Regulation of proteins affecting NMDA receptor-induced excitotoxicity in a Huntington’s mouse model

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Summary
Symptoms of Huntington’s disease may be caused by a toxic insult triggered by the mutant human huntingtin (Htt) protein itself, by a maladaptive protective mechanism initiated in response to an insult, or by a combination of these. We observed a protection from N-methyl-D-aspartate (NMDA) receptor-induced excitotoxicity in striata of symptomatic N171-82Q mice, a new transgenic model of Huntington’s disease. The goal of this study was to determine if NMDA receptor-mediated signalling pathways are altered in these mice. Multiple proteins of NMDA receptor and dopamine D1 receptor pathways are being regulated in ways predictive of the protection we observe. Although examining NMDA receptor subunit proteins showed no change in NR1, NR2A, or NR2B in the striata of the symptomatic mice, we observed a decrease in phosphorylation of NR1 at Ser897, previously reported to decrease NMDA receptor current. The dopamine D1 receptor, responsible for protein kinase A activation and subsequent phosphorylation of Ser897 of NR1, also showed an age-related decrease. Other proteins regulated in this disease were associated with PSD-95-like scaffolding proteins of the NMDA receptor. Specifically, we observed a decrease in membrane-associated neuronal nitric oxide synthase (nNOS), a decrease in PSD-95-like proteins, which link nNOS to the NMDA receptor complex, and a decrease in citron, a protein associated with dendritic spine formation. From these data, we conclude that the N171-82Q mice seem to be regulating, in a protective direction, many of the known effector pathways of NMDA receptor-induced excitotoxicity. These regulations, although seemingly effective in decreasing neuronal death, may in fact be causing some of the symptoms associated with the disease.

Keywords: Huntington’s; excitotoxicity; NMDA receptor; dopamine receptor, PSD-95

Abbreviations: Htt = human huntingtin protein; Hdh = murine huntingtin protein; NMDA = N-methyl-D-aspartate; nNOS = neuronal nitric oxide synthase; PBS = phosphate-buffered saline; PI-3 kinase = phosphatidylinositol-3 kinase; PKA = protein kinase A; PKC = protein kinase C; QA = quinolinic acid; WT = wild-type.


Introduction
Huntington’s disease occurs when more than 39 trinucleotide repeats are present in the human huntingtin (Htt) protein. Huntington’s disease patients have dementia and involuntary choreic (dance-like) movements progressing to a late hypokinetic phase with death usually resulting from pneumonia, wasting or other sedentary-associated symptoms. Inspection of brains from patients who died from Huntington’s disease shows massive neurodegeneration in the striatum, with a lesser degree of neurodegeneration seen in the cortex and other brain regions. A review of Huntington’s disease and its underlying pathogenesis with specific discussions on excitotoxicity, oxidative stress and the apoptotic pathway has been written by Petersen et al. (1999).

Excitotoxicity has had a long association with Huntington’s disease. Neuronal death is seen in various regions of the brain including the neocortex, lateral hypothalamus and striatum, with a dramatic loss of the GABAergic medium spiny neurons of the striatum. These
neurons receive glutamatergic input from the cortex and dopaminergic input from the substantia nigra. The earliest experimental models for Huntington’s disease involved intrastriatal injections of \( N \)-methyl-D-aspartate (NMDA) receptor agonists, which produced a selective neurotoxicity of the medium spiny neurons similar to that observed in the striatum of human Huntington’s patients (Ellison et al., 1987). Studies of NMDA-induced excitotoxicity in various mouse models of Huntington’s disease have shown variable responses across mouse models and at different stages of the disease. Decreased sensitivity was reported in R6/1 (Hansson et al., 1999, 2001b) and R6/2 mice (Hansson et al., 2001b; MacGibbon et al., 2002), with decreasing toxicity observed throughout the disease process. Increased sensitivity was reported in symptomatic R6/2 mice (Levine et al., 1999), symptomatic hdhCAG94 mice (Levine et al., 1999), and symptomatic YAC72 mice (Zeron et al., 2002). No change in sensitivity was reported in tgHD100 mice (Petersen et al., 2002). The Hereditary Disease Foundation has recently highlighted the importance of examining multiple transgenic models in the study of Huntington’s disease. Thus, we chose N171-82Q mice to study neuroprotection from quinolinic acid (QA) excitotoxicity and the long-term changes in proteins associated with this excitotoxicity. Yu et al. (2003) reported that N171-82Q mice displayed more degenerated neurons than did other Huntington’s disease mouse models at late disease stages, which appears to be more similar to the degeneration seen in Huntington’s disease in humans. Chan et al. (2002) suggest that expression of short N-terminal fragments of mutant Htt in transgenic mice produce alterations in gene expression similar to those observed in human HD brain, while expression of full length mutant Htt in transgenic mice does not. An important aspect of the N171-82Q mouse model was the use of a prion promoter directing expression exclusively in the brain allowing a focus on neuronal disease.

To understand changes in protein expression which may be responsible for changes in excitotoxicity, levels of several proteins known to affect NMDA receptor-induced excitotoxicity were measured. Proteins were measured during three distinct stages of the disease to assess if any changes seen were progressive. Levels of NMDA receptor subunit proteins, phosphorylation levels of the NMDA NR1 subunit, levels of membrane-associated phosphatidylinositol -3 kinase (PI-3 kinase), membrane-associated neuronal nitric oxide synthase (nNOS), PSD-95-like proteins, citron and theSrc family of tyrosine kinases were all measured. Many of these levels were altered in the N171-82Q mice and all alterations appear to be in the direction predicted for neuroprotection. The N171-82Q mice seem to have generated a series of compensatory mechanisms to reduce some of the toxicity initiated by the expression of the mutant Htt by decreasing the functional effects of NMDA receptor activation, a known factor in many toxic insults including QA toxicity, ischemia, mitochondrial defects, and kainic acid toxicity.

### Material and methods

#### Materials

**Materials**

The NR1, NR2A, and NR2B antibodies have been previously characterized in this laboratory (Wang et al., 1995; Luo et al., 1997). Phospho-Ser\(^{897}\) NR1 antibody 3381 (Cell Signaling Technology, Beverly, MA, USA), phospho-Ser\(^{897}\) NR1 antibody 3385 (Cell Signaling), PI-3 antibody 06-497 (Upstate Biotechnology, Waltham, MA, USA), PSD-95 antibody 610496 (BD Biosciences, San Jose, CA, USA), Chapsyn-110 antibody APZ-002 (Alamone, Jerusalem, Israel), SAP-102 antibody APZ-003 (Alamone), nNOS antibody 610308 (BD Biosciences), Src antibody 05-184 (Upstate Biotechnology, Charlottesville, VA, USA), Fyn antibody sc-16 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), citron antibody sc-1848 (Santa Cruz Biotechnology) and Htt antibody MAB2166 (Chemicon, Temecula, CA, USA) were used in these experiments. Dopamine D1 receptor antibody was a gift from Robert Luedtke (University of North Texas Health Science Center) and has been previously characterized (Luedtke et al., 1999).

#### Animals

Heterozygote male transgenic N171-82Q mice (Schilling et al., 1999) and wild-type (WT) B6C3F1/J female mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). A colony was raised and maintained in the animal facility at Georgetown University following the guidelines set by the Georgetown University Animal Care and Use Committee. Breeding heterozygote male mice to WT female mice produced litters containing half heterozygote mutant HD mice and half WT mice, which were used as controls. Onset of clasping phenotype occurred between 9–14 weeks in all transgenic mice used in this study. Thus, mice of age 7 weeks were classified as presymptomatic and mice of age 15 weeks were classified as symptomatic. Between 16 and 22 weeks of age, the mice enter a hypokinetic period with death following 1–3 days later. This is similar to other transgenic Htt models and human Huntington’s patients (Nance and Sanders, 1996; Reddy et al., 1999). The number of animals in each experiment group for all western blot analyses were: 7-week N171-82Q mice \((n = 11)\), 7-week littermate controls \((n = 11)\), 15-week N171-82Q mice \((n = 10)\), 15-week littermate controls \((n = 12)\), hypokinetic N171-82Q mice \((n = 7)\), and age-matched littermate controls for the hypokinetic N171-82Q mice \((n = 8)\).

#### Genotyping animals

DNA was extracted from 1 cm tail pieces using Dneasy Tissue Kit 69506 (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) amplification was performed using AmpliTaq® DNA Polymerase, Stoffel Fragment Kit N808–0038 (Applied Biosystems, Foster City, CA, USA) and primers GAACCTTT-CAGTACCAAGAAAGACCGTGT and GTGGATACCCCTC-C CCCAGCTAGACC (Invitrogen; Rockville, MD, USA) with a final reaction volume of 33 µl and <0.033 µg experimental template DNA. PCR conditions were as follows: 94°C for 3 min followed by 38 cycles at 94°C for 35 s, 64°C for 45 s, 72°C for 45 s and a final extension at 72°C for 2 min. The DNA was then separated using agarose gel electrophoresis and was visualized using ethidium bromide. A transgene-specific product 250 bp in length was visible.
**Intrastriatal QA injections**

Animals were anaesthetized with equithesin and 15-week symptomatic N171-82Q mice and their age-matched, WT littermates were stereotaxically injected using a 10 μl Hamilton syringe (intrastriatal coordinates 0.01 mm posterior, 2.0 mm left or right, 3.2 mm ventral from bregma) with 30 nmol QA (ICN Biomedicals Inc., Aurora, OH, USA) in 1 μl phosphate-buffered saline (PBS) on one side and PBS vehicle alone on the other. The QA was injected over 2 min and the cannula was left in place for an additional 3 min. Body temperature was maintained using a heating lamp. Animals were monitored for 2 h post-injection and then returned to the animal housing facility for 72 h. Injections were performed alternating between N171-82Q and WT mice.

**Tissue preparation for QA toxicity**

Seventy-two hours after injection, mice were anaesthetized and perfused intracardially with 4% paraformaldehyde in PBS. Brains were post-fixed overnight in 4% paraformaldehyde in PBS, dehydrated overnight in 30% sucrose in PBS solution, and frozen at −70°C. Coronal sections were cut (50 μm), and every other section was prepared for Fluoro-Jade staining (see below).

**Fluoro-Jade staining and analysis**

Fluoro-Jade (Histo-Chem, Jefferson, AZ, USA) staining was performed as described by Schmued et al. (1997) with a few minor alterations. Briefly, 50 μm sections were cut, mounted on superfrost plus slides, and air-dried. Sections were then immersed in 100% ethanol (3 min), 70% ethanol (1 min), distilled water (1 min), 0.001% Fluoro-Jade/0.00005% ethidium bromide in distilled water (30 min), and distilled water (1 min) × 3. Sections were dried on a hot plate at 45°C and coverslipped using DPX neutral fluorescent mounting medium. The site of injection was identified and digital images of the sections were captured at 4× using a Nikon Diaphot microscope. Sections were analysed by marking the boundary of the striatum in the two sections closest to the injection site and counting the total number of Fluoro-Jade positive cells using Meta Morph software from Universal Image Corporation (West Chester, PA, USA). Fluoro-Jade data were analysed without knowledge of the treatment and reported as the number of Fluoro-Jade positive cells per square millimetre of cross-sectional striatum. Control sides receiving PBS injections showed no Fluoro-Jade positive cells within the striatum and were therefore not included in the analysis.

**Tissue preparation for western blots**

Mice were dissected at three phenotypically distinct time points: 7-week (presymptomatic; n = 11 for transgenic and WT; average age was 49 ± 3 days), 15-week (symptomatic; n = 11 transgenic; n = 13 WT; average age was 106 ± 5 days) and hypokinetic (n = 7 transgenic; n = 8 WT; average age was 138 ± 13 days). The hypokinetic transgenic mice were dissected within the narrow time window when the mice would no longer try to escape when picked up. An age-matched, WT littermate was dissected with each hypokinetic transgenic mouse. Brains were removed from mice, striata were dissected, immediately frozen and stored at −70°C. Striata were later homogenized in ice-cold TE buffer (10 mM TRIS-HCl, pH 7.4; and 1 mM EDTA) using a Tekmar Tissumizer. The homogenates were centrifuged at 32 000 gmax for 16 min at 4°C. The supernatant was saved as the cytosolic fraction and the membrane pellet was resuspended in TE. Protein concentrations were determined by the BCA method (Pierce; Rockford, IL, USA) using bovine serum albumin as a standard. These values were used to load 20 μg of protein in each lane.

**Western blotting and analysis**

Proteins were solubilized in 2% sodium dodecyl sulphate (SDS) containing 50 mM dithiothreitol (DTT) and boiled for 5 min, except for analysis of the dopamine D1 receptor which was solubilized in 2% SDS containing 50 mM DTT at room temperature for 1 h. In order to randomize samples for optimal analysis by western blots, these studies were not performed blinded. Twenty micrograms of protein were loaded in triplicate for each animal with lanes alternating between WT and mutant Htt mice for each given age group with each age group being a separate experiment. This allows for comparison of WT and mutant Htt mice at a given age (e.g. D1 receptor in 15-week WT versus mutant Htt mice), but not between ages (e.g. D1 receptor in 7-week WT versus 15-week WT). Differences in transfer and fluorescence exposure cause variability between separate western blot experiments, which results in an inability to make comparisons between experiments. Since experiments for the 7-week and 15-week animals were too large to perform together, it was not appropriate to perform an ANOVA (analysis of variance) to compare 7-week animals with 15-week animals. All experiments were run with a standard curve of rat striatum to define the linear range for analysis. Proteins were separated using 7.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), except for citron which was separated using 5% SDS–PAGE, and proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in TBST buffer [20 mM Tris-HCl (pH 7.4) 140 mM NaCl, and 0.1% Tween-20] for 30 min at room temperature. The immunoblots were probed with the appropriate antibodies: NR2A (1 μg/ml), NR2B (1 μg/ml), NR1 (1 μg/ml), phospho-Ser980 NR1 (1:1000), phospho-Ser897-NR1 (1:1000), D1 receptor (1 μg/ml), PI-3K (1:1000), PSD-95 (1 μg/ml), Chapsyn-110 (1:1000), SAP-102 (1:500) nNOS (1:1000), Src (1 μg/ml), Fyn (1:1000), citron (1 μg/ml) or Htt (1:1000). All blots were incubated with the primary antibody overnight at 4°C in blocking buffer. Blots were washed once for 15 min and 4× for 5 min with TBST. Blots were incubated in goat anti-mouse (Amersham, Piscataway, NJ, USA), goat anti-rabbit (Amersham), or bovine anti-goat (Santa Cruz Biotechnology) secondary antibody coupled to horseradish peroxidase at a 1:2000 dilution for 30 min in blocking buffer. Blots were washed once for 15 min and 4× for 5 min with TBST. Blots were incubated with SuperSignal West Pico or West Femto kits according to the manufacturer’s instructions (Pierce; Rockford, IL, USA) for 5 min and apposed to Hyperfilm (Amersham) for various times. Each antibody used gave a single band on a western blot. In order to control for loading in this study, after standardizing samples to have the same level of total protein, we used similar aliquots to study each protein tested and, in many cases, tested proteins of different sizes on the same lanes. Therefore, the observance of proteins that did not change, as well as proteins that increased and decreased relative to total protein and many times relative to each other in the same set of lanes act as controls for loading errors. For example, the pairs of Src and NR2A at 7 weeks, citron and PI-3 kinase in hypokinetic mice, and NR1 and NR2B in hypokinetic mice were run and analysed on the same gels and in the same lanes with changes present in some and not in others.
Stripping blots

Blots were stripped of antibodies by incubating at 60°C for 30 min in stripping buffer [62.5 mM Tris (pH 6.7), 2% SDS, 100 mM β-mercaptoethanol]. Stripped blots were washed 3× for 5 min in TBST and reanalysed as described above to verify that the stripping was complete. Each sample was blotted first with phospho-Ser897 NR1 rabbit antibody, stripped and probed with NR1 mouse antibody, and then stripped and reprobed with phospho-Ser890 NR1 rabbit antibody. The experiments were repeated using the phospho-Ser890 NR1 rabbit antibody first, and then stripped and reprobed with NR1 mouse antibody to show that there was no difference caused by the stripping process.

Data analysis and statistics

The films were analysed with the BioImage Intelligent Quantiﬁer® software and the integrated intensities, taken from the linear range of a rat striatum standard curve, were normalized for each animal as a percentage of the WT mean for comparison purposes. Mean ± SEM for the mutant Htt group and WT littermate control group is reported. Each experiment was set up to compare protein levels at a given age and not across ages, and data were analysed using unpaired, two-way Student t-test for a given speciﬁc protein at each age and P-values are reported. Statistical signiﬁcance was taken to be P < 0.05 for discussion of results.

Results

Protection from QA

The first goal was to determine the susceptibility of 15-week symptomatic N171-82Q mice to QA-induced excitotoxicity in the striatum. Littermates not carrying the Htt transgene were used as WT controls and animals were injected with QA in PBS on one side and PBS alone on the other. Three days later, the side injected with PBS showed no Fluoro-Jade-positive (dead cells) within the striatum, while injection of the NMDA receptor agonist QA resulted in significant numbers of dead cells. A representative picture of the striatum from a WT mouse and 15-week symptomatic littermate injected with QA can be seen in Fig. 1A and B. The cell death seen in the WT mice continues throughout the entire striatum. In contrast, striata of the 15-week symptomatic N171-82Q mice show only sparse cell death, diminishing with increasing distance from the injection site. Analysis of images (Fig. 1C) shows a 78% decrease in Fluoro-Jade-positive cells per mm² striatum as compared with WT mice (P = 0.006). These data demonstrate that 15-week symptomatic N171-82Q mice are protected from QA-induced excitotoxicity compared with WT littermates.

We also performed the same QA injections into the striatum of 7-week WT and N171-82Q mice to determine how the Htt transgene would affect toxicity in the presymptomatic mice. We observed a protection in this strain of mice at 7 weeks independent of the transgene, with no cell death observed in either the WT or N171-82Q mice after QA injections. Therefore, we chose to focus on the changes in toxicity observed after the onset of symptoms and used three different disease stages to determine if protein changes were progressive or isolated to the 15-week time point.

NMDA receptor subunit protein levels

One simple explanation for the decreased NMDA receptor-mediated excitotoxicity seen in N171-82Q and other, similar, transgenic Htt mice (Hansson et al., 1999, 2001b;
MacGibbon et al., 2002) could be a decrease in the level of NMDA receptor subunits. To test this hypothesis, striata from N171-82Q mice and WT littermates were taken at three phenotypically distinct ages to confirm the progressive nature of any changes observed. The striata of 7-week presymptomatic, 15-week symptomatic and 20-week hypokinetic mice (see description of groups in Material and methods) were dissected and western blots were performed using selective NR2A, NR2B and NR1 antibodies. The summary of these data is shown in Fig. 2. No significant difference was found in the expression of the NR1 subunit in symptomatic 15-week or presymptomatic 7-week N171-82Q mice, agreeing with reports studying other transgenic Htt mouse models (Cha et al., 1999; Hansson et al., 1999; Luthi-Carter et al., 2000; Cepeda et al., 2001). Interestingly, during the hypokinetic stage, which had not been examined in previous studies, a significant (P = 0.02) 20% decrease in the amount of NR1 was confirmed in two separate experiments. NR2A or NR2B subunits, not well studied in transgenic Htt mice, may be more likely to be regulated than NR1 subunits, since there appears to be a stoichiometric excess of NR1 expressed by neurons compared with NR2 (Prybylowski et al., 2002). However, the 15-week symptomatic mice had no change in either NR2A or NR2B. Surprisingly, the 7-week presymptomatic mice showed a significant but transient 19% decrease in NR2A (P = 0.01), which was confirmed in two separate experiments, with no change in NR2B. These data demonstrate that neither NR1, NR2A nor NR2B subunits are decreased in the 15-week symptomatic N171-82Q mice protected from QA.

Phosphorylation of the NR1 subunit

In addition to changes in expression levels of receptor subunits, another potential mechanism affecting NMDA receptor-induced excitotoxicity is phosphorylation. Protein kinase C (PKC) and protein kinase A (PKA) mediated phosphorylation of the C1 cassette of NR1 have been associated with increases in NMDA receptor current (Tingley et al., 1997; Logan et al., 1999; Westphal et al., 1999). Therefore, we tested the hypothesis that phosphorylation of the NR1 subunit would be decreased in the N171-82Q mouse. Phosphorylation was measured using specific phospho-Ser890 NR1 and phospho-Ser897 NR1 antibodies. Sample western blots representing average values for a given experimental group are shown in Fig. 3A.

Levels of PKC-dependent (Tingley et al., 1997) Ser890 phosphorylated NR1 did not change significantly, although a potential trend may be present (Fig. 3B). However, the PKA-dependent (Tingley et al., 1997) Ser887 phosphorylated NR1 (Fig. 3C), shows a significant, apparently progressive decrease in phosphorylated NR1 in presymptomatic 7-week mice (21%, P = 0.02), symptomatic 15-week mice (45%, P = 0.005), and hypokinetic mice (56%, P = 0.005).

Fig. 2 NMDA receptor subunits change little or not at all in transgenic Htt mice. (A–C) Protein levels were quantified as described in Material and methods, and standardized to the mean WT intensity for each group. White bar = WT littermates and grey bar = transgenic N171-82Q mice. No changes were observed in NR2A, NR2B or NR1 in 15-week symptomatic mice. A transient but significant decrease was found in NR2A in 7-week presymptomatic mice (P = 0.01, n = 11 Htt and n = 11 WT). A decrease in NR1, predictive of protection, was observed in hypokinetic mice at late stages of the disease (P = 0.02, n = 7 Htt and n = 8 WT). The experiments showing changes in NR2A and NR1 were repeated and the decreases in NR2A at 7 weeks and in NR1 in hypokinetic mice were confirmed. Sample western blots representing the average value for an experimental group are shown directly above each group. 7M = 7-week presymptomatic membranes; 15M = 15-week symptomatic membranes; HM = hypokinetic membranes. *P < 0.05.
Decreases in dopamine D1 receptors

A neuronal axis significantly affecting PKA activity in the striatum is the dopamine pathway, which contains proteins reported to decrease in several transgenic Htt models (Cha et al., 1999; Reynolds et al., 1999; Bibb et al., 2000; van Dellen et al., 2000; Ariano et al., 2002). Therefore, a further goal of this study was to look upstream of the PKA-dependent phosphorylation of Ser897 of NR1 at the dopamine D1 receptor. Western blots were performed on each experimental group and the summarized data for D1 receptor are shown in Fig. 4A.

The 15-week symptomatic mice showed a 28% decrease ($P = 0.01$), while the hypokinetic mice had a 56% decrease ($P < 0.0001$). The observed decrease in phosphorylation of Ser897 of NR1 at 7-weeks with no significant decrease in D1 receptor at this time point probably indicates that earlier down-regulation of other proteins in this pathway such as DARPP-32 and PKA (Bibb et al., 2000) are likely to be contributing to the progressive decrease in downstream phosphorylation effects of this pathway.
**Increases in PI-3 kinase**

PI-3 kinase has been shown to be a major neuroprotective pathway through activation of AKT (Dudek et al., 1997; Crowder and Freeman, 1998). A recent report demonstrates a decrease in D1 receptor association with NR1 causes an increase in PI-3 kinase association with a subsequent decrease in NMDA receptor-induced excitotoxicity (Lee et al., 2002). Therefore, we hypothesized that the decreased D1 receptor levels would result in an increase in membrane-associated PI-3 kinase, a known anti-apoptotic protein, in the N171-82Q mice, which could be an additional neuroprotective mechanism. The summarized data for PI-3 kinase are shown in Fig. 4B. Although no change is observed in the 15-week mice, there was a significant increase (23%, \( P = 0.03 \)) in membrane-associated PI-3 kinase in the hypokinetic mice. These finding in hypokinetic and 15-week mice were confirmed in two separate experiments. Analysing the cytoplasmic fraction from the hypokinetic mice demonstrated a 27% decrease (\( P = 0.008 \)) in cytoplasmic PI-3 kinase, confirmed in two separate experiments, suggesting a translocation of this protein to the membrane. Since PI-3 kinase has been shown to be neuroprotective (Zhang et al., 1998; D’Onofrio et al., 2001), this increase may be another protective mechanism activated in the Htt transgenic N171-82Q mice, but only in the late stages of the disease.

**Membrane-associated nNOS decreases**

Since nNOS is reported to mediate NMDA receptor-induced excitotoxicity (Ayata et al., 1997; Eliasson et al., 1999; Huang, 1999; Sattler et al., 1999), we hypothesized that a decrease in nNOS would be neuroprotective in N171-82Q mice. Since nNOS is reportedly activated by calcium influx through the NMDA receptor, the membrane-associated fraction of nNOS was selected as the most functional fraction when considering NMDA receptor-induced excitotoxicity. Summarized data and sample western blots representing the average value for each experimental group are shown in Fig. 5A. The 15-week symptomatic mice showed a significant 15% decrease in membrane-associated nNOS (\( P = 0.03 \)) and the hypokinetic mice also showed a significant 29% decrease in membrane-associated nNOS (\( P = 0.03 \)). In order to determine if the decrease in nNOS was associated with a subsequent increase in cytoplasmic nNOS, we analysed the level of nNOS in the cytoplasm of hypokinetic mice and found no change in nNOS levels.

Since it has been demonstrated that expression of endogenous murine huntingtin protein (Hdh) is required for development (Dragatatis et al., 1998), we hypothesized that compensatory changes in the level of Hdh could alter the responsiveness of neurons. Additionally, the expression levels of Hdh have not been reported in N171-82Q mice. Analysis of data summarized in Fig. 5B shows no change in endogenous murine Hdh protein in mice containing the transgene coding for the N-terminal portion of the Htt.
unchanged in 7-week presymptomatic mice, decrease 34% \( (P < 0.0001) \) in 15-week symptomatic mice and decrease 51% \( (P = 0.003) \) in hypokinetic mice. The levels of Chapsyn-110, Fig. 6C, however, increase 54% \( (P = 0.02) \) in 15-week symptomatic mice (confirmed in two experiments), although Chapsyn-110 levels are unchanged at the earlier 7-week presymptomatic time point and the later hypokinetic time point.

**Citron and Src/Fyn kinase**

In addition to the NMDA receptor itself and nNOS, the PSD-95-like proteins are associated with many other proteins of the NMDA receptor complex and seem to serve as scaffolding for synapse formation (Sheng and Lee, 2000). Since PSD-95 and SAP-102 were significantly decreased throughout disease progression, we hypothesized that other PSD-95 associated proteins would be decreased within membrane fractions. To test this hypothesis, three proteins that have been shown to be associated with PSD-95: citron (Zhang et al., 1999), and Fyn and Src kinase (Tezuka et al., 1999) were examined. Membrane-associated citron is a newly discovered Rho-effector in the brain that may play a role in Rho’s effects on spine and synapse formation (Furuyashiki et al., 1999; Bonhoeffer and Yuste, 2002). Since others have reported an early decrease in dendritic spine formation that precedes the onset of symptoms and mitochondrial dysfunction in both mouse models and Huntington’s disease patients (Guidetti et al., 2001), we hypothesized that a decrease in citron could be an additional neuroprotective regulation in the N171-82Q mice. We found citron decreased 20% \( (P = 0.05) \) in 15-week symptomatic mice and decreased 30% \( (P < 0.0001) \) in hypokinetic mice. In order to determine whether the significant decrease in membrane-associated citron would show up as an increase in cytoplasmic citron, we measured citron levels in the cytoplasmic fraction of the hypokinetic mice. The cytoplasmic levels of citron in the hypokinetic mice did not change. This observation may indicate that the citron in the cytoplasm is maintained at some predetermined level. The membrane-associated levels of two other PSD-95-associated proteins, Fyn and Src kinases, were also examined. A decrease in these proteins would be associated with a decrease in tyrosine phosphorylation of the NR2 subunits and a decrease in NMDA receptor current. The data for Fyn and Src are reported in Fig. 7B and Fig. 7C, respectively. Surprisingly, neither Fyn kinase nor Src kinase were decreased in membrane or cytoplasmic homogenates of N171-82Q striatum.

**Discussion**

**Protection from QA excitotoxicity in Huntington’s disease**

Various transgenic mouse models of Huntington’s disease have shown different patterns of either protection or hyper-
sensitivity to NMDA receptor agonist-induced excitotoxicity.

To more fully characterize the new N171-82Q model, we gave intrastriatal injections of QA to 15-week symptomatic N171-82Q mice and found a significant 78% decrease in excitotoxic cell death compared with WT littermates. This is similar to the protection reported in the R6/1 and R6/2 mice by Hansson et al. (1999, 2001b) and MacGibbon et al. (2002). Petersen et al. (2002), however, reported no change in excitotoxicity induced by intrastriatal injection of QA measured by Fluoro-Jade staining in the tgHD100 mouse model, which expresses a much longer portion of Htt than the R6 or N171-82Q mice. In contrast, Zeron et al. (2002) used YAC72 mice, expressing full-length Htt, and showed an increase in excitotoxicity induced by intrastriatal injection of QA measured by Fluoro-Jade. The tgHD100 and YAC72 mouse models may be indicative of a much earlier stage of the disease and therefore not reflective of the protective changes observed at later disease states. The fact that R6 mice have been shown to be resistant to other toxins including dopamine (Petersen et al., 2001), malonate (Hansson et al., 2001a), 3-nitropropionic acid (Hickey and Morton, 2000), 6-hydroxydopamine (Petersen et al., 2001), kainic acid (Morton and Leavens, 2000) and ischemia (Schiefer et al., 2002) is not surprising since NMDA receptor-induced excitotoxicity is a contributing factor in secondary aspects of neurological insults in general.

NMDA receptor subunit levels

No change in NR1 was shown in presymptomatic 7-week or symptomatic 15-week mice, which agrees with reports using other transgenic mouse models (Cha et al., 1999; Hansson et al., 1999; Luthi-Carter et al., 2000; Cepeda et al., 2001). However, examination of the previously unstudied late-term, hypokinetic phase, revealed a significant 20% decrease in NR1 levels. This may reflect cell death associated with the disease. However, only a small amount of cell death has been reported in Htt transgenic mouse models including the R6 models (Turmaine et al., 2000). Only one study to date has examined NR2 proteins and Cepeda et al. (2001) reported a decrease in NR2A/B immunohistochemical staining in symptomatic R6/2 mice. Our measurements of NR2A and NR2B in N171-82Q mice showed a significant decrease of NR2A in 7-week presymptomatic mice, with no other changes observed in either NR2A or NR2B. Overall, these data suggest that NMDA receptors, per se, are not affected by the expression of mutant Htt.

Multiple protective changes

The observation of many protective pathways being regulated in the N171-82Q mice seem to indicate a coordinated and controlled response in protein levels. In this report, a significant and progressive decrease in total Ser897 phosphorylated NR1 was observed. The D1 receptor levels in N171-82Q mice are also significantly decreased in symptomatic 15-week and hypokinetic mice. This is consistent with the hypothesis that the decrease in the NR1 Ser897 phosphorylation results from decreases in the D1 receptor.
cAMP-PKA-DARPP-32 pathway. Protective changes are also initialized in later disease stages such as an increase in the antiapoptotic protein PI-3 kinase in membrane fractions and a decrease in levels of the NR1 subunit of the NMDA receptor. The fact that PI-3 kinase changes only in late stages seems to indicate that this protein is only recruited when large amounts of D1 receptor are absent.

We also observed decreases in PSD-95 and SAP-102, which are associated with many downstream effectors of NMDA receptors. The increase in Chapsyn-110 in 15-week symptomatic animals without changes in 7-week and hypokinetic animals indicates differential regulation of the various PSD-95-like scaffolding proteins in the striatum. nNOS, a protein associated with PSD-95-like proteins, produces nitric oxide in a calcium and calmodulin dependent manner. High levels of nitric oxide are thought to react with oxygen-free radical species to form peroxynitrite, a stable free radical that destroys the cell (for a review, see Dawson and Dawson, 1996). NMDA receptor-induced cell death has been linked to this process by studies showing that both pharmacologic inhibition of nNOS as well as nNOS knockout mice are protected from NMDA receptor-induced cell death (Ayata et al., 1997; Eliasson et al., 1999; Huang, 1999; Sattler et al., 1999). Additionally, it has been shown that antisense knockdown of PSD-95 decreases NMDA receptor-induced cell death by a pathway that seems to involve nNOS (Sattler et al., 1999). We observed a significant and progressive decrease in nNOS in symptomatic and hypokinetic N171-82Q mice. Another PSD-95 associated protein, citron, was observed to progressively decrease in 15-week symptomatic and hypokinetic mice. Although this Rho effector protein has only recently been characterized in neurons, it is hypothesized to be involved in dendritic spine formation (Furuyashiki et al., 1999). The decreases we observed could be responsible for previous reports of decreases in dendritic spines in Huntington’s disease (Guidetti et al., 2001), which could also be an additional neuroprotective mechanism through decreasing synaptic input. Surprisingly, no changes were observed in Src kinase or Fyn kinase, which may indicate that the majority of these proteins are localized to the membrane by proteins other than PSD-95 and SAP-102.

Those protective changes reported in this paper which have been studied in other mice expressing the mutant Htt transgene or in Huntington’s disease patients seem to be conserved. The R6/1 and R6/2 transgenic Htt mice report a similar decrease in nNOS in symptomatic and late stage mice, although the progression was not exactly the same in the three models (Deckel et al., 2002; Perez-Severiano et al., 2002). The differences in progression may indicate a difference in the regulation of protective pathways in various transgenic models throughout the disease. These differences highlight the importance of characterizing multiple transgenic models and identifying the protective mechanisms being regulated in human Huntington’s patients. The D1 receptor protein has been studied in human patients through PET studies by Turjanski et al. (1995), who observed a decrease in D1 receptor similar to the changes reported in this study for the N171-82Q mice. Additionally, a recent paper by Sun et al. (2001) showed that co-immunoprecipitation of PSD-95 with Htt itself was decreased in Huntington’s disease patients. One possible cause for this observation could be that the substantial decrease in PSD-95 observed in our study is also present in human patients, resulting in a decrease in the amount of PSD-95 associated with Htt.

Hansson et al. (2001b) showed protection from QA in the R6/1 and R6/2 mouse models of Huntington’s disease. They also found a five-fold increase in basal calcium levels with an increased capacity to handle cytoplasmic calcium overload following QA injection. They suggest that the increased basal calcium levels might cause an adaptation resulting in an up-regulation of defence mechanisms to excitotoxic stress. The findings in this paper give support to their hypothesis with additional cellular adaptations observed. The decreases in PSD-95, SAP-102, nNOS, citron and PI-3 kinase observed in this paper would decrease the effects of intracellular calcium and the decrease in dopamine receptors, NR1 phosphorylation and eventually NR1 could also be an adaptive response to decrease the calcium coming into the cell. The fact that Hansson et al. (2001b) did not observe a change in QA-induced current and calcium influx in striatal R6/2 neurons may indicate the adaptive changes in NR1 phosphorylation observed in N171-82Q mice do not affect the calcium influx in the hyperactivated state of QA injections. However, the changes in phosphorylation may still affect the calcium influx in the physiologic setting and therefore be a long-term compensatory mechanism for increases in basal calcium levels. The cause of these changes remains an interesting question. They may be related to ischaemic preconditioning in which small, non-toxic, doses of NMDA agonists or brief ischaemic insults, but not α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) agonists, provide neuroprotection from a later severe ischaemic insult (Bond et al., 1999; Pringle et al., 1999). The protective changes observed in these mouse models of Huntington’s disease may be the result of a preconditioning similar to the protection observed in an ischaemic model.

The lack of additional studies of these proteins in human Huntington’s disease patients is probably due to the difficulty in obtaining human brains before the massive neuronal degeneration makes protein analysis less revealing. Confirming these protective changes or the lack thereof in human patients may allow for therapies to limit changes that are doing more harm than good or to activate protective pathways in patients where they have not been activated.

Neuronal death secondary to loss of function
In transgenic mouse models, unlike Huntington’s patients, only a small percentage of neurons are destroyed before the mice succumb to the lethality of the disease. It may be that the neuronal death seen in patients with Huntington’s disease is secondary to a loss in GABAergic neuronal function which is
sufficient to cause symptoms and lethality in the mice. The hypothesis that striatal neurodegeneration occurs secondary to functional loss is supported by the reversal of behavioural symptoms in a conditional transgenic Htt mouse model (Yamamoto et al., 2000). The underlying cause of this functional loss has not yet been established. The changes in the neuroprotective pathways reported in this paper for NMDA receptor and D1 receptor, which receive the two main inputs into the striatum, could be causing a functional loss in coordinated neurotransmission.

Summary
This paper shows that the N171-82Q mouse model of Huntington’s disease is protected from NMDA receptor-induced excitotoxicity. We also demonstrate that many of the known effector pathways involved in NMDA receptor-induced excitotoxicity are being regulated in the direction of protection in this model. A combination of some or all of these changes may be responsible, at least in part, for protecting the striatal neurons in the N171-82Q mice. Describing these changes furthers our understanding of the molecular events that may occur during Huntington’s disease and should provide targets for additional research into the time course of protein changes and neuroprotection in various models of Huntington’s disease. This research could provide new targets for treatment through enhancing protective mechanisms and/or correcting functional deficits caused by these mechanisms.

Acknowledgements
This research was supported by the National Institutes of Health R01-NS36246 and F31-NS42399.

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