Calcium-dependent cleavage of endogenous wild-type huntingtin
in primary cortical neurons

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ABSTRACT
Huntington’s Disease (HD) is caused by a polyglutamine expansion in the amino-terminal region of huntingtin. Mutant huntingtin is proteolytically cleaved by caspases, generating amino-terminal aggregates which are toxic for cells. Addition of calpains to total brain homogenates also leads to cleavage of wild-type huntingtin, indicating that proteolysis of mutant and wild-type huntingtin may play a role in HD. Here we report that endogenous wild-type huntingtin is promptly cleaved by calpains in primary neurons. Loss of intact full-length wild-type huntingtin occurs also after exposure of primary neurons to glutamate or 3-nitropropionic acid, which lead to increased intracellular calcium concentration, and could be prevented by calcium chelators and calpains inhibitors. Degradation of wild-type huntingtin by calcium-dependent proteases thus occurs in HD neurons leading to loss of wild-type huntingtin neuroprotective activity.
INTRODUCTION

Huntingtin is a 348 kDa cytoplasmic protein which is important for cell survival (1-5). Interest into this protein stems from the fact that mutation into the encoding gene causes HD, a slowly progressing neurodegenerative disease which is characterized by the selective death of the striatal and cortical neurons (6).

In the pathology, the amino-terminal portion of huntingtin is characterized by an expanded polyglutamine stretch conferring a newly acquired toxic function to the protein (7). Several observations implicate mutant huntingtin proteolysis in the pathogenesis of HD since amino-terminal huntingtin (N-htt) fragments aggregate into the nucleus and cytoplasm of human neurons and overexpression of N-htt fragments in vitro causes cell death (8).

Despite the genetic and experimental evidence indicating a gain of function mechanism in HD, loss of normal huntingtin function may also be important (9). Normal huntingtin is antiapoptotic (1, 5) and exerts a peculiar function for striatal neurons, given that it sustains the production of cortically-derived BDNF, which they depend on for their activity (10). Proteolysis of wild-type huntingtin may cause loss of these physiological function(s). However, most of the research studies on the role of proteolysis in HD, so far, has been directed toward the analyses of mutant huntingtin. Mutant huntingtin is cleaved by caspases at aa 513 and aa 552. Mutation at these sites impairs caspase-3 cleavage and shows reduced toxicity after transfection in neuronal cells (11). Furthermore, transfection of cortical neurons with mutant huntingtin, in the presence of the broad spectrum caspase inhibitor z-VAD-fmk, blocks cleavage of exogenous huntingtin and reduces cell toxicity (12). Moreover, in HD-transgenic mice, caspases inhibition results in delayed onset of symptoms, slowed progression and prolonged survival (13, 14).

Proteases different from caspases may also be involved. Indeed, analyses of huntingtin proteolysis in HD postmortem brains have revealed fragments in cortex and striatum not justified only by caspase cleavage (15, 16). More recently, the possibility that wild-type huntingtin may be cleaved by calpains has been brought to attention by the work of Kim et al. (17). Calpains are calcium dependent non-caspase cysteine proteases which are enriched in neuronal cells. M-calpain and μ-calpain are the two main isoforms of calpains in the brain (18) and are activated in dendrites following excitotoxic and
ischemic insults in cell culture and in vivo (19). It was found that addition of m-calpain to lysates from total mouse brain causes cleavage of endogenous wild-type huntingtin and that a calpain inhibitor is able to block the cleavage of transfected wild-type huntingtin (17).

Here we aimed at evaluating whether endogenous wild-type huntingtin found in primary cortical neurons could be cleaved by stimuli that increase intracellular calcium levels, leading to calpain activation. We show that calcium influx, induced by a calcium selective ionophore, as well as by glutamate or 3-Nitropropionic (3NP) acid exposures, leads to cleavage of wild-type huntingtin in cortical neurons. Wild-type huntingtin proteolysis, without activating caspase-3, produces specific huntingtin fragments of about 75 and 60 kDa and is inhibited by calcium chelators or calpeptin. These data indicate that stimuli that trigger calcium-dependent calpain activation lead to proteolysis of wild-type huntingtin in primary brain neurons.
EXPERIMENTAL PROCEDURES

Materials:
Calpeptin was from Calbiochem and diluted in dimethyl sulfoxide (DMSO). 3NP, BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid tetrakis (acetoxymethyl ester)), ionophore A23187, were from Sigma. z-VAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone) was from Promega. Antibody MAb2166 was from Chemicon; anti-tubuline monoclonal antibody and anti caspase-3 monoclonal antibody were from Santa Cruz.

Cells culture:
Primary neuronal cultures from cerebral cortex were obtained from E16-E18 Sprague-Dawley rats according to standard procedures (20). Pregnant animals were killed by cervical dislocation under CO₂ anesthesia, the fetuses removed and put into ice cold HBSS. After dissection of the cortices, cells were dissociated, plated on poly-L-ornithine treated 35mm plates in DMEM plus 10%FCS and incubated at 37°C, 5% CO₂. The day after the medium was replaced with differentiating medium (3/4: Neurobasal medium from Euroclone, B27 and N2 from Gibco-Life Technologies, Pen-Strep, Glutamine. 1/4 DMEM F12, B27, pen-strep)

Eighteen days after plating, neurons were exposed to various treatments. In experiments where Calpeptin, BAPTA-AM, z-VAD-fmk were used, the drugs were added to the cells in KREB solution (NaCl 118.5 mM, KCl 4.8 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 25mM, Glucose 11 mM) 30 min. before ionophore, 3NP or glutamic acid. Control cultures were mantained in KREB solution.

For measurement of intracellular free calcium [Ca²⁺]ᵢ changes after 3NP, cortical neurons were plated on poly-L-ornithine coated 12 mm glass coverslip and then incubated in 0.5 ml of differentiating medium. Analyses were conducted eighteen days after plating.

Western blot analysis:
Cells were washed with phosphate buffered saline (PBS) and lysed 60 min. on ice in a buffer containing Tris HCl 10mM, NaCl 100mM, Triton X-100 1%, EGTA 5mM, EDTA 5mM, protease inhibitors. Samples were then centrifuged 15 min. at 14000 rpm at 4°C, supernatant were recovered and protein concentration evaluated by Bradford reagent assay (Biorad).
Equal amounts of protein were separated by 7.5% SDS PAGE. The blotted protein were exposed to anti-Htt MAb2166 antibody (dilution 1:5000) or anti caspase-3 (dilution 1:500). Bands were detected using an horseradish peroxidase secondary antibody and an enhanced chemiluminescence system (ECL Amersham).

**Densitometrical analysis**

The bands on the autoradiography corresponding to full length huntingtin and β-tubulin were scanned and quantified using NIH Image software. The ratio between full length huntingtin and tubulin in the untreated sample is considered 100% of basal huntingtin.

**m-Calpain biochemical assay:**

Neuronal cells grown on a 100 mm plate were washed with PBS, resuspended in 400µl of ice cold hypotonic lysis buffer (Tris HCl 50 mM, KCl 10 mM pH 7.4, β-mercaptoethanol 2%) and lysed for 40 min. on ice. After a 30 sec. sonication, samples were centrifuged 15 min. at 14000 rpm at 4°C. The supernatant was aliquoted. Specific treatments were applied to each sample. The reactions were conducted for 15 min. at 30°C. After adding sample buffer, samples were boiled and loaded onto a 7.5% SDS PAGE.

**Intracellular calcium measurements:**

Cultured cells plated on glass coverslips were loaded for 60 min. at 37°C with 2 µM Fura-2 pentacetoxy methylester in Krebs-Ringer solution buffered with HEPES (KRH) (150 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM glucose, and 10 mM HEPES/NaOH, pH 7.4), washed in the same solution to allow the deesterification of the dye, and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss) equipped with a calcium imaging unit. A modified CAM-230 dual wavelength microfluorimeter (Jasco, Tokyo, Japan) was used as a light source for the assays. The images of Fura-2 fluorescence at two excitation wavelengths (340 and 380 nm) were collected with a PCO Super VGA SensiCam (Axon Instruments, Forest City, CA, USA) and analyzed with the Axon Imaging Workbench 2.2 software (Axon Instruments, Forest City, CA, USA). Ratio values in discrete areas of interest were calculated from sequences of ratio images to obtain temporal analyses. Images were acquired at 1-5 340/380 ratios/s. Experiments were performed in static bath at room temperature (24-25°C). Drugs were applied in 2 ml of solution. The various reagents were added by loading appropriate volumes of 100X concentrated solutions into a syringe connected to the incubation chamber via a small tube; aspiration into the syringe of 1 ml of extracellular medium.
followed by the reintroduction of the mixture into the chamber ensured accurate and rapid delivery and mixing.
RESULTS

Proteolysis of endogenous wild-type huntingtin in primary neurons follows calcium influx

We aimed at analyzing whether endogenous huntingtin expressed in cortical neurons undergoes proteolysis induced by calcium entry into the cells. Eighteen days after plating, rat cortical neurons were exposed to the calcium selective ionophore (A23187) at a final concentration of 5 µM. Lysates were collected after 30 min., 1, 2 or 3 hours of treatment and processed for western blotting analyses using MAb 2166 antibody recognizing aa 181-767 of huntingtin. A band of 349 kDa corresponding to huntingtin was detected in control cultures (Fig. 1A, first lane upper panel). Upon exposure of the cultures to the ionophore, wild-type huntingtin levels decreased in a time-dependent manner, with an 80% reduction observed at 180 min. (Fig. 1A). This data supports the hypothesis that an increase in intracellular calcium level leads to degradation of endogenous wild-type huntingtin in neuronal cells.

Importantly, Figure 1A also shows increased levels of two MAb2166 immunoreactive bands of molecular weight of 75 kDa and 60 kDa in coincidence with the time-dependent decrease in intact endogenous huntingtin. These data indicate that normal huntingtin undergoes proteolytic cleavage in cortical neurons exposed to calcium ionophores, originating two amino-terminal MAb 2166 immunoreactive fragments. In the same blot, two additional bands of 68 and 70 kDa are present in all samples, likely representing cross-reactive proteins of unknown origin.

Huntingtin has been previously demonstrated to be cleaved by caspase-3 (21). We therefore investigated whether active caspase-3 could be measured in the experimental conditions indicated above that led to huntingtin proteolysis. We therefore evaluated caspase-3 activity by two means, i.e. by fluorimetric assays using specific fluorogenic substrate (Ac-DEVD-amc) (not shown) and by western blot analyses using an anti caspase-3 antibody able to recognize both the inactive and the two active p20 and p11 caspase-3 fragments. As shown (Fig. 1A lower autoradiogram, left panel), in the absence or presence of the calcium ionophore, a single band corresponding to inactive caspase-3 is detected in all samples. To validate further the specificity of the anti caspase-3 antibody, cells were exposed to serum-deprived medium (SDM), a condition that activates caspase-3 in our cellular system, as shown by the appearance of the two
immunoreactive bands corresponding to active caspase-3 (Fig. 1A lower autoradiogram, right panel). The same bands were not present in a parallel lysate obtained from cells where the caspase-3 inhibitor, z-VAD-fmk, was present.

We conclude that in our experimental condition caspase-3 activation appears not to be a major requirement for wild-type huntingtin proteolysis. Furthermore, cells exposed to stimuli that evoke caspase-3 activation do not seem to produce fragments similar to those observed in calpain activated cells (not shown).

Proteolysis of huntingtin in primary neurons is calpain-mediated

Given that addition of calpain induces proteolytic cleavage of huntingtin in total lysates from rodent brain (17) and that calpeptin, a specific inhibitor of µ-calpain and m-calpain, prevents proteolyses of transfected huntingtin in immortalized cells (17), we explored the possibility that a similar calpain-dependent cleavage of endogenous huntingtin could occur in primary cortical neurons. We exposed cortical neurons to different doses of calpeptin for 30 min. before addition of the calcium ionophore. As shown in Fig. 1B, cleavage of endogenous wild-type huntingtin could be prevented by the calpain inhibitor in a dose dependent manner.

We then tested whether endogenous wild-type huntingtin could be cleaved by calpain in vitro. Given that calpain working temperature in vitro is 30°C, we first checked whether simple exposure of the lysates to this temperature could evoke degradation of huntingtin. Lanes 1 and 2 in Figure 2 show that similar amounts of endogenous wild-type huntingtin are present at 4°C and 30°C. We next performed the assay at 30°C by incubating equal amounts of proteins with 0.1 U/ml m-calpain in the presence or absence of CaCl₂ 5 mM, as calpain activator. As shown in Figure 2, in the presence of activated calpain, endogenous wild-type huntingtin completely disappears (lane 3). This effect could be prevented by addition of calpeptin (lane 4) or in the absence of exogenous calcium (lane 5).

Stimuli that evoke [Ca²⁺], increases lead to cleavage of endogenous huntingtin

We then evaluated whether stimuli able to evoke elevation in [Ca²⁺], into neurons lead to proteolysis of endogenous huntingtin. In Fig. 3 cortical neurons were exposed to the excitatory aminoacid glutamate at a dose of 100 µM (panel A) or 500 µM (panel B) or to 5mM 3NP (panel C), a mitochondrial toxin. [Ca²⁺], homeostasis in these conditions was analyzed by loading of the cortical neurons with FURA-2 (Fig. 4). Increased cytoplasmic
concentrations of calcium was promptly measured in single neuronal cells after exposure to 100 µM glutamate (Fig. 4A, 4C). Instead, a 60 min. latent period was observed after exposure to 3NP after which \([\text{Ca}^{2+}]_i\) reached levels comparable to those evoked by glutamate. These data indicated that both glutamate and 3NP were able to induce calcium movements in our experimental conditions.

In these same conditions we analyzed the levels of endogenous wild-type huntingtin by western blotting. As shown in Fig. 3A, exposure of primary neurons to 100 µM glutamate for 24 hours caused a 56% decrease in the amount of endogenous intact huntingtin. Given that glutamate increases intracellular calcium levels in neurons (Fig. 4A, 4C), we applied 50 µM BAPTA-AM, an intracellular calcium chelator, and found it could prevent the loss of intact wild-type huntingtin observed in the presence of glutamate. In a subsequent experiment, 500 µM glutamate was applied for different time periods. As shown in Fig. 3B, the levels of endogenous wild-type huntingtin decrease starting at 2h after glutamate addition and was maximal at 24h, when fragments of huntingtin could be detected. Also in this condition addition of calpeptin was able to prevent cleavage. Finally and as expected, given the changes in \([\text{Ca}^{2+}]_i\) levels observed with 5mM 3NP, (Fig. 4B, 4C) we found that exposure of the neuronal cultures to this toxin could also evoke changes in the levels of intact wild-type huntingtin and the appearance of huntingtin fragments, an effect that was prevented by calpeptin (Fig. 3C). Also in this condition, proteolysis of intact wild-type huntingtin originated Mab2166 immunoreactive bands. The caspase inhibitor z-VAD-fmk was partially able to prevent depletion of endogenous wild-type huntingtin (Fig. 3C).
DISCUSSION

Here we show that changes in calcium homeostasis evoked by glutamate and 3NP lead to calpain-dependent proteolysis of endogenous wild-type huntingtin in cortical neurons. Previous data by Kim et al. (17) indicated that endogenous huntingtin from total brain homogenates is cleaved in vitro by calpains and that cleavage of transfected huntingtin could be prevented by a specific calpain inhibitor. We now report that stimuli that lead to changes in calcium levels in primary neurons cause proteolyses of endogenous wild-type huntingtin, in the absence caspase-3 activation (Fig. 1A). In our conditions, exposure of neuronal cultures to 3NP was able to evoke intracellular calcium influx comparable to that observed in glutamate treated neurons (Fig. 4). In both conditions, analyses of endogenous huntingtin revealed a similar decrease in protein levels, an effect that could be prevented by addition of calpain inhibitors or calcium chelators, respectively.

We found that calpain-mediated cleavage of wild-type huntingtin results in the appearance of N-terminal fragments of about 75 and 60 kDa. Fragments of a similar m.w. were identified after in vitro calpain-mediated cleavage of huntingtin from brain homogenates (17).

The evidence of calpain-mediated cleavage of wild-type huntingtin in neuronal cells is important for several factors. On one side it supports the hypothesis that an unbalance in calcium homeostasis may lead to huntingtin proteolysis and, on the other hand, it strengthens the idea that loss of full-length wild-type huntingtin protein and of its physiological activity may occur in HD and in other brain diseases characterized by alterations in calcium levels.

Calcium mediated excitotoxicity and mitochondrial dysfunction have long been hypothesized to play a role in the pathogenesis of HD (22). In R6/2 HD transgenic mice and, to a lower extent, in YAC72 mice expressing the full-length mutant gene, an increased intracellular \([\text{Ca}^{2+}]_i\) flux evoked by selective activation of NMDA receptors was demonstrated at both presymptomatic and symptomatic stages. Parallel increases in NMDA-R1 receptors and decreases in NMDA-R2A/B subunit proteins were also reported (23–25). The latter modification is of particular interest given that calpains are able to cleave the R2A/B subunit of NMDA glutamate receptor (26). It is also worth noting that NMDA-R1 receptor, which are not calpains substrate, are enriched in striatal
neurons which colocalize somatostatin (SS), neuropeptide Y (NPY) and NADPH-diaphorase (NADPH-d) and that are selectively spared in Huntington’s Disease and are also resistant to experimentally induced excitotoxic cell death (27). Calbindin D28k level, a protein with an high capacity for buffering $\text{Ca}^{2+}$, was also increased in the distal dendrites of spiny striatal neurons in HD postmortem striatum (28). All together these and other new evidence (29) indicate that calcium handling is impaired in Huntington’s Disease, therefore possibly influencing the activation of calcium-dependent proteases.

Other data point to a striatal specific activation of calpains. Indeed, ratio of the active versus inactive calpains in different brain areas from postmortem HD patients revealed a significant increase in calpains activation in putamen, while no changes were observed in frontal cortex or cerebellum. On the contrary, in Alzheimer’s Disease patients, all brain regions were similarly characterized by an increase in calpains activation with respect to controls (30).

Proteolyses of wild-type huntingtin is expected to impact cell survival and function (9). Indeed, normal huntingtin is antiapoptotic in brain cells (1, 2, 5, 31) and it is also found to protect from toxicity induced by the mutant protein (3, 4) and to support the production of cortically-derived BDNF (10).

Earlier data pointed to caspases-mediated poly-glutamine dependent cleavage of mutant huntingtin as one of the key events leading to cell toxicity (11). In this scenario, compounds able to inhibit caspases activation have been proven able in delaying disease onset in experimental animal models of HD (13).

The discovery of important physiological function(s) of wild-type huntingtin in brain cells (9) and the demonstration reported by Kim (17) and also in this paper that wild-type huntingtin is a target for calpain-mediated proteolyses in neurons, support the idea that drugs able to inhibit calpains in humans may also lead to increased wild-type huntingtin levels, therefore potentially restoring its neuroprotective activity.
FIGURE LEGEND:

**Fig. 1 (A) Calcium dependent cleavage of wild-type huntingtin in primary neurons.** Cortical neurons were exposed to A23187 calcium ionophore at a concentration of 5 µM and then lysed. A time-dependent decrease in endogenous wild-type huntingtin is observed (upper panel). Incubation of the same membranes with MAb 2166 antibody gave rise to two immunoreactive bands of about 60 and 75 kDa which increase in levels upon exposure to the ionophore (second panel from top). Blot on the bottom, left: in our experimental conditions, bands corresponding to active caspase-3 were not detected in the blots. Right: positive control, active caspase-3 is detected in ST14A cells exposed to SDM. The same fragments are not visible in z-VAD-fmk pretreated cells. Graph: densitometrical analysis performed by normalizing huntingtin signal to the β-tubulin band. Data shown are from one out of three different experiments producing the same results.

**Fig. 1 (B) Cleavage of wild-type huntingtin in primary neurons is calpain-mediated.** Cortical neurons were pretreated for 30 min. with increasing amounts of the calpain inhibitor calpeptin before addition of the ionophore. Samples were then analyzed for the levels of intact wild-type huntingtin and cleavage products. Densitometrical analysis was performed by normalizing full length huntingtin signal to β-tubulin. Data shown are from one out of three different experiments producing the same results.

**Fig. 2 Cell-free calpain assay.** 0.1 units/ml of m-calpain were incubated with cell lysates in the presence or absence of exogenous calcium. In these conditions only calcium-dependent m-calpain cleavage of huntingtin is observed. Presence of calpeptin or absence of calcium completely prevents proteolyses. Data shown are from one out of four different experiments producing the same results.

**Fig. 3 Glutamate, 3-NP and ionophore induced cleavage of wild-type huntingtin in cortical neurons.** Panel A: neuronal cultures were treated with 100 µM glutamate for 24h and assayed for the level of endogenous huntingtin in the absence or presence of the calcium chelator (BAPTA-AM). Panel B: neuronal cultures were treated with 500 µM glutamate for different time periods in the absence or presence of calpeptin. Panel C: cortical neurons maintained in the presence of 5 mM 3NP in the absence or presence of calpeptin. The caspase inhibitor z-VAD-fmk was partially able to prevent
3NP induced huntingtin proteolysis. In the same experiment, as a control, cells were exposed to A23187.

Data shown are from one out of three different experiments producing the same results.

**Fig. 4 (A) Intracellular calcium homeostasis in glutamate and 3NP treated neurons.** Temporal analysis of \([\text{Ca}^{2+}]_i\) changes in FURA2-loaded cortical neurons in the presence of calcium ions in the extracellular saline. Traces represent recordings from the soma of distinct neurons. 3NP induces gradual and persistent increases in \([\text{Ca}^{2+}]_i\) with a preonset latency of 60-120 min (lower graph). No significant changes in \([\text{Ca}^{2+}]_i\) are observed in control conditions (upper graph). At the end of the recording, cells were challenged with 100 µM glutamate revealing the expected FURA2 profile (upper graph).

**Fig. 4 (B) Quantitative analysis of \([\text{Ca}^{2+}]_i\) changes.** Changes in \([\text{Ca}^{2+}]_i\) induced by 3NP compared to \([\text{Ca}^{2+}]_i\) increases induced by 100µM glutamate. Histograms show percent changes normalized to controls (+SE) and are derived from three independent experiments.
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MAb2166 immunoreactive bands

206 kDa

52 kDa

huntingtin

% huntingtin vs β-tubulin

0 30 60 120 180 min.

5 µM A23187 (min.) + + SDM 0 30 60 120 180

procaspase-3

active caspase-3

β-tubulin
B

MAb2166 immunoreactive bands

% huntingtin vs β-tubulin

huntingtin

β-tubulin

206 kDa
119 kDa
91 kDa
52 kDa

0            0          200        50           5          0.5     calpeptin (µM)
0           +            +            +           +           +       5µM A23187, 180 min.
Fig. 2

348 kDa

|                | 30 min. 4°C | 30 min. 30°C |
|----------------|-------------|--------------|
| 0              | 0           | +            |
| 0              | +           | +            |
| 0              | 0           | +            |
| 0              | 0           | +            |
| 0              | 0           | +            |

0.1 U/ml m-calpain

5 mM CaCl₂

200 µM calpeptin
Fig. 3

A

huntingtin

β-tubulin

% huntingtin vs β-tubulin

![Image](image1.png)

B

huntingtin

MAb2166 immunoreactive bands

β-tubulin

% huntingtin vs β-tubulin

![Image](image2.png)

C

huntingtin

MAb2166 immunoreactive bands

β-tubulin

% huntingtin vs β-tubulin

![Image](image3.png)
Fig. 4

A. Control and 100 µM glutamate

B. 3NP

C. F340/F380 (normalized values)

- control
- 5 mM 3NP
- 100 µM glutamate
Calcium-dependent cleavage of endogenous wild-type huntingtin in primary cortical neurons
Donato Goffredo, Dorotea Rigamonti, Marzia Tartari, Alberto De Micheli, Claudia Verderio, Michela Matteoli, Chiara Zuccato and Elena Cattaneo

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