Increased translation as a novel pathogenic mechanism in Huntington’s disease

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Huntington’s disease is a neurodegenerative disorder caused by a CAG repeat expansion in the exon 1 of the huntingtin gene. Striatal projection neurons are mainly affected, leading to motor symptoms, but molecular mechanisms involved in their vulnerability are not fully characterized. Here, we show that elf4E binding protein (4E-BP), a protein that inhibits translation, is inactivated in Huntington’s disease striatum by increased phosphorylation. Accordingly, we detected aberrant de novo protein synthesis. Proteomic characterization indicates that translation specifically affects sets of proteins as we observed upregulation of ribosomal and oxidative phosphorylation proteins and downregulation of proteins related to neuronal structure and function. Interestingly, treatment with the translation inhibitor 4EGI-1 prevented R6/1 mice motor deficits, although corticostriatal long-term depression was not markedly changed in behaving animals. At the molecular level, injection of 4EGI-1 normalized protein synthesis and ribosomal content in R6/1 mouse striatum. In conclusion, our results indicate that dysregulation of protein synthesis is involved in mutant huntingtin-induced striatal neurons dysfunction.

Introduction

Huntington’s disease is a progressive neurodegenerative disorder characterized by motor and cognitive impairment (Martin and Gusella, 1986). It is caused by a CAG trinucleotide repeat expansion within the exon 1 of the Huntingtin (Htt) gene, which leads to lengthening of the polyglutamine chain in the N-terminus of the translated protein (HDCRG, 1593). The result is an aberrant and misfolded mutant protein (mHtt) associated with protein...
aggregation and toxicity. Although mHtt expression is ubiquitous in the brain, the most vulnerable region is the striatum (Han et al., 2010). The molecular basis that accounts for this specific neurodegeneration is still not clear, but different mechanisms have been proposed to contribute to the neurodegenerative process (Labbadia and Morimoto, 2013). Expression of mHtt disrupts the normal signalling of many intracellular pathways (Bowles and Jones, 2014), and it also induces the activation of compensatory mechanisms to prevent or delay cell dysfunction and apoptosis (Kreiner, 2015). Many intracellular pathways altered in Huntington’s disease participate in the regulation of protein translation (Wang and Proud 2006; Osterweil et al., 2010; Wiseman et al., 2013), namely the mechanistic target of rapamycin (mTOR) (Pryor et al., 2014; Lee et al., 2015), ERK1/2 (Saavedra et al., 2011) and PKA pathways (Tycbi et al., 2015). Interestingly, increased protein synthesis has been associated with neuronal toxicity or dysfunction occurring in some neurological and neurodegenerative disorders (Sharma et al., 2010; Santini et al., 2013; Balena et al., 2014; Martin et al., 2014a; Topol et al., 2015).

The initiation of protein synthesis is controlled by the eukaryotic translation initiation factor 4E (eIF4E) complex, composed of the cap-binding protein eIF4E, the RNA helicase eIF4A and the scaffolding protein eIF4G that recruits the mRNA to the ribosome. The main regulator of this complex is the eIF4E binding protein (4EBP), which binds to and inactivates eIF4E. The phosphorylation of 4EBP disrupts its interaction with eIF4E, which can then interact with eIF4G and eIF4A to form the eIF4F complex, responsible for cap-dependent translation initiation (Pause et al., 1994). Phosphorylation of 4EBP occurs initially at Thr37/Thr46 by the mTOR complex 1 (mTORC1), which is sufficient to block its association with eIF4E. Subsequent phosphorylation occurs at Thr70 followed by Ser65 (Gingras et al., 1999). In addition to mTORC1, GSK3β can also fully phosphorylate 4EBP (Ito et al., 2016). Thus, altered 4EBP phosphorylation may cause an alteration in the translational control and, consequently, an exaggerated and aberrant protein synthesis.

In Huntington’s disease striatum, the activity of mTORC1 and GSK3β are altered (Ravikumar et al., 2004; Valencia et al., 2010; Lim et al., 2014; Pryor et al., 2014; Fernández-Nogales et al., 2015; Lee et al., 2015, 2016), which suggests a possible impact on 4EBP phosphorylation and consequently on translation. Therefore, here we investigated whether 4EBP phosphorylation is altered in the striatum of Huntington’s disease and its consequences for protein synthesis and neuronal function.

Materials and methods
Huntington’s disease mouse models
Male R6/1 and R6/2 heterozygous transgenic mice (B6CBA background) expressing the exon-1 of mHtt with 145 and 90 CAG repeats, respectively, and homozygous mutant Hdh<sup>Q111</sup>, with targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues and wild-type Hdh<sup>Q72</sup> knock-in mice were used. Genotyping and CAG repeat length determination were performed as previously described (Giralt et al., 2011). All mice were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Experiments were conducted in a blind-coded manner with respect to genotype. Animals were housed with ad libitum access to food and water in a colony room kept at 19–22°C and 40–60% humidity, under a 12:12 h light/dark cycle (lights on at 8 am). All procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of Universitat de Barcelona following European (2010/63/UE) and Spanish (RD53/2013) regulations for the care and use of laboratory animals.

Post-mortem human brain tissue
Putamen samples from patients with Huntington’s disease and control individuals were supplied by the Neurological Tissue Bank of the Biobank-Hospital Clinic-Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS; Barcelona, Catalonia) following the guidelines and approval of the local ethics committee (Hospital Clinic of Barcelona’s Clinical Research Ethics Committee). Details are provided in Supplementary Table 1.

Protein extraction and western blot analyses
Protein extraction and western blot analysis were performed as described elsewhere (Saavedra et al., 2010). Immunoblots were probed with the antibodies depicted in Supplementary Table 2. After primary antibody incubation, membranes were washed with TBS-T and incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000; Promega), and the reaction was visualized with the western blotting imaging system (Gel-Pro Analyzer version 4, Media Cybernetics).

eIF4G immunoprecipitation assay
Brain tissue was homogenized using an insulin syringe in ice-cold immunoprecipitation buffer containing 40 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM EDTA, 0.3% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 1 mM NaVO<sub>4</sub>, 2.5 mM NaN<sub>3</sub>, 2 mM PMSF and 1:1000 protease inhibitor cocktail (Sigma-Aldrich). Protein (200 μg) was incubated overnight at 4°C on a rotary mixer with 5 μg of anti-eIF4G antibody (1 μl/50 μg protein; Cell Signaling) or rabbit IgGs (Jackson Immunoresearch) as a negative control. The immune complexes were precipitated overnight at 4°C with the addition of 3% A-Sepharose Cl-4B (Sigma Aldrich). Beads were collected by centrifugation (5 min, 6000 rpm at 4°C) and washed with immunoprecipitation buffer three
times and once with wash buffer containing: 50 mM HEPES (pH 7.5), 40 mM NaCl, 2 mM EDTA. Immunocomplexes were resolved on 12% SDS-PAGE and analysed by western blot.

**SunSET method in brain slices**

5 SUnSET method was carried out as described by Santini et al. (2013). This method is based on the ability of paromycin to label the newly synthesized peptides when administered at low doses. Animals were sacrificed by decapitation and the brain was quickly removed and maintained with oxygenated artificial CSF (in mM: 125 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 26 NaHCO₃, 11 glucose). Coronal slices (400 µm) were obtained from wild-type and R6/1 mice using a conventional vibratome. Slices containing cortex and striatum were then incubated for 1 h with oxygenated artificial CSF at 37°C and subsequently treated with paromycin (5 µg/ml) for 45 min. The slices were then flash frozen, and the striatum dissected out. Finally, striatal samples were processed for western blot, as described above, using anti-paromycin antibody.

**Immunofluorescence**

Coronal brain sections (30 µm) were obtained and processed as described elsewhere (Rue et al., 2013). Free-floating brain sections were incubated overnight at 4°C with anti-Paromycin (1:500; Merck Millipore), anti-DARPP-32 (1:500; Cell Signaling), anti NeuN (1:500; Cell Signaling) or anti GEAP (1:400; Sigma Chemical) antibodies. Nuclei were stained with Hoechst 33258 (1:4000; Invitrogen; prepared in TBS). Sections were then washed in phosphate-buffered saline (PBS) and incubated for 2 h at room temperature with the corresponding fluorochrome secondary antibodies: Cy3 anti-rabbit (1:200) and Cy2 anti-mouse (1:200), both from Jackson ImmunoResearch. To count DARPP-32/paromycin positive neurons and to analyse paromycin staining levels, immunostained sections were examined by using the Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems) with Argon and HeNe lasers coupled to a Leica DMi6000 inverted microscope at 40× magnification. Confocal images were taken as stacks differing in 0.29 µm in Z axis with an HCX PL APO lambda blue 40× numerical aperture objective and standard pinhole (1 Airy disk). For image measurements, 3D Objects Counter plugin in ImageJ software (NIH, Bethesda, MD, USA) was used.

**Immunohistochemistry**

Coronal sections (30 µm) of the whole brain were obtained. Anti-EM48 (1:150; Millipore) antibody was used to detect mhtt aggregates in free-floating brain slices as described elsewhere (Girak et al., 2011). Briefly, sections were incubated with biotinylated secondary antibody (1:200; Thermo Fisher) and developed with diaminobenzidine (DAB). EM48 staining was examined and photographed at 40× using Computer-Assisted Stereology Toolbox (CAST) software (Olympus Denmark A/S). All images were analysed using CellProfiler Analyst software (Jones et al., 2008).

**PUNCH-P**

PUNCH-P was performed as described elsewhere (Avineri et al., 2014). Briefly, pools of three whole brains from 15-week-old wild-type and R6/1 mice were used to isolate active ribosomes with ice-cold polycose buffer: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and 25 mM KCl, EDTA-free complete protease inhibitor mix, 1.4 µg/ml pepstatin, 2 µg/ml leupeptin and 40 U/ml RNasin. After tissue homogenization with glass tissue grinder, the volume of the cell suspension was measured, and one tenth volume of ice-cold lysis buffer was added [11% (w/v) sodium deoxycholate (Sigma Chemical Co) and 11% (w/v) Triton X-100 (Merck Millipore) in polycose buffer]. After 20 min incubation of the lysate with the lysis buffer, it was clarified by centrifugation at 20,000 g for 10 min at 4°C. The ribosomes were isolated by centrifugation at 48,600 g using a sucrose solution. The concentration of ribosomes was determined by measuring RNA absorbance at 254 nm with a spectrophotometer. We first adjusted the concentration of bio-paromycin and the amount of resin necessary for the proper isolation of newly synthesized peptides (Supplementary Fig. 1). Bioin-paromycin was added (100 µmol per 1 OD254 units of ribosome-containing solution) and the mix was incubated at 37°C for 15 min. The newly synthesized polypeptides were captured overnight, at room temperature, by adding 5 µl streptavidin bead slurry per 1 OD254 units of ribosome. The day after, the polypeptides were washed with different buffers (urea/SDS buffer, 1 M NaCl, double-distilled water, 1 mM DTT, 50 mM iodoacetamide, 50 mM ammonium bicarbonate) to finally digest the polypeptides with trypsin and perform liquid chromatography tandem mass spectrometry analysis in collaboration with `Unitat de Proteòmica` from the University of Barcelona, Barcelona, Spain.

**Acute intracerebroventricular paromycin infusion**

Fifteen-week-old wild-type and R6/1 mice were deeply anaesthetized with a mixture of oxygen and isoflurane (5% induction and 1% maintenance) and placed in a stereotaxic apparatus for intracerebroventricular injection (coordinates: −0.22 mm antero-posterior, +1 mm mediolateral, and −2.2 mm dorsoventral) of paromycin (3 µl of paromycin solution; 9 mg/ml, 10% DMSO/90% saline), which was infused using a 10 µl Hamilton microsyringe at an infusion rate of 1 µl/min. The needle was left in place for 3 min to ensure a proper diffusion.

**Surgery for 4EGI-1 treatment**

Cannula implantation and drug treatments were performed as described by Santini et al. (2013). Briefly, wild-type and R6/1 mice were placed in the stereotaxic apparatus under isoflurane anaesthesia (5% induction and 1% maintenance), and a guide cannula (26-G, Plastics One Inc) was implanted at the following coordinates from bregma and dura matter: −0.22 mm antero-posterior, +1 mm mediolateral, and −2.4 mm dorsoventral. Mice were recovered from the surgery for 1 week and intracerebroventricular pharmacological treatments were subsequently started. Mice received daily vehicle (artificial CSF) or 4EGI-1 (0.5 µl at 20 µM) for 6 days. Drugs
were infused at 0.25 µl/min in awake and freely moving mice. After infusion, the cannula was left for an additional 3 min to ensure a proper diffusion of the drug and avoid reflux. On the last day of infusions, mice received vehicle or 4EGI-1 plus paromycin (2.5 µg in 0.5 µl) in parallel. Behavioural analysis and tissue dissection were carried out 1 h after 4EGI-1 infusions.

Electrophysiology

Surgery

Animals were anaesthetized with 0.8–3.3% isoflurane delivered from a calibrated Fluotec 5 (Fluotec-Olimeda) vaporizer at a flow rate of 1 l/min oxygen. A first batch of animals (R6/1 = 9; wild-type = 17) was implanted with two recording electrodes at the right hemisphere (0.14 mm anterior to bregma, 2.2 mm lateral from midline and 3 mm depth from brain surface) and two bipolar stimulating electrodes at the ipsilateral M2 area of the motor cortex (2.46 mm anterior to bregma, 1 mm lateral from midline and 0.8 mm depth from brain surface). Stereotaxic coordinates were selected following a recent precise description of motor cortex projections to the caudate/putamen complex in mice (Hornig et al., 2016). Electrodes were made of 50 µm Teflon-coated tungsten wire (Advent Research Materials Ltd). The final location of the recording electrode was determined using as a guide, the field potential depth profile evoked by paired (40 ms of interval) pulses presented at the M2 area. Two bare silver wires (0.1 mm in diameter) were affixed to the skull as ground. The six wires were connected to a four-pin socket that was fixed to the skull with the help of two small screws and dental cement (Grau et al., 2006). For a second experiment, animals (R6/1 = 40; wild-type = 32) were also chronically implanted with a blunted, stainless steel guide 26-G cannula (Plastic One) in the lateral ventricle contralateral to the implanted electrodes (0.5 mm posterior to bregma, 1 mm lateral, and 1.8 mm from the brain surface). The tip of the cannula was aimed to be in the centre of the ventricle. Guide cannulas were anchored to the skull by dental cement. After completing surgery stainless steel stylets were inserted into the guide cannula and left in place until injections were made.

Input/output curves, paired pulse facilitation and long-term depression in behaving mice

For input/output curves, mice were stimulated at the motor cortex with single positive-negative pulses at increasing intensities (0.02–0.4 mA). The effects of paired pulses at different interpulse intervals (20, 40, 100, 200, and 500 ms) were also checked. We used intensities corresponding to ~40% of the amount necessary to evoke a saturating response. In all the cases, the pair of pulses of a given intensity was repeated ×10 times with time intervals ×30 s, to avoid as much as possible interferences with slower short-term potentiation (augmentation) or depression processes. Moreover, to avoid any cumulative effect, intensities and intervals were presented at random (Madronal et al., 2009).

For evoking long-term depression (LTD) in behaving mice, we followed procedures described previously (Grau et al., 2006). Field potential baseline values were collected 15 min prior to LTD induction using single 100 µs, square, biphasic pulses. Pulse intensity was set at ~40% of the amount necessary to evoke a maximum field potential response (0.15–0.25 mA)—i.e., well below the threshold for evoking a population spike. For LTD induction, animals were presented with a low frequency stimulation (LFS) protocol consisting of a train of 600 pulses at 1 Hz, lasting for 10 min (Berger et al., 2017). In some preliminary experiments carried out in wild-type mice, we confirmed that this LFS protocol evoked larger and longer-lasting LTD than classically used high-frequency stimulation.
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Procedure (Dang et al., 2012; Jurado-Parras et al., 2012; Braz et al., 2017). To avoid evoking large population spikes and/or the appearance of cortical seizures, the stimulus intensity during LFS was set at the same value as used for generating baseline recordings. After the LFS session, the same single stimuli were presented every 20 s for 60 min more and for 30 min the following 3 days.

In a second series of experiments, animals were administered with vehicle or 4E-BP1, as described above. Injections took place 45 min before presenting the LFS protocol.

Histology

Once experiments were finished, mice were deeply re-anesthetized (sodium pentobarbital, 50 mg/kg) and perfused transcardially with saline and 4% phosphate-buffered PFA. Their brains were removed, post-fixed overnight at 4°C, and cryoprotected in 30% sucrose in PBS. Sections (50 μm) were obtained in a microtome (Leica) and those including the cortex and striatum were mounted on gelatinized glass slides and stained with 0.1% toluidine blue to determine the location of stimulating and recording electrodes and of the implanted cannula.

Electrophysiological data collection and analysis

Field potentials evoked at the striatum and 1–V rectangular pulses corresponding to cortical stimulation were stored digitally on a computer through an analogue/digital converter (CED 1401 Plus). Data were analysed off-line for quantification of field potential recordings with the Spike 2 program (CED). The slope of evoked striatal field responses was computed as the first derivative (Vα) of collected recordings (V). A minimum of five successive field potential responses were averaged, and the mean value of the slope during the rise-time period (i.e., the period of the slope between the initial 10% and the final 10% of the recorded field potential) was determined. Computed results were processed for statistical analysis using the IBM SPSS Statistics 18.0 (IBM, Armonk, New York, USA).

Measurement of ATP levels

Striatal frozen tissue obtained 1 h after the last infusion of vehicle or 4E1G1-1 was used to measure ATP levels using the ATP Assay Fluorometric Kit (ab83355; Abcam) according to the manufacturer's instructions.Briefly, striatal tissue was homogenized with ATP assay buffer with a dounce homogenizer. After the removal of insoluble material by centrifugation, proteins were removed from the supernatant with perchloric acid. Then, in a 96-well plate, standard and sample wells were prepared following the instructions. Finally, the ATP reaction mix was added into each well to finally measure on a microplate reader at OD 570 nm. The experiment was performed in duplicate.

Ribosome visualization

Animals were perfused and brains processed as described above. Coronal sections (30 μm) fixed on coated slides were treated with PBS plus 0.1% Triton X-100 for 10 min. After washing twice with PBS for 5 min, slices were stained with Neuroltace 300/25 Green Fluorescent Nissl Stain (N21480; Invitrogen) for 20 min at room temperature. Finally, brain slices were washed for 2 h at room temperature in PBS and covered with a coverslip using Mowiol-mounting medium (Merek). Six to eight striatal sections for animal were analysed. Signal intensity from the striatum was quantified using the Cell Profiler software 3.0.0.

Statistical analysis

In most of the graphs, each point represents data from an individual mouse. All the results were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student’s t-test or the two-way ANOVA, followed by a post hoc test as appropriate and indicated in the figure legends. A 95% confidence interval was used and values of P < 0.05 were considered as statistically significant.

Data availability

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

4E-BP1 is inactivated in the Huntington's disease striatum

To investigate the phosphorylation status of 4E-BP in cells expressing mHtt, we analysed western blot the levels of total and phosphorylated form of 4E-BP at Thr37/46 (p-4E-BP) in the striatum of R6/1, R6/2 and Hdh<sup>Q111</sup> knock-in (KI) mice, in the putamen of patients with Huntington's disease, and in their respective controls. Western blot analysis revealed increased p4E-BP and decreased 4E-BP levels in the striatum of R6/1 mice from early stages of disease in comparison with wild-type mice (Fig. 1A). Similarly, KI mice showed increased striatal levels of p4E-BP from 13 months of age and onwards (Fig. 1B), but total 4E-BP levels were not altered in comparison with wild-type animals. Moreover, we detected increased p4E-BP and decreased 4E-BP levels in the striatum of R6/2 mice at 12 weeks of age (Fig. 1C). In agreement with the results obtained in R6 mice striatums, 4E-BP total levels were decreased in the putamen of Huntington's disease patients compared with non-affected individuals (Fig. 1D).

Unfortunately, we could not detect any phosphorylated form of 4E-BP in human brain samples.

Protein translation is increased in the striatum of Huntington's disease mouse models

Our results showed that 4E-BP was hyper-phosphorylated and subsequently inactivated in the striatum of Huntington's disease models. If the 4E-BP–eIF4E interaction is reduced, then the eIF4F (eIF4E–eIF4G) complex is expected to be increased and cap-dependent translation overactivated. Thus, we evaluated the interaction between eIF4E and eIF4G in the striatum of wild-type and R6/1.
mice by co-immunoprecipitation. Immunoprecipitation of eIF4G and posterior quantitative western blotting revealed increased interaction between eIF4G and eIF4E in the striatum of R6/1 mice (Fig. 2A), while no changes in total levels of eIF4F complex forming proteins were detected (Supplementary Fig. 2). To test if this increased interaction results in increased translation, we applied the SUInSET method. In brain slices containing the corticostriatal pathway obtained from 15-week-old R6/1 and 13-month-old KI mice, we found an increase in the incorporation of puromycin by striatal cells when compared with their corresponding wild-type littermates (Fig. 2B and C). In striatal samples from the same R6/1 mice used to analyse puromycin incorporation, we measured p4E-BP protein levels (Fig. 2D) and observed a good correlation between the increase in de novo cap-dependent translation and 4E-BP1 inactivation (Fig. 2E). Importantly, we observed that increased translation in R6/1 mouse striatum occurred mainly in DARPP-32 positive neurons (Fig. 2F), while GFAP positive cells did not co-localize with puromycin (Supplementary Fig. 3). The percentage of DARPP-32 positive neurons showing puromycin staining was similar in wild-type and R6/1 mouse striata (Fig. 2F), but striatal DARPP-32 positive cells from R6/1 mice showed increased puromycin intensity (Fig. 2F).

**Increased translation in Huntington's disease striatum affects proteins from selective pathways**

To identify the proteins that are being newly synthesized in R6/1 in comparison with wild-type mice striata, we used the PUNCH-P approach (Aviner et al., 2014), which was applied in 15-week-old wild-type and R6/1 mice whole...
Figure 2. Increased levels of the eIF4F complex and translation in the R6/1 mice striatum. (A) Co-immunoprecipitation analysis of eIF4E and eIF4G in striatal lysates from 20-week-old wild-type (WT) and R6/1 mice. Quantification of eIF4E levels relative to immunoprecipitated eIF4G. Values are shown as the mean ± SEM, n = 4–5 mice per genotype. Two-tailed unpaired t-test, **P < 0.01. Quantification (left) and representative immunoblots (right) of striatal lysates obtained from brain slices (containing the cortex and striatum) from 15-week-old wild-type and R6/1 (B) and 13-month-old wild-type and Hdh<sup>CQ111</sup> (KI) mice (C) incubated with puromycin to measure basal rates of protein synthesis. α-Tubulin and Ponceau S staining were used as loading controls. Puromycin incorporation is presented as the percentage change relative to vehicle-treated wild-type slices and normalized against α-Tubulin. Mean ± SEM, n = 6–7 mice per genotype. Two-tailed unpaired t-test, *P < 0.05, **P < 0.01. (D) Quantification (left) and representative immunoblots (right) of phosphorylated and total 4E-BP1 analysis in the same striatal samples where protein synthesis was analysed. Mean ± SEM, n = 6–7 mice per genotype. Two-tailed unpaired t-test, *P < 0.05. (E) Correlation between (continued)
Table 1 Biological process related to the differently expressed proteins

<table>
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<th>Biological process</th>
<th>Stat. mean</th>
<th>Set. size</th>
<th>P_up</th>
<th>P_down</th>
<th>P-value</th>
<th>q-value</th>
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Pathway enrichment analysis was performed on the Pathview web platform that uses the KEGG pathway database. Stat. mean = mean of gene set test statistics; Set. size = the effective gene set size, i.e., the number of genes included in the gene set test; P_up = P-value for over-representation of the GO terms in the upregulated genes; P_down = P-value for over-representation of the GO term in downregulated genes; P-value = global P-value or summary of the individual P-values from multiple single array-based gene set tests. This is the default P-value being used; q-value = FDR q-value adjustment of the global P-value using the Benjamini and Hochberg procedure implemented in MultiTest package. This is the default q-value being used.

brains (Fig. 3A). We identified ~1400 proteins in each pool of animals per genotype (Fig. 3B). The summation of identified peptide sequences (peptide spectrum matches, PSMs) for proteins tend to increase, although it did not reach statistical significance, in R6/1 mice brains (Fig. 3C). This parameter was considered a quantitative measurement of the amount of detected proteins. Next, we identified 233 proteins differently expressed (146 up- and 87 down-translated) between genotypes (Fig. 3D) and Supplementary Table 3. We used the SPIA analysis method to identify the possible pathways selectively affected by the differently translated peptides. Interestingly, we detected that in R6/1 mice brains, some proteins were increased while others were decreased. Proteins related with ribosomes and oxidative phosphorylation were among the proteins increased (Table 1 and Fig. 3E) with components of complex I of the respiratory chain as the most affected, and also proteins related with ubiquinol-cytochrome C reductase complex (complex III or cytochrome b-c1 complex) and ATP V-type ATPase were increased (Supplementary Table 3). On the other hand, structural and functional neuronal-related proteins, such as proteins from the dopaminergic synapse and the endocannabinoid signalling, were decreased (Fig. 3E).

**Treatment with 4EGI-1 improves motor function in Huntington’s disease mice**

Considering that increased protein synthesis could lead to neuronal dysfunction in Huntington’s disease striatum, we took advantage of 4EGI-1, an inhibitor of eIF4G-eIF4E interaction (Moerke et al., 2007), in order to normalize protein synthesis. An infusion cannula was implanted in the lateral ventricle of 13-week-old wild-type and R6/1 mice and 1 week after the surgery, vehicle or 4EGI-1 (20 µM) was infused daily for 6 days (Fig. 4A). Motor behaviour was tested by using the open field, the accelerating rotarod test, the vertical pole task and the balance beam test. Interestingly, 4EGI-1 administration improved motor behaviour in R6/1 mice, as shown in all the tests analysed whereas it did not produce any effect in wild-type mice (Fig. 4B-E). Furthermore, 4EGI-1 did not alter spontaneous locomotor activity, anxious behaviour or body weight in both wild-type and R6/1 mice (Supplementary Fig. 4).

**Treatment with 4EGI-1 normalizes protein synthesis in R6/1 striatum**

On the last day of treatment with 4EGI-1, puromycin was co-infused together with vehicle or 4EGI-1 in wild-type and R6/1 mice. One hour later, mice were sacrificed, and protein synthesis evaluated in the striatum. As in naive mice (Fig. 2B), we detected increased translation and eIF4G-eIF4E interaction in vehicle-treated R6/1 mouse striatum that were normalized to wild-type levels after 4EGI-1 treatment (Fig. 5A and B). The PUNCH-P experiment revealed increased synthesis of ribosomal proteins in R6/1 mice brains (Fig. 3), suggesting that ribosome content could be increased in the...
Figure 3  PUNCH-P method revealed selective differences in R6/1 mice translatome. (A) Scheme showing the PUNCH-P protocol. Newly synthesized peptides were isolated and identified in pools of three 15-week-old wild-type (WT, n = 3) and R6/1 (HD, n = 4) mice brains each. (B) Total number of detected proteins/pool. Mean ± SEM. (C) Summation of peptide spectrum matches (PSMs) shows a slight increase in R6/1 mice. Mean ± SEM. (D) Heat map representation of 233 differentially expressed proteins. (E) KEGG graph generated by Pathview showing the ribosomal proteins significantly increased in R6/1 mice. MS-.

5 striatum. To examine this possibility, we used the fluorescent dye NeuroTrace Green, which labels neuronal ribosomes (Slomnicki et al., 2016), and analysed by western blot ribosomal proteins, RPL10, RPS27A and S7, that were detected as up-synthesized in the R6/1 mouse brains by the PUNCH-P experiment. Ribosome content (Fig. 5C and D), RPS27A and S7 (Fig. 5F and G), but not RPL10 (Fig. 5E) protein levels were increased in the striatum of vehicle-treated R6/1 compared with vehicle-treated wild-type mice. Interestingly, 4EGI-1 treatment normalized ribosomal content in R6/1 mice with no effects in the striata of wild-type mice (Fig. 5C). RPL10, RPS27A and S7 protein levels in 4EGI-1-treated R6/1 mice striata did not differ from levels in wild-type mice striata (Fig. 5E–G). Similarly, we detected increased levels of cyclin D1, a protein previously shown to increase when 4E-8P is inactivated (Rosenwald et al., 1993), in the striatum of vehicle-treated R6/1 mice whose levels were normalized by 4EGI-1 treatment (Supplementary Fig. 5A). In addition, we analysed the levels of striatal proteins DARPP-32 (Bibb et al., 2000; van Dellen et al., 2000) and STEP46 (Luthi-Carter et al., 2000; Desplats et al., 2006; Saavedra et al., 2011) that are decreased in the Huntington’s disease striatum, and of soluble and insoluble forms of mHtt, which has been suggested as a cap-dependent translated protein (King et al., 2008). Treatment with 4EGI-1 was unable to restore DARPP-32 and STEP46 (Supplementary Fig. 5A and B) protein levels, nor to reduce the levels of soluble and insoluble mHtt and the number of mHtt aggregates (Supplementary Fig. 6) in R6/1 mouse striatum.

Overall, our results show that 4EGI-1 treatment could be effective in the normalization of aberrant synthesized proteins in Huntington’s disease striatum.
Figure 4 4EGI-1 treatment improves R6/1 mice motor phenotype. (A) Schematic representation of the experimental design performed. 4EGI-1 (20 μM) was infused in the lateral ventricle of 15-week-old wild-type (WT) and R6/1 (HD) mice over 6 days. Each test was conducted on the indicated day of treatment. OF = open field; AR = accelerating rotarod test; VP = vertical pole; BB = balance beam. (B) Accelerating rotarod test was performed for three consecutive days (three trials per day). The latency to fall per test and group is represented as mean ± SEM (n = 6–8 mice per group). Two-way ANOVA with repeated measures followed by Bonferroni’s post hoc test. **p < 0.01; ***p < 0.001. (C) Vertical pole: time to turn (left) and time to descent (right) was recorded after placing the mice upwards towards the pole. Three trials were conducted and data represent the mean ± SEM (n = 8–9 mice per group). Two-way ANOVA followed by Bonferroni’s post hoc test, **p < 0.01. (D) Percentage of R6/1 mice that fall from the pole during the Vertical Pole test when trying to turn or descend. No falls were recorded in any wild-type mice. Two-tailed unpaired t-test, ***p < 0.001. (E) Balance beam from left to right; number of frames crossed in 2 min, time to cross 30 frames, and number of slips committed per frame in 2 min. Data represent the mean ± SEM (n = 8 mice per group). Two-way ANOVA followed by Bonferroni’s post hoc test, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. ICV = .

Normalization of protein synthesis partially improves long-term depression in R6/1 mice

As alterations in translation have been associated with impaired synaptic plasticity (Santini et al., 2013, 2017), we speculated that increased protein synthesis could be partially responsible for the corticostriatal dysfunction found in Huntington’s disease mouse models (Cepeda et al., 2003; Pariesky et al., 2017). Thus, we first evaluated corticostriatal LTD in behaving wild-type and R6/1 mice in basal conditions (Fig. 6; see Fig. 7A for placement of the electrodes). Input/output curves (IOCs as a global measure of synaptic efficacy) and paired-pulse facilitation (PPFs; presynaptic function and neuromodulation) were similar in wild-type and R6/1 mice (Fig. 6B and C). To evaluate possible post-synaptic alterations in 15-week-old R6/1 mice we compared corticostriatal field excitatory postsynaptic potentials (fEPSPs) after LFS of the motor (M2) cortex in wild-type and R6/1 mice. Baseline values were collected for 15 min while the animal was stimulated in the M2 area every 20 s. The slopes of evoked field potentials were averaged every 5 min. LTD was evoked by LFS of the motor cortex at 1 Hz for 10 min (given a total of 600 pulses). Wild-type mice presented a significant decrease in the slope of evoked field potentials following the LTD protocol [F(20,260) = 1.846; P = 0.017] during the first recording session. In contrast, R6/1 mice did not
Figure 5. 4EGI-1 treatment normalizes translation and recovers ribosomal content in R6/1 mice striatum. (A) Quantification of incorporated puromycin (left) and representative immunoblot (right) of striatal lysates from 15-week-old wild-type (WT) and R6/1 (HD) mice infused over 6 days with vehicle (Veh) or 4EGI-1. Puromycin was co-administered with 4EGI-1 at the last day of treatment and the striatum was dissected 1 h after. α-Tubulin and Ponceau S staining were used as loading controls. Puromycin incorporation is presented as percentage change relative to vehicle-treated wild-type mice after normalization against α-tubulin. Mean ± SEM. *P < 0.05, **P < 0.01, two-way ANOVA with Bonferroni’s post hoc test. (B) Co-immunoprecipitation analysis of eIF4E and eIF4G in striatal lysates from wild-type and R6/1 mice after 4EGI-1 or vehicle infusions. Mean ± SEM, n = 5–6 mice per genotype. (C) Representative images showing fluorescent dye NeuroTrace Green staining in the striatum of wild-type and R6/1 mice injected with vehicle or 4EGI-1. Scale bar = 200 μm. (D) Quantification of ribosomal content in coronal sections from wild-type and R6/1 mice after 4EGI-1 or vehicle infusions. (E–G) Quantification and representative immunoblots of ribosomal proteins RPL10 (E), RPS27A (F) and S7 (G) in striatal lysates obtained from 15-week-old wild-type and R6/1 mice after 4EGI-1 or vehicle infusions. Results are expressed as the mean ± SEM. Two-way ANOVA followed by Fisher’s LSD test, *P < 0.05; **P < 0.01. IF = immunoprecipitation.

Present any sign of LTD following the presentation of the LFS session (Fig. 6D). The slope of field potentials evoked in wild-type mice were significantly (P < 0.05) reduced in comparison with values collected from the R6/1 group during the first LTD recording session. No LTD was observed during the second recording session in either group (Fig. 6D). To evaluate whether the behavioural improvement observed in 4EGI-1-treated R6/1 mice was paralleled by an improvement of the corticostriatal function, we monitored LTD. For this, 15-week-old wild-type and R6/1 mice were implanted with stimulating electrodes in the right motor cortex (M2 area), with a recording
Figure 6 R6/1 mice present similar input/output curves and paired-pulse facilitation, but less LTD, than their wild-type littermates. (A) Wild-type and R6/1 mice were chronically implanted with stimulating electrodes in the right motor cortex (M2 area) and with a recording electrode in the ipsilateral striatum. An extra wire was attached to the bone as ground. CPU = caudate-putamen (striatum); LV = lateral ventricle. (B) Input/output curves of striatal field potentials evoked by pulses of increasing (0.02-0.4 mA) intensities presented to the ipsilateral M2 area. (C) Paired-pulse facilitation in corticostriatal synapses from wild-type and R6/1 mice. The data shown are the mean ± SEM slopes of the second field potential response expressed as a percentage of the first. Selected paired traces (40 ms of inter-pulse interval) collected from representative wild-type and R6/1 mice are illustrated in the inset. (D) The graph illustrates the time course of LTD evoked at corticostriatal synapses from wild-type and R6/1 mice following an LFS session. The LFS, indicated by the dashed line, was presented after 15 min of baseline recordings. At the right, there are illustrated representative examples of field potentials collected at the times indicated below the graph. Field potential slopes are given as a percentage of slope values collected during baseline recordings (100%). *P < 0.05 compared with field potential slopes collected from R6/1 mice (Student's t-test).

electrode in the ipsilateral striatum and a cannula in the contralateral ventricle to start infusing 4EGI-1 (0.5 μl at 20 μM) 2 days before LTD induction. The correct locations of stimulating and recording electrodes, and of the implanted cannula, were examined by toluidine blue staining (Fig. 7A). We found that input/output curves evoked in 4EGI-1-treated wild-type mice presented significantly larger [F(57,304) = 2.609; P < 0.001; two-way repeated measures ANOVA followed by Holm-Sidak multiple comparison procedures] values than wild-type vehicle and the two R6/1 groups, at high (>0.25 mA) stimulus intensities (Fig. 7B). In contrast, there were no significant differences (P > 0.11) between wild-type vehicle mice and the two R6/1 groups (Fig. 7B). Concerning paired-pulse facilitation, 4EGI-1 injection in wild-type and R6/1 mice 45 min before the test did not modify the effects observed...
in vehicle-injected groups (Fig. 7C). Interestingly, the infusion of 4EGI-1 altered LTD results when compared with vehicle-injected animals. As illustrated in Fig. 7D, although the wild-type vehicle injected group presented a significant ($\chi^2 = 33.322$ with 20 degrees of freedom, $P = 0.031$) LTD in comparison with baseline values, the wild-type 4EGI-1 group presented a longer-lasting LTD ($\chi^2 = 98.330$ with 20 degrees of freedom, $P = 0.001$). Nevertheless, no significant differences were found between the two groups across the sessions ($F(20,240) = 0.264; P = 0.999$). In addition, the intraventricular injection of 4EGI-1 slightly increased the LTD effect in the R6/1 group compared with that evoked in vehicle-treated mice, but without reaching significant values ($F(20,100) = 1.11; P = 0.332$). Overall, the local administration of 4EGI-1 did not modify some basic functional properties of striatal neurons as input/output and paired-pulse facilitation in both wild-type and R6/1 mice, but promoted a significant increase in the corticostriatal LTD evoked in wild-type mice, and a non-significant tendency in R6/1 animals.

**Discussion**

Here, we show that 4E-BP is hypofunctional in Huntington’s disease striatum leading to increased formation of the eIF4F complex and, consequently, to exaggerated translation. Increased protein synthesis affects selective pathways including proteins from ribosomes and from the oxidative phosphorylation pathway. Interestingly, pharmacological normalization of protein synthesis in R6/1 mice brains ameliorates motor disturbances and normalizes ribosomal content in the striatum. In addition, we observed a tendency to correct corticostriatal LTD, although it did not reach statistical significance.

Increased p4E-BP and decreased total 4E-BP1 were detected in R6/1 mice striatum from 12 weeks of age onwards. Similarly, 4E-BP levels were reduced in the putamen of Huntington’s disease patients. Although mTORC1 has been classically defined as the main kinase phosphorylating 4E-BP at Thr37/46 (Brunn et al., 1997; Gingras et al., 1999), many other kinases have the capability to phosphorylate 4E-BP in vitro (Lawrence et al., 1997; Heesom et al., 1999).
suggesting that the regulation of 4E-BP phosphorylation is cell type- and physiological context-dependent. The activity of many of these kinases is altered in Huntington’s disease striatum such as ERK, p38, PKC or GSK-3β (Bowles and Jones, 2014), and controversial data regarding mTORC1 activity in the striatum of Huntington’s disease mouse models has been reported (Ravikumar et al., 2004; Troyer et al., 2014; Lee et al., 2015; Creus-Muncunill et al., 2018). In addition, other mechanisms such as phosphatases (Gardner et al., 2015) and the Rho GTPase RhoE regulate p4E-BP levels (Villalonga et al., 2009). Therefore, the mechanism by which p4E-BP is increased in the presence of mHtt deserves further investigations. Interestingly, 4E-BP dysregulation takes place in brains affected by other neurodegenerative disorders. Alzheimer’s disease brains present increased p4E-BP and decreased total levels correlating with tau pathology (Li et al., 2005), and amyloid-β oligomers induce inactivation of 4E-BP (Algarza et al., 2012; Basker et al., 2014). In the same line, different Parkinson’s disease causative mutations lead to the inactivation of 4E-BP in vitro and in vivo (Imai et al., 2008). Thus, our results support the view that altered 4E-BP function could be a common dysregulated mechanism involved in different neurodegenerative disorders.

The inactivation of 4E-BP in R6/1 mice striatum correlated with increased formation of the elf4F complex and with exaggerated translation, which seems to occur in DARPP-32-positive neurons. Different transcripts can be specifically up-translated when 4E-BP is inactivated (Musa et al., 2016). In fact, the proteomic analysis revealed that ribosomal and oxidative phosphorylation pathway proteins are mainly affected. Increased levels of ribosomal proteins was not fully surprising as inactivation of 4E-BP induces selective enhanced translation of transcripts containing a 5’TOP tract (Jeffries et al., 1994; Avni et al., 1996; Meyuhas, 2000), which encode for components of the translational machinery including ribosomal proteins (Avni et al., 1996; Meyuhas, 2000). Furthermore, 4E-BP has been shown to regulate the translation of mitochondria-related mRNAs (Ziel et al., 2009; Goo et al., 2012; Morita et al., 2013) and mRNAs encoding components of mitochondrial ribosomal proteins (also observed in our ribosomal set of proteins) are among them (Morita et al., 2013). On the other hand, previous results have shown complex I defects in patients with Huntington’s disease (Parker et al., 1990), but R6/1 mouse striatum showed increased complex I respiration at late stages of disease progression (Herbst and Holloway, 2015) that can be explained by the increase in complex I proteins that we observed. Nonetheless, as previously proposed by others (Herbst and Holloway, 2015), enhanced complex I respiration could be responsible for oxidative stress observed in patients with Huntington’s disease (Kumar and Ratan, 2016). Therefore, we could not discard that increased translation of striatal complex I proteins is playing a pathogenic role in Huntington’s disease. Taken together, it is tempting to speculate that striatal cells expressing mHtt attempt to compensate the deficit in proteins, such as synaptic proteins, by synthesizing more ribosomes to then increase the global rate of translation (Sormnacki et al., 2016), which is known to be neurotoxic for neurons (Martin et al., 2014a,b). In addition, they activate the synthesis of mitochondrial-related proteins to alleviate energy deficits, which seems insufficient to ameliorate mitochondrial dysfunction occurring in Huntington’s disease striatum (Jodeiri Farshbaf and Ghaedi, 2017).

We hypothesized that increased translation in R6/1 mice striatums could be involved in neuronal dysfunction and contribute to motor impairment. Our results support this proposal because infusion of 4EGI-1, an inhibitor of elf4F-elf4E interaction (Moorke et al., 2007), normalized protein synthesis and improved motor symptoms. Although the amelioration of motor symptoms did not correlate with a recovery of striatal markers, nor with changes in mHtt levels, 4EGI-1 treatment normalized cyclin D1 levels, previously proposed as a possible contributor to neuronal dysfunction in Huntington’s disease (Polegri et al., 2008) and re-established ribosomal protein content. Hence, another attractive explanation for the amelioration of motor symptoms is that 4EGI-1 normalization of translation improves protein homeostasis, thereby diminishing energy consumption in striatal cells. Our results also show that the alteration in cortico-striatal LTD detected in R6/1 behaving mice showed a trend toward recovery after 4EGI-1 treatment. The lack of LTD induction observed in R6/1 mice supports the dysfunctional connectivity along the cortico-striatal pathway previously proposed by others (Puigdoménech et al., 2015; Buren et al., 2016; Hintsanen et al., 2016; Rangel-Barajas and Rebic, 2016). Although many studies demonstrated electrophysiological alterations in this pathway (Cepeda et al., 2003; Parievsky et al., 2017), LTD alterations in Huntington’s disease were described in hippocampal (Milenkovic et al., 2006) and perihinal cortex (Cummings et al., 2006) brain slices and our results extend this observation to the cortico-striatal pathway in behaving mice. In agreement with our results, selective mutation in the LRRK2 gene, which induces an enhancement of translation, impairs cortico-striatal LTD in mice (Chou et al., 2014). Overall, our findings are in accordance with previous work reporting that increased protein synthesis is a direct contributor to neuronal dysfunction and behavioural abnormalities that can be tackled pharmacologically with 4EGI-1 treatment (Cloghesy et al., 2012; Santini et al., 2013, 2017; Martin et al., 2014a,b).

Protein synthesis normalization in Huntington’s disease could be achieved in many ways. We speculate that normalization of exaggerated translation might contribute to the beneficial effect of potential Huntington’s disease therapies. For instance, Ravikumar et al. (2004), showed that the administration of rapamycin to a mouse model of Huntington’s disease yielded a dramatic amelioration of motor impairment, accompanied by a reduction in the
aggregation of mHtt. From our results and knowing that rapamycin activates 4E-BP (Beretta et al., 1996; Tain et al., 2009), the normalization of protein synthesis could be contributing to rapamycin-mediated neuroprotection in mouse models as has been shown in cellular models of Huntington's disease (King et al., 2008). Interestingly, metformin activation of AMPK has been proposed as a therapeutic strategy for Huntington’s disease (Vazquez-Manrique et al., 2015; Jin et al., 2016; Herras et al., 2017) and AMPK activation inhibits protein synthesis (Chan et al., 2004; Reiter et al., 2008). In addition, among its different mechanisms of action, fingolimod, a drug with beneficial effects in Huntington’s disease mouse models (Di Pardo et al., 2014; Miguez et al., 2015), activates PP2A (Yang et al., 2012), a phosphatase that dephosphorylates 4E-BP (Nanahoshi et al., 1998; Nho and Peterson, 2011). Furthermore, prostaglandin E2 EP2 receptor activation has been shown to improve R6/1 phenotype (Anglada-Huguet et al., 2016), which could be attributed to normalization of translation, since prostaglandin E2 suppresses mTOR signalling and inhibits protein synthesis (Okanishi et al., 2014). Finally, 2-methyl-6-(phenylethyl)pyridine hydrochloride, a drug that has been shown to normalize translation in models of Fragile X Syndrome (Aschrafi et al., 2005; Osterweil et al., 2010), extends lifespan and ameliorates neurological disturbances in R6/2 mice (Scheick et al., 2004). Altogether, this evidence suggests that different therapeutic options proposed for Huntington’s disease converge in the normalization of exaggerated protein synthesis.

In conclusion, this study shows increased translation in Huntington’s disease striatum affecting the rate of synthesis of selective proteins, such as ribosomes and proteins from the oxidative phosphorylation pathway. Importantly, this aberrant protein synthesis plays a role in motor phenotype associated with Huntington’s disease. Therefore, the 4E-BP pathway emerges as a therapeutic target in Huntington’s disease.

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Increased protein translation in Huntington’s disease


