Polyglutamine-expanded Huntingtin Promotes Sensitization of N-Methyl-D-aspartate Receptors via Post-synaptic Density 95*

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Increased glutamate-mediated excitotoxicity seems to play an important role in the pathogenesis of Huntington’s disease (Tabrizi, S. J., Cleeter, M. W., Xuereb, J., Taaman, J. W., Cooper, J. M., and Schapira, A. H. (1999) Ann. Neurol. 45, 25–32). However, how polyglutamine expansion in huntingtin promotes glutamate-mediated excitotoxicity remains a mystery. In this study we provide evidence that (i) normal huntingtin is associated with N-methyl-D-aspartate (NMDA) and kainate receptors via postsynaptic density 95 (PSD-95), (ii) the SH3 domain of PSD-95 mediates its binding to huntingtin, and (iii) polyglutamine expansion interferes with the ability of huntingtin to interact with PSD-95. The expression of polyglutamine-expanded huntingtin causes sensitization of NMDA receptors and promotes neuronal apoptosis induced by glutamate. The addition of the NMDA receptor antagonist significantly attenuates neuronal toxicity induced by glutamate and polyglutamine-expanded huntingtin. The overexpression of normal huntingtin significantly inhibits neuronal toxicity mediated by NMDA or kainate receptors. Our results demonstrate that polyglutamine expansion impairs the ability of huntingtin to bind PSD-95 and inhibits glutamate-mediated excitotoxicity. These changes may be essential for the pathogenesis of Huntington’s disease.

Huntington’s disease (HD)‡ is a dominant inherited neurodegenerative disorder characterized by choreiform movement, psychiatric disturbance, and cognitive decline (2). The HD gene encodes a 350-kDa protein designated as huntingtin (3), which is richly expressed in dendrites and nerve terminals, where huntingtin is associated with synaptic vesicles and microtubule complexes (4–5). The defect of the HD gene is the expansion of a CAG repeat encoding polyglutamine at its 5’ end, and the length of the repeat is correlated with the age of onset and the severity of the disease (6).

Although the HD gene has been identified for many years, how polyglutamine-expanded huntingtin causes neurons to die remains unclear. Increased glutamate-mediated excitotoxicity in HD has been a very popular hypothesis for the last 25 years (1). The hypothesis is generated from findings that the intrastrial injection of glutamate or kainic acid in rat causes selective loss of medium spiny neurons that are also selectively affected in HD (7–8). This hypothesis is supported further by the findings that NMDA receptors are hyperactive, and excitotoxicity mediated by these receptors is enhanced significantly in HD transgenic mice (9–10). These results suggest that overactivation of glutamate receptors may play a significant role in the pathogenesis of HD. However, over 95% of normal or polyglutamine-expanded huntingtin is located in the cytoplasm (6–7), whereas glutamate receptors are cell surface receptors. How a cytoplasmic protein alters glutamate receptors on the cell surface membrane is an intriguing question.

PSD-95 is a scaffold protein that contains an SH3 domain, a GK domain, and three PDZ domains that bind to the NMDA receptor NR2 subunits and kainate receptor GluR6 subunit (11–12). The binding of PSD-95 to NMDA or kainate receptors causes the clustering of the receptors in the postsynaptic membrane and regulates NMDA-dependent long term potentiation and long term depression (12). PSD-95 also binds to cytoplasmic signaling proteins and links the receptors to cellular signaling cascades (13–14). In transgenic mice lacking PSD-95, the frequency function of NMDA-dependent long term potentiation and long term depression is shifted, and spatial learning is impaired severely (15). Suppression of PSD-95 expression inhibits NMDA receptor-mediated activation of nitric-oxide synthase and excitotoxicity (16). These studies suggest that PSD-95 regulates glutamate receptor-mediated excitotoxicity and plays an important role in spatial learning, which is severely impaired in HD patients and HD transgenic mice (17–19). In previous studies, we observed that the overexpression of polyglutamine-expanded huntingtin caused neuronal apoptosis via activation of the mixed lineage kinase/c-Jun N-terminal kinase signaling pathway (20–21), and mixed lineage kinase is also involved in neuronal toxicity mediated by GluR6 receptors via interaction with PSD-95 (22). The present study is intended to investigate the “missing link” protein between glutamate receptors and huntingtin and to determine its pathological significance in HD.

MATERIALS AND METHODS

Cell Culture and Transient Transfection—HN33 (an immortalized rat hippocampal neuronal cell line) (20) and 293T cells (human embryonic kidney cells expressing SV40 large T antigen) were maintained in Dulbecco’s modified Eagle’s medium/F-12 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection was performed in serum- and Mg²⁺-free medium using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. Between 10 and 50 μg of plasmids with 10–20 μl of Lipofectin/10-cm plate were used in transfection experiments.

Western Blotting and Immunoprecipitation—48–72 h after transfection, 293T cells were harvested and lysed in 1% Nonidet P-40 lysis
buffer, and co-immunoprecipitation experiments were conducted as described previously (23). Human cortex tissues were obtained from Dr. J.-P. Vonsattel or the Human Brain Bank at McLean Hospital (Belmont, MA) with institutional review board approval. Post-mortem time was between 16 and 24 h. The diagnosis of HD was confirmed with neuropathological and genetic phenotype analysis. Human cortex tissues from normal subjects or HD patients were homogenized in detergent-free lysis buffer, and SDS was added to the final concentration of 2%. The mixture was diluted 1:5 with 1% Nonidet P-40 lysis buffer. After removal of the insoluble fractions by centrifugation, brain lysates were co-immunoprecipitated with 437, a rabbit polyclonal antibody against the first 17 amino acids of huntingtin that has been previously characterized (23), or other antibodies as indicated in the figure legends.

**RESULTS**

Initially, we studied the interaction of normal huntingtin with PSD-95 in 293T cells, which are rich in huntingtin (23). Full-length PSD-95 expressing vector was transiently transfected in 293T cells. 48 h post-transfection, cell lysates were prepared, and co-immunoprecipitation was conducted using 437, an anti-huntingtin antibody (23), or a monoclonal antibody specific for PSD-95. The resulting blot was then probed with the anti-PSD-95 antibody. As shown in Fig. 1A, PSD-95 was detected in both 437 or PSD-95 (positive control) immunoprecipitates of 293T cell lysates transfected with PSD-95 but not in 437 immunoprecipitates of wild-type or vector-transfected 293T cell lysates. We then explored the association of huntingtin with PSD-95 in human cortex tissues. Brain lysates were immunoprecipitated with 437 or negative control, protein A-Sepharose beads alone, 437 pre-immune serum, or 437 pre-absorbed with its peptide antigen. The blot was analyzed with an anti-PSD-95 antibody. As shown in Fig. 1B, PSD-95 was only detected in 437 immunoprecipitates but not in any of the negative controls. Conversely, huntingtin was also present in
PSD-95 immunoprecipitates (Fig. 1C). The data suggest that huntingtin is associated with PSD-95 in human cortex.

PSD-95 is known to bind to the C terminus of the NMDA receptor NR2 subunit and the kainate receptor GluR6 subunit (11–12). If huntingtin is associated with PSD-95, it may also assemble complexes with NMDA and GluR6 receptors. Thus, we investigated whether huntingtin is associated with NMDA and kainate receptors. Human cortex lysates were incubated with anti-NR1, -NR2A, -NR2B, -GluR6, or -dopamine D4 receptor or with 437 or 437 pre-immune serum. The blot was probed with 437. As shown in Fig. 1C, huntingtin was found in the anti-NR1, -NR2A, -NR2B, -GluR6, and 437 immunoprecipitates but not in the anti-D4 receptor and 437 pre-immune serum precipitates. The data suggest that huntingtin assembles complexes with NMDA and GluR6 receptors in human cortex.

Because PSD-95 has a type II SH3 domain, we examined whether normal huntingtin can bind to the SH3 domain of PSD-95. GST alone or various PSD-95-GST fusion proteins were prepared; 1–2 μg of these PSD-95-GST fusion proteins were incubated with wild-type 293T cell lysates rich in normal huntingtin. As shown in Fig. 2, huntingtin only binds to the SH3 or SH3-GK domain, but it does not bind to GST alone, the GK domain, or any of the three PDZ domains of PSD-95. The data suggest that the SH3 domain of PSD-95 mediates its binding to huntingtin.

We next determined whether expansion of the polyglutamine repeat in huntingtin would alter its interaction with PSD-95. The N-terminal proline-rich region adjacent to the polyglutamine repeat has been reported to bind to SH3 domain-containing proteins (21, 24). To determine whether expansion of the polyglutamine repeat in huntingtin would alter its interaction with PSD-95, we examined the binding of PSD-95 to the huntingtin N terminus containing either a normal or expanded polyglutamine stretch. The huntingtin N terminus GST fusion proteins containing 16 or 56 polyglutamine repeats were generated and purified as described previously (21). These GST fusion proteins were incubated with 293T cell lysates expressing full-length PSD-95. As shown in Fig. 3A, PSD-95 binds to the huntingtin N terminus containing 16 polyglutamine repeats. Because the proline region is the only SH3 domain-binding site in this small N-terminal segment of huntingtin, these data suggest that the N-terminal proline region of huntingtin mediates its interaction with PSD-95. The amount of PSD-95 bound to the huntingtin N terminus with 56 polyglutamine repeats was significantly reduced, ~70% less than that associated with normal huntingtin (Fig. 3A). This finding suggests that polyglutamine expansion inhibits the ability of huntingtin to bind to the SH3 domain of PSD-95.

Next, we explored the association of huntingtin with PSD-95 in the brains of HD patients. Lysates of human cortex tissues from two normal subjects and two HD patients with mid-age onset were prepared. These lysates were incubated with 437, and the resulting blots were analyzed with an anti-PSD-95 antibody. As shown in Fig. 3B, huntingtin was associated with PSD-95 in the cortex tissues of both normal subjects and HD patients. However, the association of huntingtin with PSD-95 in HD patients is significantly weaker than that in normal subjects. Although it might be expected that the heterozygous nature of the huntingtin in HD patients would result in a PSD-95 associated with huntingtin of 50% compared with normal subjects; the amount of PSD-95 associated with huntingtin in the HD patients is much less, ~80% less than that in normal subjects (not 50% lower as one would expect, given the heterozygous nature of these HD patients). The brain lysates were precipitated with anti-PSD-95, and the blot was probed with 437. Normal huntingtin in the brains of HD patients remained to interact with PSD-95; however, the amount of huntingtin proteins associated with PSD-95 was below 50% (Fig. 3C). We also found that polyglutamine-expanded huntingtin is not present in the anti-PSD-95 immunoprecipitates in the brains of HD patients (Fig. 3C). The data suggest that polyglutamine-expanded huntingtin fails to bind to PSD-95 and that the normal huntingtin in the brains of these patients may be re-distributed. Similar results were obtained with three other normal subjects, and HD patients with different post-mortem times obtained similar results (data not shown). We conclude that the difference in the extent of the association of huntingtin with PSD-95 between normal subjects and HD patients is not because of the difference of post-mortem time but reflects a genuine change in the ability of huntingtin to bind to PSD-95 in HD patients.

The physiological relevance of the association of huntingtin with PSD-95 was examined because PSD-95 is known to regulate NMDA receptor- or GluR6-operated channels by clustering these receptors at post-synaptic membranes (11–12). Our hypothesis is that normal huntingtin modulates these receptor-operated channels by binding and sequestering PSD-95 and thereby regulates the clustering of these receptors. Because the ability of huntingtin to bind to PSD-95 is impaired severely upon its polyglutamine expansion, more PSD-95 proteins may be available to cluster NMDA receptors, leading to overactivation or sensitization of these receptors and excitotoxicity. If our hypothesis is correct, the expression of polyglutamine-expanded huntingtin may enhance, and overexpression of normal huntingtin may inhibit, neuronal toxicity mediated by NMDA or GluR6 receptors. In our previous studies, we observed that the overexpression of polyglutamine-expanded huntingtin in HN33 cells (which expresses both NR1 and NR2A receptors (data not shown)) induces apoptotic cell death (20–21). To test our hypothesis, we examined whether expression of the mutated huntingtin may promote activation of NMDA receptors in HN33 cells. Full-length huntingtin containing 16 or 48 polyglutamine repeats (pFL16HD or pFL48HD) was transiently expressed in HN33 cells; 1 mM glutamate, 10 μM D-AP5 (a selective NMDA antagonist), or glutamate + D-AP5 was included in the transfection medium. 24 or 48 h post-transfection, cells were fixed, TUNEL stain-detecting was conducted on apoptotic cells, and TUNEL-negative cells (living cells) were counted. Consistent with our previous reports, HN33 cells transfected with pFL48HD began to undergo apoptosis at ~24 h post-transfection (Fig. 4A). At 48 h, ~60% of HN33 cells were

Fig. 2. The SH3 domain of PSD-95 mediates its binding to huntingtin. 293T cell lysates were incubated with ~2 μg of GST alone or different PSD-95-GST fusion proteins, and the blot was analyzed with 437. SH3, SH3-GK, GK, PDZ1, PDZ2, and PDZ3 indicate different PSD-95-GST fusion proteins containing SH3, SH3-GK, GK, or a PDZ1, PDZ2, or PDZ3 domain. 437IP, immunoprecipitation with 437. The data represent a typical experiment that has been repeated three times with similar results.
apoptotic (Fig. 4A). However, the treatment of wild-type HN33 cells or cells transfected with pFL16HD with glutamate did not alter cell viability (Fig. 4A). Also, treatment of HN33 cells transfected with pFL48HD with glutamate significantly promoted neuronal toxicity. At 24 h post-transfection, 35–40% of HN33 cells were apoptotic when glutamate was included in the transfection medium, compared with 10–15% that were apoptotic when glutamate was not included in the transfection medium without glutamate (Fig. 4A). At 48 h, 85% of HN33 cells were apoptotic when treated with glutamate as compared with ~60% of apoptotic cells in the glutamate-free transfection medium (Fig. 4A). The addition of N-A-P5 significantly attenuated neuronal toxicity mediated by both glutamate and the mutated huntingtin. At 48 h, the number of apoptotic cells induced by the mutated huntingtin alone was reduced to 30% and in the presence of N-A-P5, the number of apoptotic cells induced by both mutated huntingtin and glutamate was reduced to 36% (Fig. 4B). The data suggest that the expression of polyglutamine-expanded huntingtin may sensitize and activate NMDA receptors, leading to neuronal toxicity.

Our hypothesis predicts that overexpression of the normal huntingtin N terminus (which binds and sequesters PSD-95) may inhibit neuronal toxicity induced by both mutated huntingtin and NMDA receptors. To test this possibility, pFL48HD was co-transfected with pN16HD (truncated huntingtin encoded by the first three exons with 16 polyglutamine repeats) into HN33 cells, and 1 mM glutamate was added to the transfection medium. As shown in Fig. 4C, the number of apoptotic cells induced by glutamate and mutated huntingtin was significantly reduced when pN16HD was co-expressed. This neuroprotective action mediated by normal huntingtin was abolished when full-length PSD-95 was co-introduced (Fig. 4C). The data support the view that normal huntingtin sequesters PSD-95 and thereby inhibits neuronal toxicity mediated by glutamate receptors.

**DISCUSSION**

Striatal medium spiny neurons are the first population of neurons affected in HD. It seems that the activation of NMDA or kainate receptors in the striatum also selectively induces the death of the same population despite the fact that these receptors are widely distributed in other types of striatal neurons (25). Mice transgenic for the HD gene exhibit hyperactive NMDA receptors in the brain and are deficient in long term depression and spatial learning (9–10), suggesting that expression of the mutated huntingtin can cause sensitization and activation of membrane NMDA receptors. However, full-length polyglutamine-expanded huntingtin displays perinuclear localization (26–27). PSD-95 is known to bind and regulate the activity of glutamate receptors (11–12). Thus, the association of huntingtin with PSD-95 clearly provides a crucial link between glutamate receptors and the mutated huntingtin.

Our current study indicates that normal huntingtin binds to PSD-95 and sequesters the scaffold protein, resulting in the inhibition of NMDA receptor activity. Overexpression of the normal huntingtin N terminus significantly attenuates neuronal toxicity induced by both NMDA receptors and the mutated huntingtin.
The mutated huntingtin. HN33 cells were co-transfected with pcDNA1 normal huntingtin inhibits neuronal toxicity induced by glutamate and designated as 100%. of TUNEL-negative cells in the control (transfected with pcDNA1) was stain was conducted; TUNEL-negative cells were counted. The number receptors. HN33 cells were transfected with pFL48HD in the Mg2 expanded huntingtin promotes neuronal toxicity mediated by NMDA glutamate receptors. 

A

FIG. 4. Polyglutamine-expanded huntingtin enhances, and normal huntingtin inhibits, neuronal toxicity mediated by glutamate receptors. The data presented are the average of three or four independent experiments. A and B, the expression of polyglutamine-expanded huntingtin promotes neuronal toxicity mediated by NMDA receptors. HN33 cells were transfected with pFL48HD in the Mg2+-free medium. Glutamate (Glu) (1 mM), D-AP5 (10 µM), or glutamate + D-AP5 (Glu + D-AP5) was added into the transfection medium. Cells were fixed at (A) or 48 h after (B) the time of post-transfection, and the TUNEL stain was conducted. TUNEL-negative cells were counted. The number of TUNEL-negative cells in the control (transfected with pcDNA1) was designated as 100%. con, control. C, overexpression of the N terminus of normal huntingtin inhibits neuronal toxicity induced by glutamate and the mutated huntingtin. HN33 cells were co-transfected with pcDNA1 (mock) or pFL48HD and pN16HD in the Mg2+-free medium in the absence (control) or presence of glutamate (Glu) (1 mM), and the TUNEL stain was performed 48 h after transfection.

huntingtin, and co-expression of wild-type PSD-95 inhibits the neuroprotective action of normal huntingtin. This suggests that PSD-95 is a mediator of neuronal toxicity induced by NMDA receptors and mutated huntingtin. The data are consistent with our recent report (23), as well as studies from other groups (16). Gain of function has been a popular hypothesis in the HD field in the last 20 years (28). Our previous and current studies show that loss of function may play an important role in the induction of neuronal death in HD (21). Consistent with our results, other groups have also shown that the expression of normal huntingtin in HD transgenic mice attenuates neuronal toxicity induced by the mutated huntingtin (29). Thus, the competitive loss of function of normal huntingtin may initiate the pathogenesis of HD. An interesting question is why heterozygous patients exhibit a similar HD phenotype with homozygous patients. We found that the ability of normal huntingtin to bind to PSD-95 in HD patients is also impaired and that the amount of huntingtin associated with the scaffold protein is well below 50%. In other words, the mutated huntingtin not only fails to bind to PSD-95 but also inhibits the binding of normal huntingtin to the scaffold protein in the human post-mortem brains. These observations reflect the dominant nature of the huntingtin mutation and may explain the clinical similarity between homozygous and heterozygous HD patients (29).

Clinically, HD is characterized by movement disorders, cognitive decline, and psychiatric disturbance (4, 17). If a protein were essential for the pathogenesis of HD, one would assume that disruption of the biological function of this protein might lead to these clinical symptoms. PSD-95 fits the role of such a protein because PSD-95 is known to be involved in excitotoxicity mediated by glutamate receptors (16, 22). Increased glutamate-mediated excitotoxicity plays a critical role in neuronal loss in HD (1–3, 9–10). Gene-targeted knockout of PSD-95 severely impairs spatial learning, which is also a feature of HD patients and transgenic mice (17–19). The alteration of NMDA receptor-mediated function causes schizophrenic behavior (30), and many HD patients have schizophrenia-like psychiatric disturbances (4, 17). Thus, our present study may reflect an important molecular mechanism underlying pathological changes in HD.

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