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Increased Sensitivity to N-Methyl-D-Aspartate Receptor-Mediated Excitotoxicity in a Mouse Model of Huntington's Disease

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Summary

Previous work suggests N-methyl-D-aspartate receptor (NMDAR) activation may be involved in degeneration of medium-sized spiny striatal neurons in Huntington's disease (HD). Here we show that these neurons are more vulnerable to NMDAR-mediated death in a YAC transgenic FVB/N mouse model of HD expressing full-length mutant huntingtin, compared with wild-type FVB/N mice. Excitotoxic death of these neurons was increased after intrastriatal injection of quinolinate in vivo, and after NMDA but not AMPA exposure in culture. NMDA-induced cell death was abolished by an NR2B subtype-specific antagonist. In contrast, NMDAR-mediated death of cerebellar granule neurons was not enhanced, consistent with cell-type and NMDAR subtype specificity. Moreover, increased NMDA-evoked current amplitude and caspase-3 activity were observed in transgenic striatal neurons. Our data support a role for NR2B-subtype NMDAR activation as a trigger for selective neuronal degeneration in HD.

Introduction

Huntington's disease (HD), inherited in an autosomal dominant fashion, is characterized by progressive neurodegeneration resulting in a movement disorder, emotional disturbances, and cognitive decline (Harper, 1991). HD is caused by a polyglutamine (polyQ) expansion in the N-terminal region of the protein huntingtin (htt) (HDCRG, 1993). Although it is not known why the GABAergic, medium-sized spiny neurons (MSNs) of the striatum are preferentially targeted for degeneration in HD, a body of evidence supports a role for excitotoxic cell death mediated by the release of glutamate from cortical afferents and activation of the ionotropic glutamate receptors: the N-methyl-D-aspartate (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPAR), and kainic acid (KAR) (DiFiglia, 1990; Beal, 1992). Results of radiolabeled ligand binding assays in human postmortem tissue indicated that striatal glutamate receptors show disproportionate loss in HD, and NMDAR binding was significantly decreased in this region even in the presymptomatic stage of the disease, suggesting that neurons which highly express these receptors may be most vulnerable (Young et al., 1988; Albin et al., 1990). In rodents, intrastriatal injection of kainic acid resulted in death of most neurons with preservation of glia and afferents (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976), while NMDAR agonists selectively destroyed MSNs but spared interneurons that are known to be resistant to degeneration in HD (Ferrante et al., 1985; Beal et al., 1986). Furthermore, injection of the NMDAR agonist quinolinic acid in the striata of nonhuman primates resulted in behavioral, neurochemical, and neuropathological abnormalities that mimicked the changes seen in HD (Hantraye et al., 1990). Finally, systemic injection of mitochondrial toxins such as malonate also caused striatal degeneration typical of HD; this neuronal loss was blocked by the NMDAR antagonist MK-801 (Greene et al., 1993). Although these data support the hypothesis that NMDAR-mediated excitotoxicity may play a critical role in the pathogenesis of HD, the evidence until now has been indirect.

The results of studies outlined above also raise the question of whether mutant htt expression can cause increased activity of NMDARs or their downstream effectors of cell death and how such interactions might explain selective neuronal vulnerability. Recent data strongly support roles for caspase activation, and/or formation of toxic aggregates of htt containing the polyQ tract, in the neuronal degeneration underlying HD and related neurodegenerative diseases caused by genes with a triplet repeat expansion (reviewed by Wellington and Hayden, 2000). However, each of these diseases has a distinct pattern of selective neuronal death, so the upstream events that trigger caspase activation and protein aggregation may be unique for each disease and related to interactions between mutant protein and other proteins that are selectively expressed in vulnerable neuronal populations. We have previously reported enhancement of NMDAR-mediated current amplitude and apoptosis in cell lines expressing full-length mutant htt and the NR1A/NR2B but not NR1A/NR2A subtype of NMDARs (Chen et al., 1999; Zeron et al., 2001). Notably, MSNs primarily express the NR1A and NR2B subunits (Landwehrmeyer et al., 1995; Kuppenbender et al., 1999), whereas other forebrain regions express combinations of both NR2A and NR2B with a variety of NR1 splice variants, and the cerebellum and brain stem lack NR2B (Hollmann and Heinemann, 1994; Monyer et al., 1994; Thompson et al., 2000; Kovacs et al., 2001). Therefore, we hypothesized that in MSNs, full-length mutant htt acts to augment NMDAR activity, resulting in increased caspase activation and cell death.

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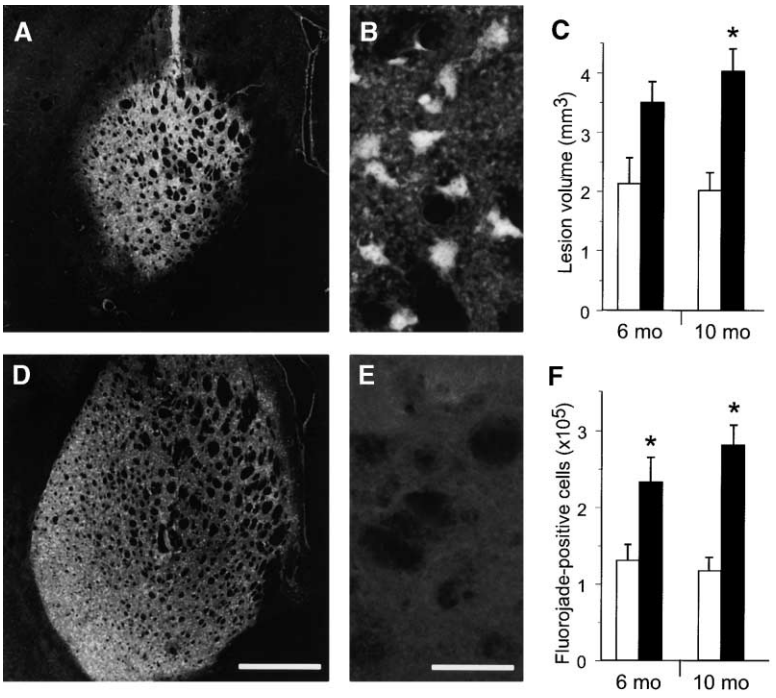

Figure 1. Increased Number of Dying Neurons in YAC72 Mice after NMDAR-Induced Excitotoxicity In Vivo

The number of dying neurons 72 hr after intrastriatal injection of quinolinic acid was detected using the fluorescent neuronal death marker Fluoro-Jade. Photomicrographs show coronal sections at the level of the striatum of 10-month-old wild-type (A) and YAC72 mice (D). (B) Fluoro-Jade stained neurons at higher magnification in a lesioned striatum. (E) Very low level of background staining in the contralateral unlesioned side of the striatum. (Scale bars = 710 μm [A and D] and 23 μm [B and E]). Quantification revealed increases in lesion volume (C) and numbers of Fluoro-Jade stained, dying neurons (F) in YAC72 mice (closed bars) compared to wild-type mice (open bars). Data are presented as mean ± SEM (n = 3 per group for 6-month-old mice, and n = 6–7 per group for 10-month-old mice). *Significantly different compared to littermate control, $p < 0.025$.

We tested our hypothesis in a transgenic mouse model, described by Hodgson et al. (1999), in which FVB/N mice express a yeast artificial chromosome (YAC) containing the full-length human HD gene including all endogenous regulatory elements with an expanded (46 or 72) CAG repeat (YAC46 or YAC72 mice). This model optimizes normal developmental and cell-specific regulation of full-length htt expression, and YAC72 mice show progressive neurobehavioral changes and selective striatal neurodegeneration similar to that seen in HD (Hodgson et al., 1999). In this study we tested whether MSNs from YAC46 and/or YAC72 mice display enhanced NMDA-mediated excitotoxic cell death, current influx, and caspase activation when compared with MSNs from wild-type FVB/N mice.

Results

Enhanced Excitotoxicity in MSNs Expressing Mutant htt In Vivo

To compare susceptibility to NMDAR-mediated excitotoxicity, the NMDAR agonist quinolinic acid was injected unilaterally into the striatum of 6- and 10-month-old YAC72 mice and their wild-type littermates. Large numbers of striatal neurons died after intrastriatal injection of quinolinic acid in both wild-type and YAC72 transgenic mice, as shown in sections stained with Fluoro-Jade (Figure 1), a fluorescent marker of dying neurons (Schmued et al., 1997; Hansson et al., 1999). However, the number of dying striatal cells was significantly greater in YAC72 transgenic compared to wild-type mice (Figure 1). As previously reported by Beal et al. (1986), we found that quinolinic acid predominantly targeted the same population of striatal neurons (MSNs) that degenerate in human HD. Interestingly, following quinolinate injection, the number of surviving MSNs (DARPP-32 immunoreactive), as well as nonpyknotic neurons (cresyl violet), was significantly reduced in the striatum of YAC72 mice compared with wild-type (Figure 2). These data support our hypothesis that striatal neurons expressing the mutant form of htt are more vulnerable to NMDAR-mediated cell death.

Enhanced Excitotoxicity in Cultured Neonatal MSNs Expressing Mutant htt

Increased vulnerability to NMDAR-mediated striatal cell death in 6- and 10-month-old YAC72 mice might be a consequence of other cellular changes resulting from mutant htt expression, since around this age, the mice show electrophysiological abnormalities and an HD-like phenotype (Hodgson et al., 1999). To help determine whether augmented sensitivity to NMDAR activation is a primary mechanism underlying increased striatal neuronal degeneration in vivo, we tested whether MSNs from young (early postnatal) YAC72 mice also exhibit enhanced vulnerability to NMDAR agonists. We used an in vitro model of excitotoxicity, treating primary neostriatal cultures with NMDA and glycine for 10 min and assessing cell survival 24 hr later, similarly to models used by others to investigate mechanisms of excitotoxicity (reviewed by Choi, 1988). Numbers of MSNs (assessed by morphological criteria) remaining attached to the dish were similar between the cultures from YAC72 and wild-type mice (data not shown). NMDA-induced cell death, as measured by percentage of MSNs with trypan blue inclusion, was dose dependent [two-factor ANOVA, effect of concentration $F(2,29) = 41.2$, $p < 0.0001$]; it was nearly 2-fold larger after a supramaximal concentration of 3 mM than after 100 μM (Figure 3A), which is close to the measured EC_{50} for NMDAR currents recorded from murine cortical and diencephalic neurons (Sather et al., 1992). As well, brief (20 min) exposures to NMDA

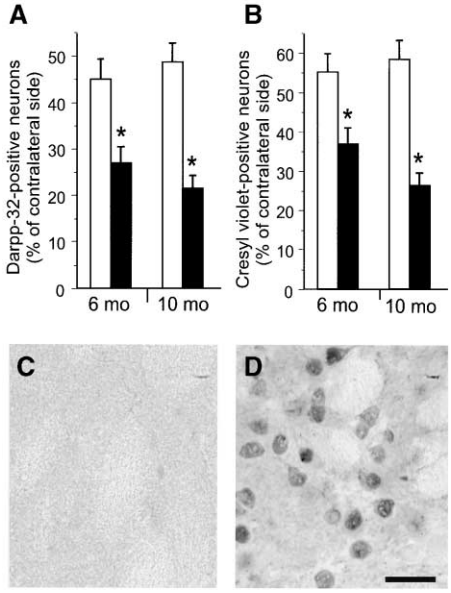

Figure 2. Decreased Number of Surviving MSNs in YAC72 Mice after NMDAR-Induced Excitotoxicity In Vivo

The numbers of surviving MSNs and nonpyknotic neurons 72 hr after intrastriatal injection of quinolinic acid were analyzed using DARPP-32 immunohistochemistry (A, C, and D) and cresyl violet (B), respectively. Photomicrographs show DARPP-32 immunostained coronal sections at the level of the striatum on the lesion (C) and contralateral intact (D) sides (scale bar = 40 μm). Data are presented as mean percentage of the number of neurons on the contralateral unlesioned side (A and B). There was a decreased number of surviving MSNs in both 6- and 10-month-old YAC72 mice (closed bars) compared to wild-type mice (open bars). n = 3 per group for 6-month-old mice, and n = 6–7 per group for 10-month-old mice. Error bars = SEM. *Significantly different compared to littermate control, $p < 0.025$.

concentrations in this range have been shown previously to induce apoptotic death in cultured cortical neurons (Budd et al., 2000). Similarly to our results in vivo, MSNs expressing mutant htt were found to be more sensitive to NMDA-mediated cell death in vitro, where a larger percentage of MSNs were dead 24 hr after NMDA exposure in cultures from YAC72 compared to wild-type mice [two-factor ANOVA, effect of genotype $F(1,29) = 6.9$, $p < 0.05$] (Figure 3A). Next, we used the terminal deoxynucleotidyl transferase-mediated dUTP (TUNEL) fluorescein tagged staining technique to examine NMDAR-induced apoptosis in our cultures, since previous work has suggested that apoptosis may be the primary mode of cell death in HD (reviewed by Wellington and Hayden, 2000). In some cases, advanced stages of degraded necrotic cells can also stain using the TUNEL technique (Trump et al., 1997); therefore, we only considered as apoptotic TUNEL-positive cells that also had small, condensed nuclei, as visualized with propidium iodide. We found a marked enhancement of NMDAR-induced apoptosis among MSNs cultured from YAC mice expressing mutant htt compared with wild-type mice [two-factor ANOVA, effect of genotype $F(2,40) = 14.9$, $p < 0.001$], and this enhancement was NMDA concentration dependent [genotype × concentration, $F(4,40) = 3.4$, $p < 0.05$]. Further analysis indicated that at 3 mM NMDA treatment, both the mutant htt groups displayed significantly more apoptosis than wild-type cultures (Bonferroni post hoc test; wild-type versus 46Q, $p < 0.05$; wild-type versus 72Q, $p < 0.001$) and the 72Q group exhibited significantly more apoptosis than the 46Q group (Bonferroni post hoc test, $p < 0.05$) (Figures 3C and 3D). These in vitro data indicate that MSNs expressing mutant htt show an increased sensitivity to NMDAR activation even from birth, providing support for the idea that cellular dysfunction could begin early and that NMDAR activation may be a trigger of apoptotic cell death in HD.

Enhanced Excitotoxicity Is Neuronal Type and NMDAR Subtype Specific

To examine whether this enhanced cell death effect was selective for neurons affected in HD, we repeated these experiments in cultured cerebellar granule neurons (CGNs). As found for MSNs, NMDAR-mediated CGN cell death was concentration dependent [two-factor ANOVA, effect of concentration $F(2,17) = 28.6$, $p < 0.0001$] (Figure 3B). In contrast to MSNs, however, primary CGNs showed no mutant htt-dependent enhancement in NMDAR-mediated cell death as measured by the trypan blue assay [two-factor ANOVA, effect of genotype $F(1,17) = 1.6$, $p > 0.05$; genotype × concentration interaction $F(2,17) = 0.4$, $p > 0.05$] (Figure 3B). It is interesting that developing CGNs express NR2B, but with maturation in vivo and in vitro (i.e., by ~9 DIV), these neurons express predominantly the NR2A and NR2C NMDAR subtypes (Hollmann and Heinemann, 1994; Monyer et al., 1994; Vallano et al., 1996; Thompson et al., 2000; Kovacs et al., 2001). Thus, our data are consistent with the pathology of HD, in which the cerebellum is largely spared, and support the idea that the excitotoxic enhancement seen in the mutant mice is NMDAR subtype dependent.

To ensure that the high NMDA concentration we used was not activating other lower affinity receptors, we treated MSNs with the use-dependent NMDAR antagonist MK-801 together with 3 mM NMDA to specifically eliminate the contribution of NMDAR activation to cell death. Under these conditions, cell death was reduced nearly to baseline levels (i.e., similar to cultures treated with MK-801 alone), confirming that >80% of the cell death observed in response to 3 mM NMDA resulted from NMDAR activation in our system (Table 1). Moreover, we found that a similar proportion (>80%) of 3 mM NMDA-induced cell death was inhibited by the NR1/NR2B subtype-specific antagonist ifenprodil (Williams, 1993) for cultured striatal neurons from both YAC72 and wild-type mice (Table 1). These results are consistent with the notion that mutant htt can enhance excitotoxicity mediated specifically by the NR1/NR2B subtype of NMDAR.

Expression of mutant htt might increase MSN vulnerability to a variety of stresses, even though in our experiments cultures were made from early postnatal mice. Therefore, we investigated the effect of another excitotoxic stress—activation of AMPA receptors—to determine whether the increased vulnerability to cell death found for mutant htt-expressing MSNs was specific for NMDAR activation. We treated cultured MSNs with 1

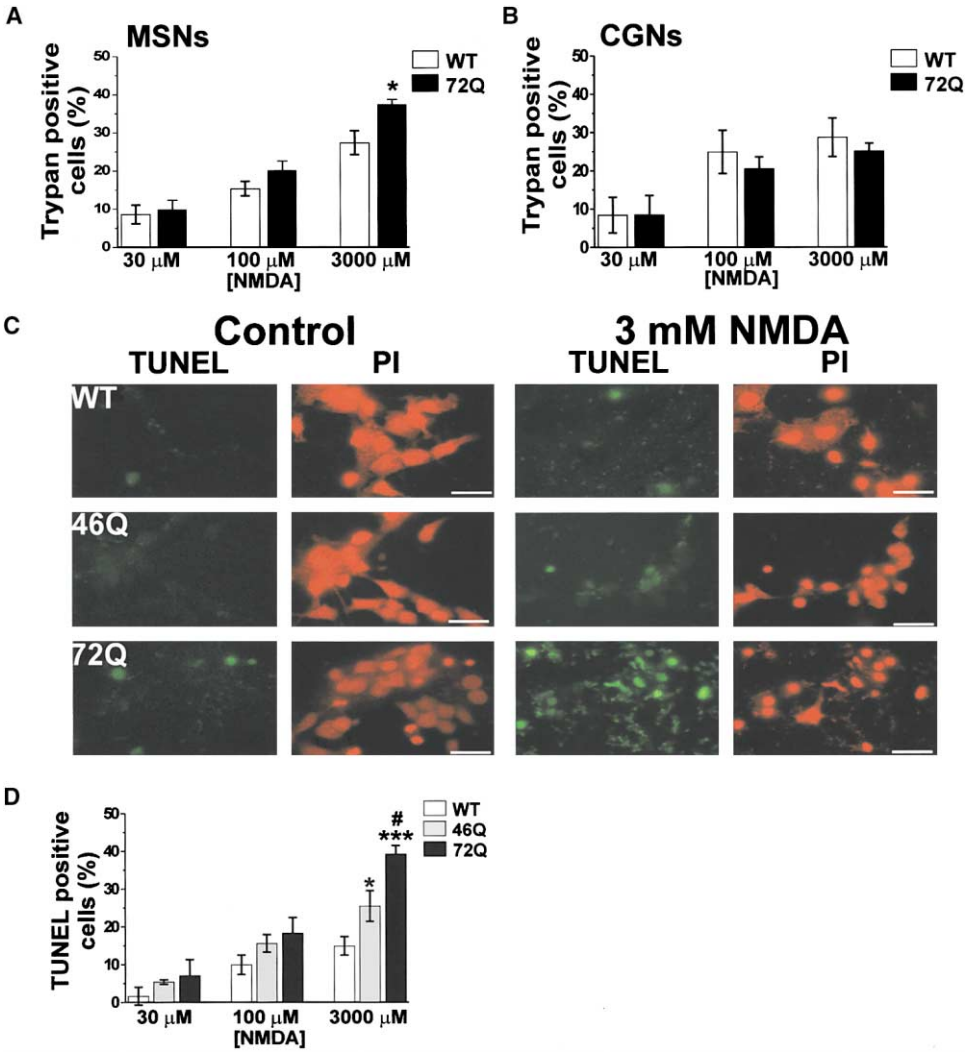

Figure 3. Enhancement of NMDAR-Mediated Cell Death in MSNs Expressing Full-Length Mutant htt Compared to Wild-Type Control MSNs

(A) Survival of MSNs was assessed using the trypan blue exclusion assay 24 hr after exposure to NMDA compared to survival of MSNs that underwent exposure to salt solution alone. *$p < 0.05$ versus wild-type (WT) at same NMDA concentration. $n = 4$–6 different batches of cultured neurons for each treatment.

(B) No trend of enhanced NMDAR-mediated cell death in primary cultured CGNs expressing mutant htt compared to wild-type (WT) CGNs. Survival of CGNs was assessed using the trypan blue exclusion assay 24 hr after exposure to NMDA compared to survival of CGNs that underwent exposure to salt solution alone. $n = 3$ different batches of cultured neurons for each treatment.

(C and D) The trend in enhancement of NMDAR-mediated apoptosis in MSNs expressing full-length mutant htt compared to wild-type (WT) MSNs is polyQ-dependent. Representative fields of striatal neurons are shown 24 hr after exposure to salt solution alone (control) or 3 mM NMDA, followed by TUNEL staining and propidium iodide (PI) counterstaining (C) (scale bar = 25 μm). In (D), apoptotic MSNs (>500) were assessed using TUNEL staining and morphological criteria as shown by propidium iodide (PI) counterstaining 24 hr after exposure to NMDA and compared to apoptosis observed in MSNs exposed to salt solution alone. *$p < 0.05$, ***$p < 0.001$ versus wild-type at same NMDA concentration. #$p < 0.05$ versus 46Q at same concentration. $n = 3$–6 different batches of cultured neurons for each treatment. Bars represent mean ± SEM.

mM AMPA and 50 μM cyclothiazide (to eliminate AMPAR desensitization and thereby increase toxicity [Brorson et al., 1995]) in the presence or absence of 10 μM ifenprodil for 2 hr. Cell death was measured 24 hr later by the trypan blue assay and the TUNEL assay. Interestingly, AMPA-stimulated cell death was reduced by 10 μM ifenprodil and therefore partially mediated by NR2B-subtype NMDARs (Table 2). However, there was no increase in AMPAR-mediated cell death in MSNs expressing mutant htt compared to wild-type either in the presence or absence of ifenprodil (Table 2). NMDAR stimulation likely resulted from glutamate released from dying neurons. Notably, the ifenprodil-sensitive component showed a trend toward increased cell death for YAC72 compared with wild-type or YAC46 MSNs, but this difference was not significant. These data confirm that mutant htt enhances excitotoxicity mediated by NMDARs but not AMPARs.

Table 1. Elimination of Excitotoxicity in MSNs after Exposure to NMDAR Antagonists or a Caspase-3-like Inhibitor

	3 mM NMDA	3 mM NMDA + MK-801	3 mM NMDA +IFN	3 mM NMDA + z-DEVD-fmk	Number of Trials
Trypan-positive cells (%)					
WT	11.4 ± 0.6	1.4 ± 2.2	1.9 ± 2.3	1.7 ± 2.2	3
72Q	29.8 ± 2.8	3.4 ± 2.1	2.3 ± 1.3	2.6 ± 1.2	3–4
TUNEL-positive cells (%)					
WT	14.0 ± 0.8	1.9 ± 0.6	1.5 ± 0.6	1.5 ± 1.9	3
46Q	28.5 ± 1.9	3.8 ± 1.1	2.8 ± 0.4	4.2 ± 0.4	3
72Q	33.5 ± 2.6	4.4 ± 1.2	4.9 ± 1.4	2.0 ± 4.2	3–4

NMDAR-Mediated Caspase-3 Activation in MSNs Expressing Mutant htt In Vitro

Because caspase-3 activation plays a central role in the regulation of neuronal death in the CNS, we examined caspase-3 activity in the primary striatal cultures after exposure to NMDA or glutamate. Using immunocytochemistry to detect the active form of caspase-3, we observed a significantly larger increase in its expression 6 hr after a brief application of 3 mM NMDA in YAC46 and YAC72 MSNs compared to wild-type controls [two-factor ANOVA, effect of genotype $F(2,47) = 7.1$, $p < 0.01$] (Figures 4A and 4B). As well, there was a trend toward a larger increase in NMDAR-induced caspase-3 activity for YAC72 versus YAC46 MSNs, which was not significant because of the variability in absolute percentages of immunopositive neurons between different batches of cultured MSNs. The difference between wild-type and YAC72 MSNs was replicated using a fluorometric assay for caspase activity; we found a significant enhancement of caspase activation in cultured striatal cell lysates from YAC72 compared to wild-type mice 3 and 6 hr after exposure to 3 mM NMDA [two-factor ANOVA, effect of genotype $F(1,14) = 34.8$, $p < 0.001$ at 3 hr and $F(1,14) = 7.1$, $p < 0.05$ at 6 hr] (Figure 4C). Together, these results (Figure 4) are consistent with the TUNEL staining data (Figures 3C and 3D), indicating that NMDA-induced apoptotic death is significantly higher for MSNs cultured from YAC72 mice compared with wild-type mice. Notably, the ratio of caspase activity measurements in wild-type controls to 72Q controls measured 6 hr after exposure to salt solution alone was 1.01 ± 0.12 (n = 4), indicating that cells expressing mutant htt and those expressing wild-type htt have similar basal caspase activity. Furthermore, in sister cultures treated with the caspase inhibitor z-DEVD-fmk just before, during, and after NMDAR stimulation, we noted a significant decrease in activation of caspase-3 (Figures 4A and 4B) and in cell death of MSNs (Table 1). Our results demonstrate that NMDAR activation is an effective stimulus of caspase-3 activity in cultured MSNs, which is required for mediating NMDA-induced cell death. Moreover, the enhanced NMDAR-induced caspase-3 activity observed in MSNs expressing mutant htt compared with wild-type controls correlates well with enhanced apoptosis shown by the TUNEL staining technique (see Figures 3C and 3D).

Table 2. No Trend in Enhancement of Non-NMDAR-Mediated Excitotoxicity in MSNs Expressing Mutant htt Compared to WT MSNs

	1 mM AMPA + cyclothiazide	1 mM AMPA + cyclothiazide + IFN	Number of Trials
Trypan-positive cells (%)			
WT	29.8 ± 3.0	16.4 ± 1.8	4
46Q	26.8 ± 4.3	14.0 ± 3.8	4
72Q	34.2 ± 1.3	14.8 ± 1.3	2
TUNEL-positive cells (%)			
WT	37.8 ± 10.2	18.8 ± 5.1	4
46Q	37.5 ± 4.6	21.1 ± 3.1	5
72Q	47.5 ± 1.1	12.6 ± 6.2	2

Analysis of trypan blue data: two-factor ANOVA; effect of ifenprodil $F(1,19) = 26.5$, $p < 0.001$; effect of genotype $F(2,19) = 0.7$, $p > 0.05$; genotype × ifenprodil interaction $F = 0.4$, $p > 0.05$. Analysis of TUNEL data: effect of ifenprodil $F(1,21) = 17.8$, $p < 0.001$; effect of genotype $F(2,21) = 0.03$, $p > 0.05$; genotype × ifenprodil interaction $F = 0.9$, $p > 0.05$.

Enhanced NMDAR-Mediated Peak Current Density in Acutely Dissociated MSNs Expressing Mutant htt

Increased sensitivity to NMDAR-mediated excitotoxicity might be secondary to mutant htt: (1) increasing activity of NMDARs in response to stimulation, or (2) modulating processes downstream of NMDAR activation (e.g., mitochondrial function or calcium homeostasis). Our previous work in a nonneuronal cell line indicated that expression of mutant htt increased NMDAR-evoked current (Chen et al., 1999). Therefore, we tested the hypothesis that an increase in NMDAR activity in MSNs of YAC72 mice underlies the increased sensitivity to excitotoxic cell death by recording NMDA-evoked current in a blinded fashion from striatal neurons acutely dissociated from the brains of 6- to 11-week-old YAC72 mice versus wild-type littermates. Using the whole-cell patch clamp recording technique under voltage clamp, we found that >50% of the peak current evoked by application of 1 mM NMDA and 50 μM glycine was blocked by 10 μM ifenprodil (e.g., Figure 5A), a dose that selectively inhibits current mediated by NR1/NR2B-type but not NR1/NR2A-type NMDARs (Williams, 1993). Strikingly, we observed significantly larger NMDAR peak current amplitudes and current density (amplitude normalized to membrane capacitance to take into account variability in cell size) in MSNs from the YAC72 mice (Figure 5B). Mean cell capacitance measured for the two groups was not significantly different (3.27 ± 0.19, n = 42 and 3.07 ± 0.18, n = 39 for YAC72 and wild-type, respectively). These data are consistent with our previous findings in a nonneuronal cell line (Chen et al., 1999) and suggest that increased NMDAR-induced sodium and calcium influx may play a role in enhanced susceptibility to cell death. This result does not rule out that other

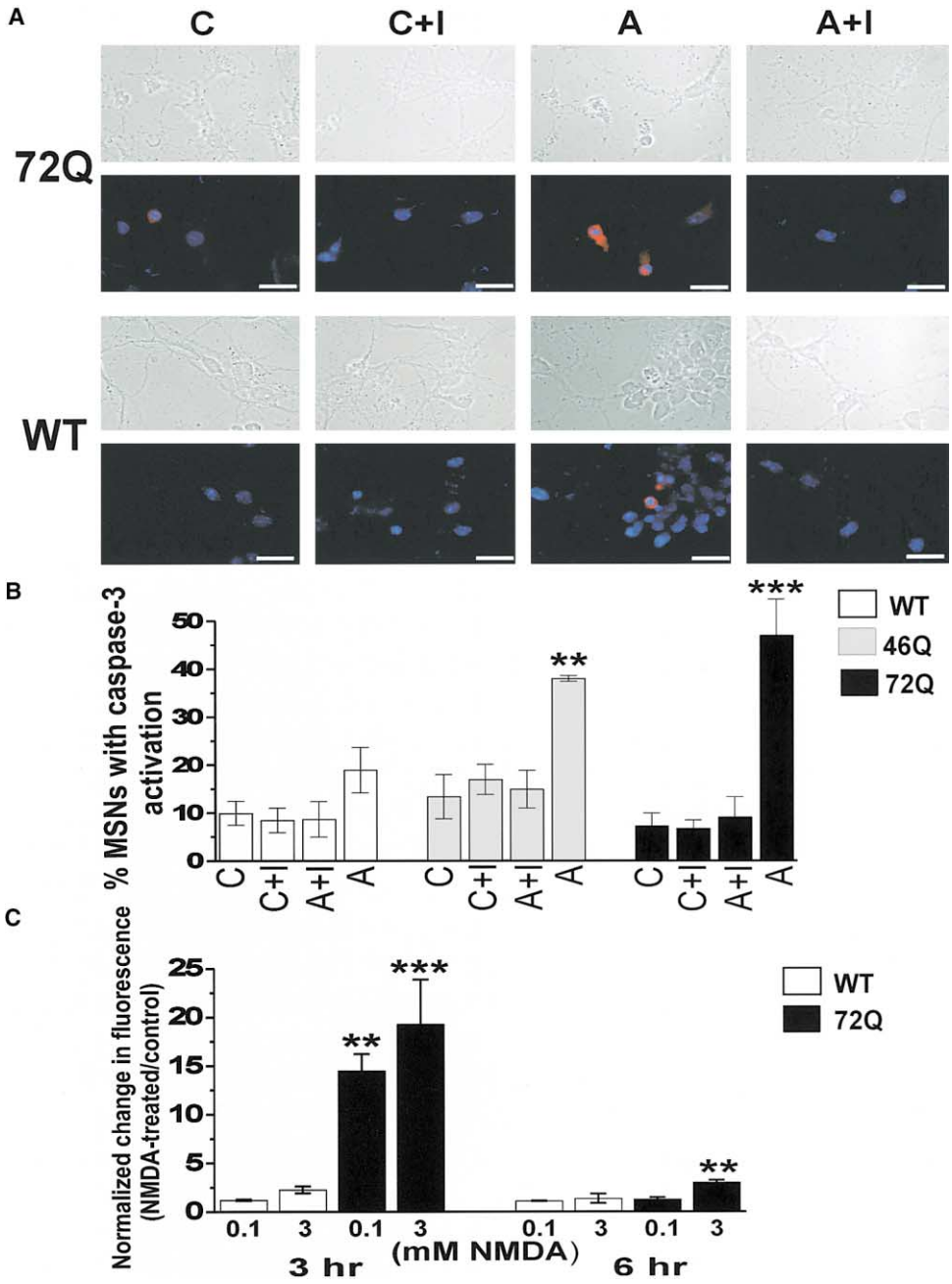

Figure 4. Increase in NMDA-Mediated Caspase-3 Activation in MSNs Expressing Full-Length Mutant htt Compared to Wild-Type Controls

(A) Caspase-3 activation was assessed by in situ immunofluorescence 6 hr after exposure to salt solution [C] or 3 mM NMDA [A], with or without 10 μM z-DEVD-fmk [I], in MSNs expressing mutant htt (72Q) compared to control MSNs. DAPI staining in blue represents total nuclei present, and red indicates staining for activated caspase-3. MSNs are shown in phase contrast (upper panels) or in fluorescence (lower panels). Bar = 20 μm.

(B) Enhancement of NMDAR-dependent caspase-3 activation is also polyQ length-dependent, as assessed by in situ immunofluorescence 6 hr after exposure to experimental solutions. $^{**}p < 0.01$, $^{***}p < 0.001$ versus wild-type (WT) at same NMDA concentration. n = 3–5 different batches of cultured neurons for each treatment.

(C) Enhancement of NMDAR-dependent activation of caspase proteolysis in cultured striatal cells expressing full-length mutant htt (72Q) compared to wild-type (WT) control. Caspase activation was assessed fluorometrically by cleavage of Ac-DEVD-AFC 3 and 6 hr after exposure to NMDA, normalized to background fluorescence and amount of protein/sample, and compared to sister cultures that underwent exposure to salt solution alone. $^{**}p < 0.01$, $^{***}p < 0.001$ versus wild-type at same NMDA concentration. n = 3–5 different batches of cultured neurons for each treatment and time point.

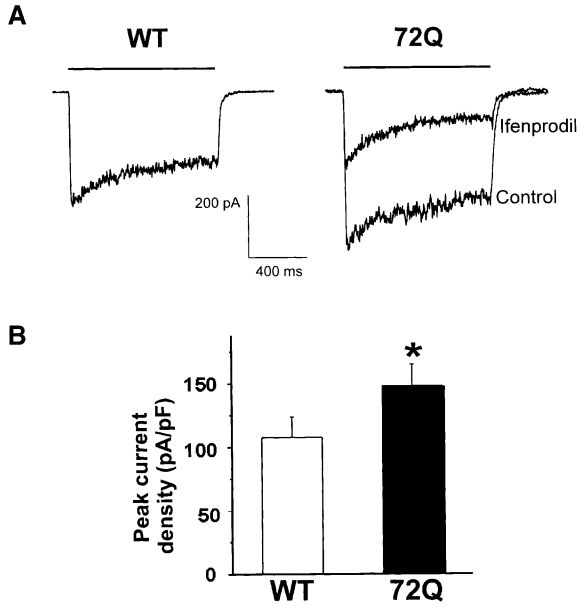

Figure 5. Increased Peak NMDA-Evoked Current Recorded from Acutely Dissociated YAC72 Striatal Neurons Compared with Wild-Type Controls

(A) Representative traces of NMDA-evoked current recorded from a single wild-type (WT) or 72Q MSN. Bar represents application of 1 mM NMDA in the presence of 50 μM glycine. In the right panel, "control" indicates application of NMDA alone, and "ifenprodil" indicates application of NMDA with 10 μM ifenprodil. Note that >50% of peak current is inhibited by ifenprodil, indicating that the majority of current is carried by NR1/NR2B-type NMDARs.

(B) Peak current density for 1 mM NMDA-evoked currents recorded from MSNs acutely dissociated from the striata of 6- to 11-week-old animals. Bars represent mean ± SEM measured from n = 39 and 42 different cells from 10 and 13 different animals for wild-type (WT) and YAC72, respectively. *p = 0.015 compared with wild-type, using unpaired t test.

intracellular processes downstream of NMDAR activation might also be altered by mutant htt and contribute to increased sensitivity to excitotoxicity.

Discussion

Enhanced NMDAR Activity May Contribute to the Mutant Steady State in HD

Here, we have demonstrated that mutant htt increases sensitivity of striatal MSNs to excitotoxic cell death induced by NMDAR activation in a transgenic mouse model of HD, addressing a long-standing question regarding the role of excitotoxicity in the pathogenesis of HD. One potential mechanism underlying this enhanced susceptibility of MSNs to NMDA is the increase in NMDAR current we observed in MSNs expressing mutant htt. YAC72 neurons would be expected to be as healthy as wild-type neurons in the young (6- to 11-week-old) mice used for these studies, since neuronal degeneration does not occur in this line until after 6 months of age. Therefore, our results are unlikely to be caused by secondary effects of cell stress due to other pathways. In the R6/2 mice, reduced membrane area and truncated dendritic trees and spines have been observed for medium-sized spiny striatal neurons (Levine et al., 1999; Laforet et al., 2001). In contrast, we found no significant difference in mean cell capacitance measured for wild-type versus YAC72 neurons, indicating that the total membrane area recorded from was equivalent, and we observed that the dendritic trees remaining after acute dissociation appeared similar for the two groups.

In previous studies, electrophysiological recordings from MSNs in acute striatal slices have suggested only a very minor role for NMDARs in routine cortico-striatal synaptic activity, due to the hyperpolarized resting membrane potential measured for these neurons (Calabresi et al., 1987) and voltage-dependent Mg^{2+} block of NMDARs (Hollmann and Heinemann, 1994). On the other hand, measurements made in vivo indicate that MSNs show spontaneous depolarizations of membrane potential and that the role of NMDARs in cortico-striatal MSN synaptic transmission may be substantial during such times (Cepeda and Levine, 1998; Calabresi et al., 2000). Therefore, it is plausible that an increase in NMDAR-mediated current in response to synaptic stimulation, resulting in an increased intracellular calcium load, may chronically stress MSNs expressing mutant htt. The increase in cell stress resulting from the mutant htt-induced increase in NMDAR activity would be one candidate mechanism for contributing to the "mutant steady-state" in human HD suggested by Clarke et al. (2000). As well, this increased stress from the earliest ages could be consistent with late onset neurodegeneration, since the aging process is associated with a progressive decrease in the ability of cells to compensate for oxidative stress (reviewed by Beal, 1992).

It has been proposed that presynaptic mechanisms, such as aberrant activity and/or increased release of glutamate from cortical afferents, might contribute to degeneration of striatal neurons in mice expressing mutant htt (Gutekunst et al., 1999; Li et al., 2000). Recent data indicate mitochondrial dysfunction in YAC72 mice (T. Greenamyre, personal communication), and MSNs from another model of HD, the R6 mice, tend to have depolarized resting membrane potentials (Levine et al., 1999), which would facilitate activation of NMDARs. As well, previous studies in late-stage symptomatic mice expressing N-terminal fragments of mutant htt, and in older (1–2 years) YAC72 mice, have also shown a trend toward increased NMDAR currents recorded from striatal neurons (Cepeda et al., 2001a; Laforet et al., 2001). Our data strongly support a role for mutant htt in enhancing responsiveness of postsynaptic NMDARs in MSNs, even in very young, presymptomatic animals; this mechanism could act synergistically with presynaptic processes, mitochondrial dysfunction, and postsynaptic membrane depolarization to facilitate degeneration of MSNs.

Potential Molecular Interactions between NMDARs and htt

Our data (see Figure 3) suggest that mutant htt effects little, if any, change in the EC_{50} for NMDA-induced cell death, but a robust increase in maximal toxicity. This result parallels studies of modulation of glutamate receptor currents by protein kinases, which also induce

a large potentiation of the maximal current response with little or no change in EC_{50} (Wang et al., 1993, 1994). The mechanism of NMDAR current potentiation by mutant htt has not been addressed in the present studies; however, preliminary results suggest that expression levels of NR1 and NR2B are not increased in striatal tissue from 6- to 12-week-old YAC46 or YAC72 mice compared with wild-type (C.D. Icton and L.A.R., unpublished data). On the other hand, recent data indicate that htt may associate with NMDARs within a larger complex via direct interaction with postsynaptic density protein-95 (PSD-95) (Sun et al., 2001) or through interactions with huntingtin-interacting protein 1 (HIP-1) and α-actinin (C.D. Icton and L.A.R., unpublished data). It is possible that mutant htt increases NMDAR current and sensitivity to NMDAR-mediated excitotoxicity by interacting with these cytoskeletal associated proteins, which are known to modulate NMDAR channel function and/or subcellular localization (Scannevin and Huganir, 2000).

Differential Sensitivity to NMDAR-Induced Excitotoxicity of R6 and YAC72 Mice

In contrast to the present results obtained in YAC72 mice, intrastriatal injection of quinolinic acid in the transgenic R6 mouse models of HD (Mangiarini et al., 1996) resulted in significantly less neuronal degeneration and apoptosis compared with wild-type littermates (Hansson et al., 1999). The resistance developed gradually with age and more rapidly in R6/2 (150 CAG repeats) than R6/1 mice (115 CAG repeats) (Hansson et al., 2001). R6 mice and YAC72 mice differ in many respects, including: (1) R6 mice express only a small fragment ($\sim$3% of the total length) of the htt protein, whereas YAC transgenics express the full-length protein; (2) htt expression is controlled by some but not all of the regulatory elements contained in the 5′ untranslated region of the human gene for R6 mice, but all regulatory elements are included in controlling expression of htt in YAC mice; (3) the number of CAG repeats in the HD gene is twice as large for R6/2 mice as for YAC72 transgenics; and (4) R6 mice show widespread and abundant intranuclear inclusions, whereas those found in brains of YAC transgenics are more sparse and restricted in distribution. We have shown previously that while expression of full-length mutant htt with 138Q significantly increased NMDAR-mediated cell death in a cell line, an N-terminal fragment ($\sim$540 amino acids) of htt had less effect (Zeron et al., 2001). Thus, it is possible that the full context of the mutant htt protein is required to increase NMDAR-mediated cell death and that these initial steps of pathogenesis in HD are bypassed in the R6 mice. On the other hand, recent data suggest that NMDARs in striatal neurons of R6/2 mice show increased activity, in part due to decreased sensitivity to Mg^{2+} block, at ages when symptoms and htt aggregates are present (Cepeda et al., 2001a). Why then are the R6 striatal neurons resistant to NMDAR-mediated excitotoxicity? One likely explanation is the widespread intranuclear aggregation of htt in up to 90% of these neurons (Davies et al., 1997), since we have recently correlated the development of nuclear inclusions and more efficient handling of Ca^{2+} increases with the appearance of resistance to NMDAR-induced toxicity in R6 mice (Hansson et al., 2001). These data suggest that R6 neurons are under chronic stress and have developed compensatory mechanisms that diminish the damage of excitotoxic insults (Hansson et al., 2001). YAC72 mice and patients with advanced HD exhibit intranuclear inclusions in a small percentage of striatal neurons (Hodgson et al., 1999; Sapp et al., 1999), and, therefore, these mice may constitute a more relevant disease model.

Enhanced Excitotoxicity as a Trigger of Downstream Events in the Pathogenesis of HD

The data presented here identify NMDAR activation as a trigger for enhanced caspase activation and cell death in striatal MSNs expressing mutant versus wild-type htt. In this regard, a variety of studies suggest a strong role for caspase activation in neuronal degeneration in HD (Ona et al., 1999; Sanchez et al., 1999; Wellington and Hayden, 2000). Furthermore, minocycline, an inhibitor of caspase-1 and -3, delays death in the R6 mice (Chen et al., 2000), and intracerebral infusion of caspase inhibitors or crossing R6/2 mice with a mutant mouse dominant-negative for caspase 1 have both been reported to inhibit development of the R6 phenotype (Ona et al. 1999). Our results indicate that increased NMDA-induced caspase-3 activation and cell death, as well as increased NMDAR-mediated currents, occur in mutant htt-expressing MSNs as early as the first few weeks postnatal, strongly supporting a primary role for excessive NMDAR stimulation in MSN degeneration in HD.

A large body of evidence in in vitro models suggests that cellular accumulation of short fragments of the N-terminal region of htt containing the polyQ tract can cause neuronal dysfunction and/or death (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998; Li et al., 1999). Huntingtin is cleaved by caspase-3 and -6, as well as calpains (Wellington et al., 2000; Kim et al., 2001; J. Gafni and L.M. Ellerby, submitted; C.L.W., unpublished data) in the brains of normal controls as well as patients symptomatic with HD. Our data showing enhanced NMDAR-induced caspase-3 activation in mutant htt-expressing striatal neurons suggest NMDAR activity as a potential trigger for increased htt cleavage. We have observed a high basal level of htt cleavage products and little difference in the extent of cleavage in cultured murine MSNs across different genotypes (wild-type, YAC46, and YAC72) or treatment conditions (NMDA versus buffered salt solution) by Western blot analysis using multiple N-terminal htt antibodies (our unpublished data). Additionally, we have observed no increase in immunodetection of caspase-2/3 htt cleavage fragments in striatal tissue in vivo following quinolinate injection (B.R.L. and C.L.W., unpublished data). However, because the sites for many of the proteases that cleave htt are tightly clustered and some are indistinguishable (i.e., caspase-3 and caspase-2), it may not be possible to use htt as the sole marker to detect a shift from one proteolytic pathway to another upon NMDAR activation using currently available antibodies. Rather, our results suggest that the mutant htt-induced increase in caspase-3 activation following NMDA treatment in our model of acute excitotoxicity may result in cleavage of many additional substrates that together lead to death

of MSNs, consistent with the accepted role for caspase-3 in neuronal cell death in the CNS.

Increased NR2B-Subtype NMDAR Activity May Explain Selective Neuronal Degeneration

Our results shed light on the issue of how a family of autosomal dominant hereditary neurodegenerative disorders, each caused by a (different) gene encoding an expanded polyglutamine repeat within a protein of wide CNS distribution, may show selective neuronal vulnerability in distinct brain regions (for review, see Tobin and Signer, 2000). We propose that the upstream events triggering the probable final common pathway in these diseases may be unique for each disease and related to interactions between the mutant protein and other proteins that are selectively expressed in vulnerable neuronal populations. By using the YAC transgenic mouse model of HD, in which selective neuronal degeneration so closely parallels that found in the human disease (Hodgson et al., 1999), we have been able to address this issue.

Within the striatum, the vulnerable MSNs display important differences in NMDAR subunit composition and electrophysiological characteristics compared to the population of aspiny cholinergic neurons, which are relatively resistant to degeneration in HD. MSNs express mainly NR1A and NR2B, a receptor complex previously shown to be modulated by mutant htt, whereas cholinergic interneurons express mainly NR2D with NR1 (Landwehrmeyer et al., 1995; Chen et al., 1999; Kuppenbender et al., 1999). As well, MSNs respond with markedly larger amplitude membrane depolarization to exogenous application of AMPA, kainate, and NMDA receptor agonists compared with striatal cholinergic neurons; this increased responsiveness may be one mechanism for increased MSN vulnerability to cell death in ischemia or neurodegenerative diseases (reviewed by Calabresi et al., 2000; Cepeda et al., 2001b).

It is interesting that the regional variation in severity of neuronal degeneration in HD—striatum $>$ cortex $>>$ cerebellum and brainstem (Vonsattel and DiFiglia, 1998)—correlates well with the relative expression levels of NR2B compared with other NR2 subunits (Hollmann and Heinemann, 1994; Monyer et al., 1994; Portera-Cailliau et al., 1996; Thompson et al., 2000). We would predict that neurons expressing NR2B at lower ratios to other NR2 subunits than MSNs, such as pyramidal neurons of the cortex and hippocampus, might show a smaller increase in NMDAR-mediated current and excitotoxicity in the YAC72 mice. Further experiments are required to test this prediction, and the results would help determine whether NR2B expression is sufficient or if other downstream factors contribute to selective neuronal vulnerability in HD.

In conclusion, we propose that mutant htt selectively enhances activity and toxicity of NR1/NR2B-type NMDARs, as supported by our data in neuronal cultures showing: (1) no difference between wild-type and YAC72 striatal medium spiny neuron death upon selective activation of AMPARs; (2) no difference between wild-type and YAC72 cerebellar granule neuron death upon stimulation of NMDARs, which are composed of NR1 with NR2A and/or NR2C, but do not include NR2B (Vallano et al., 1996; Thompson et al., 2000; Kovacs et al., 2001); and (3) the majority of NMDA-evoked current in MSNs was sensitive to the NR2B-specific antagonist ifenprodil, and NMDA-mediated excitotoxic cell death was nearly eliminated by treatment with this antagonist. Selective enhancement of NR1/NR2B-type NMDAR activity by mutant htt may, in part, explain selective vulnerability of striatal MSNs to neurodegeneration in HD. Moreover, our results suggest that NMDAR antagonists specific for the NR1/NR2B subtype may show higher efficacy in slowing progression of this disease than the less specific inhibitors of glutamatergic transmission tested thus far.

Experimental Procedures

YAC Transgenic Mice

In vivo experiments were performed using heterozygous YAC72 mice from line 2511 (as described by Hodgson et al., 1999), at ages 6 and 10 months, derived from a pure FVBN/NJ strain background. The control mice were nontransgenic littermates. Only crosses between two homozygous YAC transgenic mice, designed as described previously by Hodgson et al. (1999), or between homozygous YAC and wild-type mice were used to make neostriatal cultures. YAC46 (46Q; 668 line) or YAC72 (72Q; 2511 line) mice were used as models expressing full-length mutant htt and compared to FVB wild-type mice.

Lesion Surgery

Quinolinic acid (Sigma, Sweden) was dissolved in 0.1 M phosphate buffered saline (pH 7.4). Under halothane anesthesia, the mice received intrastriatal injections of 8 nmol of quinolinic acid (0.8 μl). Using a 2 μl Hamilton microsyringe fitted with a glass micropipette (outer diameter 60–80 μm), injections were made into the right striatum at the following stereotaxic coordinates: 0.9 mm rostral to bregma, 2.0 mm lateral to midline, 3.2 mm ventral from the bone surface, with the tooth-bar set at zero. The toxin was injected over 5 min, and thereafter, the cannula was left in place for an additional 5 min to minimize the risk of the retrograde leakage of toxin. Body temperature was controlled using a heating pad set at 37°C. There was no mortality of mice in conjunction with surgery.

Neuronal Culture

Anterior striata were dissected, using landmarks previously described (Howe and Surmeier, 1995), from postnatal day 0–1 (P0–P1) mice in ice-cold divalent-free Hank's Balanced Salt Solution (GIBCO), then diced and dissociated with 0.25% trypsin-EDTA (GIBCO) using a series of reducing bore-size Pasteur pipettes. After enzyme inhibition with 10% heat-inactivated fetal bovine serum (GIBCO) in Neurobasal medium (GIBCO), cells were plated at a density of $\sim 1 \times 10^6$ cells/mL on poly-D-lysine (MW = 30,000–70,000 g/mol; 250 μg/mL final concentration) coated dishes with or without nitric acid-treated 12 mm round coverslips in plating medium containing Neurobasal media, B27 (GIBCO), 100 units/ml penicillin-streptomycin (GIBCO), and 2 mM α-glutamine and maintained at 37°C, 5% CO_2 with humidity. Cells were fed $\sim$3 days after plating with medium lacking α-glutamine.

Cerebella were dissected from P8 mice following a similar protocol as for the MSNs, with a few changes. Trypsin action was ended with plating medium containing Basal Medium Eagle with Earle's Salts and $NaHCO_3$, 10% heat inactivated fetal calf serum (GIBCO), 100 units/mL penicillin-streptomycin, 2 mM α-glutamine, 17 mM D-glucose, and 25 mM KCl. Cells were plated at 2.0×10^6 cells/mL and examined 8–9 DIV.

Induction of Cell Death

After 9–12 DIV, cultured MSNs were exposed for 10 min to varying NMDA concentrations (30, 100, and 3000 μM) in triplicates or greater in the presence of 50 μM glycine in a physiological salt solution: 134 mM NaCl, 2.5 mM KCl, 4 mM $NaHCO_3$, 5 mM HEPES, 2.3 mM $CaCl_2$, 5.5 mM D-glucose, and phenol red, or exposed to salt solution

alone (control). Cells were then rinsed with plating media, the original (conditioned) plating media was replaced, and cells were maintained in humidified 5% CO_2 at 37°C. An additional set of sister cultures that did not undergo the procedure were also maintained under the same conditions (untreated). Some cultures underwent exposure to 20 μM MK-801 or 10 μM ifenprodil in conditioned media for 30 min before agonist exposure, in the agonist solution, as well as after exposure. Other experiments utilized 10 μM caspase inhibitor z-DEVD-fmk (Calbiochem), requiring a 1 hr pretreatment, with additions during and after agonist exposure. For experiments examining non-NMDAR-mediated cell death, MSNs underwent a 2 hr exposure to 1 mM AMPA (Tocris) and 50 μM cyclothiazide, with or without 10 μM ifenprodil, and were examined for cell death 24 hr later. At 8–9 DIV, CGNs underwent exposure to NMDA for 30 min, then were refreshed with serum-omitted media, and examined for cell death 24 hr later.

Cell Death Assays

YAC72 mice and littermate controls were transcardially perfused with 4% paraformaldehyde 72 hr after intrastriatal injection of QA. Coronal brain sections were cut (30 μm) and stained for Fluoro-Jade as previously described by Hansson et al. (1999). Fluoro-Jade is a marker for degenerating neurons (Schmued et al., 1997) and correlates strongly with the number of TUNEL-positive cells after intrastriatal injection of quinolinic acid in mice (Hansson et al., 1999). The sections were also processed for DARPP-32 immunohistochemistry to evaluate the number of surviving MSN (Hansson et al., 1999). In addition, the number of surviving nonpyknotic cells was investigated on cresyl violet-stained sections. The quantification of cells was performed using an unbiased stereology method and the CAST-Grid system (Olympus Denmark A/S, Albertslund, Denmark) (West et al., 1991). Cell loss in the striatum was examined in every fifth section (120 μm apart) in a region starting caudally at the level of the ventral hippocampal commissure and extending rostrally up to the level of the genu of the corpus callosum. This encompasses the major portion of the head and tail of the caudate-putamen, and typically 12 sections were examined per brain.

In striatal cultures, the trypan blue inclusion assay was done 24 hr after exposure to NMDA. Trypan blue dye (0.4%) was added to the cultures, and 10 random photographs of different brightfield (250×) images per dish were taken. The photographs were blind coded and quantified for total cell numbers and number of trypan blue-positive cells; only cells that met the morphological criteria for MSNs were scored. Values were averaged per condition, and total cell numbers were compared within a condition group and between conditions. The percentage of trypan blue-positive cells in cultures that underwent exposure to salt solution alone was subtracted from the percentage of trypan blue cells measured after NMDA treatment. Neuronal loss in the control (salt solution alone) was minimal (~5%) and comparable with that of the untreated group.

For the fluorescent TUNEL assay, 24 hr after the MSN cultures grown on coverslips were exposed to NMDA, they were fixed for 1 hr in a 4% paraformaldehyde solution (pH 7.3). Cells were stained with TUNEL mixture as described by Roche Diagnostics or with label solution alone (negative control), counterstained with 4 μM propidium iodide (PI) (Molecular Probes), and incubated with SlowFade from the Antifade Kit (Molecular Probes) according to manufacturer's instructions. The number of TUNEL-positive cells was compared to the total number (>500) of PI-positive cells in randomly chosen microscopic fields after blindly counting only cells showing condensed nuclei and resembling MSNs. The percentage of apoptotic cells in the control solution group was subtracted from the percentage of apoptotic cells after exposure to NMDA for each respective genotype.

Caspase-3 Immunofluorescence

MSNs were cultured on coverslips and treated for the caspase activity measurements as above. After recovery for 6 hr, cells were rinsed with PBS and fixed in freshly prepared 4% paraformaldehyde in PBS, rinsed in PBS, permeabilized with 0.5% Triton-X100 in PBS, washed in PBS, and blocked with 4% normal goat serum (NGS) in PBS for 30 min. Coverslips were then incubated with a polyclonal antibody specific for the cleaved and activated form of caspase-3 in 4% NGS in PBS (a kind gift of Drs. Donald Nicholson and Sophie Roy) overnight at 4°C in a humidified chamber. After washing three times in PBS, coverslips were then incubated with Alexa 594-conjugated goat anti-rabbit secondary antibody in 4% NGS in PBS for 30 min at room temperature, followed by three washes in PBS. Cells were counterstained with DAPI and mounted on glass slides. Images were captured using a CCD camera using Prince (Northern Eclipse) software.

Caspase Activity Assay

As described by Wellington et al. (2000), we measured DEVD-ase activity with the ApoAlert fluorometric kit (Clontech) in MSN primary cultures. At various time points following NMDA exposure, we harvested the cells, lysed them with cell lysis buffer (Clontech), and collected the supernatant. Protein concentration in the supernatant was measured using the Lowry assay. The supernatant was incubated with reaction mixture containing DEVD-AFC from Clontech and compared to control samples that were inhibited with DEVD-CHO and to substrate blanks. Caspase activity was measured fluorometrically every 5 min for 1 hr at 37°C at excitation wavelength 390 nm and emission wavelength 510 nm. The linear range of rate of change in fluorescence was normalized to protein concentration and expressed as relative levels and compared to measurements from cells that underwent exposure to salt solution alone.

Electrophysiology

Acute dissociation of neostriatal neurons was performed as previously described (Howe and Surmeier, 1995) with a few changes. Using a Leica microtome (Leica VT 1000 S, Germany), 400 μm slices were made from freshly dissected brain tissue bathed in slicing buffer (SB) that contained 127 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 17.5 mM glucose, 19.5 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 1 mM kynurenic acid, 1 mM pyruvic acid, 0.1 mM N-nitroarginine, and 5 mM glutathione (pH 7.4 and osmolarity ~300 mosm/l). Slices were incubated for 1–5 hr at room temperature in SB continuously bubbled with 95% O_2 and 5% CO_2. Then, in triturating buffer (TB) containing 140 mM Na isethionate, 2 mM KCl, 4 mM $MgCl_2$, 23 mM Glucose, 15 mM HEPES, 5 mM glutathione, 1 mM kynurenic acid, 1 mM pyruvic acid, and 0.1 mM N-nitroarginine, regions of the dorsal neostriatum were carefully microdissected and kept in humidified 5% CO_2 at 37°C. After 10 min digestion with papain, tissue was rinsed in enzyme free TB and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. Cells were used for electrophysiological analysis 5–10 min after plating onto coverslips. Patch clamp recording techniques were similar to those previously described (Chen et al., 1997). Coverslipped acutely dissociated striatal neurons were set in the recording chamber on the stage of an inverted microscope (Aviovert 100; Carl Zeiss, Thornburg, NY). During whole-cell recording mode under voltage clamp ($V_H = -60$ mV), neurons were lifted to achieve fast perfusion achieved by a piezo-driven theta tube (Hilgenburg, Malsfeld, Germany). In all experiments, 50 μM glycine was added to both control and NMDA-containing extracellular solutions. The intracellular recording solution contained 120 mM $CsMeSO_4$, 5 mM 1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetra-acetic acid, 3 mM MgATP, 1 mM $MgCl_2$, 0.3 mM GTP-tris, and 10 mM HEPES (titrated to pH 7.25 with CsOH). Extracellular recording solution contained 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES (titrated to pH 7.35 with 10 M NaOH). 10 μM CNQX and 0.1 μM TTX were added to the extracellular recording solution immediately before each experiment to block AMPA/kainate-type glutamate receptors and voltage-gated sodium currents, respectively. Currents were sampled at 2 kHz and acquired and analyzed using pCLAMP software and the Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Current amplitude measurement and kinetics fitting were conducted with Clampfit software.

Statistical Analysis

Significant differences were determined using the two-factor ANOVA followed by Bonferroni-Dunn's post hoc test for pairwise comparisons, unless otherwise noted.

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