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Mutant huntingtin affects endocytosis in striatal cells by altering the binding of AP-2 to membranes

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ABSTRACT

Clathrin-mediated endocytosis plays an important role in the maintenance of neuronal integrity in the synaptic terminals. Here we studied the effect of anomalous polyglutamine expansion in huntingtin on the interaction of coat proteins with membranes, in areas of mouse brain or in cultured striatal cells. We observed that this anomaly induces a redistribution of AP-2, but not other coat proteins, from the membrane to the cytosol in the striatum, and in the cultured striatal cells. It was also noted that huntingtin associates with AP-2, and that this association decreases due to the mutation in huntingtin. This decreased receptor-mediated endocytosis, measured by the internalization of transferrin in the mutated cells. It was also confirmed that huntingtin mutation made the cells more vulnerable to the action of quinolinic acid, with an increasing degradation of the AP-2 alpha subunits. On the basis of these results, we conclude that abnormal polyglutamine expansion in huntingtin affects clathrin-mediated endocytosis, and may be one of the pathogenic mechanisms of neurodegeneration.

Introduction

Clathrin-mediated endocytosis (CME) is one of the main routes for the incorporation of nutrients and for the regulation of surface receptors in eukaryotic cells. The formation of clathrin coated vesicles (CCVs) requires spatial and temporal coordination of multiple endocytic proteins (Loerke et al., 2009; Mettlen et al., 2009). Although clathrin is the major component of the coat, there are other proteins that are essential to confer selectivity and allow the correct formation of CCVs (Schmid and McMahon, 2007). The adaptor complex-2 (AP-2), AP-180, syndapin-1, dynamin and the huntingtin-interacting protein (HIP-1) are among the many proteins involved in the biogenesis of CCVs (Ford et al., 2001; Waelter et al., 2001; Wendland, 2002). An important early event in the formation of CCVs is the recruitment of AP-2 to the plasma membrane. The AP-2 complex is a heterotetramer composed of two large subunits (α and β2), in association with a 50 kDa (μ2) and a 17 kDa (δ2) subunit (Ahle et al., 1988). This complex plays a central role in the endocytic pathway since it mediates the selection of the protein "cargo", and interacts with other endocytic proteins (Mousavi et al., 2004). Although AP-2 is mostly located in the cytosol, under appropriate stimulus it is recruited to sites rich in PtIns-4,5-P_2 at the plasma membrane (Krauss et al., 2006). The redistribution of endocytic proteins to membranes is generally associated with a change in their phosphorylation status (Korolchuk and Banting, 2002, 2003; Ricotta et al., 2002; Zhang et al., 2004). Most of the endocytic proteins interact, through specific domains, with membrane phospholipids (De Camilli et al., 2002; Legendre-Guillemin et al., 2004; Peter et al., 2004). AP-2, however, needs to be associated with "docking" proteins to reinforce its interaction with membranes. There is evidence that synaptotagmin and the small GTPase ARF6 participate in the recruitment of AP-2 to the membrane (Mousavi et al., 2004; Poupart et al., 2007) although other, unidentified, proteins may contribute to this process.

Although CME is a common pathway for the internalization of molecules in different cell types, it appears to be critical in neurons; after fusion of synaptic vesicles (SVs) with the pre-synaptic plasmalemma, the homeostasis of membrane is maintained by internalization of lipids and proteins via CCVs (Brodin et al., 2000; Granseth et al., 2006). Additionally, CME modulates synaptic efficacy by mediating the internalization of NMDA-R and AMPA-R at the post-synaptic terminals (Lavezzari et al., 2004; Metzler et al., 2003; Teng et al., 1999). Alterations in this pathway have been associated with Alzheimer's disease and schizophrenia, which exhibit increased internalization of NMDA-R (Gu et al., 2005; Kurup et al., 2010). Huntington´s disease (HD) is a neurodegenerative disorder caused by an abnormal expansion of CAG repeats in the huntingtin (htt) gene, which adds more than 36 poly-glutamine residues

Abbreviations: AP-2, adaptor complex-2; CCVs, clathrin coated vesicles; CME, clathrin-mediated endocytosis; HIP-1, huntingtin interacting protein-1; htt, huntingtin; mHtt, mutated huntingtin; QA, quinolinic acid.

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to its amino-terminal sequence. Consequently, the protein becomes insoluble and forms cytoplasmic aggregates (DiFiglia et al., 1995; Hilditch-Maguire et al., 2000). Since the genetic basis of HD is well known, several research groups have attempted to elucidate the function of htt. From studies of protein-protein interaction, it has been demonstrated that htt is involved in several processes; in vesicular transport along microtubules, in post-Golgi traffic, and it also has anti-apoptotic activity (Del Toro et al., 2006, 2009; Pal et al., 2006). Recent evidence suggests that htt interacts with HIP-1, and both proteins are present in CCVs (Velier et al., 1998; Waelter et al., 2001). Moreover, htt binds to acidic phospholipids of the plasma membrane (Kegel et al., 2005, 2009). However, it is not known whether htt is part of the endocytic machinery.

Although htt is expressed in all tissues, the striatum is the brain area primarily affected in HD, showing a selective death of striatal medium spiny neurons (Vonsattel and DiFiglia, 1998) and cortical neurons (Reiner et al., 1988). In addition, increased levels of quinolinic acid (AQ) have been observed in the striatum of HD patients (Schwarcz et al., 1988), thereby inducing neuronal death mediated by over-stimulation of NMDA-R (Brown et al., 1998; de Carvalho et al., 1996; Schwarcz and Pellicciari, 2002). The main focus of this study was to determine whether abnormal polyglutamine expansion in htt affects the endocytic machinery of nerve cells. Thus, we studied the distribution of coat proteins in certain brain areas of transgenic mice and in striatal cells that express the abnormal polyglutamine in htt, as occurs in HD. From the results, we suggest that htt is part of the endocytic machinery in nerve cells. In turn, endocytosis would be affected due to the mutation and this alteration could be one of the causes of the pathogenesis of HD.

Materials and methods

Reagents and antibodies

The rabbit polyclonal anti-α-subunit for AP-2, goat anti-AP180, mouse polyclonal anti-dynamin I, goat anti-HIP-1 and goat anti-syndapin-1 antibodies were purchased from Santa Cruz Biotechnology (California, CA, USA). The mouse anti-actin antibody was from BD Transduction Laboratories (Chicago, USA). The antibody against N-terminal Ab1 (amino acids 1–17) of htt, developed in rabbits, was a gift from Dr. DiFiglia (Charlestown, USA) and the mouse monoclonal against the N-terminus R11–29 (amino acids 11–29) of the anti-μ2 subunit was kindly provided by Dr. Bonifacino (Bethesda, USA). The biotin- or FITC-conjugated antibodies were purchased from Sigma (St. Louis, MO, USA). Chemiluminescent reagents were from Pearce (Rockford, IL, USA). The transferrin-Alexa Fluor 568 conjugate and dextran-FITC conjugate were from Molecular Pobes, Inc. (OR, USA).

Animals

Tet/HD94 mice (ten months old) used in this study were generated as previously described (Yamamoto et al., 2000), and bred and maintained at the Centro de Biología Molecular "Severo Ochoa" (Madrid, Spain). The mice were maintained in a temperature-controlled environment on a 12/12 h light/dark cycle, with food and water available ad libitum. All experiments were carried out in accordance with institutional guidelines approved by ethical committee of Consejo Superior de Investigaciones Científicas (CSIC-Universidad Autónoma de Madrid, Spain).

Processing of tissue

Tissues from striatum, hippocampus, cerebellum, and cortex of HD94 or wt mice, were homogenized in (1:3 w/v) ice-cold lysis buffer (20 mM HEPES, pH: 7.4, containing 0.25 M sucrose, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 1 mM pepstatin A, 100 mM leupeptin, 5 mM sodium glycerophosphate and 1 mM sodium orthovanadate) with a Teflon pestle homogenizer, and centrifuged at 1500 *g* for 10 min. The postnuclear supernatants were subjected to further centrifugation at 100,000 *g* for 30 min to obtain a membrane fraction and cytosol. The samples were stored at −20 °C until use.

Cell culture

Conditionally immortalized wild-type (STHdhQ7/Q7) and mutant (STHdhQ111/Q111) striatal neuronal progenitor cell lines expressing endogenous levels of normal and mhtt with 7 and 111 glutamines, respectively, have been described elsewhere (Trettel et al., 2000; Gines et al., 2003). StHdhQ cells (with 7 or 111 repeats) have the advantage because they come from the striatum, the area most damaged in the pathology of HD, and they express the "full-length" htt (normal or mutated) to physiological levels. The cells were grown at 33 °C with a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids (1×) and 400 μg/ml geneticin.

Processing of cells

Striatal cells (StHdhQ7 or StHdhQ111) were grown to 80% confluence and detached from the flasks with 0.25% trypsin for 2 min. The cells were washed twice with 20 mM phosphate buffered saline (PBS) and resuspended in lysis buffer. They were then broken by multiple passages through a needle and syringe and subsequent homogenization with a glass homogenizer with Teflon pestle. After that, the homogenates were centrifuged at 1500 *g* for 10 min and the postnuclear supernatant was subjected to further centrifugation at 30,000 *g* for 30 min. As with the tissue, a membrane fraction was separated from cytosol and both stored at −20 °C until use.

Immunoblotting

All procedures were carried out as described by Alberdi et al. (2005). Briefly, 50 μg of protein of each sample (or 30 μg for the homogenates) was boiled in sample buffer (Laemmli, 1970) and loaded to 8% SDS-PAGE gels. Subsequently, proteins were electrotransferred onto nitrocellulose membrane (Sartorius, Göttingen, Germany) and the proteins under study were detected with the corresponding specific antibody. Antibodies were used at the following dilutions: anti α-subunit of AP-2 (1/5000), anti htt (1/750), anti HIP-1 (1/5000), anti dynamin (1/2500), anti syndapin (1/2500), anti AP180 (1/2500), anti actin (1/5000), and anti μ2 subunit of AP-2 (1/1000), all prepared in PBS supplemented with 0.05% Tween (PBS-Tween). After incubating overnight with the primary antibodies, the membrane was washed with PBS-Tween three times and then incubated with the corresponding biotin-conjugated secondary antibody. After three washes with PBS-Tween, membranes were incubated with avidin-peroxidase and finally the protein bands were detected by chemiluminescence (Pearce), following the advices of manufacturers. The luminescent signals were developed and quantified with LAS-4000 imaging system (Fujifilm Lifescience, USA).

Immunoprecipitation

For immunoprecipitation, 50 μl of protein A-Sepharose (Sigma) was incubated with 2 μg of anti α-subunit (AP-2) at 4 °C for 2 h, with continuous gentle agitation. The beads were then centrifuged at 1000 *g* for 5 min, washed three times with PBS (to remove excess of antibody) and incubated with homogenates (250 μg of proteins) from either StHdhQ7 or StHdhQ111 cells for 12 h (at 4 °C) with gentle rotation. Finally, the beads were centrifuged at 1000 *g*, washed twice with PBS and solubilized with sample buffer at 95 °C (10 min). The α-subunits of AP-2 and htt were detected from immunoprecipitates by immunoblot with the corresponding antibodies, as described above. The bands were detected by chemiluminescence and quantified by

densitometry using the LAS-4000 imaging system. The optical density values for htt were corrected for AP-2 detection in the immunoprecipitates.

Immunofluorescence

The cells were grown on coverslips seated at the bottom of culture wells, under the conditions described above. Cells were washed three times with PBS and then fixed with 4% paraformaldehyde for 10 min. The coverslips were incubated for 30 min with 1 M glycine, and the cells were permeabilized with 0.1% Triton X-100 for 15 min, and blocked for 1 h with 1% bovine serum albumin (BSA). Then, cells were incubated for 12 h with anti-AP-2 (1/100) and subsequently washed three times with PBS and incubated with the corresponding secondary antibody (FITC-conjugated anti rabbit; 1/100), for 1 h at room temperature. Cells were observed with an Olympus FV1000 confocal microscope and the images were saved with the program FV 10-ASW 1.7 (Olympus, Japan). The cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride), incorporated into the mounting solution (Ultra Cruz, Santa Cruz Biotechnology, Inc.).

Uptake assays

StHdhQ7 and StHdhQ111 cells were grown on coverslips as detailed above, and incubated with 100 μg/ml Alexa 568- conjugated transferrin (Tf) at 4 °C for 60 min (binding to Tf receptors). After that, 1 μg/ml FITC-conjugated dextran 70 was added to the cultures and incubated for 10 min at 33 °C to allow internalization of the molecules. Finally, the coverslips were mounted on slides and observed by confocal microscopy. Fluorescence intensity was quantified by Image J software. The fluorescence intensity of Tf or dextran was normalized to the nuclei fluorescence intensity (stained with DAPI).

Stimulation of endocytosis

Striatal cells (StHdhQ111 or StHdhQ7) grown on 24 well plates, were pre-incubated in DMEM supplemented with 2 mM HEPES and 0.2% BSA at 33 °C for 45 min. The cells were washed twice with cold 20 mM HEPES buffer (pH 7.0) containing 100 mM potassium acetate, 85 mM sucrose and 1 mM magnesium acetate (buffer KSHM), and were then incubated with 100 μg/ml holotransferrin (in buffer KSHM) for 10 min at 33 °C. Subsequently, cells were processed to obtain cytosol and membrane, as detailed above. These fractions were used for AP-2 detection by immunoblot.

Treatment of cells with quinolinic acid (QA)

Striatal cells (StHdhQ7 or StHdhQ111), grown under the conditions described above, were exposed to 250 μM of QA (dissolved in PBS) for 1, 3 or 6 h. Then, cells were homogenized, and the homogenates were used for AP-2 detection by immunoblot. Non treated cells were used as controls.

Cell viability

Mitochondrial activity in cells

This assay is based on metabolic reduction of Methylthiazolyldiphenyltetrazolium bromide (MTT), catalyzed by the mitochondrial enzyme succinate deshydrogenase, to a blue compound (formazan) as an index of mitochondrial function and cell viability. The number of living cells is proportional to the amount of formazan produced. Striatal cells (StHdhQ7 or StHdhQ111), grown under the conditions described above, were treated with 250 μM of QA for 1, 3 or 6 h. Then, the medium was replaced by a fresh one, containing 5 mg/ml MTT, and the cells were incubated at 33 °C for 4 h. The crystals (formazan) were then dissolved with 10% SDS containing 0.01 M HCl for12 h at 33 °C. The supernatants were recovered, and centrifuged at 800 g for 5 min to remove debris. The formazan released was quantified by spectrophotometry at 570 nm. As a positive control, cell cultures were treated with 1% sodium azide.

Detachment of cells (as a mortality index)

Striatal cells (StHdhQ7 or StHdhQ111) were exposed to 250 μM of AQ for 1, 3 or 6 h. After that, the detached cells were rescued from the medium and counted in a Neubauer hemocytometer. They were considered as dead cells. This was confirmed because all of detached cells were stained with 1 mg/ml propidium iodide (PI).

Statistical analysis

The data obtained were subjected to the non-parametric one-way ANOVA and the Tukey´s multiple comparison *post hoc* test. For comparisons between two groups of data a Student`s test was used. The level of significance was set at $p \leq 0.01$.

Results

Polyglutamine expansion in htt (exon 1) alters the recruitment of AP-2 to the membranes in the striatum of HD94 mice

Most of the proteins involved in the endocytic machinery are predominantly cytosolic, but under stimulation they are recruited to the membrane to form CCVs. To determine whether htt is involved in the recruitment of proteins to membrane, we studied the distribution of proteins relevant for the CME, in models of transgenic mice HD94, expressing exon 1 mutant htt with 94 CAG repeats. Different brain areas: the striatum, prefrontal cortex, hippocampus and cerebellum were dissected and studied separately for the distribution of proteins involved in CME. We first corroborated that expression of this mutant does not affect protein synthesis in any brain area studied. In the striatum of HD94 mice (10 months old), we observed that AP-2 is redistributed from membranes to the cytosol (Figs. 1A and B). Other endocytic proteins such as syndapin-1 and HIP-1 (involved in early and late steps of CCV formation, respectively), and dynamin (involved in the fission of coated pits), did not change their distribution in the studied areas, when compared between control and HD94 mice (Fig. 1D). Although the amount of HIP-1 associated with membranes tended to diminish in the striatum of HD94 mice, this difference would not be significant (Fig. 1E).

Expression of mHtt alters interaction of AP-2 with striatal cell membranes

We wondered whether the changes in the distribution of AP-2 observed in striatum of transgenic mice could be reproduced in a cell model of striatal neurons. We observed that AP-2 (α and μ2 subunits) associated with membranes decreased significantly in SHdhQ111 cells compared to controls (StHdhQ7) (Figs. 2A and B). By immunofluorescence, we observed that AP-2 has a slightly different distribution between cell types, as these proteins were distributed in vesicular structures or scattered in the cytoplasm in StHdhQ7 cells, whereas a slightly more dispersed distribution was observed in StHdhQ111 (Fig. 2C). This may be related to redistribution of AP-2 to the cytosol as observed by immunoblot. In addition, these changes were selective for AP-2, as the expression or distribution of other coat proteins, such as dynamin, AP180 or HIP-1 did not change with htt mutation (Fig. 2D). As occurred with HD94 mice, HIP-1 associated with membranes showed a slight decrease in the mutated cells, although it would not be significant. These results suggest that the mutation in htt leads to a selective alteration in the recruitment of AP-2 to membranes.

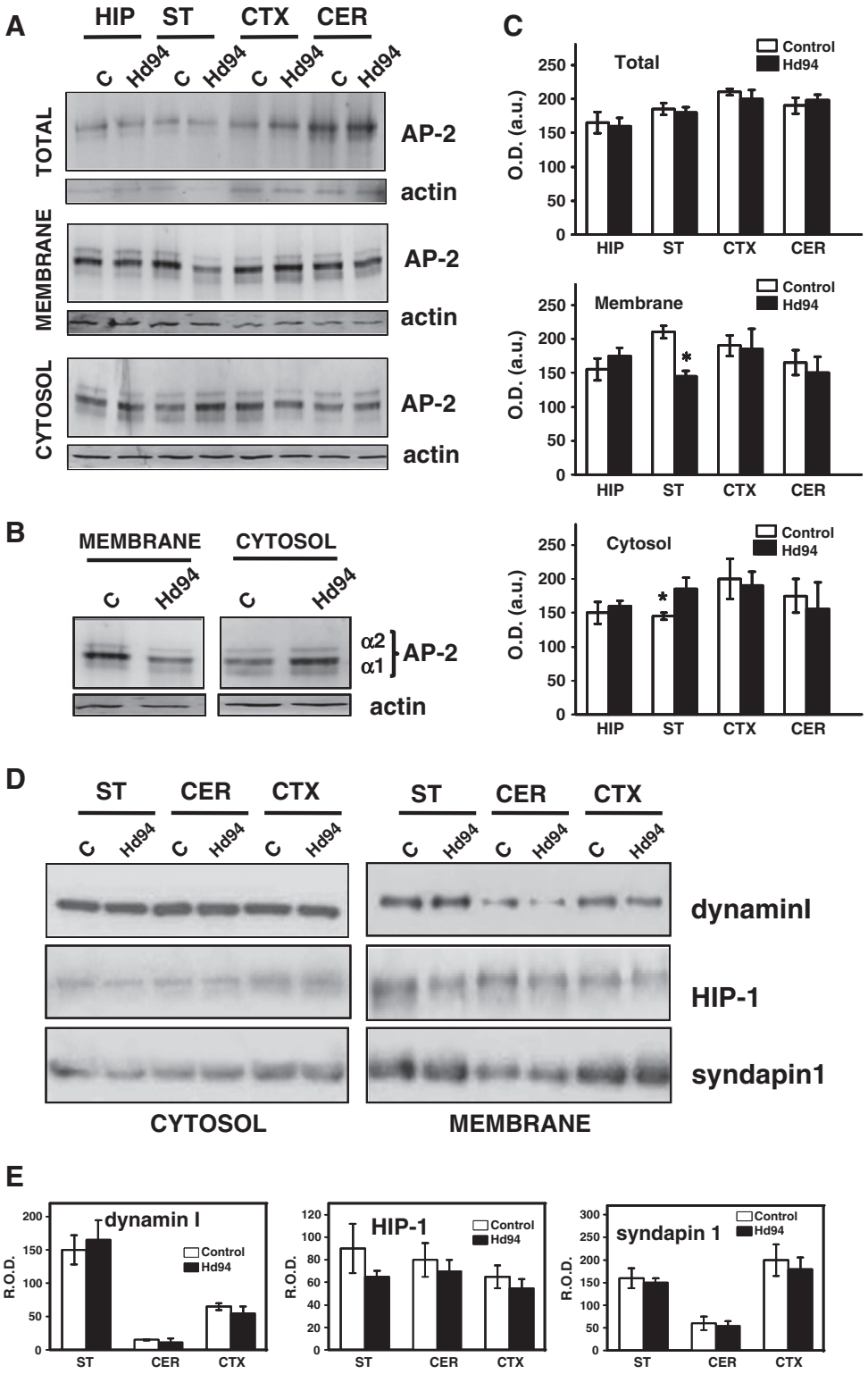

Fig. 1. A) Distribution of AP-2 between cytosol and membrane in different brain areas of the control and HD94 mice. B) Amplification of the bands observed in A for the striatum. C) Quantification of the bands by densitometry. Bars represent the means of relative optical densities (O.D.) ± SD from three independent experiments. (*) Significantly different ($p<0.05$) with respect to the corresponding control. D) Distribution of dynamin I, HIP-1 and syndapin-1 between cytosol and membrane in different brain areas of control and HD94 mice. E) Quantification of the proteins associated to membranes as observed in D, from two independent experiments. In all cases, detection of actin was used as loading control. HIP: hippocampus; ST: striatum; CTX: prefrontal cortex; CER: cerebellum.

AP-2 interacts with htt

It is known that HIP-1 interacts with htt and also with AP-2. However it is not well known whether AP-2 can interact (directly or indirectly) with htt. The fact that mHtt induces an alteration in the recruitment of AP-2, but not HIP-1, to membranes, would suggest that htt interacts with AP-2. To evaluate this possibility, immunoprecipitation (IP) assays were performed, using an antibody against the α-subunit of AP-2. Fig. 3A shows that htt co-precipitated with AP-2 and the interaction is reduced in StHdhQ111 cells. Htt that co-precipitated with AP-2 was

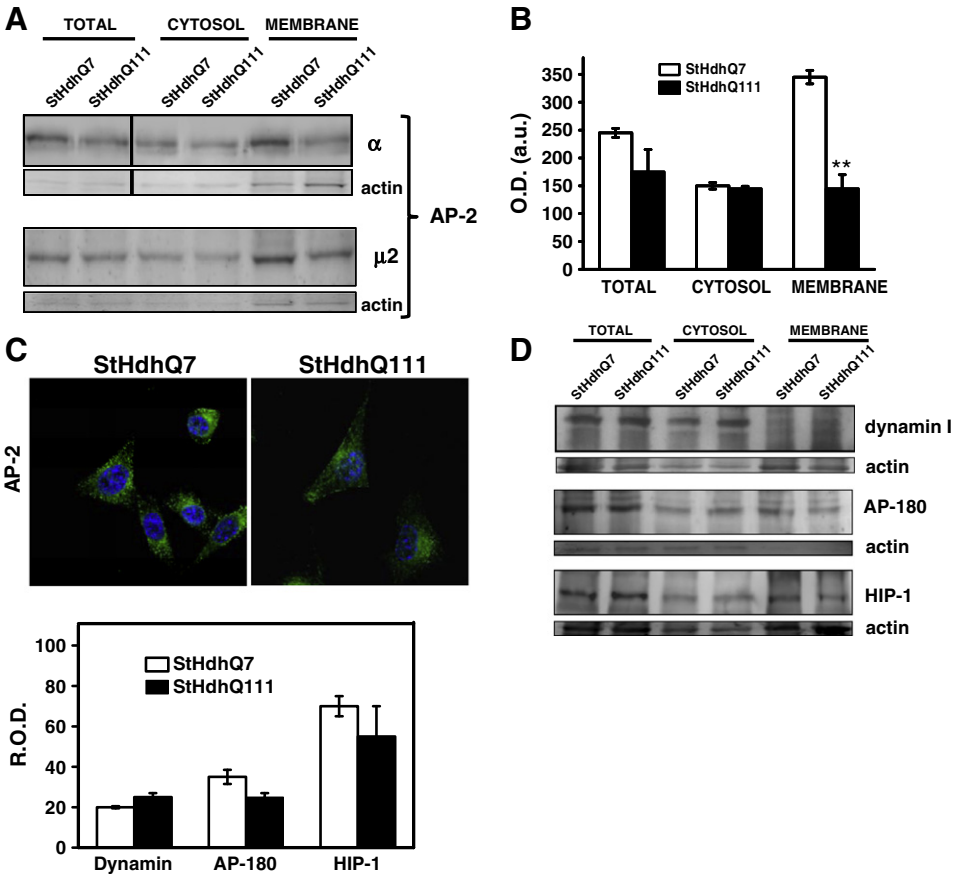

Fig. 2. A) Distribution of AP-2 (α and μ2 subunits) between cytosol and membrane of StHdhQ7 and StHdhQ111 striatal cells. B) Quantification of α subunit by densitometry, the bars represent the means of relative optical density (O.D.) from three independent experiments ± SD. (**) Significantly different ($p<0.01$). C) Immunofluorescence for detection of AP-2 in the cells. D) Distribution of dynamin, AP180 and HIP-1 between cytosol and membrane of striatal cells. E) Quantification of the proteins associated to membranes as observed in D, from two independent experiments.

standardized according to expression levels of AP-2 in StHdhQ7 or StHdhQ111 cells, since the expression of both proteins is affected in StHdhQ111 cells (Fig. 3B, input). These results reinforce the hypothesis that htt may act as a docking protein for recruitment of AP-2 to membranes, and this function is lost when htt is mutated.

Clathrin-mediated endocytosis is altered in StHdhQ111 cells

The loss of AP-2 associated with membranes in the StHdhQ111 cells, could lead to alterations in CME. To assess this, we have studied incorporation of transferrin (Tf), a known ligand that is internalized via CME. First, it was observed that stimulation of endocytic activity by holotransferrin slightly increased the affinity of AP-2 for membranes in control cells, whereas StHdhQ111 cells did not respond to the stimulus (Figs. 4, A and B). Subsequently, we assessed the ability of cells to internalize the ligand (Tf). By using Alexa-568 conjugated-holotransferrin, the internalization of this ligand was evaluated by fluorescence microscopy. As shown in Fig. 5A, binding of Tf to surface receptors (cells maintained at 4 °C) was not affected by the mHtt, when compared to controls. However, when cells were incubated at 33 °C for 10 min, a

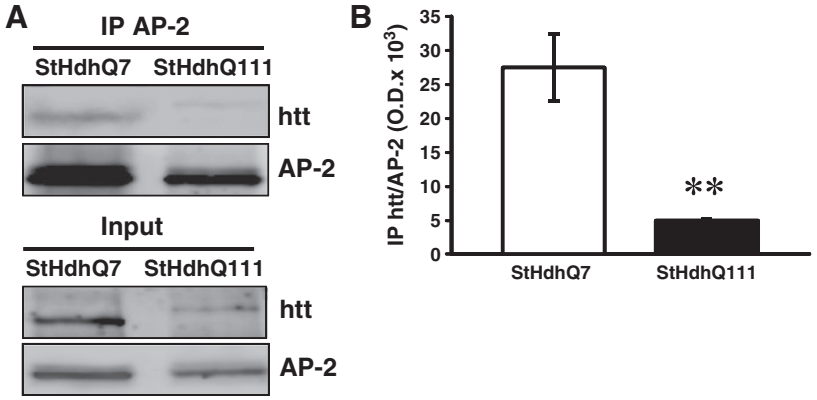

Fig. 3. A) Immunoprecipitation with anti-α subunit of AP-2 from homogenates of StHdhQ7 and StHdhQ111 striatal cells, and detection (immunoblot) of htt in the precipitates. B) Quantification of htt immunoprecipitated, normalized for AP-2 expression. Bars represent the means of relative optical densities (O.D.) ± SD from three independent experiments. (**) significantly different ($p<0.01$).

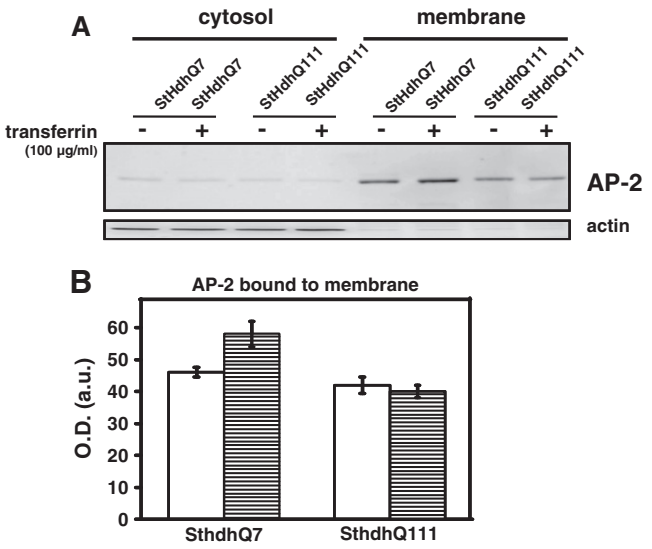

Fig. 4. A) Distribution of AP-2 between cytosol and membrane in StHdhQ7 and StHdhQ111 striatal cells after stimulation with holotransferrin, as detailed in the Materials and methods section. B) Quantification of AP-2 (α-subunit) associated to membranes of striatal cells, either stimulated (hatched bars) or not stimulated (white bars) with holotransferrin. Values represent the means of O.D. ± SD from three independent experiments.

marked decrease in the internalization of Tf was observed in StHdhQ111 cells (Figs. 5, B and C). This effect is selective for the CME, since the internalization of FITC-conjugated dextran was not altered due to htt mutation (Figs. 5, B and C).

mHtt increases vulnerability of neurons to quinolinic acid (QA)

StHdhQ111 and StHhdQ7 cells were exposed to 250 μM of QA at different time points to evaluate if mutation in htt affects the vulnerability of the cells to this excitotoxic agent. It is known that this endogenous metabolite increases abnormally in the striatum of HD patients and is believed to be responsible, in part, of the excessive neuronal death observed in this pathology. As shown in Fig. 6, treatment with QA reduced levels of the α-subunit of AP-2, simultaneously with an increase of a lower molecular weight fragment (Fig. 6A). This fragment coincides with that described by other authors, so we assume that this is a cleavage product of α-subunit. Interestingly, we found that α-subunit is more susceptible to cleavage in StHdhQ111 cells, since the fragments appear earlier and in higher amounts when compared to control cells. As expected, the mutated cells were more vulnerable to excitotoxic effects of QA than controls, since mitochondrial activity, measured with MTT, was drastically reduced in the mutated cells (Fig. 7A). The vulnerability of the mutated cells to excitotoxicity was confirmed by loss of adhesion to the plates, and increased staining with propidium iodide (Figs. 7, B and C).

Discussion

In order to understand the molecular basis of Huntington's disease and thus develop appropriate treatments, the functions of htt through the structural and functional alterations have been studied for decades.

It has been established that htt is involved in various cellular events through its multiple domains that interact with other proteins. Although it has been demonstrated that htt is present in the CCVs, and interacts with the endocytic protein HIP-1 (Mishra et al., 2001; Waelter et al., 2001), it is still controversial whether htt is a component of the endocytic machinery, and how this protein could participate in CME.

An early event in the biogenesis of CCVs is the recruitment of AP-2 to the plasma membrane. However, if at this stage a critical mass of AP-2 and other accessory proteins is not achieved, the initial nucleus of the coat is destabilized (Loerke et al., 2009; Mettlen et al., 2009). Although the α-subunit of AP-2 has binding sites for phosphoinositides, recruitment of this complex to the membrane requires "docking" proteins to reinforce the interaction. For example, synaptotagmin

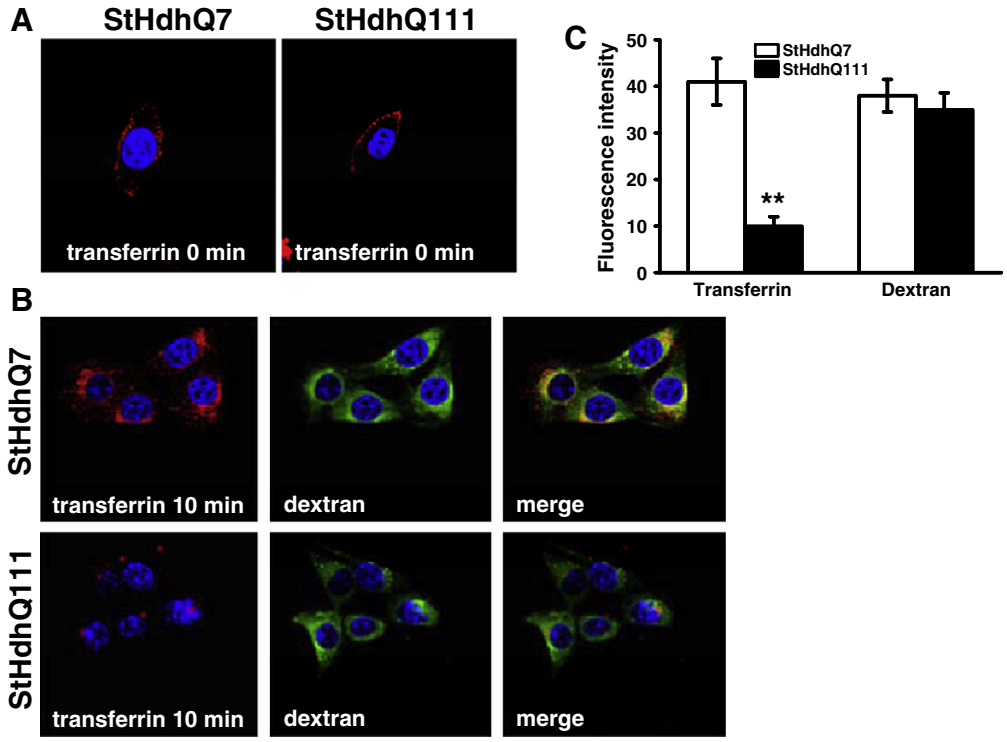

Fig. 5. Internalization of transferrin conjugated with Alexa-568 and dextran conjugated with FITC, by the StHdhQ7 and StHdhQ111 striatal cells. After incubation with transferrin at 4 °C, as detailed in the Materials and methods section, cells were warmed at 33 °C, and observed with a confocal microscope at 0 or 10 min after change (A and B respectively). C) Quantification of cell-associated fluorescence in B. Values represent the means of fluorescence intensity ± SD from three independent experiments. (**) Significantly different ($p<0.001$).

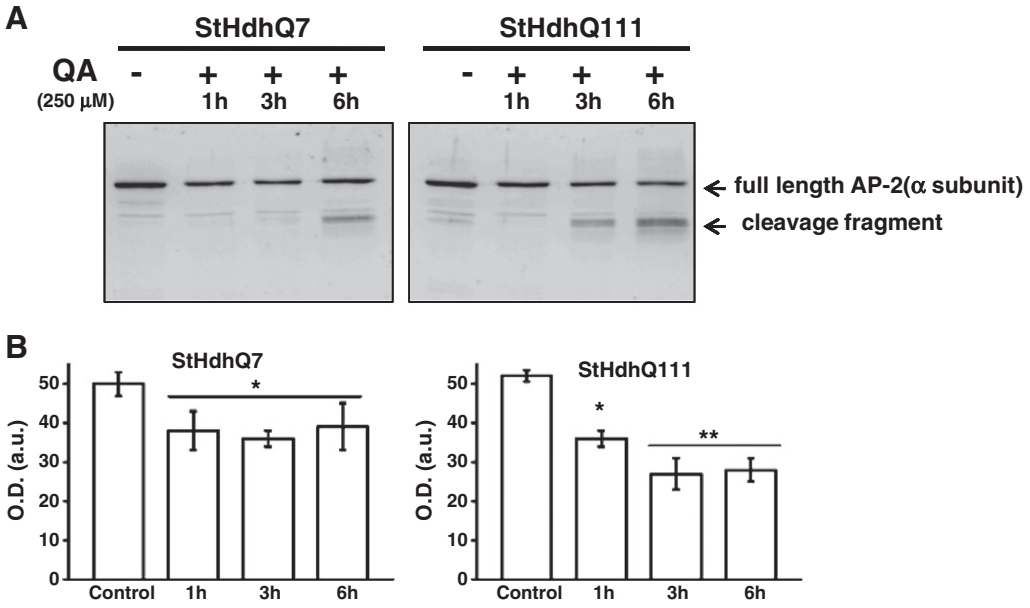

Fig. 6. Effect of quinolinic acid (QA) on the integrity of the α-subunit of AP-2. A) Immunoblot for detection of the protein from homogenates of StHdhQ7 and StHdhQ111 striatal cells, after incubation with QA at different times. B) Quantification of the band (full length protein) by densitometry. The bars represent the means of relative optical densities (O.D.) ± SD from three independent experiments. (*) and (**) significantly different with respect to controls ($p<0.01$ and $p<0.001$ respectively).

mediates recruitment of AP-2 to the plasma membrane (Mousavi et al., 2004). However, it is likely that many other proteins are necessary to ensure the recruitment of AP-2 and trigger the formation of CCVs.

In this study, we demonstrated that htt is somehow associated with AP-2, one of the key proteins of the endocytic machinery, and that anomalous polyglutamine expansion in htt affects the association between the two proteins. However, we should not rule out the possibility that the interaction between AP-2 and htt is mediated by HIP-1, despite the fact that the distribution of HIP-1 did not change with htt mutation. In the striatum of HD94 mice and in StHdhQ111 cells (with polyglutamine expansion in htt), AP-2 redistributes from the membranes to the cytosol. These results show that htt may act as a "docking" protein to allow the recruitment of AP-2 to membranes, whereas that function is lost in mHtt. Furthermore, we observed that alterations in the distribution of AP-2 in HD94 mice occur selectively in striatal neurons, consistent with the susceptibility of this brain area to neurodegeneration in the course of Huntington's disease. Other authors have demonstrated that cortical neurons are also significantly affected in HD (Reiner et al., 1988). This discrepancy with our results in transgenic mice could be due to differences in

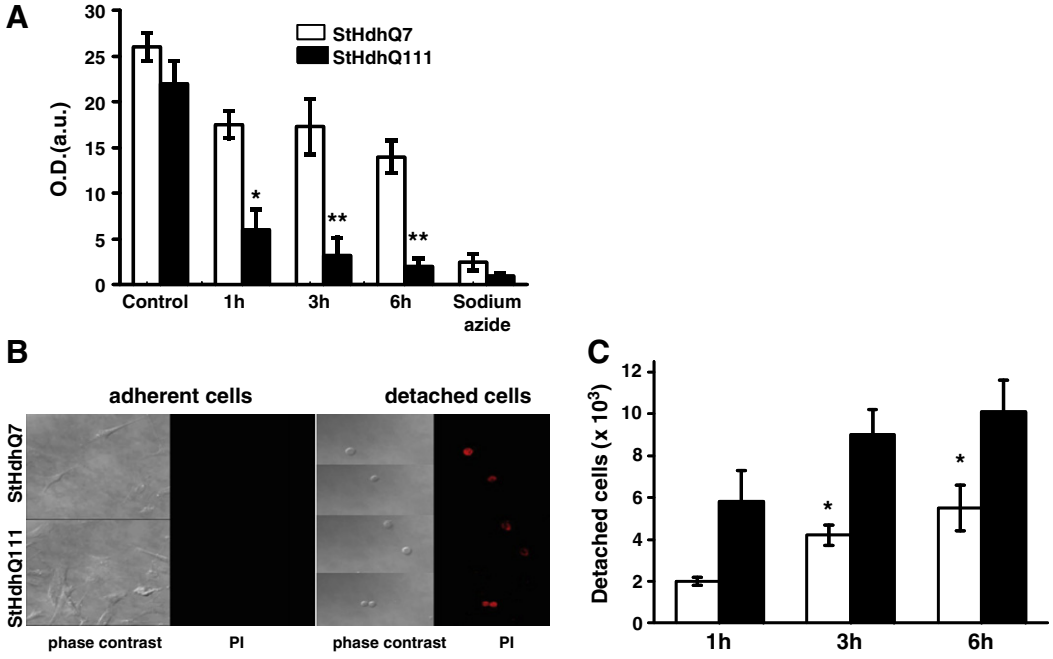

Fig. 7. Excitotoxic effect of QA on striatal neurons. A) The mitochondrial activity was measured with MTT, in StHdhQ7 and StHdhQ111 striatal cells after incubation with QA at different times. B) and C) Detachment of cells and staining with propidium iodide (as index of cell mortality) after treatment with QA, and quantification of detached cells. The bars represent the number of detached cells ± SD from three independent experiments. (*) and (**) significantly different ($p<0.05$ and $p<0.001$ respectively).

experimental models. The effect of the mutation in htt is selective for AP-2, since other proteins involved in CCV formation are not affected. This fact may reflect that these proteins have domains to interact with membranes independently of AP-2 recruitment (Legendre-Guillemin et al., 2004; Metzler et al., 2003; Pérez-Otaño et al., 2006; Roux et al., 2006). In this sense, it is known that HIP-1 and AP-180 have Anth/ENTH domains in their NH2-terminus, which are inserted into the lipid bilayer (Szymkiewicz et al., 2004), and dynamin has a PH domain that interacts with PtIns-4,5-P_2 (Qualmann et al., 1999).

The alteration in the recruitment of AP-2 to membranes in StHdhQ111 cells induces a decrease in CME, as uptake of transferrin is significantly reduced. Furthermore, the recruitment of AP-2 to membrane was not increased in these cells after stimulation with holotransferrin.

We also noted that StHdhQ111 cells are more vulnerable to excitotoxic effects of QA. It is currently unknown if there is a direct relationship between the expression of mHtt and increased levels of QA in HD, although it has been suggested that excitotoxic mechanisms are involved in the pathogenesis of HD. QA is an endogenous agonist for the NMDA-R (Brown et al., 1998; de Carvalho et al., 1996), produced as an intermediate metabolite from tryptophan (Schwarcz and Pellicciari, 2002). High levels of QA induce hyper-activation of NMDA-Rs, with a consequent increase in intracellular Ca^{2+}, and allow the activation of some enzymes that contribute to cell damage. In turn, a massive influx of Ca^{2+} produces an accumulation of this ion in the mitochondria, leading to dysfunction and subsequent decrease in ATP levels (Dugan and Kim-Han, 2006). Activation of proteases such as calpain explains the decrease of AP-2 in StHdhQ111 cells treated with QA, as α and β2 subunits of AP-2 are substrates for these proteases (Rudinskiy et al., 2009). We also observed increased susceptibility of the major subunits of AP-2 to cleavage by proteases in StHdhQ111 cells. This could be attributed to the redistribution of AP-2 into the cytosol, where these proteins would be exposed to the action of proteases. To counteract the mechanisms of excitotoxicity, the neurons regulate the number of NMDA-R on the cell surface. Some evidence indicates that these receptors are internalized via CCVs (Lavezzari et al., 2004). From our observations, we can infer that a dysfunction in the endocytic machinery leads to cellular damage due to increased exposure of NMDA-Rs to the toxic action of QA.

From these findings, we propose that htt could act as a "docking protein" to recruit AP-2 complexes to membranes. Subsequently, the protein "cargo" and surface receptors are recognized by AP-2 and internalized by CCVs. When htt is mutated, AP-2 accumulates in the cytosol, where it can be phosphorylated and can also become vulnerable to the action of proteases. Thus, the cells (striatal medium spiny neurons) could increase the number of NMDA-Rs on the surface, which would be hyperactivated by exposure to increased levels of QA observed in Huntington's disease. In conclusion, it is suggested here that alterations in the endocytic machinery, due to mutation in htt, may be involved in increasing cell vulnerability in Huntington's disease.

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