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ESTUDIO DE LOS MECANISMOS IMPLICADOS EN LA NEURODEGENERACIÓN ESTRIATAL EN MODELOS MURINOS DE LA ENFERMEDAD DE HUNTINGTON

Tesis presentada por Jesús Fernando Torres Peraza

para optar al título de Doctor por la Universidad de Barcelona

RESULTADOS

TRABAJO 3

Disruption of striatal glutamatergic transmission induced by mutant huntingtin involves remodeling of both postsynaptic density and NMDA receptor signalling.

En preparación.

Disruption of striatal glutamatergic transmission induced by mutant huntingtin involves remodeling of both postsynaptic density and NMDA receptor signalling.

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ABSTRACT

Here, we study the striatal susceptibility to NMDA receptor (NMDAR)-mediated injury of two Huntington's disease (HD) transgenic mice, R6/1 and R6/1:BDNF+/- (with normal or reduced BDNF levels, respectively). We found that R6/1:BDNF+/- mice were more resistant than R6/1 mice to intra-striatal injection of quinolinate. This increased resistance was related to reduced expression of NMDAR scaffolding proteins MAGUKS (PSD-95, PSD-93, SAP-102 and SAP-97), but not with altered levels or synaptic location of NMDAR. Furthermore, NMDAR signalling was differentially affected by BDNF levels in HD mice, as we found a reduction in synaptic αCaMKII only in R6/1:BDNF+/- mice, while nNOS was not affected. Interestingly, we detect a deep reorganization of post-synaptic density (PSD) in HD transgenic mice, as we show a switch of PSD-93 by PSD-95 in PSD. The specific regulation of MAGUKS and αCaMKII in medium spiny neurons may reflect protective mechanism against expression of mutant huntingtin.

INTRODUCTION

Huntington's disease (HD) is due to an abnormal expansion of CAG codon in exon 1 of huntingtin (htt) gene resulting in a devastating cognitive and motor disorder (HDCRG, 1993), which is related mainly with a selective degeneration of striatal GABAergic medium spiny neurons (Reiner et al.,1988). The underlying cause of this selective neuronal loss has not yet been established; however exists growing evidence that links excitotoxicity with degeneration of striatal medium spiny neurons in HD (reviewed in Perez-Navarro et al., 2006). The earliest studies showed that intrastriatal injections of NMDAR agonists in rats (Beal et al., 1986) and primates (Hantraye et al., 1990; Ferrante et al., 1993) mimicked behavioural, neurochemical and neuropathological abnormalities observed in HD. Moreover, NMDAR levels are reduced in the putamen of patients with HD (Young et al., 1988; Dure et al., 1991). The generation of transgenic mouse models of HD confirmed the view that mutant huntingtin modifies NMDA-induced excitotoxicity, although the response is variable depending on the mouse model used. Thus, NR2B-type NMDAR currents are selectively potentiated by mutant htt in transfected non-neuronal cells and acutely dissociated striatal neurons from the YAC72 mice expressing full length mutant htt (Chen et al., 1999; Zeron et al., 2001; Li et al., 2004). Furthermore, these cells have higher intra-cellular Ca^{2+} overload after glutamate application (Tang et al., 2005). In different transgenic mouse model expressing exon 1 of mutant htt, it has been described enhanced NMDAR-mediated current densities (Cepeda et al., 2001) and intracellular Ca^{2+} concentration (Cepeda et al., 2001; Hansson et al., 2001). However, *in vivo* studies of NMDAR agonist-induced injury in HD transgenic mouse models expressing exon-1 or full length mutant htt announce disagreeing findings: YAC72 HD transgenic mice show increased vulnerability to high doses of NMDA (Zeron et al 2002; Tang et al., 2005), while three different HD transgenic mice (R6/1, R6/2 and N171-82Q) are resistant to striatal excitotoxicity induced by the

NMDAR agonist, quinolinic acid (QUIN) (Hansson et al., 1999, 2001; Jarabek et al., 2004), and no change in sensitivity is observed in tgHD100 mice (Petersen et al., 2002). These results suggest the involvement of some kind of compensatory mechanisms in NMDAR properties and intracellular signalling after the chronic excitotoxic stress initiated by mutant huntingtin.

NMDAR properties like synaptic targeting, clustering and trafficking, as well as appropriate interaction with down-stream signalling proteins, are modulated by members of membrane-associated guanilate kinase (MAGUKS) family proteins (Kim and Sheng, 2004). MAGUKS are key proteins enriched in post synaptic densities (PSD) and consist of PSD-95/synapse associated protein (SAP)-90 (Cho et al., 1992; Kistner et al., 1993), SAP-102 (Muller et al., 1996), PSD-93/Chapsyn-110 (Brenman et al., 1996; Kim et al., 1996) and SAP-97/hdlg (Muller et al., 1995). Expression of MAGUKS and NMDAR are temporally and spatially regulated (Sans et al., 2000), supporting their specialized function in controlling MAGUKS-dependent NMDAR properties. Thus, PSD-95 and PSD-93 modulate synaptic properties of NMDAR in adulthood (Sans et al., 2000; Elias et al., 2006) while, SAP-102 controls traffic and synaptic expression of NMDAR during early post-natal development (Sans et al., 2000). Although protein binding domains conserve a large homology between four MAGUKS, a preferential pattern of binding with NMDAR subunits has been described. Thus, NR2A and NR2B subunits preferably bind to PSD-95 and SAP-102 respectively (Sans et al., 2000; Van Zundert et al., 2004). Furthermore, altered MAGUKS experssion has been involved in the pathophysiology of several neurodegenerative disorders of the basal ganglia (Gardoni et al., 2006; Nash et al., 2005), including HD (Jarabek et al., 2004; Luthi-Carter et al., 2002).

Another mechanisms involved in the specific vulnerability of striatal neurons in HD is the regulation of the activity of neurotrophic factors. Between them, BDNF is the most potent trophic factor for striatal projection neurons. Thus, administration of exogenous BDNF protects projection neurons in the excitotoxic (Perez-Navarro et al., 1999, 2000) or transgenic model of HD (Canals et al., 2004). Moreover, reduced levels of endogenous BDNF increase mutant huntingtin-induced degeneration of striatal neurons inducing an accelerated onset and increased severity of motor abnormalities (Canals et al., 2004). However, the mechanism by which BDNF levels modulates mutant-huntingtin induced striatal neuropathology remains unclear. Although some properties and activity of NMDAR are modulated by MAGUKS, a relationship between NMDAR-mediated neurotoxicity and altered MAGUK expression has not been established in HD. In this order we studied whether impaired BDNF trophic support in HD modulates abnormalities of NMDAR signalling in striatal neurons and the possible mechanism implicated in these abnormalities. Our results show that the reduction of BDNF levels in mutant htt-expressing mice increase the disruption of NMDAR signalling in striatal spiny neurons by modulating scaffolding and signalling proteins of NMDAR in the PSD.

EXPERIMENTAL PROCEDURES

Animal model. In the present work we used R6/1 mice (*Mangiarini et al., 1996*), expressing transgenic exon-1 of mutant htt with 115 CAG repeats and normal levels of BDNF (*Canals et al., 2004*) and R6/1 mice with the same transgene but with reduced levels of BDNF (R6/1:BDNF+/-; Canals et al., 2004). To obtain these mice, we cross-mated R6/1 mice with BDNF heterozygous (BDNF+/-) mice (*Ernfors et al., 1994*). We used wild type (wt) and BDNF+/- littermates as controls. For all experiments were used male littermates from the F3 population to avoid strain and sex differences. Mice

were housed together in numerical birth order in groups of mixed genotypes with access to food and water ad libitum in a colony room kept at a constant temperature (19–22°C) and humidity (40–50%) on a 12 hr light/dark cycle. All experiments were conducted in a blind-coded manner with respect to genotype, and data were recorded for analysis by microchip mouse number. All animal-related procedures were in accordance 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 and by the Generalitat de Catalunya. All efforts were made for minimizing suffering of animals. For genotyping, DNA was obtained from tail biopsy and processed for PCR. The primers used for DNA amplifications have been described previously (Mangiarini et al., 1996; Agerman et al., 2003). PCR fragments were resolved in agarose gels of 2% and 1% for bdnf and mutant htt, respectively.

Striatal homogenates and subcellular fractionation. To obtain proteins from total striatal extracts, animals (*n*: 4 to 6 per genotype and time point) were deeply anesthetised in a CO_2 chamber, brains quickly removed and straitum was dissected. For total striatal proteins, tissue was frozen using CO_2 pellets and stored at -80ºC until use. Next, it was sonicated in 200 µl of lysis buffer (phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 µg/ml Aprotinin, 1 µg/ml Leupeptin, 2 mM sodium orthovanadate), centrifuged at 12,000 r.p.m for 20 min, supernatants were collected and frozen until use. Synaptic plasma membrane (SPM) preparation was obtained as reported previously (Perez-Otano et al., 2006). Striatum was homogenized in lysis buffer (4 mM HEPES, 0.32M sucrose, 1 mM PMSF, 10 µg/ml Aprotinin, 1 µg/ml Leupeptin, 2 mM sodium orthovanadate, Benzamidine 0.1mg/ml) and centrifuged at 1000 x g for 10 min. The resulting supernatant was centrifuged at 10.000 x *g* for 15 min to obtain a crude membrane fraction and cytosol/light membrane supernatant (S2). The pellet was resuspended in

deionized H_2O (1 mM PMSF, 10 µg/ml Aprotinin, 1 µg/ml Leupeptin, 2 mM sodium orthovanadate, Benzamidine 0.1mg/ml) in a teflon–glass potter and centrifuged at 25,000 x g for 20 mim. Next, the pellet was resuspended in lysis buffer and centrifuged on a discontinuous sucrose gradient (0.8M, 1M and 1.2M) at 150.000 x g for 2 hr. SPM were collected from 1:1.2M interface and resuspended in 50mM HEPES (2mM EDTA, 1 mM PMSF, 10 µg/ml Aprotinin, 1 µg/ml Leupeptin, 2 mM sodium orthovanadate, Benzamidine 0.1mg/ml). S2 was centrifuged at 100.000 x g for 15 mim to obtain cytosolic (supernatant) and light membrane fractions (pellet).

Western blot analysis. Proteins, from total striatal extracts (15 µg) or from striatal SPM, without head denaturation were loaded in a 7.5% SDS PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked in TBS-T (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.05% Tween 20) with 5% non fat dry milk and 5% BSA. Immunoblots were probed with the appropriate antibodies: Anti-NR1 (1:500), anti-NR2A (1:1000), anti-NR2B (1:1000) (AB1516, AB1555P and AB1557P respectively; Chemicon, Temecula); anti-GluR1 (1:4000), anti-GluR2/3 (1:2000), anti-GluR4 (1:1250), anti-GluR5 (1:2000), anti-GluR6/7 (1:2000) (06-306, 07-598, 06-308, 07-258 and 06-309 respectively; UPSTATE Biotech, NY); mouse anti-PSD-95/SAP-90 (1:1000) and rabbit anti-SAP-97/hdlg (1:1000) (MA1-046 and PA1-741 respectively, Affinity BioReagents, Golden, CO); rabbit anti-SAP-102 (1:1000) (124202, Synaptic System, Germany); rabbit anti-PSD-93 (1:1000) (APZ-002 Alomone Labs, Jerusalen), rabbit anti-nNOS (1:1000) (610310, BD Transduction Laboratories, San Diego, CA), anti-CaMKinase II (1:1000) (sc-9035, Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-NR1 serine 896 (ser^{896}; 1:1000) that recognise NR1 phosphorylated at ser^{896} and anti-phospho NR1 serine 896 (ser^{897}; 1:1000) that recognise NR1 phosphorylated at ser^{897} and dually phosphorylated on ser^{896} and ser^{897} (06-640 and 06-641 respectively, UPSTATE Biotechnology, NY); anti-p38 kinase (1:500) and anti- phopho p38 Kinase (1:1000) (612168, BD Transduction

Laboratories and 9211s, Cell Signalling Technology, respectively). All blots were incubated with the primary anibody overnight at 4ºC in PBS with 0.02% sodium azide. After several washes in TBS-T, blots were incubated with 1:3000 of anti-mouse or anti-rabbit IgG HRP-conjugated (Promega, Madison, WI) and developed by ECL Western blotting analysis system (Bioscience Europe, Freiburg, Germany). As loading controls, we reincubated the membranes with a monoclonal anti-β-tubulin antibody (1:100000, Sigma, St. Louis, MO).

Stripping blots. Blots were stripped of antibodies by incubating at 55°C for 30 min in stripping buffer (62.5 mM Tris pH 6.7, 2% SDS, 100 mM β-mercaptoethanol). Stripped blots were washed 3 times for 5 min in TBS-T and incubed 1 hour with secondary antibody as described above to verify that the stripping was complete. Each sample was immunoblotted first with anti-phospho-NR1ser^{896} or anti-phospho-NR1ser^{897}, stripped and probed with anti-NR1 antibody.

Striatal lesions. 12 weeks-old mice (*n*: 3-5 per genotype) were anaesthetized with pentobarbital (40 mg/kg) and 30 nmoles of QUIN (320-4, Aldrich) in 0.5 µl of sterile PBS were stereotaxically injected into the striatum nuclei (coordinates + 0.6 mm posterior, 2.0 mm left, 2.7 mm ventral from bregma) using a 10 µl Hamilton syringe. QUIN was injected over 2 min and the cannula was left in place for additional 3 min. Animals were monitored for 2 h post-injection and then returned to the animal housing facility for 7 days.

Immunohistochemistry. This assay was performed on paraformaldehyde fixed material as described previously (Torres-Peraza et al., 2007) antibody used was anti-Neu-N (1:100), (MAB377, Chemicon, Temecula). No signal was detected in controls avoiding primary antibodies.

Cell counting. Striatal volumes were measured by Cavaliery's method. Unbiased stereological counts of striatal neurons were performed blind with respect to genotype

and obtained using the Computer Assisted Stereology Toolbox (CAST) software (Olympus Danmark A/S, Ballerup, Denmark) as described previously (Torres-Peraza et al., 2007). The dissector counting method was employed to determine cell density in coronal sections spaced 150 μm apart in the striatal lesion area from bregma 1.05 to 0.15 mm according to Franklin and Paxinos (1997).

All results were expressed as the mean and the s.e.m. of different genotypes and time points. Statistical analysis was performed using ANOVA followed by the L.S.D. post-hoc test.

RESULTS

R6/1:BDNF+/- are more resistant than R6/1 mice to intrastriatal injection of QUIN.

To study the involvement of mutant huntingtin and endogenous BDNF levels in the differential vulnerability of striatal neurons to excitotoxicity, intrastriatal injection of QUIN was performed in 12 week old R6/1 (with normal BDNF levels) and R6/1:BDNF+/- (with reduced BDNF levels), and 7 days later striatal volume and cell density was measured in the lesion area. Intrastriatal injection of QUIN induced a similar reduction of neuronal density in wt and BDNF heterozygous mice (wt: 83±4% and bdnf $^{+/-}$: 82±7 with respect to contralateral striatum, fig. 1A-B). However, this reduction was lower in animals with mutant htt, and interestingly the mice with decreased BDNF levels (R6/1:BDNF+/-; 43±13% respect to contralateral striatum) were more resistant than the mice with normal BDNF levels (R6/1; 14±4% respect to contralateral striatum) to excitotoxic damage (Fig. 1A-B). Since an alteration in striatal volume or neuronal density was previously described in these model at 30 weeks of age (Canals et al., 2004), we analyzed whether exist differences in density of striatal neurons previous to lesion in 12 week old mice. Our results show that R6/1 and

R6/1:BDNF+/- mice had the same striatal volume and density of striatal neurons at 12 weeks of age (data not shown).

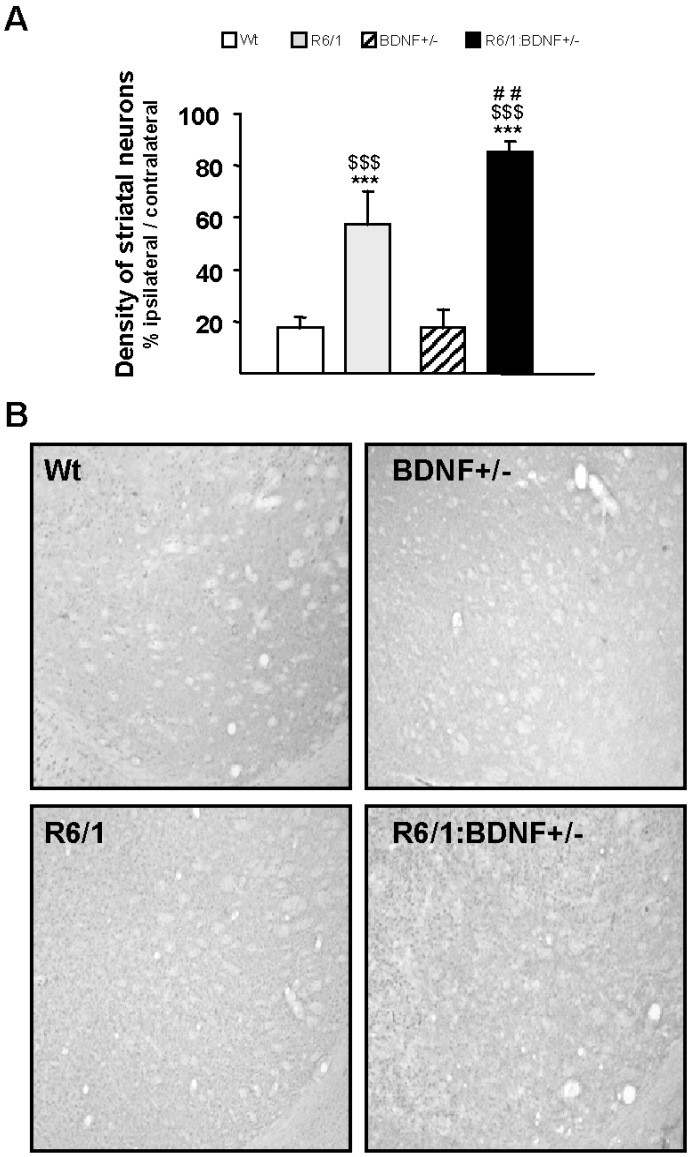

Figure 1: R6/1:BDNF+/- mice are more resistant to QUIN-induced lesion. A: Density of striatal NeuN-positive cells was stereologically determined seven days after unilateral intrastriatal injection of QUIN. Graph shows neuronal density of injected striatum as% respect to non-injected striatum. Bars represent mean±S.E.M. B: Representative microphotograph of striatal NeuN immunohistochemistry 7 days after QUIN injection at 12 weeks of age. ***: p‹ 0,005 respect to wt; $$$: p‹ 0,005 respect to bdnf $^{+/-}$; # #: p‹ 0,01 respect to R6/1; *n*: 3-5 mice per genotype.

Excitotoxic resistance does not depend on abnormal expression of NMDA or AMPA receptor subunits

Expression of NMDAR subunits is known to control NMDAR activity (Koles et al., 2001) and neuronal susceptibility to excitotoxicity (Torres-Peraza et al., 2007, Kovacs et

al., 2001). To study whether reduced NMDAR-mediated injury showed in R6/1 and R6/1:BDNF+/- mice depends on differential expression of NMDAR subunits, we measured striatal levels of NMDAR subunits in pre- and symptomatic stages of disease. We did not observe changes in protein levels of NR1, NR2A and NR2B in wt, BDNF+/- and R6/1 at 8, 12 and 30 week of age (Fig. 2). However, a progressive reduction of NR2B protein levels was observed in R6/1:BDNF+/- mice. Normal levels were detected at presymptomatic stages (8 and 12 weeks), whereas NR2B levels were reduced (35±7% compared with wt) in 30 week old animals (Fig. 2E-F). This is a late symptomatic stage that shows loss of striatal projection neurons (Canals et al., 2004).

On the other hand, we did not find differences in total levels of AMPA receptor subunits (GluR1, wt: 100±4%, R6/1: 87±2%, bdnf $^{+/-}$: 90±0% and R6/1:BDNF+/-: 93±8% respect to wt; GluR2/3, wt: 100±5%, R6/1: 89±9%, bdnf $^{+/-}$: 94±6% and R6/1:BDNF+/-: 87±2% respect to wt; and GluR4, wt: 100±8%, R6/1: 112±13%, bdnf $^{+/-}$: 117±13% and R6/1:BDNF+/-: 102±4% respect to wt).

We next analyzed expression of NMDAR and AMPA receptor subunits in the striatal synaptic plasma membrane (SPM) fraction of 12 week-old mice. We did not observe differences in synaptic expression of NR1 (wt: 100±20%, R6/1: 80±4%, BDNF $^{+/-}$: 93±5% and R6/1:BDNF+/-: 83±3% respect to wt; fig. 3A, D), NR2A (wt: 100±22%, R6/1: 113±9%, BDNF $^{+/-}$: 113±7% and R6/1:BDNF+/-: 110±18% respect to wt, fig. 3B, D) nor NR2B (wt: ±%, R6/1: ±%, bdnf $^{+/-}$: ±% and R6/1:BDNF+/-: ±% respect to wt, fig. 3C, D). In the same way, AMPA receptor subunits show no changes in the expression in SPM preparations (GluR1, wt: 100±5%, R6/1: 105±6%, bdnf $^{+/-}$: 111±5% and R6/1:BDNF+/-: 110±4% respect to wt; GluR2,/3wt: 100±19%, R6/1: 99±26%, bdnf $^{+/-}$:

93±23% and R6/1:BDNF+/-: 111±23% respect to wt and GluR4, wt: 100±7%, R6/1: 103±9%, bdnf $^{+/-}$: 123±4% and R6/1:BDNF+/-: 100±13% respect to wt).

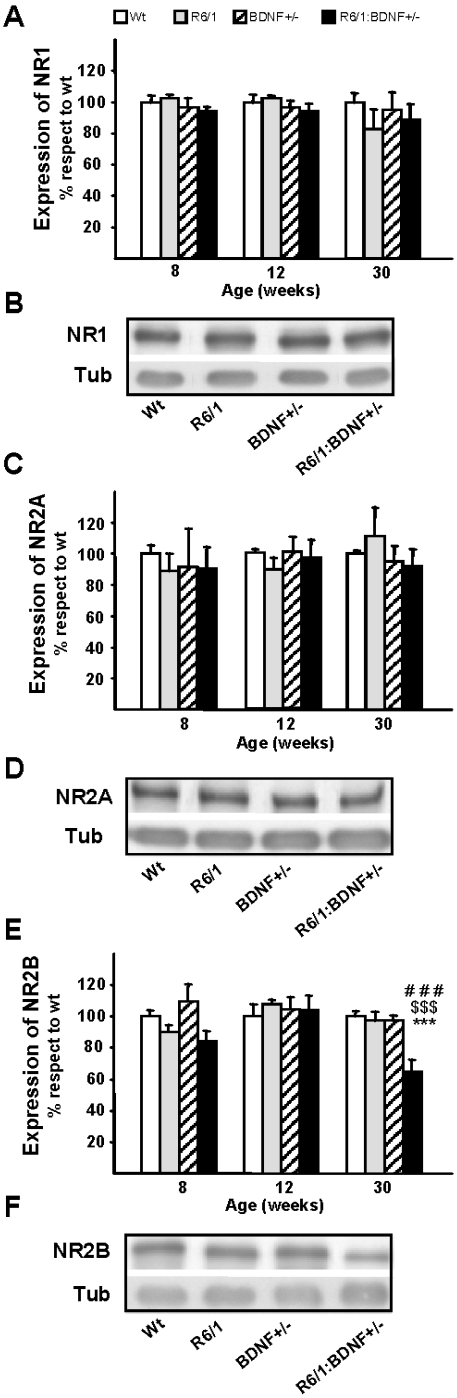

Figure 2: Reduced susceptibility to QUIN-induced lesion of R6/1 and R6/1:BDNF+/- mice does not depend on NMDAR subunit expression. Striatal expression of NR1, NR2A and NR2B subunits was accessed by western blot in total protein extract at 8, 12 and 30 weeks of age. A, C, E: Graphs show quantification of striatal expression of NR1 (A), NR2A (C) and NR2B (E) at ages indicated. Representative blots of total NR1 (B), NR2A (D) and NR2B (F) subunits at 30 weeks of age. Interestingly, R6/1:BDNF+/- mice present a specific down-regulation of NR2B subunit at 30 weeks of age. Bars represent mean±S.E.M, ***: p‹ 0,005 respect to wt; $$$: p‹ 0,005 respect to bdnf $^{+/-}$; # # #: p‹ 0,005 respect to R6/1.

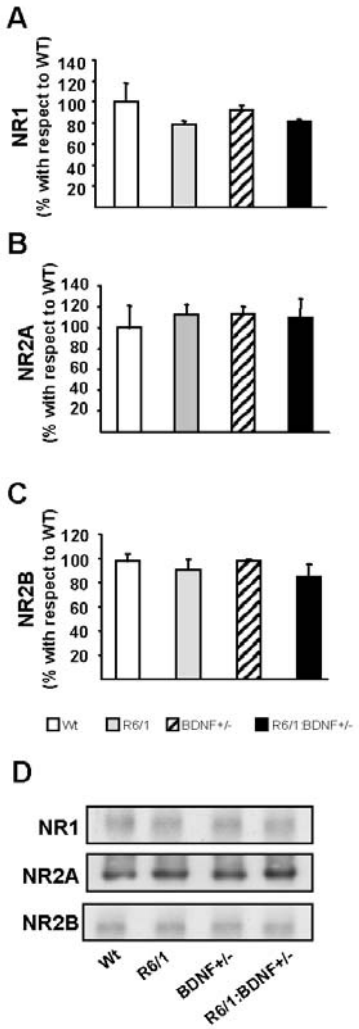

Figure 3: Synaptic expression of NMDAR is not altered in mutant htt-expressing mice. Synaptic expression of NMDAR subunits in striatum was determined by Western blots of synaptic plasma membrane preparations (1µg of SPM protein per lane) at 12 weeks of age. A-C: Graphs show quantification of synaptic NR1 (A), NR2A (B) and NR2B (C). D: Representative blots of synaptic NR1, NR2A and NR2B subunits at 12 weeks of age. For synaptic plasma membrane preparation striatum from 3 mice by each *n* were employed (*n*: 3).

All these result together, demonstrate that striatal resistance observed in R6/1 and R6/1:BDNF+/- mice at 12 week of age does not depend on differences in levels and/or synaptic expression of NMDAR or AMPA receptor subunits. However, we observe that in striatum from mice expressing mutant htt mice both ser^{896} and ser^{897} of NR1 subunit were more phosphorylated, but only phospho-NR1ser^{897} levels were selectively modulated by BDNF. At 12 week of age, R6/1 mice showed an increase of 46±12% in phospho-NR1ser^{897} levels with respect to wt. Interestingly, age matched

R6/1:BDNF+/- mice showed a 99±13% of increase in phospho-NR1ser^{897} levels with respect to wt, which was statistically different from those found in R6/1 mice. In addition, both R6/1 and R6/1:BDNF+/- mice showed increased levels of phospho-NR1ser^{896} (R6/1: 98±26%, R6/1:BDNF+/-: 111±21% with respect to wt), but in contrast to phospho-NR1ser^{897}, mutant htt-induced phosphorylation of NR1 ser^{896} was not affected by BNDF levels, because there was not statistic difference in the levels of phospho- NR1 ser^{896} between R6/1 and R6/1:BDNF+/- mice.

Mutant htt-induced abnormalities in striatal expression of MAGUKS are differentially modulated by BDNF levels

It is well known that MAGUKS interact with NMDAR modulating their traffic and surface expression and linking NMDAR to intracellular signalling pathways. Thus, we quantified striatal levels of PSD-95, SAP-97, SAP-102 and PSD-93 from R6/1 and R6/1:BDNF+/- mice at 8, 12 and 30 weeks of age. In accordance with increased resistance found in mutant-htt expressing mice (Fig.1), we observed reduced levels of MAGUKS in striatum from R6/1 and R6/1:BDNF+/- mice (Fig. 4). Mutant-htt induced a reduction of PSD-95 protein levels in any age analyzed (Fig. 4A-B). However, in late stages (30 weeks) R6/1 mice with low levels of BDNF showed higher reduction of PSD-95 levels (54%) than R6/1 mice with normal levels of BDNF (32%).

SAP-97 protein levels began to decrease at 12 weeks of age in mutant htt-expressing mice (R6/1: 68±12%, R6/1:BDNF+/-: 68±5% respect to wt, figure 4C-D) and showed no differences between R6/1 and R6/1:BDNF+/- mice in older ages. Interestingly, decrease in BDNF levels produced a selective decrease of SAP-102 and PSD-93 levels (figure 4E-H). Thus, R6/1:BDNF+/- mice showed a progressive decrease of SAP-102 from 8 weeks of age (8 weeks, R6/1: 94±11%, R6/1:BDNF+/-: 81±2%; 12

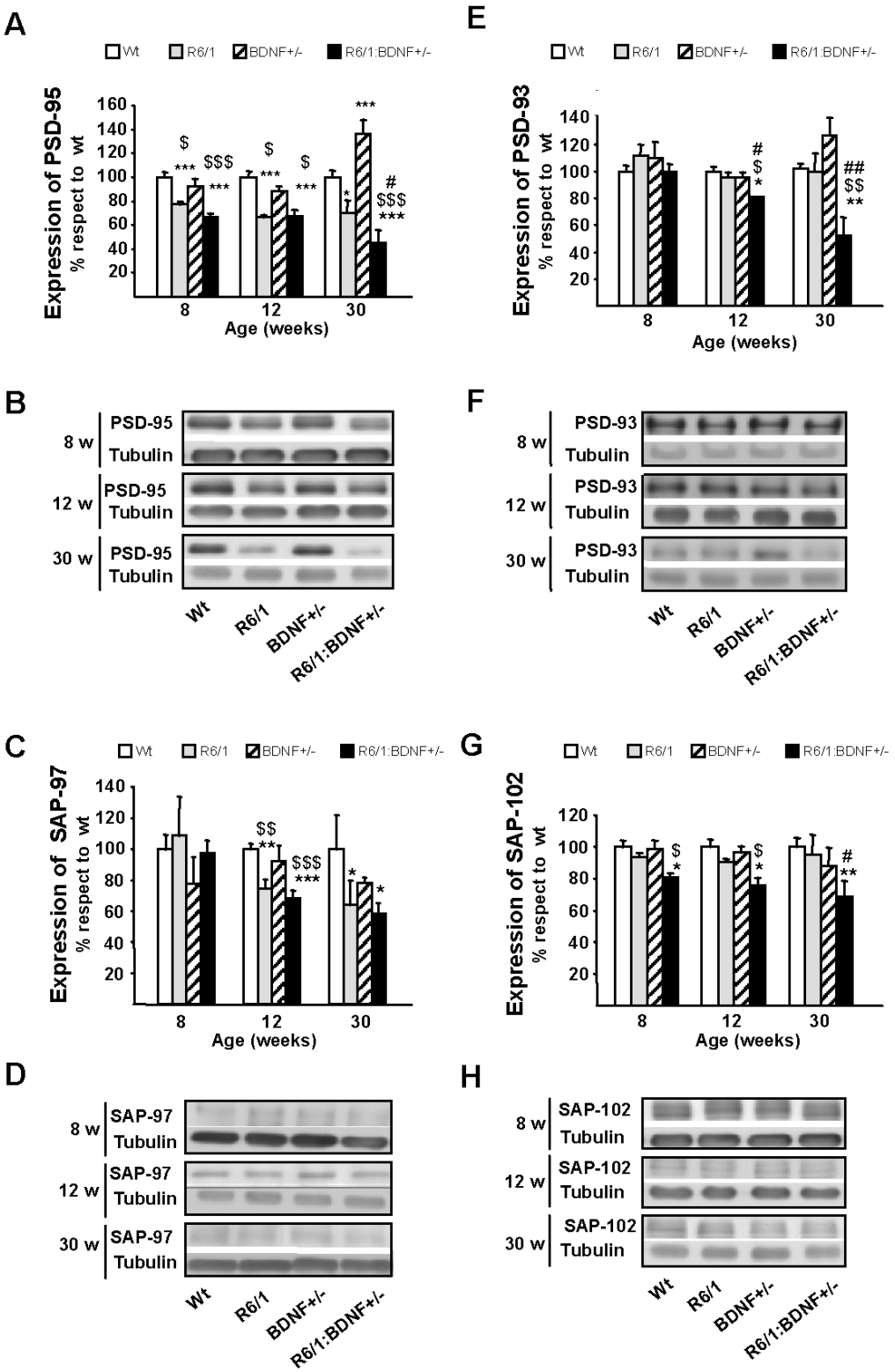

Figure 4: BDNF differentially modulates total expression of MAGUKS in striatum of mutant htt-expressing mice. Graphs show quantification by western blot of striatal PSD-95 (A-B), SAP-97 (C-D), PSD-93 (E-F) and SAP-102 (G-H) at 8, 12 and 30 weeks of age. Mutant htt expressing mice (R6/1 and R6/1:BDNF+/-) present reduced levels of PSD-95 (A) and SAP-97 (C) in striatum. However, total extract from R6/1:BDNF+/- mice present a specific down-regulations of PSD-93 (E) and SAP-102 (G) from 12 and 8 weeks of age, respectively. B, D, F and H: Representative blots of PSD-95 (B), SAP-97 (D), PSD-93 (F) and SAP-102 (H) at ages indicated. Bars represent mean±S.E.M., *: p‹ 0,05, **: p‹ 0,01 and *** p‹ 0,005 respect to wt; $: p‹ 0,05, $$: p‹0,01, $$$: p‹ 0,005 respect to bdnf $^{+/-}$; #: p‹ 0,05 and # #: p‹ 0,01 respect to R6/1; w: weeks of age, *n*: 4-6 mice per age and genotype.

weeks: R6/1: 90± 5%, R6/1:BDNF+/-: 76±7%; 30 weeks, R6/1: 95±4%, R6/1:BDNF+/-: 69±7% respect to wt; figure 4E-F) and PSD-93 from 12 weeks of age (8 weeks, R6/1: 112 ±8%, R6/1:BDNF+/-: 100±5%; 12 weeks, R6/1: 95±4%, R6/1:BDNF+/-: 81±0.3% ; 30 weeks, R6/1: 98±14%, R6/1:BDNF+/-: 53±13%; respect to wt; figure 4G-H). It is important to note that R6/1 and bdnf $^{+/-}$ mice presented no differences in SAP-102 or PSD-93 levels respect to wt mice at any age analyzed (fig. 4E-H), showing that changes in the expression of these two MAGUKS does not depend on expression of mutant htt or reduced levels of BDNF alone.

Differential regulation of synaptic MAGUKS in mutant htt-expressing mice

We next determined synaptic expression of MAGUKS in SPM and cytosol preparations from 12 weeks-old mice. Interestingly, only synaptic expression of PSD-95 was reduced in R6/1 and R6/1:BDNF+/- mice. Thus, mutant htt induced a significant decrease of PSD-95 levels in both SPM (R6/1: 71±4%, R6/1:BDNF+/-: 74±9% with respect to wt; fig. 5A, E) and cytosol (R6/1: 51±7%, R6/1:BDNF+/-: 57±8% with respect to wt; fig. 5F, J). Interestingly, we found a switch between synaptic PSD-95 and PSD-93, since R6/1 and R6/1:BDNF+/- mice showed a significant increase in synaptic levels of PSD-93 (R6/1: 143±7%, R6/1:BDNF+/-: 133±10% with respect to wt; fig. 5B, E). At the same time, cytosolic levels of PSD-93 fall down in both mutant htt-expressing mice (R6/1: 60±11%, R6/1:BDNF+/-: 67±7% with respect to wt; fig. 5G, J). This redistribution of PSD-93 may represent compensatory mechanisms in front to reduction of PSD-95 and it helps to explain normal synaptic expression of NMDAR and AMPA receptor subunits.

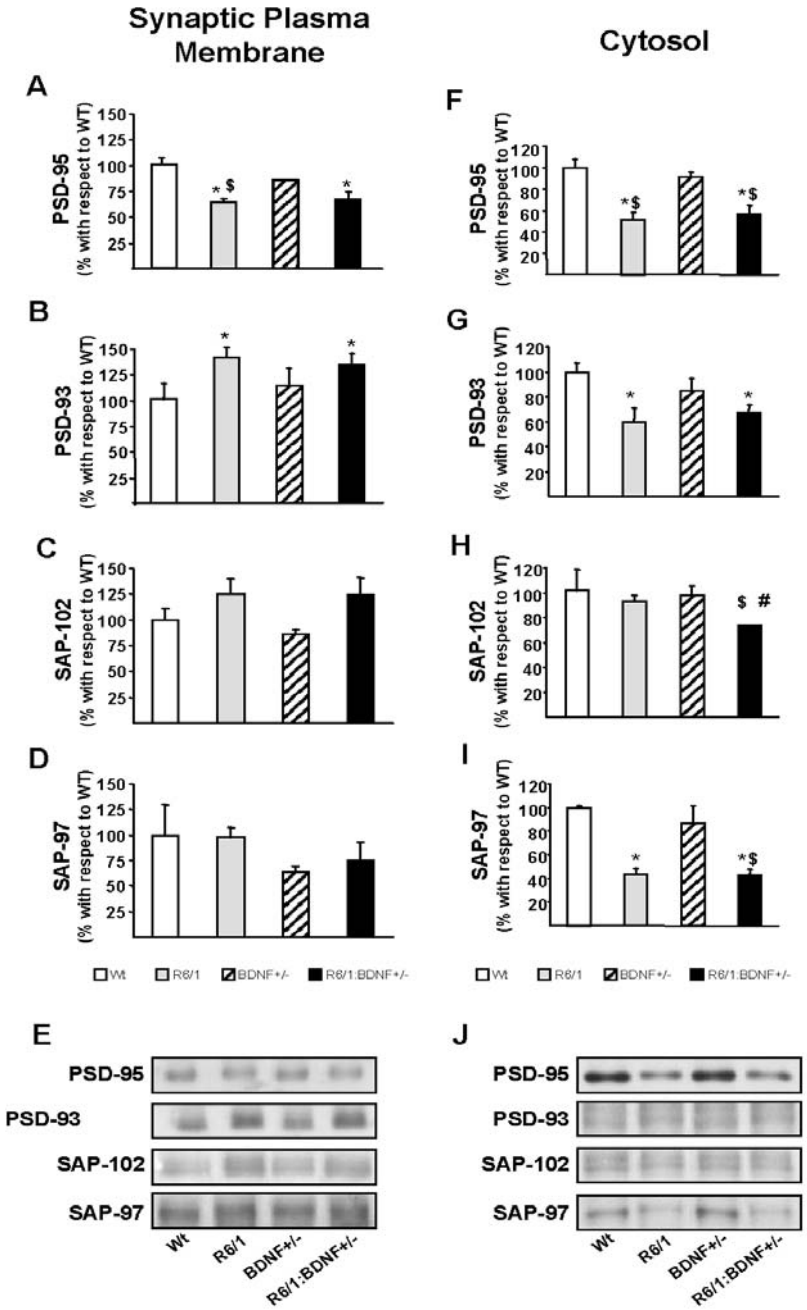

Figure 5: HD transgenic mice show specific alterations in synaptic and cytosolic expression of MAGUKS. Synaptic and cytosolic expression of MAGUKS in striatum were determined by Western blots employing synaptic plasma membrane (1µg of SPM protein per lane) and cytosoloc preparations from 12 week-old mice. A-D: Graphs show that synaptic levels of PSD-95 (A) are significantly reduced in both R6/1 and R6/1:BDNF+/- mice. By contrast, synaptic levels of PSD-93 (B) are increased in these mice with respect to controls. Other MAGUKS, like SAP-102 (C) and SAP-97 (D), present no statistic differences in their synaptic expression with respect to controls. E: Representative blots of synaptic PSD-95, PSD-93, SAP-102 and SAP-97. F-I: Cytosolic expression of MAGUKS is differentially affected in R6/1 and R6/1:BDNF+/- mice. Western blots of cytosolic fractions show that R6/1 mice present reduced levels of PSD-95 (F), PSD-93 (G) and SAP-97 (I), while cytosolic SAP-102 (H) is reduced only in R6/1:BDNF+/- mice. J: Representative blots of cytosolic PSD-95, PSD-93, SAP-102 and SAP-97. Tubulin was employed as load control in cytosolic fraction. Bars represent mean±S.E.M., *: p‹ 0,05 respect to wt; $: p‹ 0,05 respect to bdnf $^{+/-}$; #: p‹ 0,05 respect to R6/1. For synaptic plasma membrane and cytosolic fraction prepration striatum from 3 mice by each *n* were employed (*n*: 3).

On other hand, synaptic levels of SAP-102 show a tendency to increase (but not statistically significant) in R6/1 and R6/1:BDNF+/- mice (R6/1: 125±14%, R6/1:BDNF+/-: 125±16% with respect to wt; fig. 5C, E) while, cytosolic levels of SAP-102 were reduced only in R6/1:BDNF+/- mice (72±1% with respect to wt; fig. 5H, J) that correlate with decreased expression found in total extract (fig. 4G, H). In the same way, SAP-97 showed no significant differences between genotypes in SPM (fig. 5D, E) while, R6/1 and R6/1:BDNF+/- mice have reduced levels in cytosol (R6/1: 44±5%, R6/1:BDNF+/-: 42±6% with respect to wt; fig. 5I, J) that correlates with decreased expression found in total extract (fig. 4C, D).

Specific alteration in NMDAR signalling in R6/1:BDNF+/- mice: Role of αCAMKII.

To test whether aforementioned changes in synaptic expression of MAGUKS compensate PSD-95 deficit and maintain integrity of NMDAR:nNOS complex, we quantified nNOS in both total extract and SPM. The expression of nNOS presented no changes between genotypes in total extract (wt: 100±5%, R6/1: 98±10%, bdnf $^{+/-}$: 109±19% and R6/1:BDNF+/-: 92±5% with respect to wt; figure 6A, C) and SPM (wt: 100±20%, R6/1: 100±12%, bdnf $^{+/-}$: 100±12% and R6/1:BDNF+/-: 109±4% respect to wt; figure 6D, F). Nex, we analysed phosphorylation levels of p38, a protein kinase activated by phosphorylation downstream to nNOS (Cao et al., 2005). In accordance with normal levels of nNOS, we found no differences in levels of phospho-p38 between genotypes (wt: 100±7%, R6/1: 104±10%, bdnf $^{+/-}$: 94±8% and R6/1:BDNF+/-: 105±11% respect to wt). Next, we analyzed striatal expression of αCAMKII since, it is a NMDAR signalling protein implicated in both NMDA and AMPA receptors-mediated neurotoxic pathway (Gardoni et al., 2002). We found that total levels of αCAMKII showed a small (but not significant) tendency to decrease in R6/1:BDNF+/- mice (wt: 100±6%, R6/1: 100±4%, bdnf $^{+/-}$: 101± 7% and R6/1:BDNF+/-: 88±6% with respect to

wt; figure 6B, C). However, synaptic expression of αCAMKII showed a specific decrease in R6/1:BDNF+/- mice (wt: 100±12%, R6/1: 86±8%, bdnf $^{+/-}$: 101±15% and R6/1:BDNF+/-: 62±2% with respect to wt; figure 6E, F) that correlate with increased resistance to NMDAR-mediated injury found in R6/1:BDNF+/- mice (fig. 1).

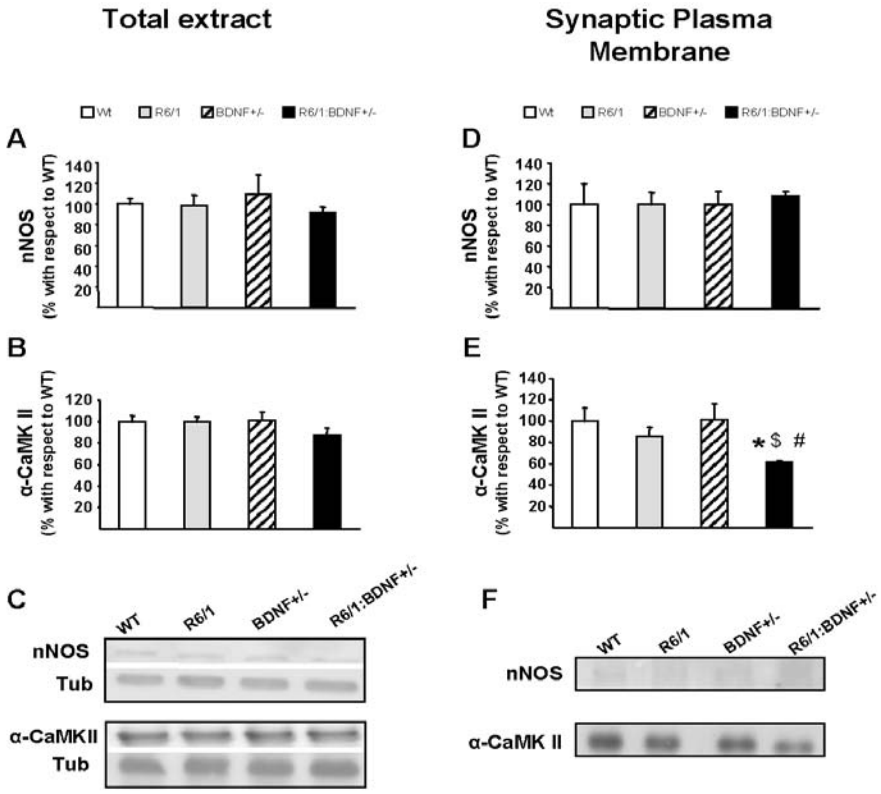

Figure 6: Signalling of NMDAR is selectively impaired in R6/1:BDNF+/- mice. A-F: Striatal expression of nNOS and αCaMKII was accessed by western blot in total protein extract and synaptic plasma membrane preparation (1 and 5µg of protein from synaptic plasma membrane per lane for αCaMKII and nNOS, respectively) from 12 weeks-old mice. Striatal expression of nNOS presents no changes in total extract (A, C) or SPM (D, F). Meanwhile, striatal expression of αCaMKII (B,C) present no changes between genotypes. By contrast, only R6/1:BDNF+/- mice express reduced levels of αCaMKII in SPM (E, F). Bars represent mean±S.E.M., *: p‹ 0,05 with respect to wt; $: p‹ 0,05 with respect to bdnf $^{+/-}$; #: p‹ 0,05 with respect to R6/1; *n*: 4-6 mice per genotype for total extract, and for synaptic plasma membrane purification striatum from 3 mice by each *n* were employed (*n*: 3).

Levels of SAP-102 and PSD-93 are decreased in caudate and putamen nuclei from HD patients.

We next examined levels of MAGUKs in those brain regions from HD patients that are more affected in HD, to determine whether down-regulation of SAP-102 and PSD-93 occur currently in HD. As expected, levels of PSD-95 were decreased in both

caudate and putamen nuclei from HD patients (caudate: 10 ± 3 respect to controls, fig. 7A,C; putamen: 18 ± 7 % respect to controls, fig. 7B-C). In addition, levels of SAP-102 and PSD-93 were significantly decreased in caudate nucleus (SAP-102: 57 ± 9 %, PSD-93: 55 ± 6 % respect to controls, fig. 7A,B), while in putamen nucleus we found a decrease but it did not reach statistical significance (SAP-102: 63 ± 10 % respect to controls, p:0.07; PSD-93: 45 ± 14 % respect to controls, p:0.011; fig. 7B-C).

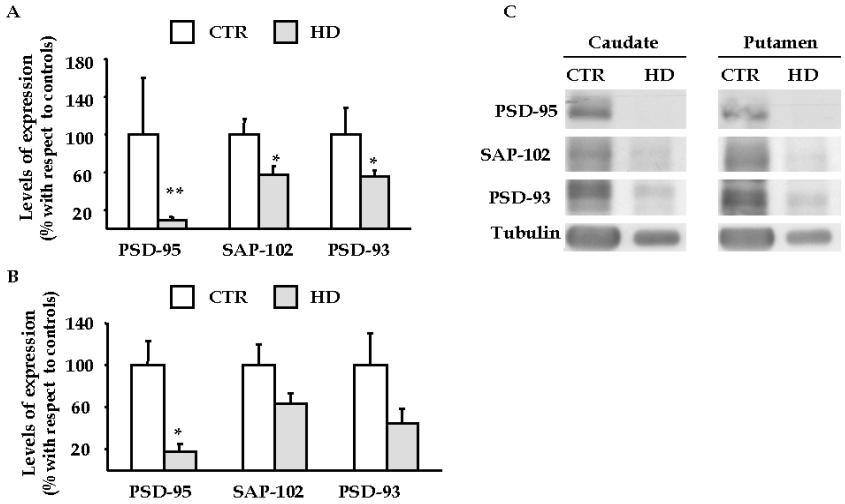

Figure 7: Levels of MAGUKs protein in caudate and putamen nuclei from HD patients were accessed by western blot. A-B: In caudate nucleus (A) as well as in putamen nucleus (B) levels of PSD-95 SAP-102 and CHAPSYN-110 were decreased with respect to controls. C: Representative blots of MAGUKs from caudate and putamen nuclei of controls and HD patients. CTR: controls (*n*:3) ; HD: HD patients (*n*:4). *: p‹ 0,5; ** p‹ 0,005).

DISCUSION

Here we show that mutant htt modifies NMDAR function activating protective compensatory mechanisms against excitotoxic damage. Thus, R6/1 mice are resistant to intrastriatal injection of the NMDAR agonist, QUIN. This resistance is dependent on the severity of the pathology, since R6/1 mice with low BDNF levels (R6/1:BDNF+/-), which have striatal neurodegeneration and worst motor activity performance, were more resistant to QUIN lesion than R6/1 with normal BDNF levels. The levels of NMDAR subunits were not affected in the striatum of mice expressing mutant htt,

except NR2B that was decreased in late stages in R6/1:BDNF+/- mice. However, their signaling pathways were severely altered. Mutant htt induced a disorganization of NMDAR scaffolding proteins. A differential age-dependent decrease in the levels of PSD-95, PSD-93, SAP-97 and SAP-102 was observed in total extracts. However, in SPM only PSD-95 and PSD-93 were altered with a different pattern: A decrease in PSD-95 and an increase in PSD-93. These changes were similar in R6/1 and R6/1:BDNF+/- mice. Furthermore, a reduction in αCaMK II levels in SPM were observed only in animals with severe pathology (R6/1:BDNF+/-). This selective regulation of MAGUKS and αCaMKII may be correlated with the lack of QUIN effect on striatal neurons in R6/1 mice.

Abnormal glutamate receptor function can underlie some of the motor and cognitive impairments found in transgenic models of HD. Thus, the excitotoxic hypothesis is based on evidence that mutant htt is able to increase NMDAR function from the pre-symptomatic stage of disease (Cepeda et al., 2001), producing an increased NMDAR-dependent Ca^{2+} influx (Hansson et al., 2001) that in turn, gives rise to neuronal degeneration (Zeron et al., 2002). Here, we show that R6/1 mice show increased levels of phosphorylated NR1 NMDAR subunit and it was higher in R6/1 mice that express reduced levels of BDNF. These results correlates with increased activity and currents found in pre-symptomatic HD transgenic mice (Cepeda et al., 2001; Hansson et al., 2001), since PKA- and PKC-meditated serine phosphorylation of NR1 subunit increase currents and open rate of NMDAR (Westphal et al., 1999; Logan et al., 1999; Rosenblum et al., 1996; Rostas et al., 1996). However, R6 mice exhibit striatal resistance to QUIN (Hansson et al., 1999, 2001; and present results) that progress as the symptoms worsen (Hansson et al., 2001). In the present work, our results demonstrate that this resistance is

directly related to the severity since diminution of BDNF levels in R6/1 enhance the severity of motor dysfunction (Canals et al., 2004) and reduce QUIN-induced striatal damage (present results). A similar relationship between the severity of the disease and striatal resistance was observed when striatal susceptibility to QUIN of R6/1 and R6/2 was compared (Hansson et al., 2001). However, increased susceptibility to NMDAR-mediated striatal neurotoxicity has been observed in HD transgenic mice expressing mutant full length htt (YAC72 and 1YAC128), which show slower progression of motor impairment that R6 models (Zeron et al., 2002; Tang et al., 2005). Taken together, these results suggest that mutant htt triggers different compensatory mechanisms leading to a reduced striatal susceptibility to QUIN that may be related to the degree of striatal neuronal dysfunction.

Previous works by other groups support the idea that expression of mutant htt causes conditioning changes resulting in an up-regulation of defense mechanism to excitotoxic stress (Hansson et al., 2001; Gines et al., 2003; Jarabek et al., 2004). This mechanism may be related to neuronal pre-conditioning, which is a phenomenon whereby a non-toxic over activation of NMDAR provides tolerance against damage induced by a major insult (Llado et al., 1999; Ogita et al., 2003; Tarabal et al., 2005). Interestingly, at 30 weeks of age, mice showed reduced levels of NR2B subunit without affectation of other subunits. These results suggest a specific affection of those neurons that express NR2B subunits which correlates with a selective degeneration of striatal projection neurons (Canals et al., 2004) which mainly express NR1/NR2B receptor complex (Standaert et al., 1999; Landwehrmeyer et al., 1995). Similarly, NMDAR expression is reduced in post-mortem samples of human HD patients (Young et al., 1988; Dure et al., 1991), suggesting that NMDAR-expressing cells are the most affected in HD. In

agreement with these results, it has been previously shown that NR2B subunit receptor is implicated in the initial degeneration of striatal projection neurons in another mouse model YAC 72 (Zeron et al., 2002). Our results also demonstrate that this decrease in the levels of NR2B does not correspond with striatal resistance to NMDA since no affection of this subunit receptor has been observed in pre-symptomatic mice neither in total or SPM levels. However, total levels of MAGUKS are reduced in young mice when they show resistance to QUIN. These findings suggest that MAGUKS may be more determining than NMDA receptors *per se* in the vulnerability of striatal neurons. Decreased levels of MAGUKS may be consequence of over-activation of NMDAR induced by their increased phophorylation. In support of these view, over-activation of NMDAR give rise to dissociation of NMDAR:PSD-95 complex and leads to PSD-95 degradation by the ubiquitin-proteome system (Colledge et al., 2003). Interestingly, we also observed that the total levels of MAGUKS are reduced in samples of postmortem HD human patients. It has been shown that reduction of protein levels of both PSD-95 and PSD-93, reduce both AMPA and NMDAR excitatory post-synaptic currents (Elias et al., 2006). Among all MAGUKS, it seems that PSD-95 may be the most important involved in HD since this is reduced in both cytosol and SPM. In agreement, it has been shown that down-regulation of PSD-95 by PSD-95 anti-sense *in vitro* (Sattler et al., 1999*)* and *in vivo* (Hou et al., 2005) confer increased resistance to excitotoxicity and ischemia, respectively.

It is well demonstrated that MAGUKS interact with different NR2 subunits early from endoplasmic reticulum and that they are involved in trafficking, synaptic targeting and clustering of NMDAR (Kim and Sheng, 2004). In this sense, it is noteworthy that synaptic levels of NMDAR subunits and NR2B levels in light

membrane fraction (corresponding to endomembranes, data not shown) remain unchanged in spite of altered level of MAGUKS. Thus, normal synaptic levels of NMDAR found in HD mice reinforce the hypothesis that synaptic expression of NMDAR is affected only when the levels of MAGUKS falls bellow a critical threshold. Knockdown of SAP-102 *in vivo* does not alter NMDAR-dependent synaptic transmission, but reduces NMDAR excitatory post-synaptic currents only when a lack of both PSD-95 and PSD-93 occurs (Elias et al., 2006). In this sense, it is possible that kinetic of MAGUKS-dependent traffic and/or synaptic expression of NMDAR must be affected in more advances stages of disease, when strongest reduction in SAP-102, PSD-93 and PSD-95 levels occurs in R6/1:BDNF+/- mice. All these results together may reflect compensatory mechanism between MAGUKS. Thus, we show that synaptic levels of PSD-95 were reduced in SPM of HD transgenic mice, while synaptic expression of PSD-93 increases while its cytosolic expression decrease, suggesting that PSD-93 trans-locates from cytosol to SPM. It has been shown that functional compensation by molecular redundancy among MAGUKS may occur as mutant mice with simultaneous lack of PSD-95 and PSD-93 (Elias et al., 2006), or expressing mutant PSD-95 (Vickers et al., 2006), show compensatory increase of SAP-102.

The ability of MAGUKS to regulate NMDAR pathway may depend, at less in part, on possibility of binding both NMDAR and nNOS in a ternary complex (Sattler et al., 1999; Dawson and Dawson, 1996; Aarts et al., 202; Cao et al., 2005). However, our results demonstrate that synaptic levels of nNOS and levels of phospho-p38 kinase remain unaltered in spite of reduced synaptic levels of PSD-95, suggesting that in our models the nNOS-p38 pathway may not be involved in striatal resistance to QUIN. Interestingly, reduced synaptic expression of αCaMKII correlates with increased

striatal resistance to QUIN found in R6/1:BDNF+/- mice. In agreement, it has been demonstrated that αCaMKII is involved in excitotoxic pathway (Hajimohammadreza et al., 1995; Gardoni et al., 2002). Thus, increased phosphorylation of αCaMKII occurs after brain ischemia (Yan et al., 2004) and traumatic brain injury (Atkins et al., 2006) and previous treatment with a specific CaMKII inhibitor provides neuroprotection against NMDA induced excitotoxicity and hypoxia/hypoglycemia-induced neuronal insult (Hajimohammadreza et al., 1995)., In addition, recent findings showed that activation of αCaMKII potentiates AMPA receptors-mediated Ca^{+2} influx (Gardoni et al., 2002). These results also suggest that BDNF modulates changes in NMDAR signaling in PSD induced by mutant htt, as synaptic levels of αCaMKII are specifically reduced in R6/1:BDNF+/- mice, but not in BDNF+/- or R6/1 mice.

Thus, present results show molecular evidence that reorganization of NMDAR signaling proteins occurs in PSD of HD transgenic mice. These changes may reflect important basis underlying dysfunctions of NMDAR and their intracellular signaling in HD neuropathology.

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