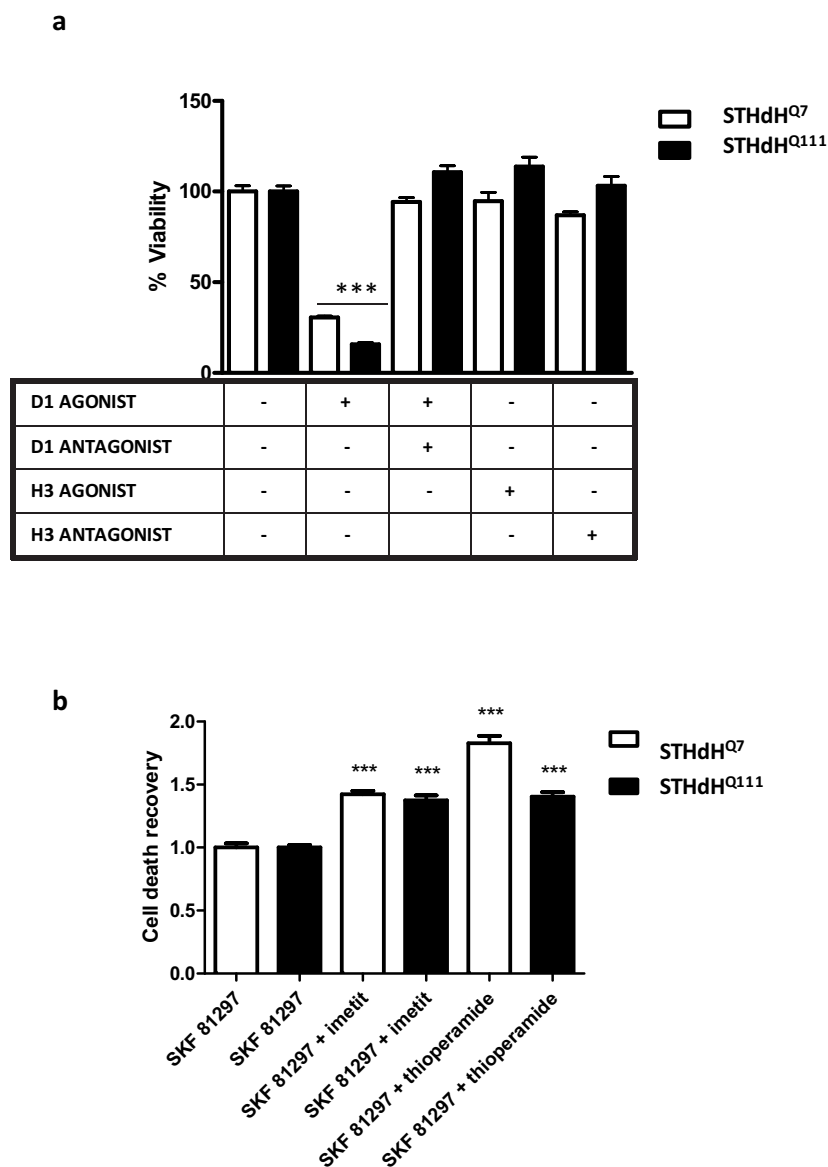


Fig. S3. Effect of D₁R antagonist, H₃R ligands and silencing vector transfection in striatal cells viability. Wild type STHdH^{Q7} (white columns) and HD model STHdH^{Q111} (black columns) cells were not infected (a) or infected with GIPZ Non-silencing Lentiviral shRNA Control plasmid (b). Cells were pretreated for 1 hour with vehicle, SCH 23390 (10 μ M), imetit (10 μ M) or thioperamide (10 μ M) prior overstimulation with SKF 81297 (30 μ M; 24 hours). Values represent mean \pm SEM of percentage of viable cells respect to vehicle-treated cells (a) or the cell viability recovery expressed as in-fold respect to SKF 81297 treated cells (b). Student's t test showed a significant (***) effect over not treated cells (a) or SKF 81297 treated cells (b).

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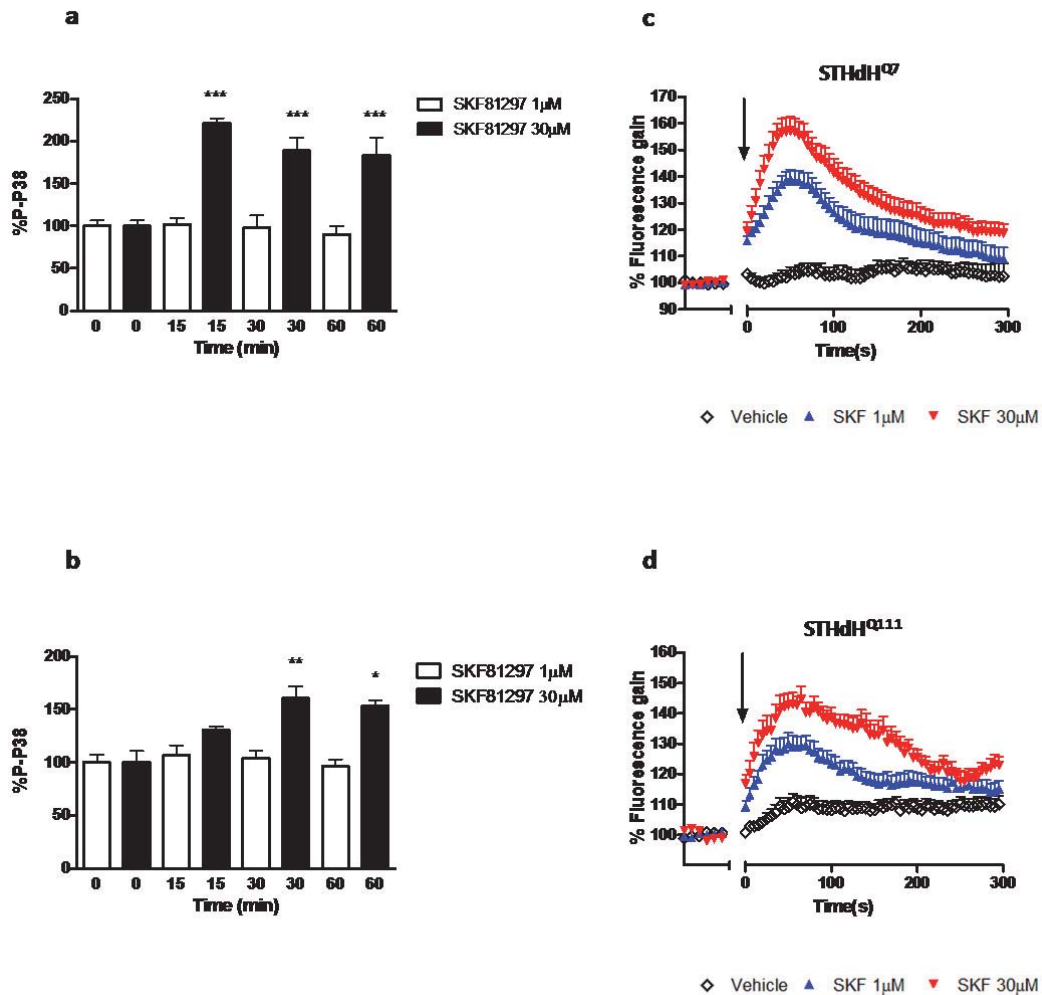


Fig. S4. Effect of low and high SKF 81297 concentrations in p-38 and intracellular calcium release. Wild type STHdH^{Q7} (a, c) and HD model STHdH^{Q111} cells (b, c) were time-dependent stimulated with 1 μM or 10 μM SKF 81297 and p-38 phosphorylation (a, b) or intracellular calcium release (c) was determined. In (a and b) values represent mean ± SEM of percentage of phosphorylation respect to vehicle-treated cells. Student's t test showed a significant (*p < 0.05, **p < 0.01, ***p < 0.001) effect over not treated cells. In (c), values are expressed as a percentage of increase respected to non treated cells and curves are mean ± SEM of three independent experiments.

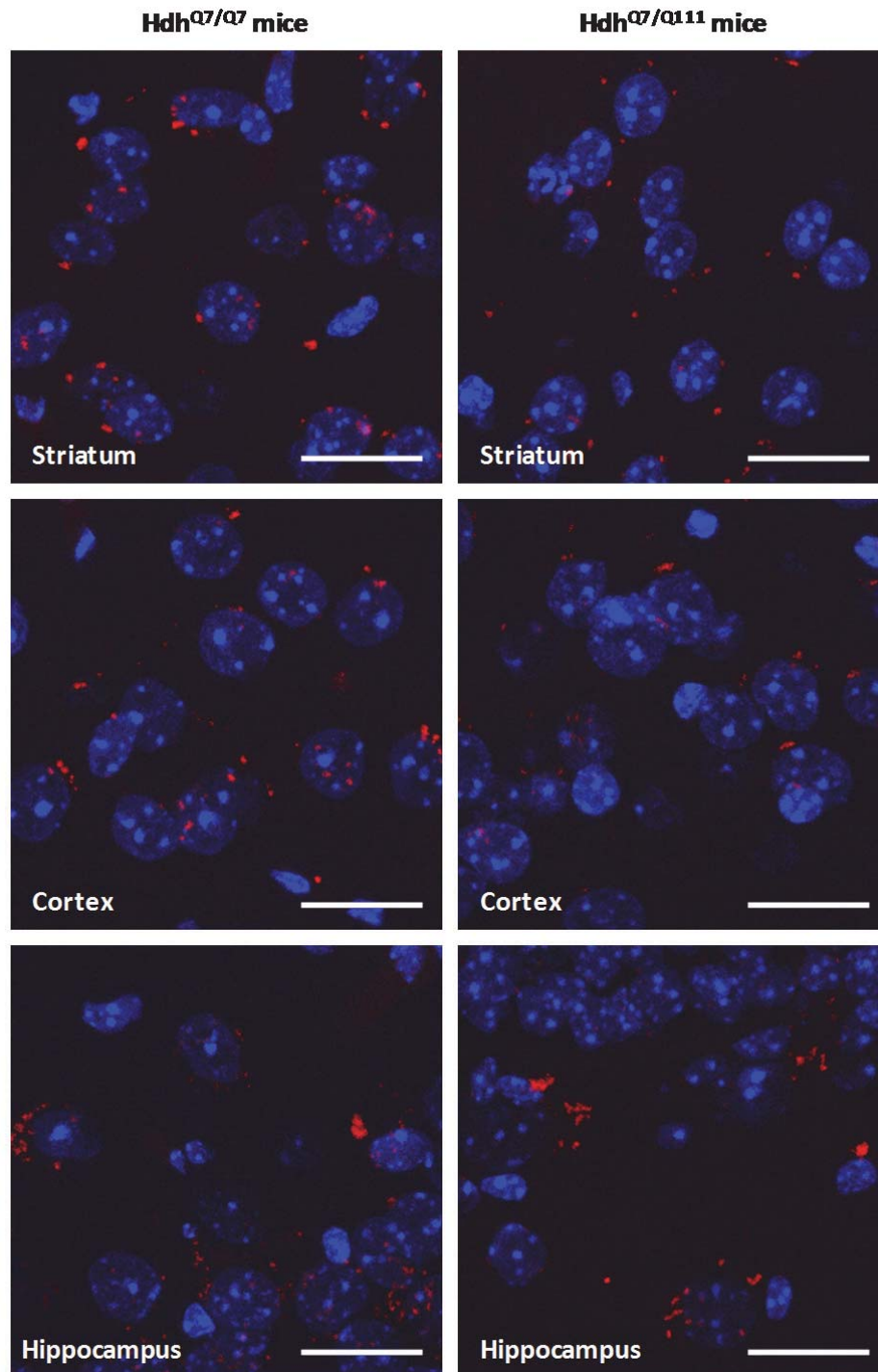


Fig. S5. D₁R-H₃R heteromers are expressed in 2 months old wt Hdh^{Q7} and HD model Hdh^{Q111} mice. Proximity Ligation Assays (PLA) were performed using striatal, cortical or hippocampal slices from two months old Hdh^{Q7} and Hdh^{Q111} mice. D₁R-H₃R heteromers were visualized in all slices as red spots around blue colored DAPI stained nucleus. Scale bar: 20 μ m.

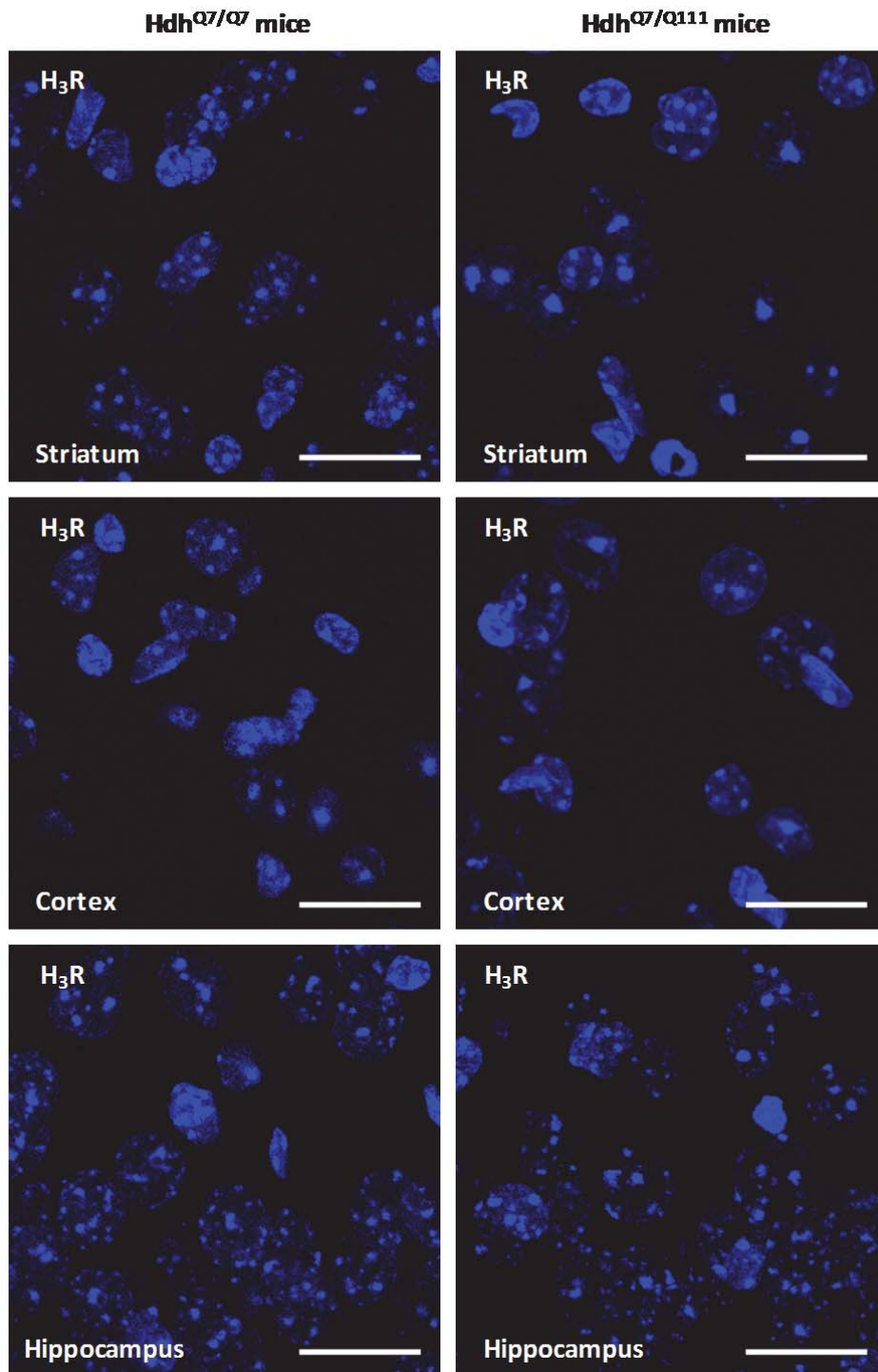


Fig. S6. Negative controls for Proximity Ligation Assays (PLA) in mouse brain slices. Proximity Ligation Assays (PLA) were performed in the absence of the primary antibody using striatal, cortical or hippocampal slices from four months old Hdh^{Q7} and Hdh^{Q111} mice. In all slices a lack of red spots around blue colored DAPI stained nucleus was observed. Scale bar: 20 μ m.

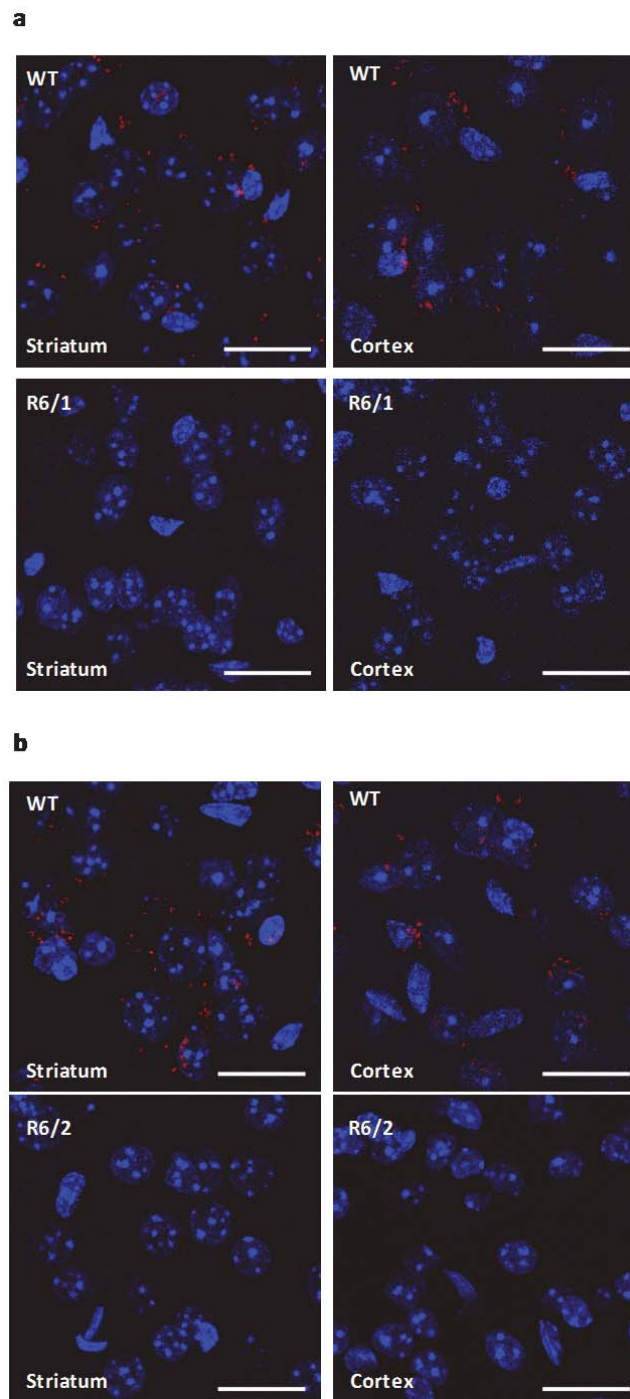
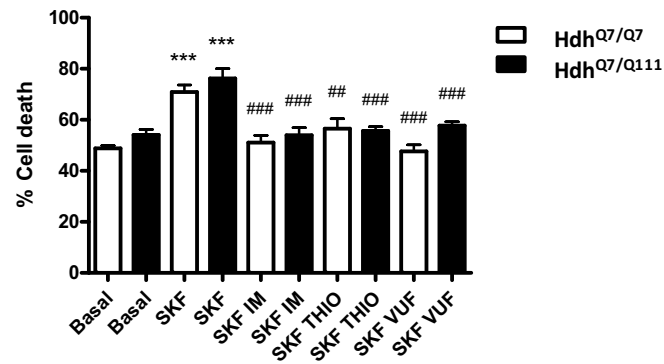


Fig. S7. D₁R-H₃R heteromers are not expressed in HD R6/1 and R6/2 mouse models. Proximity Ligation Assays (PLA) were performed using striatal or cortical slices from age matched wild type littermates (WT) and 12 weeks old R6/1 (a) or 4 months old R6/2 mice (b). D₁R-H₃R heteromers were visualized only in wild type mouse slices as red spots around blue colored DAPI stained nucleus. Scale bar: 20 μ m.

a



b

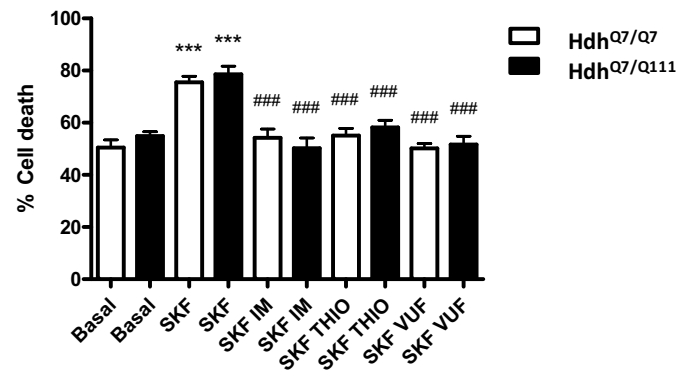


Fig. S8. Functional D₁R-H₃R heteromers are expressed in 5 months old wt Hdh^{Q7} and Hdh^{Q111} mice. Striatal (a) and cortical (b) organotypic cultures from 5 months old Hdh^{Q7} and Hdh^{Q111} mice were treated for 1 hour with medium, the H₃R agonist imetit (10 μ M) or the H₃R antagonists thioperamide (10 μ M) or VUF (10 μ M) before the addition of SKF 81297 (50 μ M) and cell death was determined. Values represent mean \pm SEM of percentage of cell death. One-way ANOVA followed by Bonferroni post hoc tests showed a significant effect over non-treated organotypic cultures (***) $p < 0.001$) or of the H₃R ligands plus SKF 81297 treatment over the SKF 81297 treatment (### $p < 0.001$).

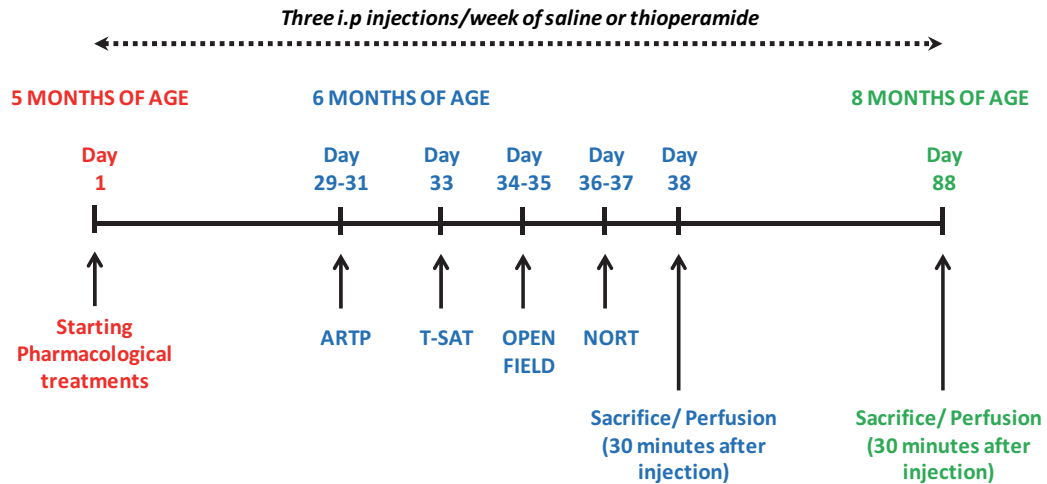


Fig. S9. Schematic representation of pharmacological treatments and behavioral analysis performed after chronic treatment with saline (NaCl 0.9% saline) or thioperamide (10mg/Kg). Three intraperitoneal injections per week of saline or thioperamide were performed from 5 months of age until the animals were sacrifice and perfused. Behavioral assessment started at 6 months of age with the evaluation of the ARTP, T-SAT, Open field and NORT. One cohort of animals was sacrifice and perfused 30 minutes after the last injection to evaluate PLA at 6 months of age. A second cohort of animals was sacrifice and perfused 30 minutes after the last injection to evaluate PLA at 8 months of age.

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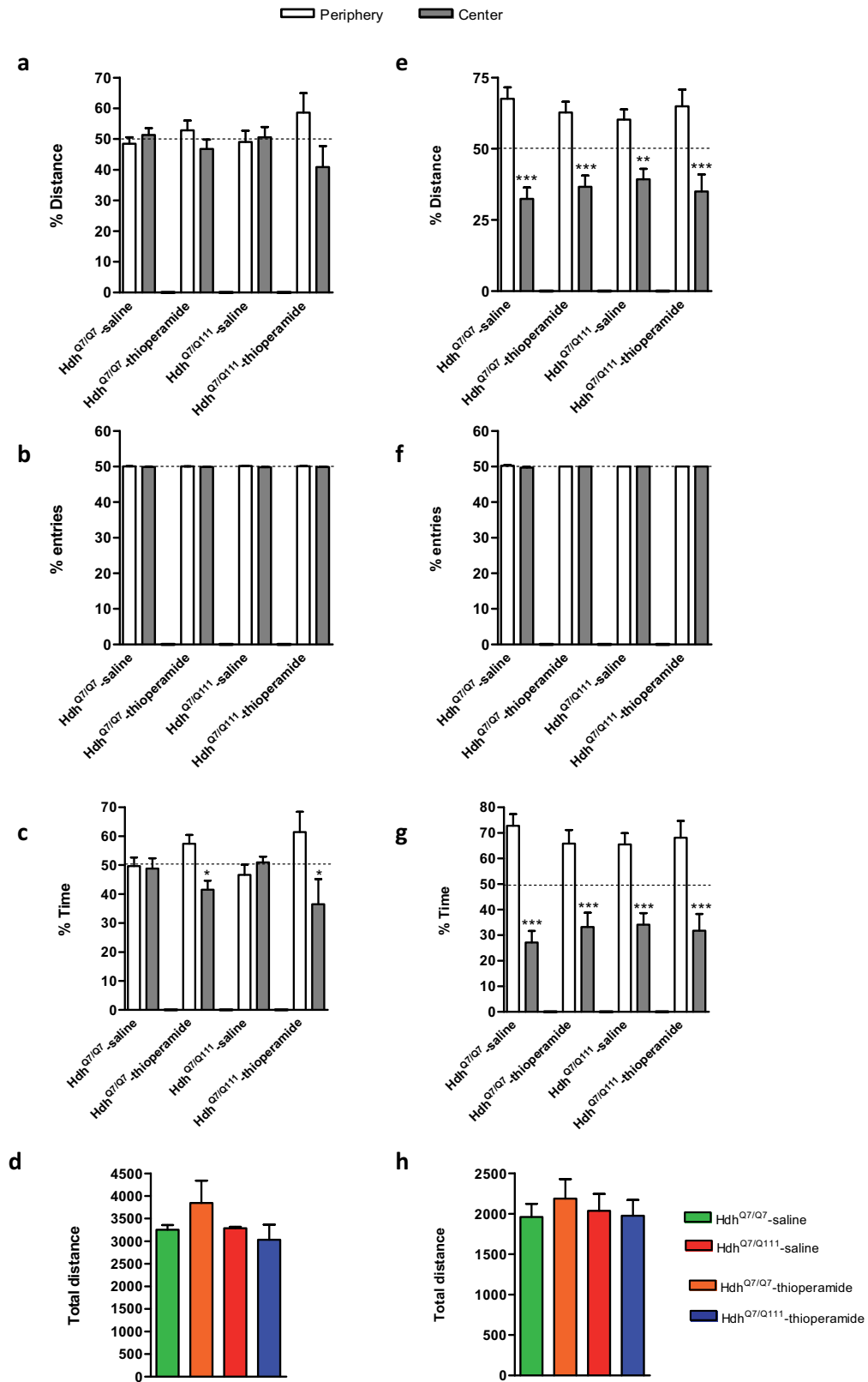


Fig. S10. No significant differences in the open field habituation were found between treatments and genotypes. To avoid motivation and anxiety differences between genotypes and treatments, the percentage of distance (A,E), the percentage of entries (B,F) and the percentage of time (C,G) between the periphery and the center in the open field arena were analysed in the first (A,B,C) and second (E,F,G) day of habituation in the open field arena. To avoid spontaneous locomotor activity differences between genotypes and treatments the total distance rove for each animal was analysed in the first (D) and second day (H) of habituation in the open field arena. After two days of habituation in the open field arena, all mice behaves equal, indicating that the results showed in the NORT (training and testing sessions) are exclusively linked with recognition long-term memory but not with differences in anxiety or spontaneous locomotor activity between genotypes or treatments. Statistical analysis was performed using one-way ANOVA with Bonferroni *post hoc* comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the periphery.

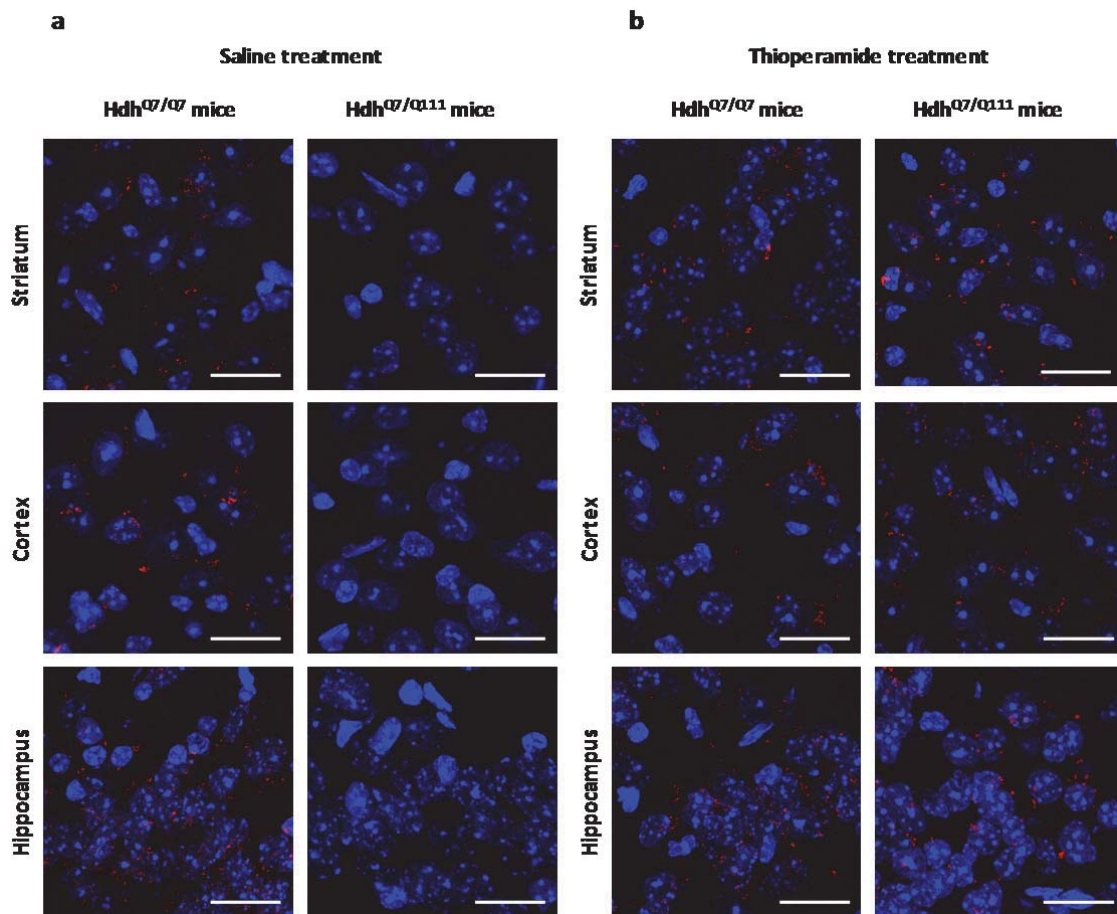


Fig. S11. Expression of D₁R-H₃R heteromers in 6 months old Hdh^{Q7} and Hdh^{Q111} mice not treated or chronically treated with thioperamide. Proximity Ligation Assays (PLA) were performed with striatal, cortical or hippocampal slices from 6 months old Hdh^{Q7} and Hdh^{Q111} mice not treated (a) or chronically treated (b) with thioperamide. D₁R-H₃R heteromers were visualized as red spots around blue colored DAPI stained nucleus in thioperamide treated or not treated Hdh^{Q7} mice (a, b) and in thioperamide treated Hdh^{Q111} mice (b) but were very diminished in thioperamide non-treat Hdh^{Q111} mice (a). Scale bar: 20 μ m.

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IV. DISCUSSION

Therapeutic research in HD is a major issue as there is currently no treatment available for delaying or preventing the progression of the disease. Neuronal dysfunction and neuronal death in HD are caused by a combination of interrelated pathogenic processes. Understanding how mutant huntingtin impacts on this plethora of cellular functions and identifying new molecular targets involved in all these aberrant processes are goals of increasing urgency for scientific community. An ambitious goal of HD research resides in finding new molecular targets that contribute not only to restore cognitive deficits but also to prevent neuronal death. Therefore, in this Thesis we have focused on the identification of new therapeutical targets that could prevent both neurodegeneration and cognitive decline in HD.

Based on decades of HD research, we have focused on some established mechanisms of neurodegeneration and neuronal dysfunction, including alterations in transcriptional machinery, neurotrophic support, corticostriatal communication, synaptic plasticity, as well as kinase and neurotransmitter signaling (Labbadia and Morimoto, 2013; Zuccato et al., 2005; Zuccato et al., 2010). From these altered neuronal mechanisms, we have studied and identified specific targets to be potential candidates in developing pharmacological therapeutics in HD.

1. PHENOTYPIC CHARACTERIZATION OF MUTANT KNOCK-IN Hdh^{Q7/Q111} MICE: AN ACCURATE HD MOUSE MODEL TO STUDY DISEASE PROGRESSION.

Cell culture and genetic HD mouse models represent good tools to test several chemical compounds as potential treatments that could be translated to HD patients. In this Thesis we have evaluated HD pathology using different HD models: stable striatal cell lines from Hdh^{Q111/Q111} mice, neuronal primary cultures from Hdh^{Q7/Q111} and R6/1 mice, and R6/1, homozygous Hdh^{Q111/Q111} and heterozygous Hdh^{Q7/Q111} mice.

Knock-in mouse models represent the most accurate HD genetic models as they carry the mutation in its appropriate genomic context. However, lack of studies regarding its behavioral function difficult their use to test possible therapeutic interventions. Therefore, we have evaluated cognitive function, motor coordination and synaptic transmission and plasticity in Hdh^{Q7/Q111} mouse, to determine and

validate whether this HD mice could represent a valuable model for studying HD progression (Fig. 25).

Evidence of altered acquisition of new motor skills, paired attention, planning and memory has been demonstrated in HD patients before the onset of motor symptoms (Berrios et al., 2002;Foroud et al., 1995;Lawrence et al., 2000;Lemiere et al., 2004;Majerova et al., 2012). Accordingly, in this Thesis, we have demonstrated for the first time that Hdh^{Q7/Q111} mice exhibit motor learning deficits in the accelerated rotarod task as early as 2 months of age getting worse with HD progression. At middle disease stages (6 months of age), motor learning deficits are accompanied by procedural learning memory disturbances in the reversal phase of swimming T-maze test, revealing that Hdh^{Q7/Q111} mice, as human HD patients, have difficulty in changing strategies. Importantly, nor at 2 neither at 6 months of age, Hdh^{Q7/Q111} mice display motor coordination deficits as demonstrated by equal performance in the fixed rotarod and/or in the open field compared to wild type Hdh^{Q7/Q7} mice. These results are in agreement with previous data in other HD mouse models (van Raamsdonk et al., 2005) and human patients (Gabrieli et al., 1997;Heindel et al., 1988;Heindel et al., 1989;Lawrence et al., 1996;Lawrence et al., 1998a;Lawrence et al., 2000;Schmidtke et al., 2002), in which deficits in procedural learning appear prior to other cognitive symptoms and certainly prior to motor coordination impairments. Spatial and recognition memory decline have also been reported in HD patients (Berrios et al., 2002;Lawrence et al., 2000;Majerova et al., 2012) and in several HD mouse models (Giralt et al., 2009;Giralt et al., 2011a;Giralt et al., 2011b;Lione et al., 1999;Ramaswamy et al., 2007;van Raamsdonk et al., 2005). These studies prompted us to investigate whether spatial and recognition memory deficits were also present in Hdh^{Q7/Q111} mice. Consistent with data in other HD mouse models, Hdh^{Q7/Q111} mice exhibit several deficits in spatial and recognition memories in the T-SAT and NORT paradigms at 6 and 8 months of age. Importantly, no memory-related deficits appear before, at 4 months of age, revealing that motor learning decline is present in Hdh^{Q7/Q111} mice months before spatial and recognition memory impairments.

Altered synaptic transmission and plasticity has been shown to contribute to behavioral deficits in HD (Cepeda et al., 2007;Cybulska-Klosowicz et al., 2004). Indeed,

functional magnetic resonance imaging studies in HD patients revealed impairments in the activity of frontal cortex and putamen brain regions (Kim et al., 2004a) and different studies in HD mouse models have demonstrated altered corticostriatal connectivity (Cybulska-Klosowicz et al., 2004;Levine et al., 1999;Milnerwood et al., 2006;Milnerwood and Raymond, 2007), and hippocampal LTP disturbances (Giralt et al., 2009;Hodgson et al., 1999;Lynch et al., 2007;Murphy et al., 2000;Usdin et al., 1999). In agreement with these previous studies, we have demonstrated that $Hdh^{Q7/Q111}$ mice exhibit altered corticostriatal synaptic transmission as early as 2-3 months of age, while hippocampal LTP deficits at 6 months of age, which correlates with the initial detection of cognitive impairments.

As previously mentioned in the introduction of this Thesis, how the brain processes environment information is intricately controlled by synaptic machinery in synapses. Pre and postsynaptic elements form this fundamental unit that supports information processing. Glutamatergic excitatory synapses mainly occur on dendritic spines, dynamic structures that play a crucial role in synaptic transmission and plasticity (Nithianantharajah and Hannan, 2013b;Segal, 2005;Shepherd and Erulkar, 1997;van der Zee, 2014;von Bohlen Und, 2009). Because cognitive deficits and synaptic transmission are altered in our $Hdh^{Q7/Q111}$ mice at early and mild-disease stages, we have explored whether these alterations could correlate with altered dendritic spine population. We have demonstrated that dendritic spine density and morphology are altered in cortical, hippocampal and striatal slices from $Hdh^{Q7/Q111}$ mice. Importantly, we have demonstrated that decreased spine-like structures analyzed by spinophilin and PSD95 immunostaining appear in the cortex, but not striatum, from $Hdh^{Q7/Q111}$ mice at the ages where we detected motor learning deficits and altered corticostriatal synaptic transmission, while at more advanced disease stages also striatum shows a decrease in spine-like structures, which is consistent with the observed motor coordination deficits. Importantly, although no accurate morphological experiments have been performed in the cortex and striatum of $Hdh^{Q7/Q111}$ mice, decreased number of spine-like structures correlate with changes in the mean size of these particles, suggesting that both altered density and size contributes to modify spine population function. Interestingly, the decrease in dendritic spine density and the altered

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percentage of spine type population in the hippocampus do not appear in $Hdh^{Q7/Q111}$ mice until 6 months of age. Accordingly, at this age, $Hdh^{Q7/Q111}$ mice exhibit spatial, recognition and associative memory deficits as well as impaired hippocampal LTP.

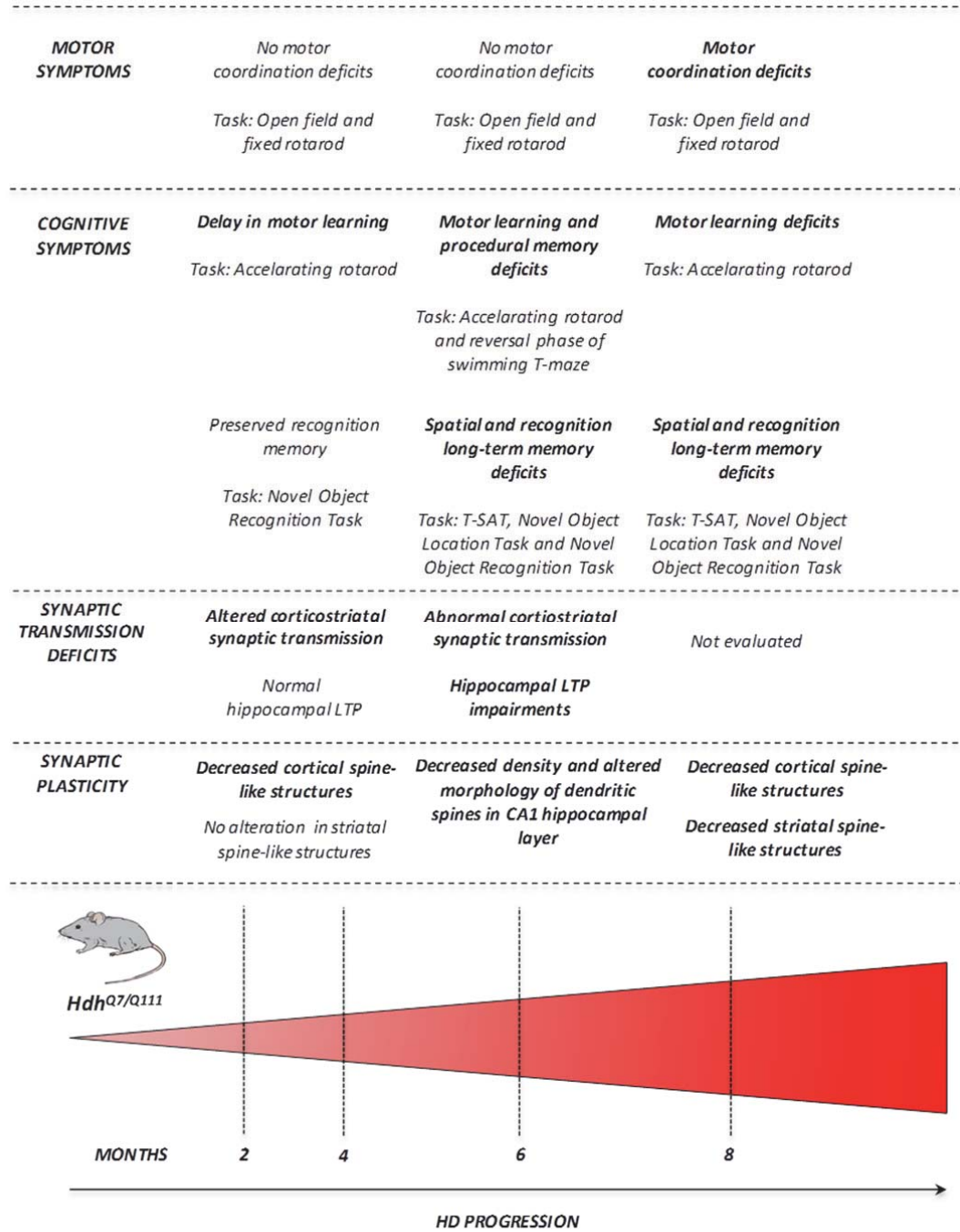


Figure 25. Schematic representation showing $Hdh^{Q7/Q111}$ phenotype along disease progression. See the text for details.

In agreement with these results showing cortical, hippocampal and striatal dendritic spine alterations in Hdh^{Q7/Q111} mice, it has been described that dendritic spine alterations are also present in other HD mouse models (Guidetti et al., 2001; Heck et al., 2012; Klapstein et al., 2001; Nithianantharajah et al., 2009; Spires et al., 2004; Xie et al., 2010) and HD patients (Ferrante et al., 1991; Graveland et al., 1985; Sotrel et al., 1993).

Altogether, these findings provide evidence for the first time of early cognitive, synaptic plasticity and transmission deficits in Hdh^{Q7/Q111} mice, prior to motor coordination symptomatology. Importantly, these data highlight the potential benefits of using this mouse model for studying HD pathology as they reproduce accurately the genetic properties of human condition and exhibit an early and progressive HD phenotype.

2. NEW THERAPEUTIC TARGETS WITH A DUAL EFFECT: REDUCING STRIATAL VULNERABILITY AND RESTORING COGNITIVE FUNCTION IN HD

The identification of proteins and signaling pathways specifically altered by the expression of mutant huntingtin is a priority to find new potential candidates to develop pharmacological strategies to treat or delay motor and cognitive deficits in HD.

Although HD is characterized by degeneration of the striatum, cerebral cortex (Reiner et al., 1988; Vonsattel and DiFiglia, 1998), and other brain areas such as the hippocampus, thalamus, globus pallidus, substantia nigra (Herndon et al., 2009; Ille et al., 2011; Kassubek et al., 2004; Morton et al., 2000; Politis et al., 2008; Rosas et al., 2003; Spargo et al., 1993; Vonsattel and DiFiglia, 1998), the pathogenic mechanisms whereby the CAG repeat expansion finally produce disease symptoms are poorly understood. Importantly, evidence from both patients (Caramins et al., 2003) and mouse models (Levine et al., 2004; Turmaine et al., 2000) indicate that cell death does not occur until late stages of the disease, suggesting that neuronal dysfunction and abnormal synaptic plasticity are the earliest in the disease process rather than

neuronal death. Taking into account this evidence, one could focus their research in finding common molecular targets that could restore cognitive function and synaptic plasticity at the earliest stages of the disease that in turn could represent a positive potential regulator in preventing neuronal death in the late stages of the disease.

2.1 FROM HUNTINGTIN DYSFUNCTION TO THERAPEUTICAL TARGETS: MOLECULAR CHANGES UNDERLYING COGNITIVE DEFICITS AND NEURODEGENERATION

In this Thesis we got new insights in understanding early pathophysiological changes in HD by identifying proteins and signaling pathways altered by the expression of mutant huntingtin in different HD models. We have tested in Hdh^{Q7/Q111} mice how the modulation of these targets could represent good approximations for the design of new therapeutic interventions in HD.

Extended literature has helped us in selecting potential candidates to be good therapeutic targets. Our main goal was built on work that these candidates could exert a dual effect in HD pathology by restoring cognitive and synaptic dysfunction at early disease stages leading thereafter to strength neuronal circuitries and prevent late neuronal death.

Although HD is characterized by a progressive degeneration of MSNs (Ferrante et al., 1991; Vonsattel et al., 1985), functional and morphological changes in the neocortex has been proposed as the initial triggers of striatal pathology. In this context, HD patients exhibit impaired sensory discrimination, learning memory, planning and decision strategies, all of them related with neocortical dysfunction (Hahn-Barma et al., 1998; Ho et al., 2001; Lawrence et al., 1998a; Paulsen et al., 2001a; Rosenberg et al., 1995; Schneider et al., 2010). Moreover, marked nuclear and cytoplasmic accumulation of mutant huntingtin has been reported in cortical pyramidal neurons, which overlap with cortical dendritic abnormalities in postmortem brain of patients with low-grade of striatal pathology (DiFiglia et al., 1997; Sapp et al., 1997). Indeed, abundant mutant huntingtin is evident in numerous cortical dystrophic axons that project to the striatum (Sapp et al., 1999). Moreover, it has also been reported that stage I and II HD patients exhibit specific thinning of the neocortex (Kassubek et al., 2004; Rosas et al., 2010) while cortical metabolic dysfunction has been

proposed to occur early in the course of the disease (Sax et al., 1996). More recent evidence demonstrate reduced corticostriatal functional connectivity in preHD patients when compared to controls (Unschuld et al., 2012), and a recent reports further confirms functional connectivity reductions in both preHD and manifest HD patients (Poudel et al., 2014b;Poudel et al., 2014a). Importantly, an early alteration of cortical function has also been reported in HD mouse models as demonstrated by impaired learning-dependent cortical plasticity (Cybulska-Klosowicz et al., 2004;Mazarakis et al., 2005), altered excitability of cortical pyramidal neurons (Cepeda et al., 2007;Cummings et al., 2009) and abnormal cortical dendritic morphology (Guidetti et al., 2001;Laforet et al., 2001;Spires et al., 2004) that could contribute to the reduced synaptic responsiveness of striatal neurons to cortical stimuli (Laforet et al., 2001). In agreement, an early and progressive reduction in the frequency of spontaneous EPSCs in MSNs has been shown to match with the emergence of an overt behavioral phenotype in a HD mouse model (Cepeda et al., 2003), which is accompanied by decreases in presynaptic and postsynaptic marker proteins, supporting the hypothesis that alterations in cortical neurons and therefore in corticostriatal projections may precede or coincide with postsynaptic changes in MSNs. Overall, these findings indicate that cortical changes in HD human and mice are fundamental to the onset and progression of HD phenotype, evidencing a central role of the cortex in the early stages of HD. However, to our knowledge none of these works have elucidated which underlying mechanisms could contribute to this early cortical dysfunction, although strongly suggested that the relevant changes could occur in the synapses. These evidences together with our data on procedural learning deficits, impaired corticostriatal synaptic transmission and early cortical dendritic spine alteration in 2 months-old $Hdh^{Q7/Q111}$ mice leads us to work on early synaptic-related candidates. Following an extended analysis of synaptic-related proteins such as NMDA (GluN1 and GluN2B) and AMPA (GluA1) receptors, presynaptic (Vglut1 and Synaptophysin) and postsynaptic (Kal7, PSD95, Shank3 and CamKII) scaffolding and signaling proteins, Kalirin-7 was the only analyzed protein that showed an early, significant and specific reduction in the cortex, but not striatum, of $Hdh^{Q7/Q111}$ and R6/1 mice (Work 4), which correlates with the earliest manifestation of corticostriatal behavioral deficits in these mice (Work 4). As mentioned in the introduction of this

DISCUSSION

This Thesis, Kalirin-7 is a postsynaptic brain-specific RhoGEF protein that emerged as a key regulator of excitatory synapses (Ma et al., 2003; Ma et al., 2008; Penzes et al., 2001a; Penzes et al., 2001b), leading us to hypothesize that Kalirin-7 loss in the cortex of young HD mice, could be associated with the early loss of excitatory synapses in HD. To validate our hypothesis, we overexpressed Kalirin-7 in cortical primary neurons from HD mice in an attempt to restore the number of glutamatergic excitatory synapses. Our data demonstrates that Kalirin-7 overexpression restores the number of cortical glutamatergic synapses in cortical neurons. This is in agreement with previous studies showing that Kalirin-7 overexpression causes an increase in dendritic spine density, spine size and excitatory synapse number, while knock-down of Kalirin-7 promotes spine shrinkage and loss in cultured hippocampal and cortical neurons (Ma et al., 2003; Ma et al., 2008; Penzes et al., 2001a; Penzes et al., 2001b). Although we were not able to study the *in vivo* impact of overexpressing Kalirin-7 in the adult mice due to methodological limitations, our data suggest that the early decrease in this RhoGEF protein could contribute to HD cortical and in turns corticostriatal dysfunction in HD (Fig. 26). Indeed, supporting the relevance of Kalirin-7 in the adult mice brain, *in vivo* studies conducted in Kal7^{KO} and Kal^{GEF1-KO} mice demonstrate that loss of Kal7 results in decreased spine density in the CA1 hippocampal neurons and reduced number of excitatory synapses in pyramidal cortical neurons (Cahill et al., 2009; Ma et al., 2008; Xie et al., 2011). Importantly, these alterations in excitatory synapses correlate with a decline in the magnitude of the hippocampal LTP and in reduced glutamatergic synaptic transmission in the cortex, as well as with impaired cognitive functions (Cahill et al., 2009; Ma et al., 2008; Xie et al., 2011), suggesting that the loss of Kal7 in our HD mice could contribute not only to decrease spine density, but probably to the alteration of corticostriatal synaptic transmission and cognitive deficits. In this Thesis, the identification of Kalirin-7 downregulation at early HD stages and its role in modulating cortical excitatory synapses (Work 4), allow us to hypothesize that cortical dysfunction could be restored by increasing the levels of Kalirin-7 in the cerebral cortex. Such increase could be a first step to prevent loss of later corticostriatal connectivity. In this context, we strongly believe that by restoring cortical and corticostriatal dysfunction, striatal dysfunction could be, at least in part, prevented or delayed. To study whether Kalirin-7 overexpression in the adult mouse brain could

restore corticostriatal function in HD, we propose the use of High capacity adenovirus. This strategy could serve to evaluate specifically the functional impact on Kalirin7 overexpression in HD pathology and validate Kalirin-7 as a new molecular target to restore cortical dysfunction in HD.

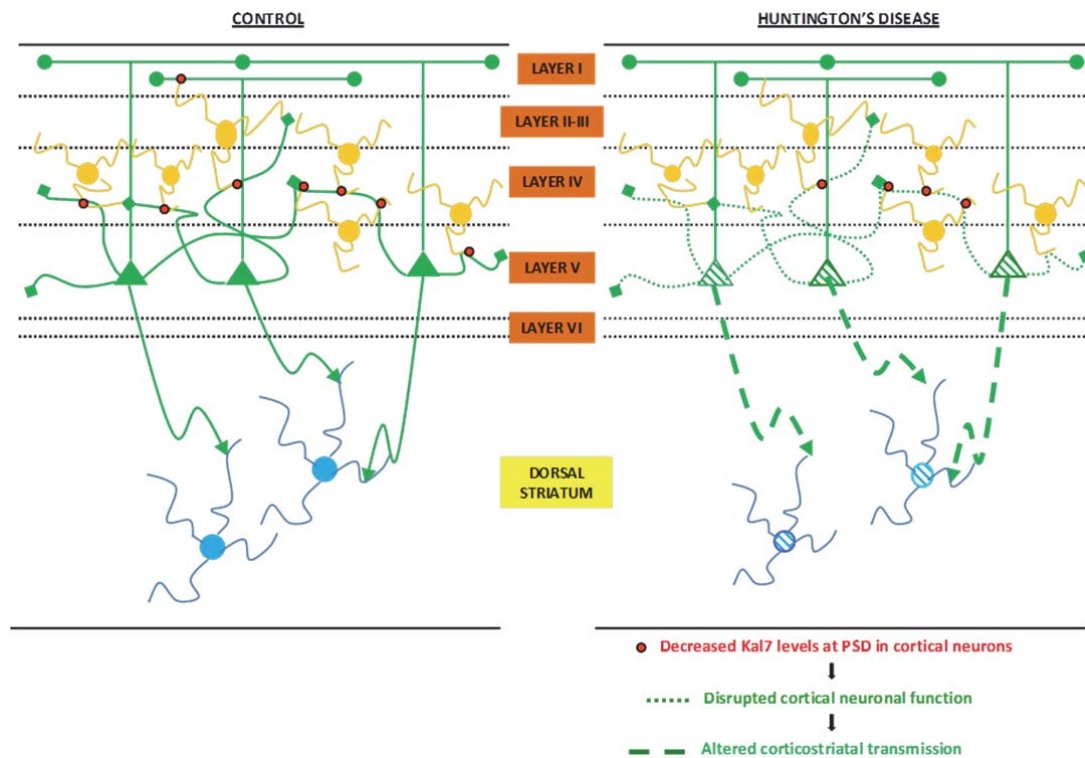


Figure 26. Schematic representation of impaired corticostriatal connectivity in HD: The role of Kalirin-7. In the presence of mutant huntingtin, cortical neurons expressed diminished kalirin-7 levels at the post-synaptic site, resulting in altered cortical connectivity and function. This disruption in cortical functionality affected corticostriatal transmission.

Cortical and subsequently corticostriatal dysfunction could have important negative effects on intrinsic striatal function. Indeed, BDNF, a crucial regulator of neuronal survival and synaptic plasticity (Binder and Scharfman, 2004) that has been extensively shown to be compromised in HD pathology (Ferrer et al., 2000; Gines et al., 2003b; Zuccato et al., 2001; Zuccato et al., 2005; Zuccato and Cattaneo, 2007; Zuccato and Cattaneo, 2009), is mainly produced in the cerebral cortex and is anterogradely transported along the corticostriatal pathway to the MSNs, the most vulnerable neuronal population in HD (Altar et al., 1997; Baquet et al., 2004). Indeed, decreased

BDNF-mediated signaling has been shown to cause dendritic abnormalities and neuronal loss (Baquet et al., 2004; Gorski et al., 2003) and its downregulation is a common phenomenon in different regions of HD brains (Ferrer et al., 2000; Gines et al., 2003b; Zuccato et al., 2001; Zuccato et al., 2005; Zuccato and Cattaneo, 2007; Zuccato et al., 2008; Zuccato and Cattaneo, 2009), suggesting that BDNF therapy could represent an excellent strategy for treating the clinical hallmarks of HD (Alberch et al., 2004; Zuccato et al., 2005; Zuccato et al., 2010). However, BDNF administration as a therapy for HD has shown important methodological drawbacks in HD models (Zuccato and Cattaneo, 2009), and several studies have shown that increasing BDNF levels only ameliorate or partially improve HD phenotype (Arregui et al., 2011; Gharami et al., 2008; Giralt et al., 2011a; Simmons et al., 2011; Xie et al., 2010). BDNF exerts trophic effects through its binding to its receptors TrkB and p75^{NTR}. In this view, BDNF binding to TrkB receptor has been shown to promote neuronal survival (Numakawa et al., 2010; Skaper, 2012), while BDNF binding to p75^{NTR} could either potentiate Trk function (Bibel et al., 1999; Ceni et al., 2010; MacPhee and Barker, 1997; Song et al., 2010) or activate cell death cascades (Coulson et al., 2008; Friedman, 2000; Troy et al., 2002). Previous studies conducted by our and other groups demonstrated reduced TrkB levels in different HD models and HD patients (Gines et al., 2006; Gines et al., 2010; Zuccato et al., 2008), as well as increased p75^{NTR} mRNA expression in the caudate nucleus of HD patients (Zuccato et al., 2008), suggesting that deregulation of BDNF receptors in HD could contribute to avoid complete reversion of HD pathology by the administration of the neurotrophin. In this Thesis, we have demonstrated an imbalance in TrkB and p75^{NTR} expression in the striatum and hippocampus, but not in the cortex, of HD mouse models as early as 2-3 months of age, and in HD patients (Work 2 and 3), supporting the hypothesis that not only BDNF reduction but also an imbalance in the expression of its receptors could contribute to early and progressive HD pathology. Moreover, our results have demonstrated reduced cell survival in STHdh^{Q111} mutant cells and corticostriatal slices from Hdh^{Q111} mutant mice after BDNF treatment (Work 2). These results support the hypothesis that altered expression of TrkB and p75^{NTR} receptors could contribute in disturbing BDNF neuronal protection. In agreement with our data, it has been reported that hippocampal neurons expressing increased p75^{NTR} levels and lacking Trk expression exhibit increased BDNF-mediated cell death

(Friedman, 2000; Troy et al., 2002). Importantly, our results demonstrate that p75^{NTR} reduction in corticostriatal slices of Hdh^{Q111/Q111} mice not only increase cell survival but also prevents the cell death induced by BDNF (Work 2), indicating that normalization of p75^{NTR} levels could represent an excellent approach to restore BDNF-mediated signaling in HD (Fig. 27).

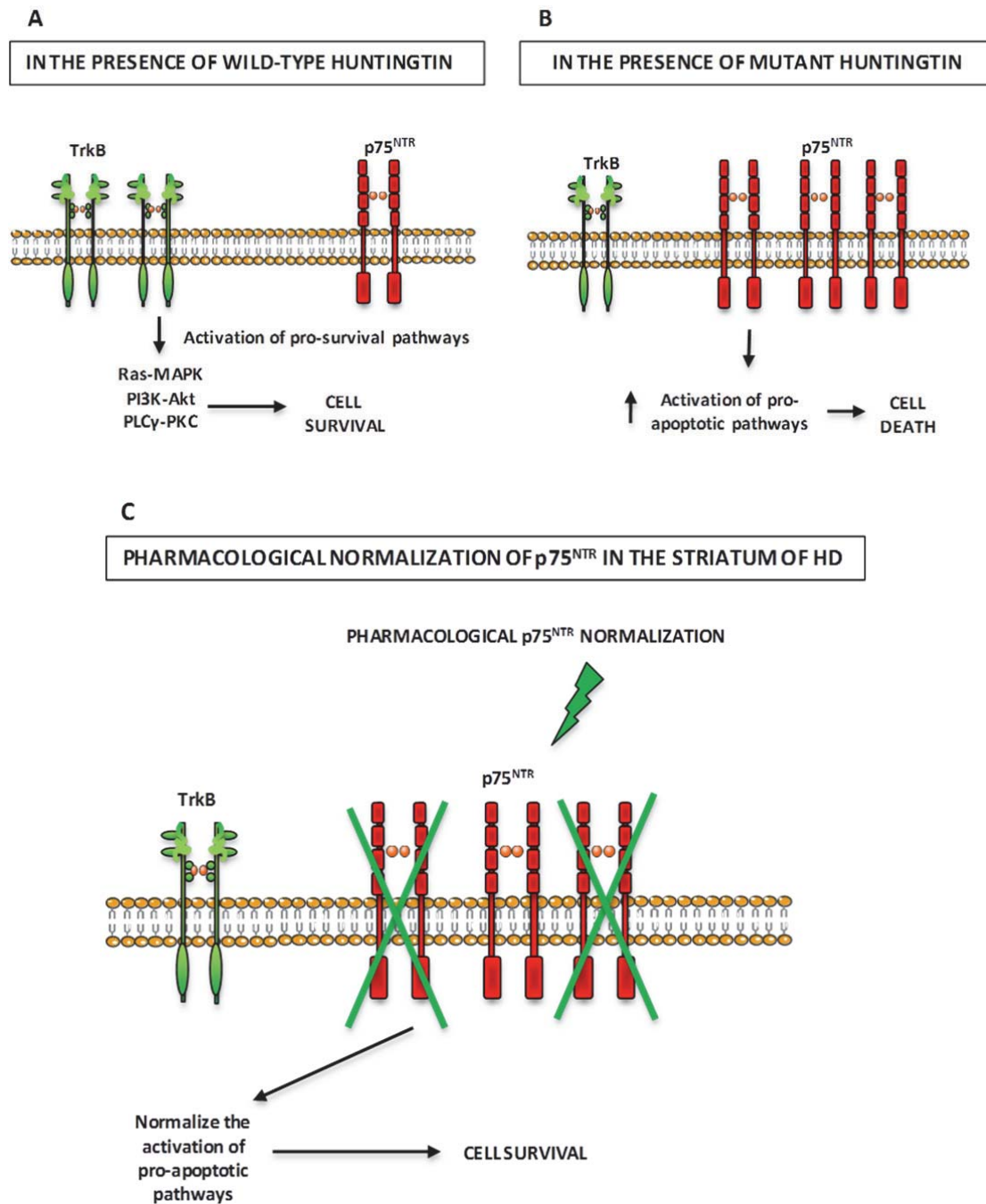


Figure 27. Consequences of TrkB/p75^{NTR} imbalance in the striatum of HD. (A) Whereas in the presence of wild type huntingtin, BDNF stimulates pro-survival pathways, **(B)** the presence of mutant huntingtin disrupts TrkB/p75^{NTR} balance, by decreasing TrkB and increasing p75^{NTR} expression levels, promoting the activation of pro-apoptotic pathways that finally results in cell death. **(C)** p75^{NTR} inhibition decreased the activation of pro-apoptotic pathways in corticostriatal organotypic slices from HD mice, thereby inhibiting cell death and promoting BDNF-mediated cell-survival.

To validate the functional impact of increased p75^{NTR} expression in HD pathology and determine whether p75^{NTR} normalization could improve not only cell survival but also cognitive deficits in HD mice, in this Thesis we have also evaluated the functional impact of p75^{NTR} normalization in the cognitive dysfunction of Hdh^{Q7/Q111} mice.

First, we have analyzed whether p75^{NTR} normalization could restore corticostriatal-dependent cognitive and synaptic transmission deficits in HD mice. Interestingly, general genetic reduction of p75^{NTR} levels in the mouse brain did not prevent motor learning deficits or corticostriatal LTP abnormalities, although a recovery of DARPP-32 levels, a striatal marker known to be reduced in HD mice from early HD stages (Bibb et al., 2000), was found (Work 3). These results suggest that striatal neuronal dysfunction could be slightly ameliorated but not prevented by downregulation of aberrant p75^{NTR} expression in Hdh^{Q7/Q111} mice brain. In this context, it is important to note that p75^{NTR} levels are upregulated in the striatum but not in the cerebral cortex from HD mice or human HD patients (Work 2 and 3), suggesting that decreasing p75^{NTR} levels in the cortex of HD mice can be deleterious for normal forms of synaptic structural plasticity and cognitive processes. These findings lead us to hypothesize that normalization of p75^{NTR} levels specifically in the striatum but not in the cortex of HD mice could help to understand whether an optimal level of p75^{NTR} will be critical in the activation of certain transduction pathways important for corticostriatal synaptic transmission and cognition. In this view, lack of *in vivo* studies regarding the role of p75^{NTR} in corticostriatal-dependent transmission and cognitive processes difficult to elucidate the role of this receptor in corticostriatal deficits in HD. Therefore, we consider that studies aim to evaluate the functional effects of p75^{NTR} inhibition or normalization specifically in the striatum of HD mice will be necessary and

could represent a crucial step to consider p75^{NTR} modulation as a new therapeutic strategy to restore corticostriatal-dependent cognitive and synaptic function in HD.

Second, recent studies revealed a critical role for p75^{NTR} in hippocampal-dependent synaptic plasticity (Barrett et al., 2010; Greferath et al., 2000). The volume of the hippocampus is known to be reduced in premanifest HD individuals (van den Bogaard et al., 2011), while spatial and recognition memory deficits have been reported in moderately advanced HD patients (Berrios et al., 2002; Lawrence et al., 2000; Majerova et al., 2012), supporting the idea that besides an atrophy in corticostriatal connectivity, a more general neurodegenerative process that involves hippocampal function contributes to HD memory deficits, in middle disease stages. Indeed, in this Thesis we have further validated hippocampal dysfunction in our Hdh^{Q7/Q111} mice as previously discussed in the first section of this discussion. Thus, abnormal hippocampal LTP, decreased dendritic spine density in the CA1 region of the hippocampus, as well as hippocampal memory-related deficits appear in HD mice at middle-disease stages (Work 1, 3, 5 and 6), revealing that hippocampal dysfunction is an important hallmark of HD pathology and suggesting that restoring hippocampal function in HD at middle disease stages could represent a promising alternative strategy to prevent memory loss in HD. Therefore, we have also evaluated whether p75^{NTR} normalization in the hippocampus of Hdh^{Q7/Q111} mice could improve hippocampal function in HD. Interestingly, we have found that genetic normalization of p75^{NTR} in Hdh^{Q7/Q111} mice rescues hippocampal synaptic plasticity and memory deficits as well as hippocampal dendritic spine alterations, likely by normalization of the RhoA GTPase activity (Work 3) (Fig. 28 and 29). To further explore whether these improvements were due to a normalization of p75^{NTR} specifically in the hippocampus, or due to a general reduction of p75^{NTR} in all the mouse brain, we have evaluated HD-related cognitive deficits in wild-type mice overexpressing p75^{NTR} specifically in the hippocampus and in Hdh^{Q7/Q111} mice in which p75^{NTR} was specifically decreased in this brain region. Interestingly, our results show that overexpression of p75^{NTR} in the hippocampus of wild type animals reproduces those memory deficits observed in HD mice, while specific p75^{NTR} knockdown prevents the manifestation of cognitive impairments (Work 3). Together, these findings provide strong evidence of

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hippocampal dysfunction in HD and demonstrate that p75^{NTR} upregulation in HD mediates synaptic, learning and memory deficits observed in these mice. In agreement with our data, it has been found that overexpression of p75^{NTR} in hippocampal neurons decreases spine density (Zagrebelsky et al., 2005), while null p75^{NTR} mice exhibit increased hippocampal dendritic spine density, improved spatial learning and enhanced LTP (Barrett et al., 2010; Greferath et al., 2000), which also supports an antagonistic role of p75^{NTR} in synaptic plasticity. Moreover, and in support of our studies, the inhibition of aberrant p75^{NTR} signaling by small molecules has been demonstrated to prevent memory decline and neuritic dystrophy in Alzheimer's disease (Knowles et al., 2009; Knowles et al., 2013). Overall, these data suggests that genetic or pharmacological normalization of p75^{NTR} levels in the striatum and in the hippocampus of HD animals prevents striatal neuronal death (Work 2), ameliorates striatal dysfunction and rescues for hippocampal-dependent HD deficits (Work 3). Although further studies will be needed to determine whether specific normalization of p75^{NTR} levels in the striatum of HD mice could improve corticostriatal function in HD, our data uncover a new role of p75^{NTR} as a potential candidate to treat hippocampal-related HD memory and plasticity deficits as well as to prevent striatal neuronal death at more advanced disease stages.

IN THE PRESENCE OF MUTANT HUNTINGTIN

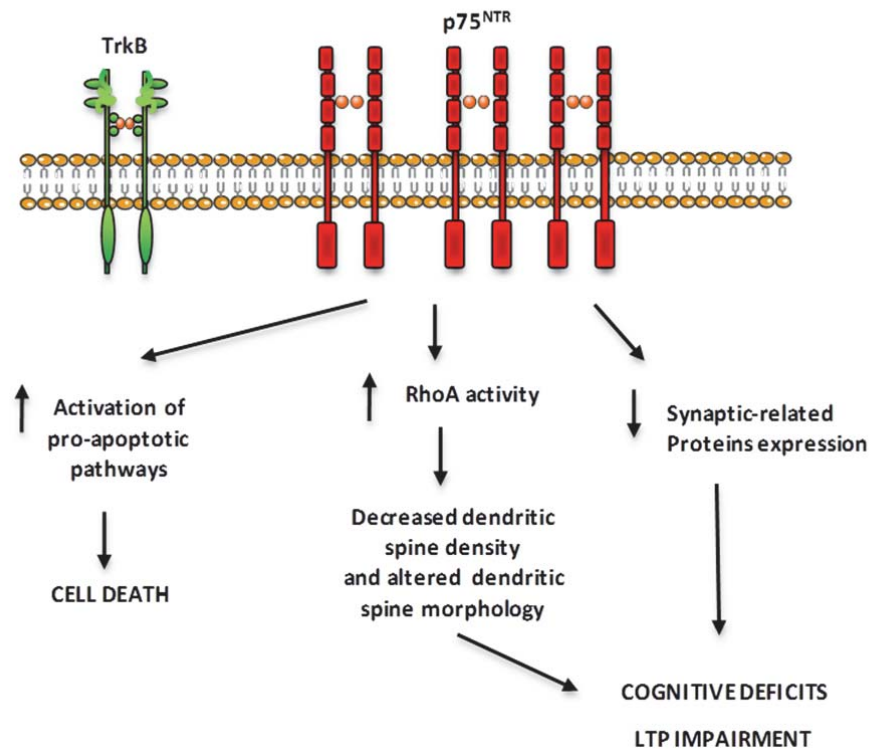


Figure 28. Consequences of TrkB/p75^{NTR} imbalance in the striatum and hippocampus of HD. The presence of mutant huntingtin disrupts TrkB/p75^{NTR} balance not only in the striatum of HD mice promoting the activation of pro-apoptotic pathways leading to cell death, but also in the hippocampus of HD mice. In the hippocampus of HD mice, the upregulation of p75^{NTR} levels induces the activation of RhoA leading to a spine shrinkage and loss. The loss of dendritic spines and the downregulation of synaptic-related proteins by the presence of mutant huntingtin contributes to cognitive and hippocampal LTP impairments.

GENETIC OR PHARMACOLOGICAL NORMALIZATION OF p75^{NTR} IN HD

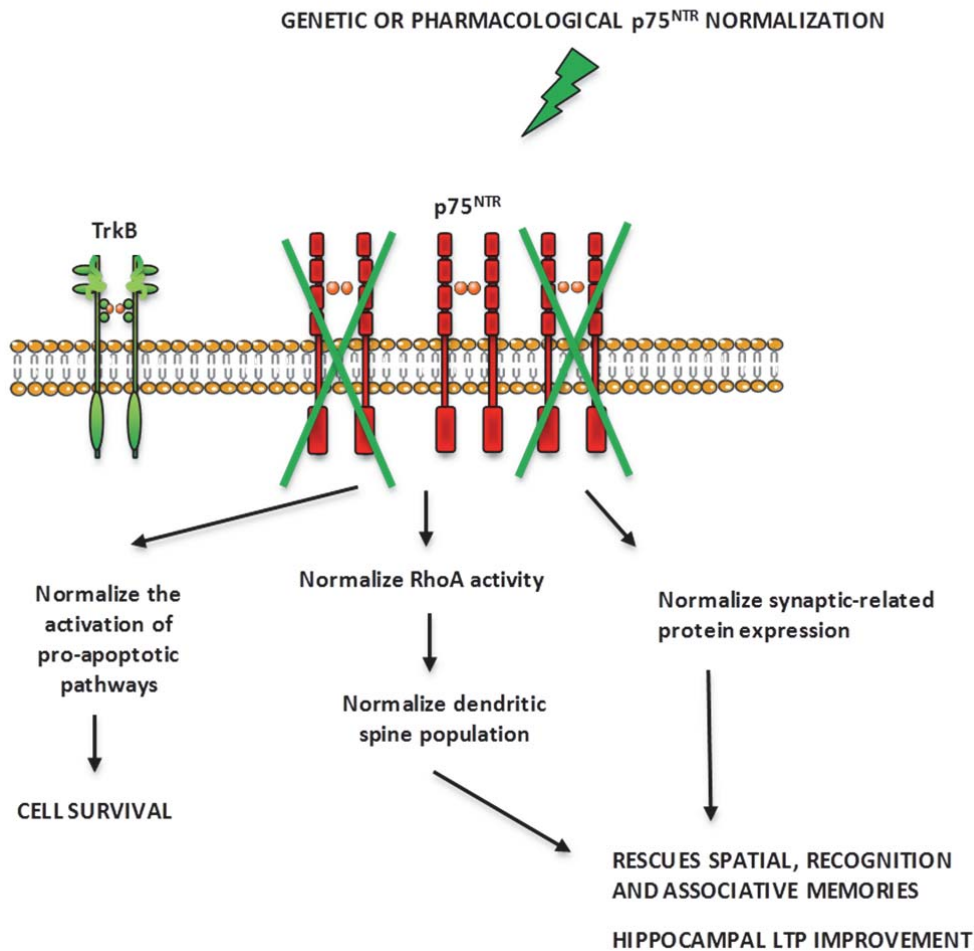


Figure 29. Genetic or pharmacological normalization of p75^{NTR} levels prevents cell death and restores cognitive and plasticity deficits in HD. p75^{NTR} inhibition decreased the activation of pro-apoptotic pathways in corticostriatal organotypic slices from HD mice, preventing striatal cell death. Moreover, genetic normalization of p75^{NTR} levels in Hdh^{Q7/Q111} mice rescues memory and hippocampal LTP deficits by restoring the activation of RhoA activity that in turns restore dendritic spine population and the expression of synaptic-related proteins at the levels of Hdh^{Q7/Q7} mice.

Another therapeutic approach to restore corticostriatal and hippocampal deficits in HD could be the modulation of glutamatergic and dopaminergic neurotransmitter systems due to its implication in HD pathology (Fan and Raymond, 2007; Mota et al., 2014; Perez-Navarro et al., 2006; Stojanovic et al., 2014). In this view, a prevalent theory is that alterations in DA stimuli from the substantia nigra cause an imbalance in striatal neurotransmission, promoting the activation of different cascades

that induce cell death (Paoletti et al., 2008; Ross and Tabrizi, 2011). Interestingly, our group has previously demonstrated that D₁R activation increases neuronal death in mutant STHdh^{Q111} cells, and that NMDA enhances dopamine D₁R-mediated cell death selectively in mutant cells (Paoletti et al., 2008). As an underlying mechanism the kinase Cdk5 was identified, as treatment with Roscovitine (Cdk5 inhibitor) prevents cellular death induced by NMDAR and D₁R activation. Moreover, an aberrant Cdk5 kinase activity has been demonstrated in mutant STHdh^{Q111} cells as well as in the striatum from Hdh^{Q111/Q111} mice and HD patients (Paoletti et al., 2008), suggesting that aberrant Cdk5 activity/signaling contributes to HD pathology. Because Cdk5 inhibition shows potential effects in protecting mutant STHdh^{Q111} cells against neurotoxicity, and Cdk5 has emerged as a key regulator of synaptic plasticity (Cheung et al., 2006; Hawasli and Bibb, 2007; Lai and Ip, 2003; Su and Tsai, 2011), in this Thesis we further explored its potential effects in cognitive dysfunction in HD. To this aim we generated a new transgenic animal expressing mutant huntingtin and heterozygous for Cdk5 (Hdh^{Q7/Q111}; Cdk5^{+/-}). We demonstrate that genetic Cdk5 reduction in Hdh^{Q7/Q111} mice completely restores corticostriatal deficits, such as motor learning and procedural memory deficits (Work 5), the first phenotypic deficits observed in our HD mice (Work 4). Lack of studies regarding the role of Cdk5 activity in modulating corticostriatal-dependent cognition, evidenced that our data is the first one linking Cdk5 modulation and corticostriatal-dependent cognition as well as in showing that reduction (but not completely inhibition) of Cdk5 in Hdh^{Q7/Q111} mice restores corticostriatal learning deficits in HD. To our knowledge, the only study that has provided a link between Cdk5 and corticostriatal transmission is a recent work in which authors demonstrated that pharmacological inhibition of Cdk5 (by Roscovitine) facilitates the induction of LTP in glutamatergic corticostriatal synapses (Miranda-Barrientos et al., 2014), which agree with our data in Hdh^{Q7/Q111}; Cdk5^{+/-} mice showing improved performance in corticostriatal-dependent tasks. Other interesting studies have revealed the important role for Cdk5 in locomotor responses to cocaine. Thus, it has been reported that Cdk5 conditional KO mice exhibit increased excitability of MSNs in the nucleus accumbens (NAc) (Benavides et al., 2007), suggesting that Cdk5 acts as a negative regulator of neuronal excitability not only in the corticostriatal pathway but also in the NAc. Moreover, restricted loss of Cdk5 in NAc neurons facilitates cocaine-induced

locomotor sensitization and conditioned place preference for cocaine (Benavides et al., 2007). In this view, pharmacological or transgenic inhibition of Cdk5 enhances locomotor responses to high-doses of cocaine as well as the development of locomotor sensitization (Benavides et al., 2007; Bibb et al., 2001; Taylor et al., 2007). From our results, together with these evidences it will be interesting to study whether Cdk5 reduction in $Hdh^{Q7/Q111}$ mice restores corticostriatal-dependent learning by rescuing impairments in corticostriatal connectivity.

Importantly, in this Thesis we have demonstrated that Cdk5 reduction in $Hdh^{Q7/Q111}$ mice not only restores corticostriatal dependent cognitive deficits, but also improves the performance of spatial and recognition memory tasks at 6 months of age (Work 5). In agreement with our data, Cdk5 conditional KO mice exhibit enhanced LTP and improved performance in hippocampal behavioral tasks (Hawasli et al., 2007). Moreover, our data suggest that improved cognition in $Hdh^{Q7/Q111}; Cdk5^{+/-}$ mice could be mediated, at least in part, by the modulation of the Cdk5/Src/pTyr1472-GluN2B pathway. Consistently, improvements in synaptic plasticity in conditional Cdk5 KO mice has been attributed to increased GluN2B levels in the membrane surface and decreased GluN2B degradation by calpains (Hawasli and Bibb, 2007). However, we cannot rule out that other underlying mechanisms could contribute to the cognitive improvements observed in $Hdh^{Q7/Q111}; Cdk5^{+/-}$ mice. Since partial reduction of Cdk5 results in improved corticostriatal and hippocampal function in $Hdh^{Q7/Q111}$ mice (Work 5), we propose a genetic or pharmacological modulation of Cdk5 function as a new promising therapeutic strategy with dual effects: in restoring corticostriatal dysfunction, hippocampal dysfunction and preventing striatal vulnerability at late stages of the disease (Fig. 30), but not its complete inhibition, because Cdk5 expression is required in the adult brain for the regulation of different synaptic and memory functions (Fischer et al., 2002; Guan et al., 2011; Hawasli and Bibb, 2007; Ohshima et al., 2005),

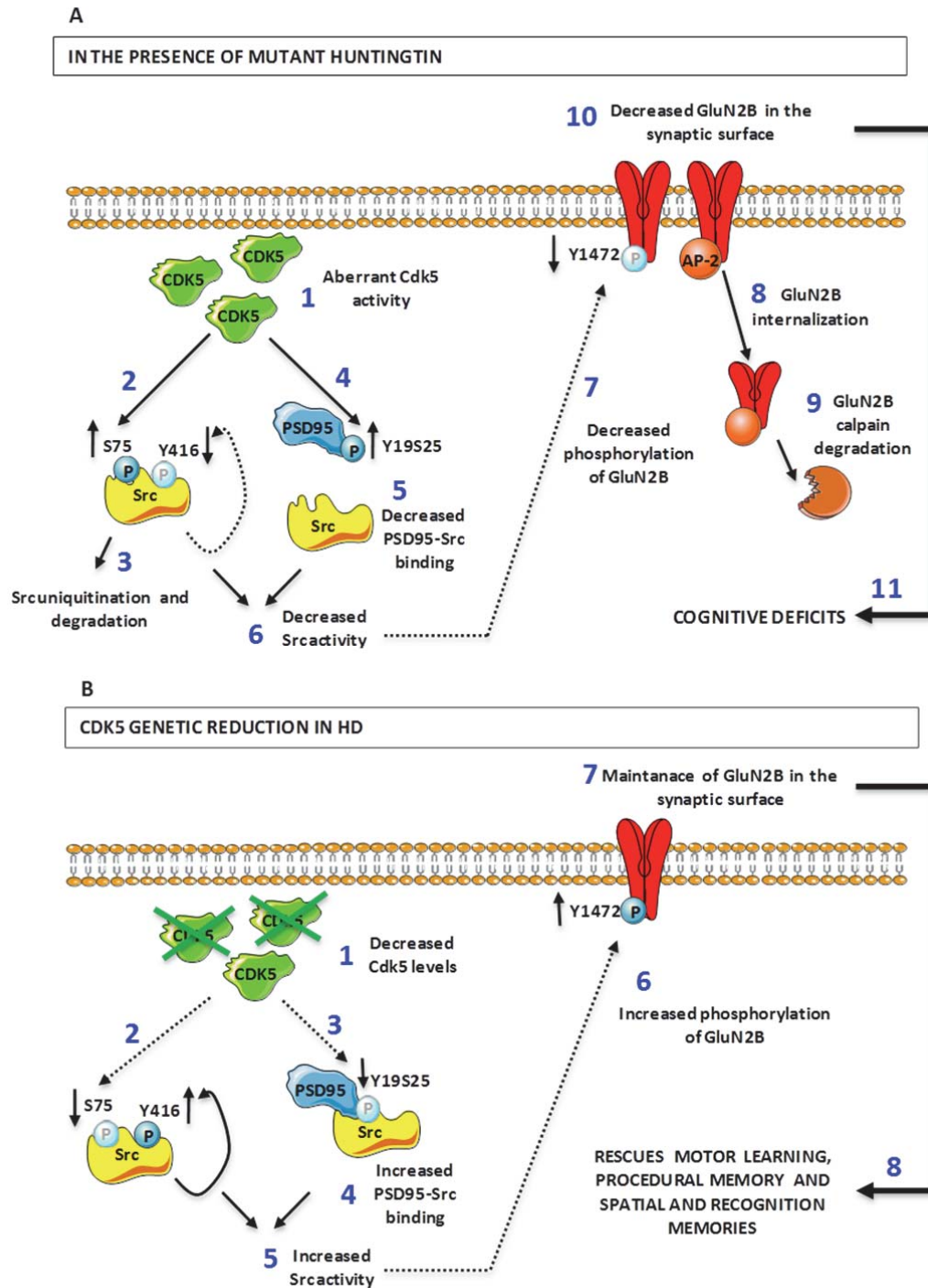


Figure 30. Genetic reduction of Cdk5 levels in *Hdh*^{Q7/Q111} mice rescues corticostriatal and hippocampal cognitive deficits. (A) In the presence of mutant huntingtin: (1) aberrant Cdk5 activity promotes (2) the phosphorylation of Src at S75 leading to (3) Src ubiquitination and degradation (Pan et al., 2011). Moreover, Cdk5 activity can also promote (4) the phosphorylation of PSD95 at Y15S25 (Morabito et al., 2004), (5) decreasing the binding of PSD95 to Src (Zhang et al., 2008). By these mechanisms, an aberrant Cdk5 activity finally results in (6) decreased Src activity. Decreased Src activation results in (7) decreased phosphorylation of GluN2B at Y1472, facilitating AP-2 binding to GluN2B that promotes (8) GluN2B internalization and (9) degradation by calpains. (10) Decreased GluN2B expression in the synaptic

DISCUSSION

surface alters NMDAR function that contributes to (11) the manifestation of cognitive deficits. **(B)** New transgenic mice expressing mutant huntingtin but heterozygous for Cdk5 exhibit (1) decreased Cdk5 levels that probably leads to decreased kinase activity that in turns (2) avoids an aberrant phosphorylation of Src at S75. Moreover, decreased Cdk5 activity (3) reduces the phosphorylation of PSD95 at Y19S25, (4) facilitating the interaction between PSD95 and Src proteins. (5) Increased Src activity as demonstrated by increased phosphorylation of Src at autophosphorylated residu (Y416) results in (6) increased phosphorylation of GluN2B at (Y1472) that (7) preserves GluN2B expression in the synaptic surface that facilitates normal NMDAR function and (8) improves cognitive function.

In the same signaling pathway, we can also modulate the dopaminergic system not only by targeting a particular target for D₁R-mediated signaling, such as Cdk5, but selecting the first step that drives to the alteration in its signaling cascades, the modulation of the main executor: D₁R. As extensively reviewed in the introduction of this Thesis, dopaminergic signaling in HD is deregulated and strategies that might reduce D₁R signaling have been proposed as valuable in preventing neuronal cell death (Paoletti et al., 2008;Tang et al., 2007), aberrant synaptic transmission (Andre et al., 2011b;Andre et al., 2011a) and motor symptoms (Mestre et al., 2009;Tang et al., 2007). Indeed, a randomized controlled trial provided encouraging evidence in favor of the use of Tetrabenazine (TBZ), a dopaminergic inhibitor, for the control of choreic movements in HD patients (Huntington Study Group, 2006;Frank et al., 2008). However, general inhibition of D₁R has side effects such as depression, parkinsonism and sedation in HD patients and HD mouse models (Huntington Study Group, 2006;Frank et al., 2008;Gimenez-Llort et al., 1997), suggesting that new research avenues aim to modulate aberrant dopaminergic signaling in HD are needed. In this Thesis, we have worked on new strategies to modulate D₁R-mediated signaling. Whereas most drug strategies target one single receptor, we take advantage for the recently described GPCRs heteromers, by which one receptor cooperate and physically contact to other receptor thereby conferring novel functions (Kamal and Jockers, 2011). Particularly, it has been recently demonstrated that D₁R form heteromers with H₃R and dopamine D₁R signaling has been shown to be modulated via its association with H₃R (Ferrada et al., 2009;Moreno et al., 2011;Moreno et al., 2014). In this context, it has been shown that H₃R ligands act as a “molecular brake” for D₁R signaling (Moreno et al., 2011), suggesting that the use of H₃R ligands in HD could be useful in modulating aberrant D₁R signaling, thereby preventing D₁R-mediated cell death and

preserving cognitive function. By *in vitro* and *ex vivo* approximations, our results have demonstrated that co-treatment with D₁R agonist and H₃R antagonist completely abrogates D₁R-induced cell death likely by inhibition of D₁R-mediated calcium influx and p38 activation (Work 6), both pathways highly related with neuronal cell death in HD (Cepeda and Levine, 1998;Cepeda et al., 2001;Fan et al., 2012;Gianfriddo et al., 2004;Saavedra et al., 2011;Seong et al., 2005;Starling et al., 2005;Tang et al., 2007) (Fig. 31B). Importantly, we have demonstrated for first time that mutant huntingtin expression induces the loss of D₁R-H₃R heteromers in all analyzed brain regions at mild and advanced disease stages in Hdh^{Q7/Q111} mice (Fig. 31A), which correlates with impairments in corticostriatal and hippocampal cognitive functions (Work 6). Importantly, the loss of D₁R-H₃R heteromers at middle and advanced disease stages is not exclusive for Hdh^{Q7/Q111} mice, as it was also observed in R6/1 and R6/2 mice, as well as in Grade 3 and 4 HD patients, indicating that the loss of heteromerization is a general hallmark in HD condition. Because the heteromer is present in several HD mice at early disease stages and in low grade of human HD patients, we have studied whether the used of H₃R antagonist at early stages could improve corticostriatal and hippocampal cognitive function in HD mice. Our data demonstrates that chronic treatment with thioperamide, an H₃R antagonist, starting at 5 months of age where heteromers are still present and functional, prevents the disruption of the heteromer at advanced HD progression stages and improves the performance of corticostriatal and hippocampal tasks (Fig. 31C), which highlight the need of functional D₁R-H₃R heteromers to restore cognitive function in HD mice. However, we cannot discard that the positive effects observed in our HD mice by the use of H₃R antagonist are exclusive for the modulation of D₁R-H₃R heteromers, as H₃R antagonists can exert effects on other heterodimers or even in H₃R homodimers (Ferrada et al., 2008;Ferrada et al., 2009;Moreno et al., 2011), suggesting that a better characterization of H₃R interaction with other GPCRs will be necessary. Nonetheless, the data presented in this Thesis open a new and promising pharmacological strategy for treating HD pathology at early disease stages: The modulation of aberrant D₁R signaling by targeting D₁R-H₃R heteromers instead of single receptor.

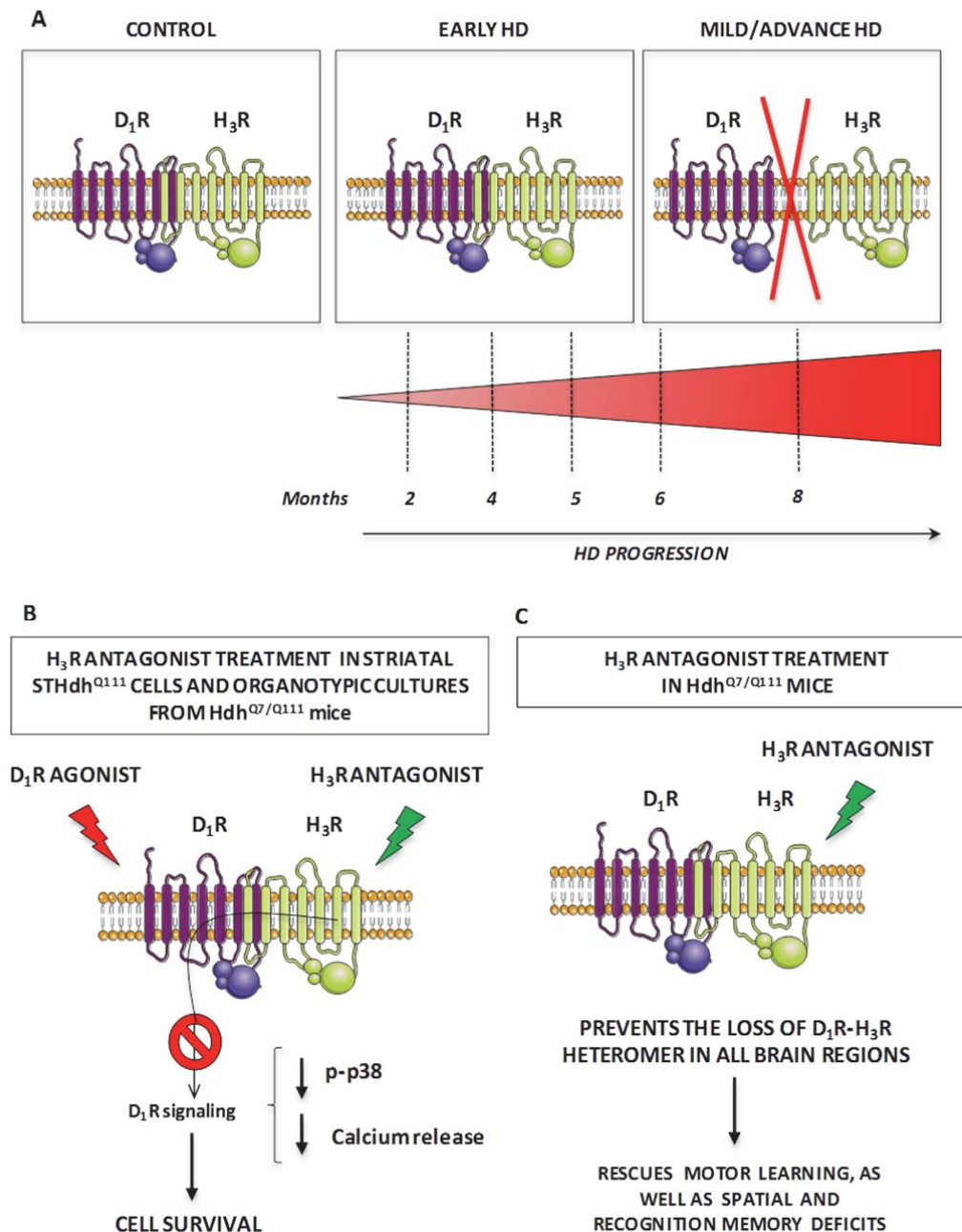


Figure 31. H₃R antagonist treatment prevents neuronal death and rescues cognitive decline in HD. (A) D₁R-H₃R heteromers are expressed in control and early HD disease stages, but disappears at mild and advance disease stages in Hdh^{Q7/Q111} mice. (B) Co-treatment with D₁R agonist and H₃R antagonist decreased D₁R signaling, as demonstrated by decreased phosphorylation of p38 and decreased in calcium release, promoting cell survival in mutant STHdh^{Q111} cells and striatal, hippocampal and cortical organotypic cultures. (C) Chronic H₃R antagonist treatment started at 5 months of age until 6 or 8 months of age prevents the loss of D₁R-H₃R heteromer in all studied brain regions and rescues motor learning as well as spatial and recognition memory deficits in Hdh^{Q7/Q111} mice.

Finally as an alternative strategy to restore hippocampal dysfunction in HD at middle-advanced disease stages, we propose the modulation of CBP levels and/or activity. Activity-induced gene transcription is required for hippocampal synaptic plasticity and memory consolidation (Barco et al., 2003;Kandel, 2001), and among other transcription factors CREB is a well known mediator of synaptic-related gene expression (Silva et al., 1998). Expression of CREB-related target genes are downregulated in several *in vitro* and *in vivo* models of HD (Gines et al., 2003b;Nucifora, Jr. et al., 2001;Sugars et al., 2004). CBP has emerged as a key regulator of CREB-mediated transcription by acting as a CREB transcriptional co-activator and as a histone acetyltransferase (HAT) (Kalkhoven, 2004;Ogryzko et al., 1996;Vo and Goodman, 2001). Several studies have involved CBP in striatal HD pathology, as demonstrated by 1) reduction of striatal CBP levels in HD models (Cong et al., 2005;Jiang et al., 2003;Jiang et al., 2006;McC Campbell et al., 2000;Nucifora, Jr. et al., 2001;Sadri-Vakili et al., 2007), and 2) the blockage of CBP acetyltransferase activity by direct interaction between CBP and mutant huntingtin (Steffan et al., 2001). However, although compelling evidence indicate that CBP exerts an important function in memory and cognitive-related processes (Alarcon et al., 2004;Chen et al., 2010;Valor et al., 2011), the role of CBP in memory HD deficits has not been investigated. In this Thesis we have demonstrated reduced levels of CBP in the hippocampus of Hdh^{Q7/Q111} mice and HD patients (Work 1). Importantly, while CBP levels remain unaltered at early disease stages (4 months of age), an age in which cognitive impairments are not observed (Work 1), reduced hippocampal CBP levels in Hdh^{Q7/Q111} mice were found at 8 months of age which associated with spatial and recognition memory deficits,. In agreement with our results, loss of CBP in the hippocampus of transgenic mice expressing a truncated form of CBP, or in heterozygous CBP^{+/-} mice, as well as in different experimental mouse models of psychiatric and cognitive disorders, has been associated with deficits in synaptic plasticity and long-term memory (Alarcon et al., 2004;Chen et al., 2010;Oike et al., 1999;Saura et al., 2004;Wood et al., 2005). Moreover, our data demonstrates that the reduction in CBP levels in the hippocampus of Hdh^{Q7/Q111} mice may account for 1) its association with oligomeric forms of mutant huntingtin, thereby reducing the levels of soluble CBP, and 2) increased CBP-mediated proteosomal degradation (Work 1), in agreement with previous studies in striatal HD

DISCUSSION

cells (Cong et al., 2005; Jiang et al., 2003; McCampbell et al., 2000; Nucifora, Jr. et al., 2001; Sadri-Vakili et al., 2007). Consistent with the role of CBP in regulating memory-related gene transcription (Hardingham et al., 1999; Wood et al., 2006), our results reveal that reduced CBP levels in $Hdh^{Q7/Q111}$ mice were associated with selective deregulation of CREB/CBP-target genes related to memory and synaptic plasticity (*c-fos*, *Nr4a2* and *Arc*), while no changes in cell proliferation (*Cyr61*) or stress (*Fosb*)-related genes were observed (Work 1). This data demonstrates that CBP reduction in the hippocampus of $Hdh^{Q7/Q111}$ mice results in decreased memory-related gene transcription, suggesting that CBP loss is responsible, at least in part, for the spatial and recognition memory deficits observed in $Hdh^{Q7/Q111}$ mice. Reduced CBP expression and/or activity have been associated with diminished H3 acetylation in mouse models of cognitive dysfunction (Chen et al., 2010; Korzus et al., 2004; Rouaux et al., 2004). Consistently, we demonstrate that decreased CBP levels associates with diminished H3 acetylation in $Hdh^{Q7/Q111}$ mice, suggesting that CBP reduction but also decreased histone acetylation contributes to memory dysfunction in HD (Fig. 32).

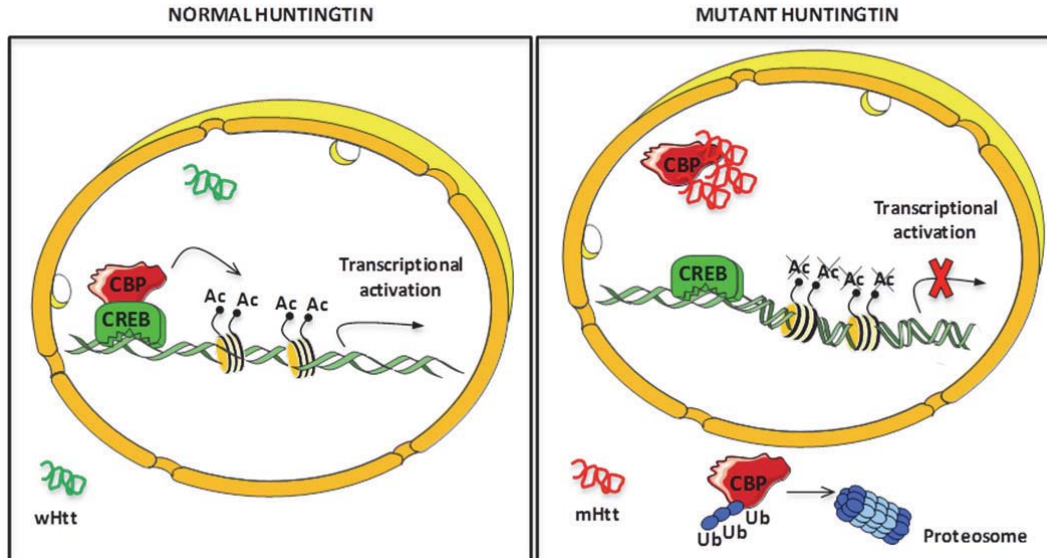


Figure 32. Deregulated CREB/CBP pathway in the hippocampus of $Hdh^{Q7/Q111}$ mice. Whereas in the presence of wild type huntingtin (wHtt), CBP binds with CREB, and by its histone acetyl transferase activity promotes the histone acetylation and activates the transcription of genes involved in neuronal survival and plasticity, the presence of mutant huntingtin (mHtt) decreases CBP levels by its recruitment into mutant huntingtin oligomeric forms and by increasing its ubiquitination and degradation by the proteasome. Moreover the presence of mutant huntingtin decreases the acetylation of the histone 3 causing a condensed chromatin state that in results in decrease transcription of synaptic-related genes, such as *c-fos*, *Nr4a2* and *Arc*.

In an attempt to restore histone acetylation and improve memory deficits in 8 months-old Hdh^{Q7/Q111} mice, we treated these mice with a general HDAC inhibitor (TSA). Our data revealed that TSA treatment reversed long-term memory impairments in Hdh^{Q7/Q111} mice possibly by increasing the expression of *c-fos* and *Arc* (Work 1) (Fig. 33). Supporting our data, previous studies have found that CBP histone acetyltransferase activity play a crucial role in memory consolidation processes (Korzus et al., 2004), and treatment with TSA enhanced hippocampal-dependent memory consolidation by increasing the expression of specific genes during memory consolidation (Vecsey et al., 2007). Because CREB/CBP have been considered potential therapeutic targets for neuronal cell death in HD, and we demonstrated a crucial role of this pathway for the maintenance of hippocampal-dependent long-term memory in Hdh^{Q7/Q111} mice, we propose that therapies aimed to increase CBP levels and/or activity by using HDAC inhibitors would be a good approach for preventing striatal neuronal death and cognitive deficits in HD. Although in this Thesis we did not evaluate striatal survival and motor function after HDAC inhibitor treatment, other studies have demonstrated the potential effects in improving striatal atrophy and survival as well as in preventing brain weight loss and motor symptoms (Chuang et al., 2009), indicating that targeting hypo-acetylation in HD could serve to ameliorate corticostriatal and hippocampal dysfunction as well as motor symptoms and neurodegeneration. Besides research in this area is still in preliminary stages and crucial issues will need to be addressed, such as the development of new potent and more effective HDAC inhibitors with excellent blood-brain-barrier permeability, less cytotoxicity or other side effects, HDAC inhibitors emerge as a promising new avenue for therapeutic interventions in HD.

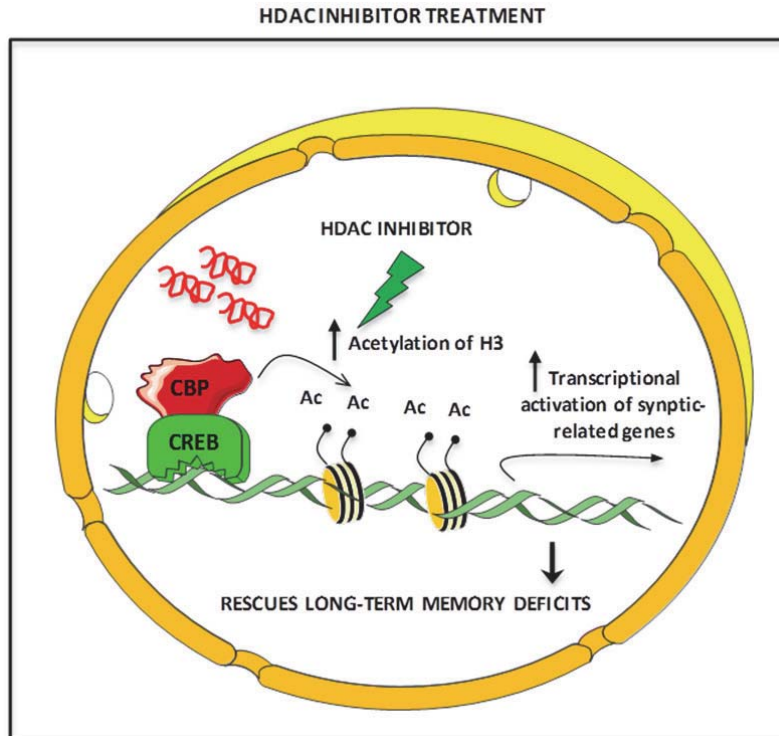


Figure 33. HDAC inhibitor treatment rescues long-term memory deficits in $Hdh^{Q7/Q111}$ mice. General inhibitor of histone deacetylases in $Hdh^{Q7/Q111}$ mice rescues long-term memory deficits by increasing the acetylation of histone 3 (H3) that in turns changes the condensed state of chromatin leading to the transcription of synaptic-related genes.

In conclusion, in this Thesis we provide new evidence for early corticostriatal dysfunction in HD mice at 2-3 months of age, that are followed by hippocampal dysfunction at 6 months of age, prior to the manifestation of motor symptoms at 8 months of age, encouraging the search of novel therapeutic approaches that improves HD pathology at different disease stages. Particularly, we propose that a first therapeutic intervention has to be focus in restoring corticostriatal dysfunction that in turns could contributes in preserving intrinsic striatal function. In this context, we have identified Kalirin-7 as an early candidate responsible, at least in part, for the early altered corticostriatal dysfunction, and place restoration of Kal7 levels as an early therapeutic intervention to restore cortical excitatory synapses. As a second step to improves not only corticostriatal dysfunction, but also hippocampal-dependent deficits in HD, we have proposed 1) the preservation of D_1R - H_3R heteromers in HD brains by antagonizing H_3R , thereby restoring cognitive dysfunction and preventing D_1R -

mediated striatal cell death, 2) genetic or pharmacological inhibition of p75^{NTR} and Cdk5 that will preserve synaptic and cognitive functions as well as will prevent striatal neuronal death, and finally 3) treatment with HDAC inhibitors that exhibit promising therapeutic effects in restoring memory decline in HD and improving striatal survival and motor coordination at more advanced disease stages (Fig. 34).

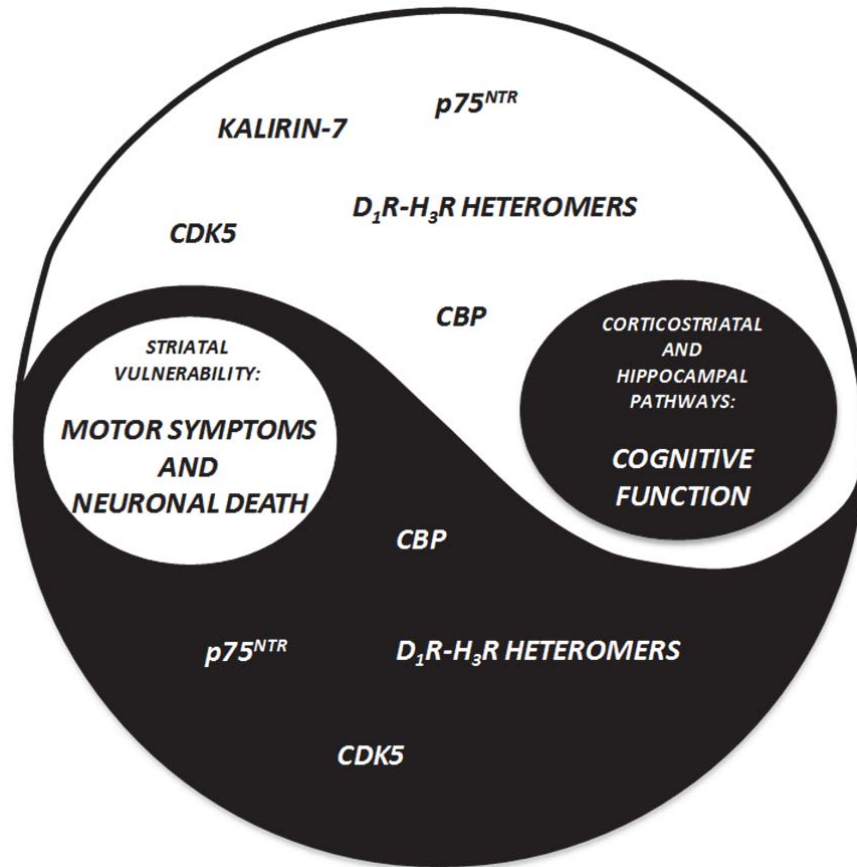


Figure 34. The yin and yang of HD pathology: finding dual targets. In this thesis we have pretended to increase our knowledge in finding common targets to restore corticostriatal and hippocampal dysfunction that finally results in the manifestation of cognitive symptoms, as well as in preventing striatal vulnerability due to mutant huntingtin expression, responsible for the manifestation of motor symptoms and neuronal death.

V. CONCLUSIONS

1. Mutant knock-in Hdh^{Q7/Q111} mice emerge as a useful HD mouse model for studying HD pathology as they accurately reproduce the genetic properties of human condition and exhibit an early and progressive HD phenotype.
2. Long-term memory deficits in Hdh^{Q7/Q111} mice are associated with reduced hippocampal expression of CREB-binding protein (CBP), diminished levels of histone H3 acetylation and reduced expression of CREB/CBP target genes related to memory such as *c-fos*, *Arc* and *Nr4a2*.
3. The administration of the histone deacetylase inhibitor trichostatin A (TSA) rescues recognition memory deficits and transcription of selective CREB/CBP target genes in Hdh^{Q7/Q111} mice, suggesting the use of histone deacetylase inhibitors as a novel therapeutic strategy for the treatment of memory deficits in Huntington's disease.
4. p75^{NTR}/TrkB imbalance induced by mutant huntingtin in striatal cells associated with an aberrant PP1 activity disturbs BDNF neuroprotection, contributing to increase striatal vulnerability in Huntington's disease. Normalization of p75^{NTR} expression improves BDNF neuroprotective effects in corticostriatal organotypic slices of HD mice.
5. Normalization of p75^{NTR} levels in Hdh^{Q7/Q111} mice prevents memory and synaptic plasticity deficits and ameliorates dendritic spine abnormalities, likely through normalization of the activity of the GTPase RhoA. The upregulation of p75^{NTR} expression specifically in the hippocampus of Hdh^{Q7/Q111} mice is a crucial player for synaptic, learning, and memory dysfunction in HD.
6. Cortical dysfunction precedes striatal pathology and mediates early corticostriatal synaptic and cognitive deficits in HD mice. Kalirin-7 downregulation in the cortical region of HD mice at early disease stages emerged as a key contributor of these cortical alterations. Importantly,

Kalirin-7 overexpression in mature cortical neurons rescues the deficits observed in the number of cortical excitatory synapses.

7. Genetic reduction of Cdk5 levels in Hdh^{Q7/Q111} mice restore corticostriatal learning deficits and improves spatial and recognition memories, likely by the modulation of the Cdk5/Src/pTyr1472-GluN2B pathway.
8. Targeting D₁R-H₃R heteromers by using H₃R antagonist reduces D₁R-induced cell death in striatal, hippocampal and cortical brain slices from Hdh^{Q7/Q111} mice. Chronic treatment with Thioperamide, an H₃R antagonist, restores corticostriatal learning deficits as well as spatial and recognition memory decline in Hdh^{Q7/Q111} mice, probably by inhibiting the loss of the D₁R-H₃R heteromer expression at middle and advanced HD stages.

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