

Expression of Polyglutamine-expanded Huntingtin Induces Tyrosine Phosphorylation of *N*-Methyl-D-aspartate Receptors*

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In our previous studies, we found that expression of polyglutamine-expanded huntingtin in HN33 cells induced sensitization of *N*-methyl-D-aspartate (NMDA) receptors (Sun, Y., Savinainen, A., and Liu, Y. F. (2001) *J. Biol. Chem.* 276, 24713–24718). Following this study, we investigated whether tyrosine phosphorylation of NMDA receptors might contribute to the altered property of the receptors. Expression of polyglutamine-expanded huntingtin induced elevation of phosphorylated or activated Src and increased targeting of PSD-95 (post-synaptic density 95) and activated Src to cell surface membrane. Expression of the mutated huntingtin also induced tyrosine phosphorylation of NR2B (NMDA receptor 2B) subunits, and co-expression of PSD-95 enhanced the phosphorylation. Treatment of SU6656 (a specific Src inhibitor) or co-expression of a mutated NR2B subunit with mutations of all three major tyrosine phosphorylation sites significantly attenuated neuronal toxicity induced by the mutated huntingtin. Addition of AP-5 did not further inhibit the neuronal toxicity. Taken together, our studies show that polyglutamine-expanded huntingtin increases tyrosine phosphorylation of NMDA receptors via PSD-95 and Src, and increased tyrosine phosphorylation may contribute to the sensitization of the receptors mediated by polyglutamine-expanded huntingtin.

of the NMDA receptor function (10). Src tyrosine kinases are anchored by PSD-95 to the proximity of NMDA receptors where they induce tyrosine phosphorylation of NR2A and NR2B subunits and enhance the receptor-mediated current (10). Ischemic or inflammatory insults increase tyrosine phosphorylation of NMDA receptors at the PSD (post-synaptic density) fraction (11–13). Inhibition of the interaction of NMDA receptors with PSD-95 significantly attenuates ischemia-induced brain damage (14). These studies indicate that PSD-95 and tyrosine phosphorylation of NMDA receptors may be involved in excitotoxicity mediated by glutamate.

Many studies have shown that increased glutamate-mediated excitotoxicity may play an important role in the pathogenesis of HD. Intraatrial injection of glutamate or kainic acid in rat causes selective loss of medium spiny neurons that are also selectively affected in HD (15). In the brains of HD patients, NMDA receptor-binding sites were disproportionately reduced even at the pre-symptomatic stage (16, 17). In neuronal cells expressing the mutated huntingtin or mice transgenic for HD, NMDA receptors are highly responsive to the receptor agonists, and the receptor-mediated current is also significantly increased (18–20). In our previous studies, we observed that expression of polyglutamine-expanded huntingtin induced sensitization of NMDA receptor via PSD-95 (1). In the present studies, we examined whether tyrosine phosphorylation of NMDA receptors may contribute to the sensitization of the receptors in HN33 cells. We found that expression of the mutated huntingtin induced tyrosine phosphorylation of NMDA receptors, and inhibition of the phosphorylation attenuated the neuronal toxicity mediated by the mutated huntingtin in HN33 cells.

MATERIALS AND METHODS

Cell Culture, Plasmids, Transfection, and Kinase Assays—Wild-type NR1 or NR2B expression vectors were provided by Dr. J. Marshall, and triply mutated NR2B and *fyn* vectors were provided by Dr. M. Greenberg (21). HN33 and 293T cells were maintained in F12/Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. We performed transient transfection using Lipofectin per instruction by the manufacturer. For detecting pSrc, HN33 or 293T cells were lysed with 1% Triton X-100 buffer (22) 24 h following the transfection. These cell lysates (30 μ g of protein) were resolved in a 10% SDS gel and then transferred. The blots were analyzed by an anti-pSrc antibody, which mainly recognizes activated 60-kDa Src (Cell Signaling). Fold increase of the kinase activity was quantified using a computer program associated with Kodak image station 440, and the level in each control was designated as 1.

Generation of Anti-phospho-NR2B Antibody—Tyr-1472 is a major tyrosine phosphorylation site for the NR2B subunit (23). Phosphospecific peptide NH₂-CSNGHV(pY)EKLSLI-COOH was conjugated to keyhole limpet hemocyanin and used as an immunogen to raise rabbit polyclonal antibody. The antibody 438 was purified from sera by successive affinity chromatography. This was done using a column of *N*-hydroxysuccinimide-activated Sepharose 4B resin conjugated to each

Huntington's disease (HD)¹ is a progressive neurodegenerative disorder with an autosomal dominant inheritance (2). The HD gene, encoding a ~350-kDa protein designated as huntingtin, is ubiquitously expressed (3, 4). The genetic defect in the HD gene involves an expansion of a polyglutamine stretch near the 5'-end of the gene (5). The length of the polyglutamine repeat is correlated with the age of onset and the severity of the disease (6, 7).

PSD-95, a scaffold protein, anchors many signaling proteins to the NMDA receptor complex and regulates biological function of the receptors (8, 9). Tyrosine phosphorylation by the Src family is an important molecular mechanism for the regulation

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¹ The abbreviations used are: HD, Huntington's disease; NMDA, *N*-methyl-D-aspartate; PSD, post-synaptic density; PSD-95, post-synaptic density 95; NR, NMDA receptor; pSrc, phospho-Src; TUNEL, terminal deoxynucleotidyltransferase dUTP fluorescein nick end-labeling.

non-phosphorylated peptide to remove IgGs against non-phosphorylated NR2B subunits and was followed by a column conjugated to each immunogen to obtain the antibody specific for the tyrosine-phosphorylated NR2B subunit.

Membrane Fraction—Membrane fraction was conducted as described by Cho *et al.* (24) with a few modifications. 36 h following transfection, transfected or non-transfected HN33 cells were homogenized with a Teflon-glass homogenizer (0.25 mm of clearance) in solution A (0.32 M sucrose, 1 mM NaHCO₃, 1 mM MgCl₂, 0.5 mM CaCl₂, 200 mM NaVO₄, 100 mM NaF, and protease inhibitor mixture). The resulting homogenates were diluted with solution A and centrifuged at 1,400 × *g* for 10 min. The pellets were homogenized again with solution A and centrifuged at 710 × *g* for 10 min. The supernatants from both centrifugations were combined and centrifuged at 13,800 × *g* for 10 min.

Co-immunoprecipitations—48 h following transfection, 293T cells were lysed with 1% Nonidet P-40 lysis buffer (22). Cell lysates were incubated with an antibody as indicated in the figure legends for 3 h at 4 °C, and a mixture of proteins A- and G-Sepharose 4B beads (Amersham Biosciences) were added. Incubation was continued for another hour. Precipitated proteins were resolved in a SDS gel, and the resulting blots were probed with antibodies as indicated in the figure legends.

Immunocytochemistry—A yellow fluorescence expression vector was included for localizing transfected cells. 24 h after transfection, HN33 cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 2 min on ice. Cells were incubated with the first antibody as indicated in the figure legends followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse secondary antibody. After washing, the cells were incubated with another antibody followed by incubation with rhodamine-conjugated anti-sheep or anti-rabbit secondary antibody. The distribution of huntingtin and activated Src or PSD-95 was visualized under a Zeiss LSM-5 confocal microscope.

TUNEL Staining—36 h post-transfection, HN33 cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 for 2 min on ice, and TUNEL staining was performed as described previously (1). Most apoptotic HN33 cells were detached from the slides, and TUNEL staining was performed on the remaining attached cells. TUNEL-stained negative cells (living cells) were counted in the 20× field in four different locations on the slides, and ~600–800 cells were counted in the control.

RESULTS

First, we investigated whether expression of polyglutamine-expanded huntingtin induced activation of Src tyrosine kinase in HN33 cells. Full-length huntingtin expression vector containing 16 or 48 CAG repeats or vector alone (pcDNA1, control) was separately transfected into HN33 cells. 24 h post-transfection, cells were lysed and the resulting blot was analyzed with an anti-phospho-Src antibody, which only recognizes activated Src. As shown in Fig. 1A, Src was activated in HN33 cells, and expression of normal huntingtin with 16 CAG repeats did not cause further elevation of pSrc. Expression of polyglutamine-expanded huntingtin with 48 CAG repeats, however, caused an ~5-fold increase of pSrc. To further investigate Src activation mediated by the mutated huntingtin, we conducted immunocytochemistry studies. 24 h following transfection, HN33 cells were fixed and stained with an anti-phospho-Src antibody. As shown in Fig. 1B, activated Src is diffusely localized in HN33 cells. Expression of normal huntingtin with 16 CAG repeats did not alter the localization of the activated kinase, whereas expression of the mutated huntingtin with 48 CAG repeats induced translocation of activated Src from cytoplasm to cell periphery. These data suggest that polyglutamine-expanded huntingtin may increase targeting of activated Src to cell surface membrane.

Src is anchored by PSD-95 to cell surface membrane (10). In our previous studies, we found that normal huntingtin binds to PSD-95, and polyglutamine expansion impairs the ability of huntingtin to interact with the scaffold protein (1). Therefore, we examined whether expression of normal or polyglutamine-expanded huntingtin altered the localization of PSD-95. As shown in Fig. 2A, PSD-95 is present in the cytoplasm, and most were co-localized with normal huntingtin. Expression of nor-

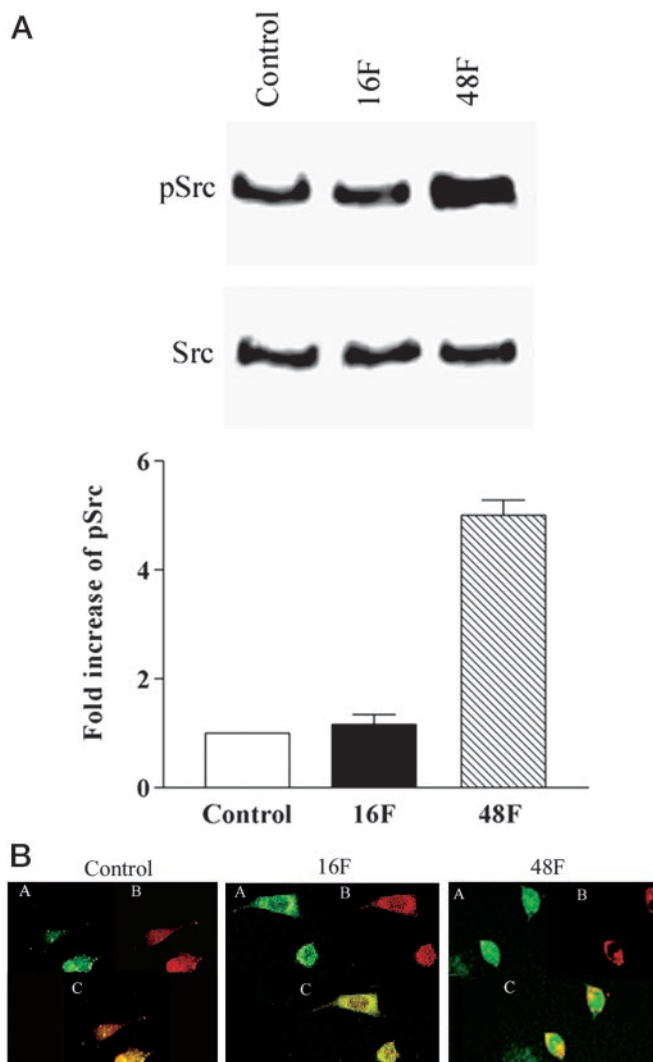


FIG. 1. Polyglutamine-expanded huntingtin induces activation and translocation of Src tyrosine kinase in HN33 cells. Panel A, pcDNA1 (Control) or a full-length huntingtin construct containing 16 (16F) or 48 (48F) CAG repeats was transfected into HN33 cells. 24 h post-transfection, cell lysates were prepared. We first analyzed the blot using an anti-phospho-Src antibody, then stripped the blot and reprobbed with an Src antibody. The data in the upper panel represent a typical experiment. The data in the lower panel are the mean of five independent experiments, and pSrc was quantified according to the density of each band using a computer program associated with a Kodak 440 image station. Panel B, pcDNA1 (Control) or a full-length huntingtin construct containing 16 (16F) or 48 (48F) CAG repeats was transfected into HN33 cells. Two cells showing in the 16F or 48F panels were transfected cells according to the presence of co-transfected yellow fluorescence vector (data not shown). 24 h post-transfection, HN33 cells were co-stained with 437, an anti-huntingtin antibody and an anti-phospho-Src antibody. The distribution of huntingtin (green) and activated Src (red) was visualized under a confocal microscope. A, huntingtin; B, activated c-Src; C, overlay of A and B. The data represent a typical experiment, which has been repeated three times with similar results.

mal huntingtin with 16 CAG repeats did not alter the co-localization, whereas expression of the mutated huntingtin with 48 CAG repeats caused translocation of PSD-95 to cell surface membrane, and the co-localization of the scaffold protein with huntingtin was mostly disrupted (Fig. 2A). These data suggest that the mutated huntingtin also increases the targeting of PSD-95 to the cell surface membrane. To further validate these results, we performed the membrane fraction and examined the amount of PSD-95 and activated Src in the membrane fraction with or without transfection of normal or

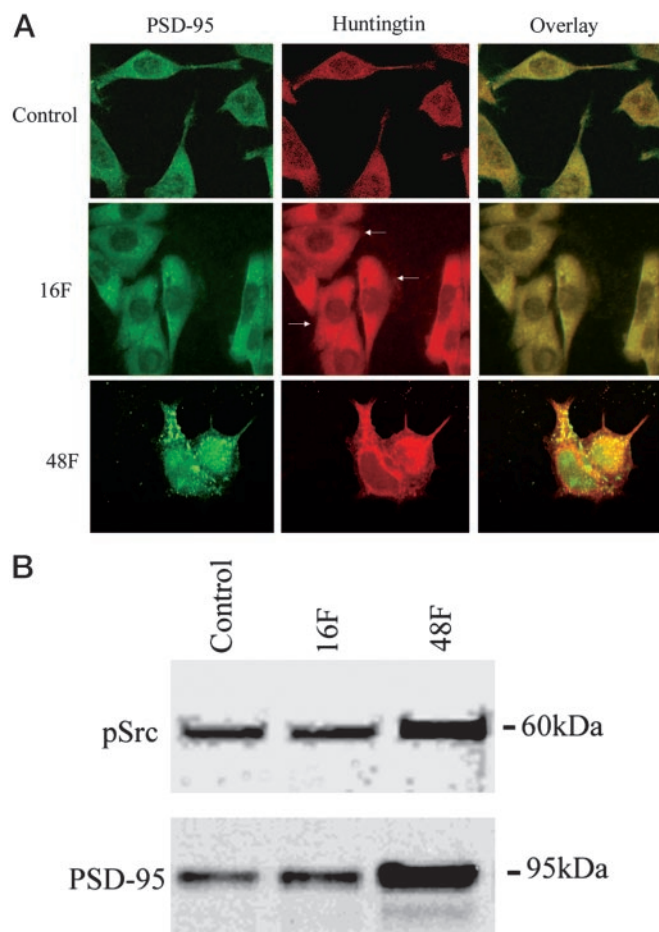


FIG. 2. Expression of polyglutamine-expanded huntingtin increased the targeting of PSD-95 and Src to cell surface membrane. Panel A, HN33 cells were transfected with a full-length huntingtin vector containing 16 or 48 CAG repeats (16F or 48F). A yellow fluorescence vector was included for identifying transfected cells, and the arrows show transfected cells. Two cells showing in 48F panels were all transfected. 24 h post-transfection, cells were fixed and co-stained with an anti-PSD-95 antibody and 437, an anti-huntingtin antibody. The distribution of PSD-95 (green) and huntingtin (red) was visualized under a confocal microscope. A, PSD-95; B, huntingtin; C, overlay of A and B. Panel B, HN33 cells transfected with full-length huntingtin with 16 or 48 CAG repeats. The membrane fraction was conducted as described under "Materials and Methods."

mutated huntingtin. As shown in Fig. 2B, expression of the mutated huntingtin with 48 CAG repeats clearly increased the amount of PSD-95 and activated Src in the membrane fraction.

We then examined whether expression of polyglutamine-expanded huntingtin induced tyrosine phosphorylation of NMDA receptors. Because expression of the mutated huntingtin induced rapid apoptotic cell death in HN33 cells, we first conducted these experiments using 293T cells. A huntingtin construct containing 16 or 48 CAG repeats was co-transfected with NR1 and NR2B with or without PSD-95 into 293T cells. 48 h following transfection, cells were lysed, and co-immunoprecipitation was conducted using an anti-NR2B antibody. The resulting blot was analyzed by PY20 anti-phosphotyrosine antibody to detect tyrosine-phosphorylated NR2B subunits, and then the blot was stripped and re-probed with an anti-NR2B antibody (Fig. 3A). 50 μ l of cell lysates from the same preparation was resolved in a SDS gel, the blot was probed with an anti-phospho-Src antibody, and the blot was stripped and re-probed with an anti-Src antibody (Fig. 3A). Changes of tyrosine-phosphorylated NR2B subunits or pSrc were quantified according to the density of each band. Expression of normal

huntingtin neither elevated the levels of pSrc nor mediated tyrosine phosphorylation of NR2B subunits (Fig. 3A). Co-transfection of PSD-95 with normal huntingtin induced an \sim 6-fold increase of tyrosine-phosphorylated NR2B subunits suggesting that PSD-95 recruited endogenous activated Src to induce tyrosine phosphorylation of NR2B subunits (Fig. 3A). Expression of the mutated huntingtin elevated the levels of pSrc and induced tyrosine phosphorylation of NR2B subunits (Fig. 3A). Co-expression of PSD-95 did not cause further elevation of pSrc but further increased the number of tyrosine-phosphorylated NR2B receptors (Fig. 3A). In comparison to the \sim 7-fold increase of tyrosine-phosphorylated NR2B receptors with expression of the mutated huntingtin, addition of PSD-95 induced an \sim 14-fold increase of these phosphorylated receptors. These data suggest that the mutated huntingtin may induce tyrosine phosphorylation of NR2B subunits via other members of the PSD-95 family endogenously expressed in 293T cells, which also anchor Src to NMDA receptors, and expression of PSD-95 recruits more Src to these receptors thereby further increasing the number of tyrosine-phosphorylated receptors.

Src induces phosphorylation of the NR2B subunit on Tyr-1252, -1336, and -1472, and mutations of these three tyrosines block the phosphorylation by Src significantly attenuating Src-mediated increased calcium influx via NMDA receptors (21). To further investigate whether expression of the mutated huntingtin induced tyrosine phosphorylation of NMDA receptors, we generated a rabbit polyclonal antibody specific for Tyr-1472-phosphorylated NR2B, 438. To determine the specificity of the antibody, we co-transfected NR1 with wild-type NR2B and mutated NR2B with substitutions of Tyr-1252, -1336, and -1472 to phenylalanine (NR2BTM) or NR2A expression vectors in the presence or absence of Fyn (a member of the Src tyrosine kinase family that has been shown to bind to PSD-95 and induce tyrosine phosphorylation of NR2B subunits) (21). As shown in Fig. 3B, co-expression of *fyn* induced tyrosine phosphorylation of wild-type NR2A and NR2B subunits, which, except for the mutated NR2B subunit, were detected by PY20. 438 recognized the wild-type tyrosine-phosphorylated NR2B subunit but not the non-phosphorylated NR2B (the mutated NR2B) and the tyrosine-phosphorylated NR2A subunits. The data indicate that the antibody 438 specifically recognizes the tyrosine-phosphorylated NR2B subunit. Thus, 438 was utilized to determine whether the mutated huntingtin induced tyrosine phosphorylation of NR2B subunits in HN33 cells. 24 h following transfection of normal or mutated huntingtin, HN33 cells were lysed, and the resulting blot was analyzed with 438. As shown in Fig. 3C, expression of the mutated huntingtin with 48 CAG repeats (but not normal huntingtin with 16 CAG repeats) induced tyrosine phosphorylation of NR2B subunits in HN33 cells.

To evaluate whether tyrosine phosphorylation of NR2B receptors is involved in the neuronal toxicity mediated by the mutated huntingtin, we added SU6656, a specific Src tyrosine kinase inhibitor, to the transfection medium. 36 h following transfection, HN33 cells were fixed, and TUNEL staining, which detects the late stage of apoptotic cells, was conducted (25). As we reported previously, expression of the mutated huntingtin with 48 CAG repeats induced rapid apoptosis in HN33 cells, and \sim 70% of the cells were apoptotic 36 h following transfection (1). Addition of the Src inhibitor significantly attenuated apoptotic cell death mediated by the mutated huntingtin (Fig. 4A). We have shown that addition of AP-5, an NMDA receptor antagonist, to the transfection medium significantly attenuated apoptotic death induced by the mutated huntingtin (1). Co-addition of AP-5 with SU6656 did not further inhibit neuronal apoptosis induced by the mutated hun-

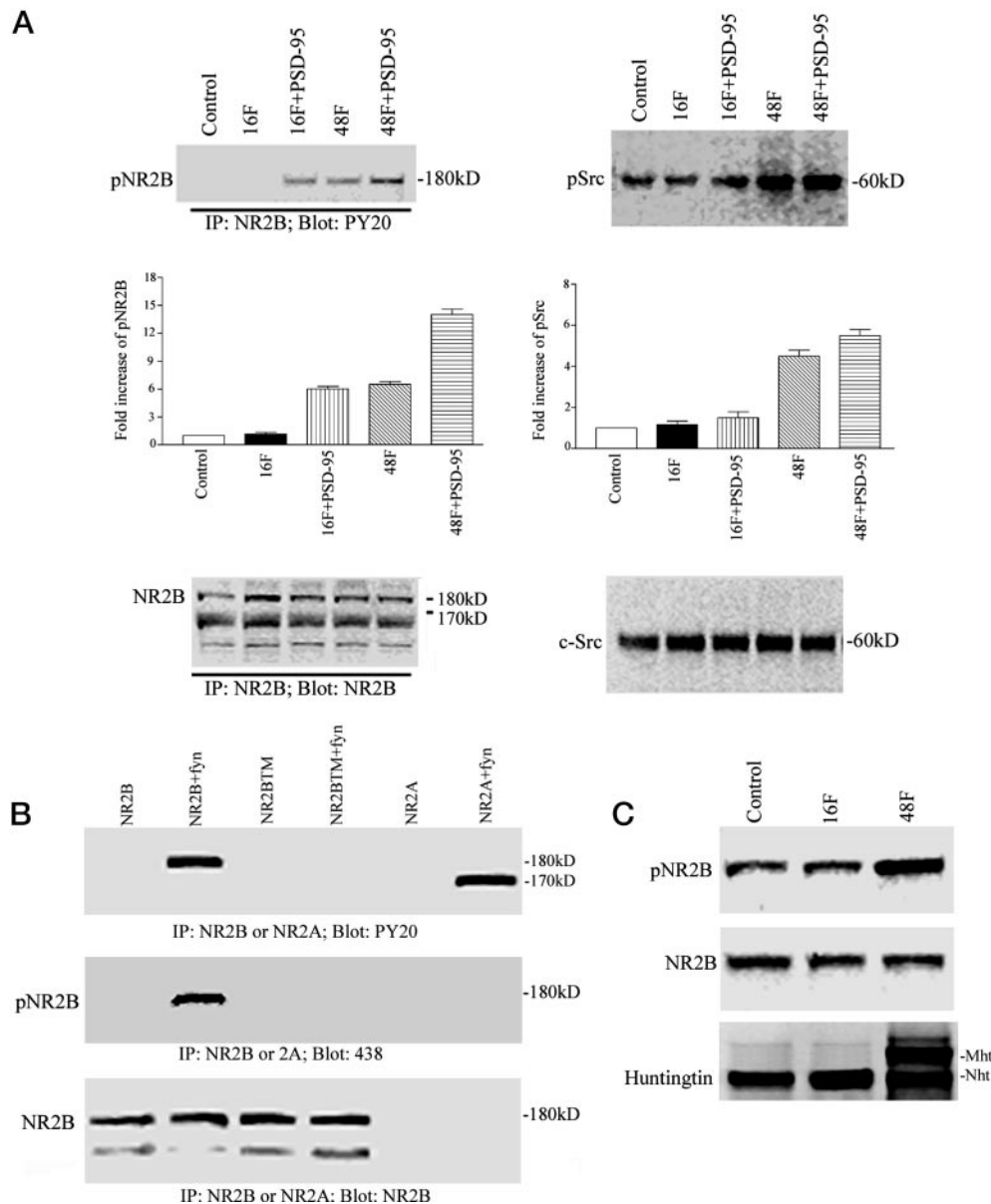


FIG. 3. Expression of polyglutamine-expanded huntingtin induces tyrosine phosphorylation of NR2B subunits. A, pcDNA1 (Control) or a full-length huntingtin construct containing 16 (16F) or 48 (48F) CAG repeats was co-introduced with NR1 and NR2B receptor cDNA with or without a PSD-95 expression vector into 293T cells. *Left upper panel*, IP with anti-NR2B antibody and blot with PY20. *Left middle panel*, the amount of phosphorylated NR2B subunits was quantified according to the density of each band. *Left lower panel*, the blot was stripped and then probed with an anti-NR2B antibody. *Right upper panel*, the blot from lysates of the same preparation was probed with an anti-phospho-Src antibody. *Right middle panel*, the amount of activated Src was quantified according to the density of each band. *Right lower panel*, the blot was stripped and then reprobed with an anti-Src antibody. The data in the *upper* and *lower panels* represent a typical experiment. The data in the *middle panels* are the mean of four independent experiments. *Panel B*, 293T cells were transfected with NR1 and NR2B or mutated NR2B or NR2A with or without *fyn*. The blot was probed with PY20 (*upper panel*) or stripped and probed with 438 (*middle panel*). The blot was stripped again and probed with an anti-NR2B antibody (*lower panel*). The data represent a typical experiment, which has been repeated three times with similar results. *C*, HN33 cells were transfected with pcDNA1 (Control) or full-length huntingtin with 16 (16F) or 48 (48F) CAG repeats. The blot was probed with 438. The data represent a typical experiment, which has been repeated two more times with similar results. *Mht*, the mutated huntingtin; *Nht*, normal huntingtin.

tingtin, suggesting that the Src inhibitor may inhibit the neuronal toxicity via the inhibition of NMDA receptors.

To further investigate the role of tyrosine phosphorylation of NMDA receptors in the induction of neuronal toxicity mediated by the mutated huntingtin, we co-transfected normal or mutated huntingtin construct with a vector alone or an NR2B mutant with triple substitutions of all three tyrosine phosphorylation sites (NR2BTM). As shown in Fig. 4B, expression of vector alone failed to influence the neuronal toxicity mediated by the mutated huntingtin, whereas co-expression of triply mutated NR2B subunit significantly attenuated the neuronal toxicity induced by the mutated huntingtin. Addition of AP-5 in

the transfection medium did not further inhibit the neuronal toxicity. These data further support that tyrosine phosphorylation of NMDA receptors may be involved in the neuronal toxicity mediated by polyglutamine-expanded huntingtin in HN33 cells.

DISCUSSION

In the present studies, we investigated a role of tyrosine phosphorylation of NMDA receptors in the induction of neuronal toxicity mediated by polyglutamine-expanded huntingtin. We found that expression of the mutated huntingtin with 48 CAG repeats caused an elevation of activated Src in both HN33

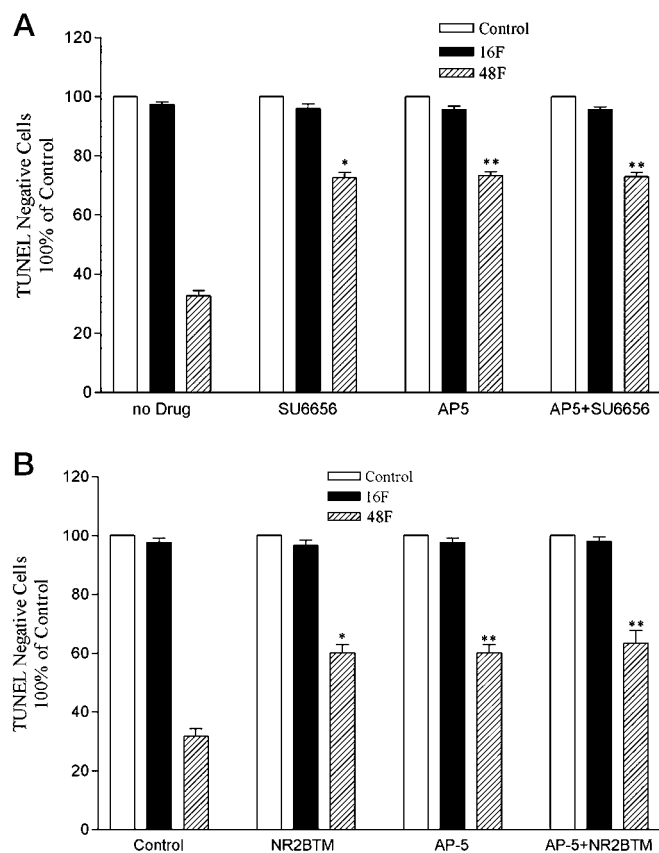


FIG. 4. Inhibition of tyrosine phosphorylation of NR2B subunits significantly attenuated apoptotic cell death induced by the mutated huntingtin in HN33 cells. *, significant difference ($p < 0.001$, analysis of variance). **, no significant difference in comparison of AP-5 or AP-5 + NR2B-TM with NR2B-TM ($p > 0.05$, analysis of variance). *A*, HN33 cells were transfected with a full-length huntingtin construct containing 16 (16F) or 48 (48F) CAG repeats in the presence or absence of SU6656 (1 μ M), AP-5 (1 μ M), or AP-5 + SU6656. 36 h following transfection, cells were fixed and TUNEL stain was conducted. TUNEL-negative cells were counted. Approximately 200–300 cells in the control cells (transfected with pcDNA1) were counted and designated as 100%. The data were the mean of five independent experiments. *B*, triply mutated NR2B subunit (NR2B-TM) was co-transfected with normal (16F) or mutated (48F) huntingtin into HN33 cells. AP-5 (1 μ M) was added as indicated in the figure. 36 h following transfection, cells were fixed and TUNEL stain was conducted. The data were the mean of five independent experiments.

and 293T cells, whereas expression of normal huntingtin failed to do so. Expression of the mutated huntingtin also induced tyrosine phosphorylation of NR2B subunits in both cell lines. We also performed these studies using full-length huntingtin vector containing 89 CAG repeats and obtained similar results. These results indicate that huntingtin mediates Src activation and tyrosine phosphorylation of NMDA receptors in a polyglutamine expansion-dependent manner. Addition of SU6656 or co-expression of an NR2B mutant with substitutions of all three tyrosine phosphorylation sites significantly attenuated neuronal toxicity mediated by the mutated huntingtin, and addition of an NMDA receptor antagonist did not further inhibit the neuronal toxicity. These data support that tyrosine phosphorylation of NMDA receptors may be involved in sensitization of the receptors and neuronal toxicity mediated by polyglutamine-expanded huntingtin.

Many studies have shown that polyglutamine-expanded huntingtin induced sensitization of NMDA receptors (1, 18–20, 26). Our studies support this notion. Normal huntingtin is a cytoplasmic protein (4, 5). An intriguing question is why polyglutamine expansion in huntingtin alters the property of

NMDA receptors at the cell surface. In our previous studies, we have shown that normal huntingtin binds to PSD-95, and polyglutamine expansion inhibits the ability of huntingtin to interact with the scaffold protein. In the present studies, we showed that normal huntingtin is co-localized with PSD-95, and expression of the mutated huntingtin causes translocation of the scaffold protein and inhibits the co-localization of these two proteins in the cytoplasm. These data demonstrate that polyglutamine expansion impairs the ability of huntingtin to retain PSD-95 in the cytoplasm and promotes the targeting of the scaffold protein to cell surface membrane (1). PSD-95 anchors Src to NMDA receptors (10); therefore, it is not surprising that expression of the mutated huntingtin also increased the targeting of Src to the cell surface membrane. In the brain, normal huntingtin and PSD-95 are mostly associated with microtubules (4, 27). One of the possibilities is that normal huntingtin regulates the amount of PSD-95 at PSD by retaining the scaffold protein in the microtubule complex, and polyglutamine-expanded huntingtin loses such a function leading to the increase of targeting of PSD-95 and PSD-95-associated proteins, such as Src, to PSD. In parallel with this study, we also examined changes of PSD-95, Src, and tyrosine-phosphorylated NMDA receptors in the PSD fraction from brain tissues of R6/2, a HD transgenic mouse model (28). We found that PSD-95, activated Src, and tyrosine-phosphorylated NR2B subunits were significantly increased in the PSD fraction.² These results further support our hypothesis.

Understanding of the pathogenesis of HD is critical for developing effective treatment to this devastating neurodegenerative disease. Many NMDA receptor antagonists have been developed. Although they are neuroprotective in many animal models, the clinical application of these NMDA receptor antagonists is limited because of their toxicity. Our studies demonstrate that polyglutamine-expanded huntingtin induces tyrosine phosphorylation of NMDA receptors. Perhaps, an NMDA receptor antagonist that selectively inhibits tyrosine-phosphorylated NMDA receptors may eliminate unwanted side effects and effectively treat this otherwise untreatable disease.

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