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Pathological Cell-Cell Interactions Elicited by a Neuropathogenic Form of Mutant Huntingtin Contribute to Cortical Pathogenesis in HD Mice

Xiaofeng Gu,[1] Chenjian Li,[2] Weizheng Wei,[3] Victor Lo,[1] Shiaoching Gong,[4] Shi-Hua Li,[5] Takuji Iwasato,[6,7] Shigeyoshi Itohara,[6] Xiao-Jiang Li,[5] Istvan Mody,[3] Nathaniel Heintz,[4] and X. William Yang[1,*]

[1]Center for Neurobehavioral Genetics
Neuropsychiatric Institute
Department of Psychiatry
and Biobehavioral Sciences
Brain Research Institute
David Geffen School of Medicine at UCLA
Los Angeles, California 90095
[2]Department of Neurology and Neuroscience
Weill Medical College of Cornell University
New York, New York 10021
[3]Department of Neurology
David Geffen School of Medicine at UCLA
Los Angeles, California 90095
[4]Laboratory of Molecular Biology
Howard Hughes Medical Institute
Rockefeller University
New York, New York 10021
[5]Department of Human Genetics
Emory University School of Medicine
Atlanta, Georgia 30322
[6]RIKEN Brain Science Institute
Saitama 351-0198
Japan
[7]PRESTO, Japan Science and Technology Agency
Saitama 332-0012
Japan

Summary

Expanded polyglutamine (polyQ) proteins in Huntington's disease (HD) as well as other polyQ disorders are known to elicit a variety of intracellular toxicities, but it remains unclear whether polyQ proteins can elicit pathological cell-cell interactions which are critical to disease pathogenesis. To test this possibility, we have created conditional HD mice expressing a neuropathogenic form of mutant huntingtin (mhtt-exon1) in discrete neuronal populations. We show that mhtt aggregation is a cell-autonomous process. However, progressive motor deficits and cortical neuropathology are only observed when mhtt expression is in multiple neuronal types, including cortical interneurons, but not when mhtt expression is restricted to cortical pyramidal neurons. We further demonstrate an early deficit in cortical inhibition, suggesting that pathological interactions between interneurons and pyramidal neurons may contribute to the cortical manifestation of HD. Our study provides genetic evidence that pathological cell-cell interactions elicited by neuropathogenic forms of mhtt can critically contribute to cortical pathogenesis in a HD mouse model.

*Correspondence: xwyang@mednet.ucla.edu

Introduction

Huntington's disease (HD) is an adult-onset, autosomal-dominant neurodegenerative disease that is clinically characterized by a triad of movement disorders (i.e., chorea and bradykinesia), psychiatric symptoms, and cognitive deficits. In afflicted patients, symptoms usually progress relentlessly until death in 15–20 years after disease onset. HD is one of nine neurodegenerative disorders caused by a CAG repeat expansion encoding a polyglutamine (polyQ) repeat in otherwise unrelated proteins (Zoghbi and Orr, 2000). In HD, the mutated Huntingtin (mhtt) protein is ubiquitously expressed in both neuronal and nonneuronal tissues (Sharp et al., 1995; Schilling et al., 1995). The polyQ repeat, located in the N terminus of *huntingtin* (*htt*), is normally less than 36, but is expanded to more than 37 in HD patients (The Huntington's Disease Collaborative Research Group, 1993). In all polyQ disorders, there is an inverse relationship between the length of polyQ and the age of disease onset. Currently, there is no effective treatment or cure for HD or any other polyQ disorder.

All polyQ disorders share a common neuropathological feature: despite the widespread expression of mutant polyQ proteins, significant neurodegeneration occurs in distinct subsets of neurons in the brain. In HD, neuordegeneration is mostly confined to striatal medium spiny neurons and, to a lesser extent, to cortical pyramidal neurons (Vonsattel and DiFiglia, 1998). These two types of neurons constitute the vast majority (about 80%–90%) of the total neurons in the respective brain regions. With longer expansion of the polyQ repeats, cell death pathology in HD as well as other polyQ disorders becomes increasingly less distinct and more overlapping (Zoghbi and Orr, 2000). The cellular and molecular mechanisms underlying the selective neuronal toxicity in HD as well as in other polyQ disorders remain largely unknown.

A number of pathogenic mechanisms have been implicated in HD. First, mhtt has been shown to exert dominant toxicity to cause disease-like phenotypes in a variety of cellular and animal models. In transgenic mouse models of HD, overexpression of mhtt resulted in late-onset behavioral and neuropathological phenotypes mimicking the disease, but deletion of the htt gene in mice resulted in an embryonic lethal phenotype and not HD-like neurodegenerative phenotypes (Duyao et al., 1995). A variety of toxic gain-of-function mechanisms have been implicated in HD pathogenesis. A common mechanism shared by HD as well as other polyQ disorders is the progressive accumulation of protein aggregates containing the mutant polyQ proteins in the affected neurons (Davies et al., 1997; DiFiglia et al., 1997). However, the pathogenic role of the polyQ protein aggregates remains unresolved (Bates, 2003; Klement et al., 1998; Arrasate et al., 2004). Other pathogenic mechanisms implicated in HD include transcriptional dysregulation, mitochondrial deficits, proteosomal dysfunction, abnormal endocytosis, axonal transport deficits, and synaptic dysfunction (Zoghbi and Orr,

2000; Dunah et al., 2002; Li et al., 2000; Steffan et al., 2004; Panov et al., 2002; Bence et al., 2001; Gunawardena et al., 2003; Zeron et al., 2002). In addition to the gain-of-function pathogenic mechanisms, recent studies using cellular models of HD also suggested that loss of normal htt function, including regulation of transcription and vesicular transport of BDNF, may also contribute to HD pathogenesis (Zuccato et al., 2001; Gauthier et al., 2004). Together, current evidence suggests that a combination of gain-of-function and loss-of-function mechanisms may be involved in pathogenesis of HD (Ross, 2004).

In HD, there is increasing evidence to support the idea that proteolysis of mhtt into toxic N-terminal fragments containing the polyQ repeat is a critical early pathogenic event. In the brains of HD patients, the full-length mhtt was shown to be proteolytically cleaved into small N-terminal fragments (110–600 amino acids) by a variety of proteases, including caspases (Martindale et al., 1998; Wellington et al., 2002), calpains (Kim et al., 2001; Gafni et al., 2004), and a yet unidentified aspartyl endopeptidase (Lunkes et al., 2002). In both HD patients and transgenic mice expressing the full-length mhtt, accumulation of small aggregated mhtt N-terminal fragments in the nucleus and cytoplasm was evident prior to the onset of motor deficits and neurodegeneration (DiFiglia et al., 1997; Gutekunst et al., 1999; Hodgson et al., 1999; Wellington et al., 2002; Zhou et al., 2003). Furthermore, in cellular models of HD, small mhtt N-terminal fragments are clearly more toxic to neurons than the full-length mhtt, suggesting that small mhtt fragments are likely to be a critical neuropathogenic entity in HD (Hackam et al., 1998). Finally, mhtt N-terminal fragments (including mhtt-exon1) can elicit a variety of HD-like phenotypes ranging from aggregation and cellular toxicity in yeast (Muchowski et al., 2000) and cultured cell models (Hackam et al., 1998; Arrasate et al., 2004) to progressive motor deficits and neuropathology in transgenic *Drosophila* (Jackson et al., 1998) and transgenic mice (Mangiarini et al., 1996; Schilling et al., 1999; Yamamoto et al., 2000; Laforet et al., 2001). Transgenic mice expressing mhtt N-terminal fragments exhibit a variety of HD-like phenotypes including progressive motor deficits, striatal and cortical atrophy, reactive gliosis, mhtt aggregation, and alteration of gene expression (ibid; Menalled and Chesselet, 2002; Yu et al., 2003; Luthi-Carter et al., 2000). Together, existing evidence supports the "toxic fragment hypothesis," which states that polyQ-containing mhtt N-terminal fragments generated by proteolysis of the full-length mhtt are the critical pathogenic entity that mediates neuronal dysfunction and degeneration in HD (Leavitt et al., 1999; Ross, 2002). Therefore, cellular and transgenic animal models expressing neuropathogenic mhtt N-terminal fragments are widely used to study HD pathogenic mechanisms downstream of the proteolysis of the full-length mhtt into the toxic N-terminal fragments.

Despite significant progress in HD research, many critical issues remain to be addressed (Tobin and Signer, 2000; Ross, 2002; Ross, 2004). One such issue is whether neuronal dysfunction and degeneration in HD are solely caused by cell-autonomous toxicities of mhtt (cell-autonomy model) or require pathological interactions between cells (cell-cell interaction model)? As summarized above, much has been learned about cell-autonomous toxicities of mhtt. However, very little is known about the possible role of mhtt in pathological cell-cell interactions and their relative contribution to the overall pathogenesis of HD. A few recent studies in HD mice have suggested that pathological cell-cell interactions may be present in vivo. Such interactions include blocking transcription and/or vesicular transport of BDNF (Zuccato et al., 2001; Gauthier et al., 2004), generating abnormal corticostriatal neurotransmission (Cepeda et al., 2003), and alterations in other nonneuronal cell types, including astrocytes (Lievens et al., 2001) and microglia (Sapp et al., 2001). Yet, there is no direct evidence to support that pathological cell-cell interactions are critical to HD pathogenesis in vivo in a mouse model. In this study, we asked the following questions: Can a neuropathogenic form of mhtt (mhtt-exon1) elicit pathological cell-cell interactions in vivo? Are such interactions critical to cortical pathogenesis in HD mice?

Results

Creating a Cre/LoxP Conditional Mouse Model of HD

Our strategy to test the cell-autonomy model versus the cell-cell interaction model was to develop conditional HD mice that express a toxic mhtt fragment in discrete neuronal populations: either in all neurons in the brain or restricted to cortical pyramidal neurons, one of the vulnerable neuronal types in HD. The cell-autonomy model predicts that restricting mhtt expression to the cortical pyramidal neurons alone should be sufficient to cause significant pathology in these neurons. The cell-cell interaction model predicts that restricting mhtt expression to cortical pyramidal neurons alone will be insufficient to cause significant pathology in these neurons. Only mhtt expression in multiple neuronal types should cause significant pathology in cortical pyramidal neurons secondary to pathological cell-cell interactions with other types of neurons. To test these models in vivo, we created conditional HD mice in which expression of mhtt-exon1 with a 103 glutamine repeat was completely dependent on Cre-mediated excision of a transcription termination (STOP) sequence flanked by two Cre binding sites (LoxP) (Nagy, 2000; Soriano, 1999; Srinivas et al., 2001) (Figure 1A). In this mouse model (*RosaHD*), *mhtt-exon1* is targeted to the murine *Rosa26* locus, hence its expression is driven by the endogenous murine *Rosa26* promoter, which directs ubiquitous transgene expression (Zambrowicz et al., 1997). *RosaHD* mice were crossed with two Cre transgenic mice: *Nestin-Cre*, which activates LoxP recombination in all neurons and glia in the brain (Tronche et al., 1999), and *Emx1-Cre*, which activates LoxP recombination in all cortical pyramidal neurons and glia, but not in cortical interneurons or striatal neurons (Iwasato et al., 2000; Gorski et al., 2002). Therefore, a critical difference between these two Cre mouse lines in the cortex is that *Nestin-Cre* is active in all pyramidal neurons (about 80% of the cortical neurons) and inhibitory interneurons (about 20% of the cortical neurons; Somogyi et al., 1998); while *Emx1-Cre* is only active in

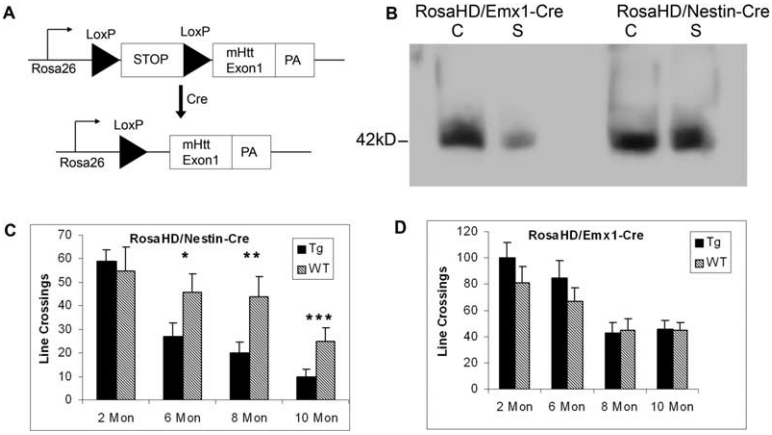

Figure 1. Generation of the Cre/LoxP Conditional Mouse Model of HD

(A) The schematics of conditional HD mice in which expression of mhtt-exon1 was completely dependent on expression of Cre recombinase. Mutant htt-exon1 was targeted to the *Rosa26* locus in ES cells. The targeted locus contains the endogenous Rosa26 promoter, a transcriptional STOP sequence, two loxP sites (black triangles), mhtt-exon1 with a polyQ repeat containing 103 glutamine, and a polyadenylation signal (PA).

(B) Western blot analyses of mhtt-exon1 expression in the conditional HD mice with the 1C2 antibody. Lane 1, *RosaHD/Emx1-Cre* cortex; lane 2, striatum; lane 3, *RosaHD/Nestin-Cre* cortex; lane 4, striatum.

(C and D) Open field tests for the conditional HD mice. *RosaHD/Nestin-Cre* and *RosaHD/Emx1-Cre* transgenic mice and their respective wild-type littermates were tested in an open field test at 2, 6, 8, and 10 months of age. Horizontal locomotor activity of a mouse over a 10 min period was quantified by counting the number of lines crossed by all four limbs. A Student's t test with a critical value of $p < 0.05$ was used for all statistical analyses. Values are mean ± SEM, *p = 0.05; **p = 0.035; ***p = 0.02.

cortical pyramidal neurons and not in interneurons. Because the *Rosa26* promoter expresses predominantly in neurons and expresses very little in glia (Anthony et al., 2004), *RosaHD/Nestin-Cre* mice expressed mhtt-exon1 in all neurons of the brain, and *RosaHD/Emx1-Cre* mice expressed mhtt-exon1 only in cortical pyramidal neurons. The cellular distribution of mhtt in these two mouse models was further confirmed by immunostaining of brain sections using a mhtt-specific antibody (see below).

To determine mhtt-exon1 expression levels in these two models, we performed Western blot analyses using the 1C2 antibody against the expanded polyQ stretch (Trottier et al., 1995) (Figure 1B). We detected the 42 kDa mhtt-exon1 bands with similar intensities in the cortices of both models, but significant amounts of mhtt-exon1 were detected only in the striata of *RosaHD/Nestin-Cre* mice. These results demonstrate that *RosaHD* mice can conditionally express mhtt-exon1 in a Cre-dependent manner.

Motor Phenotype

We next examined the mice for motor deficits using the open field test, which quantifies their locomotor activity (Menalled et al., 2002). Progressive deterioration in locomotor activity has been observed in other mouse models of HD (Menalled and Chesselet, 2002). *RosaHD/Nestin-Cre* mice displayed normal horizontal locomotor activity at 2 months, but had significantly reduced locomotor activity at 6, 8, and 10 months of age (Figure 1C). No locomotor deficits were observed in *RosaHD/Emx1-Cre* mice at any age tested (Figure 1D). Thus, in the conditional HD mice, progressive locomotor deficits were present only when mhtt was widely expressed in the brain, and not when mhtt expression was restricted to the cortical pyramidal neurons alone.

Cell-Autonomous Nuclear Accumulation and Aggregation of mhtt

Because the nuclear accumulation and aggregation of neuropathogenic mhtt N-terminal fragments is a pathological hallmark of HD patients (DiFiglia et al., 1997; Gutekunst et al., 1999) and this phenotype has also been recapitulated in existing HD mice (Davies et al., 1997; Hodgson et al., 1999; Menalled et al., 2002; Schilling et al., 1999; Laforet et al., 2001), we immunostained the conditional HD mouse brains with a rabbit polyclonal EM48 antibody which preferentially recognizes the aggregated form of mhtt (Gutekunst et al., 1999). In 6-month-old *RosaHD-Emx1-Cre* brains but not in wild-type littermate control brains, EM48(+) staining was detected throughout the cortex and striatum, as well as in several other brain regions, including the cerebellum, midbrain, and brain stem (Figures 2A–2C and Figure S1 in the Supplemental Data available with this article online). In *RosaHD/Emx1-Cre* mice, however, EM48(+) staining was highly restricted to the cortex, with only a few stained cells in the striatum and cerebellum and only background staining in other brain regions, including midbrain and brainstem (Figures 2D–2F; Figure S1). These results confirm that the expression of mhtt-exon1 is throughout the brain in *RosaHD/Nestin-Cre* mice and is highly restricted to the cortex in *RosaHD/Emx1-Cre* mice.

At the cellular level, the EM48 staining patterns in the cortex are similar between the two conditional HD mouse models. In cortical layers II and III, the EM48 staining pattern is predominantly diffuse nuclear staining plus small nuclear and cytoplasmic aggregates (Figures 2A and 2D). In the large cortical layer V pyramidal neurons, EM48 predominantly stains small nuclear and neuropil aggregates (Figures 2B and 2E). In *RosaHD/Nestin-Cre* striata, the EM48 staining pattern is predominantly diffuse nuclear staining with small nuclear aggregates (Figure 2C). Notably, very few neurons in *RosaHD/Emx1-Cre* striata are stained with the EM48 antibody (Figure 2F).

To further ascertain the mhtt expression patterns in cortical neuronal subtypes in these mice, we performed double immunofluorescent staining. To determine whether mhtt accumulation occurs in cortical pyramidal neurons, we double stained the brain sections using rabbit polyclonal EM48 antibodies and mouse monoclonal antibodies against calmodulin-dependent kinase II (Cam-

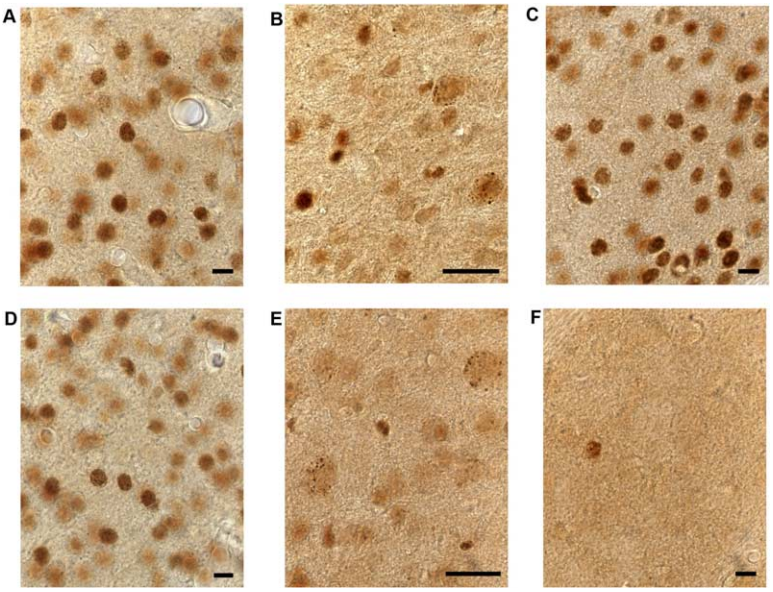

Figure 2. Cell-Autonomous Nuclear Accumulation and Aggregation of mhtt in the Cortices and Striata of the Conditional HD Mice

(A–C) EM48 antibody staining patterns in the *RosaHD/Nestin-Cre* mice. (A) Cortical layers II and III; (B) cortical layer V; (C) striatum. (D–F) EM48 staining patterns in *RosaHD/Emx1-Cre* mice. (D) Cortical layers II and III; (E) cortical layer V, (F) striatum. Scale bars, 10 μm.

KII), a marker for cortical pyramidal neurons (Benson et al., 1992). As shown in Figure 3A, in the *RosaHD/Nestin-Cre* mouse cortices, the vast majority of cells stained with EM48 are also stained with CamKII antibodies (183 CamKII-positive cells out of 214 EM48-positive cells, or 86%); in *RosaHD/Emx1-Cre* mice, nearly all the cells stained with EM48 are also stained with CamKII(+) (172 CamKII-positive cells out of 179 EM48-positive cells, or 96%). Therefore, in both conditional HD mouse models, the vast majority of neurons with nuclear accumulation of mhtt in the cortex are cortical pyramidal neurons.

To determine the extent of mhtt accumulation in cortical interneurons in these mice, we performed double immunofluorescent staining using the rabbit polyclonal EM48 antibody and a mouse monoclonal antibody against GABA, the neurotransmitter used by cortical interneurons but not by cortical pyramidal neurons. As shown in Figure 3B, in *RosaHD/Nestin-Cre* cortices, the majority of GABA(+) cortical interneurons have diffuse nuclear EM48 staining (31 EM48-positive cells out of 42 GABA-positive cells, or 74%). The intensity of EM48 staining in the GABA(+) interneurons in these mice appears to be slightly lower compared to the adjacent GABA(–) cortical pyramidal neurons, which constitute the majority of the EM48(+) cells in the cortex. In *RosaHD/Emx1-Cre* cortices, GABA(+) cortical interneurons only have faint cytoplasmic EM48 staining comparable to the background staining pattern in the wild-type mice (Figure 3B), thus, none of the GABA(+) interneurons in *RosHD/Emx1-Cre* mice exhibit significant EM48 staining (0 EM48-positive cells out of 75 GABA-positive cells). We conclude that *RosaHD/Nestin-Cre* mice but not *RosaHD/Emx1-Cre* mice accumulate aggregated forms of mhtt in the nucleus of cortical interneurons.

Since both the brain regional distribution and the cellular distribution of mhtt in the two conditional HD models match the expression patterns of the respective Cre mouse lines, these results validate the accuracy of mhtt expression patterns in both models. Furthermore, since EM48 preferentially detects the aggregated form of mhtt (Gutekunst et al., 1999), these results also demonstrate that nuclear accumulation and aggregation of mhtt are cell-autonomous processes. Since both models had very little EM48(+) staining in the brains at 2 months of age (data not shown) but had extensive staining at 6 months of age, these results are also consistent with the progressive nature of mhtt aggregation in the conditional HD mice.

Neuropathology in the Conditional HD Mice

Since cortical pyramidal neurons in both models have comparable levels of mhtt expression (both driven by the endogenous murine *Rosa26* promoter) and aggregation, a direct comparison of neuropathology in the cortices of these two models will allow us to assess whether cell-cell interactions are critical to cortical pathogenesis in the conditional HD mice. If cell-autonomous toxicities of mhtt are the only critical pathogenic mechanism in these mice, then the cortical pyramidal neuron pathology should be comparable between the two HD models. If cell-cell interactions are critical to cortical pathogenesis, then significant cortical neuropathology should only be observed in *RosaHD/Nestin-Cre* mice but not in *RosaHD/Emx1-Cre* mice. To distinguish between these two models, we examined the conditional HD mice for several cortical pathological phenotypes previously reported in existing HD mouse models.

We first examined reactive gliosis, a pathological reaction of glia secondary to neuronal dysfunction or degeneration (Ridet et al., 1997) reported in both HD patients (Vonsattel and DiFiglia, 1998) and HD mice (Yu et al., 2003). We stained 6-month-old mouse brains with anti-GFAP antibodies to label the reactive glia (Ridet et al., 1997). Surprisingly, the extent of reactive gliosis was dramatically different between the two models. In *RosaHD/Nestin-Cre* mice, the density of GFAP(+) cells

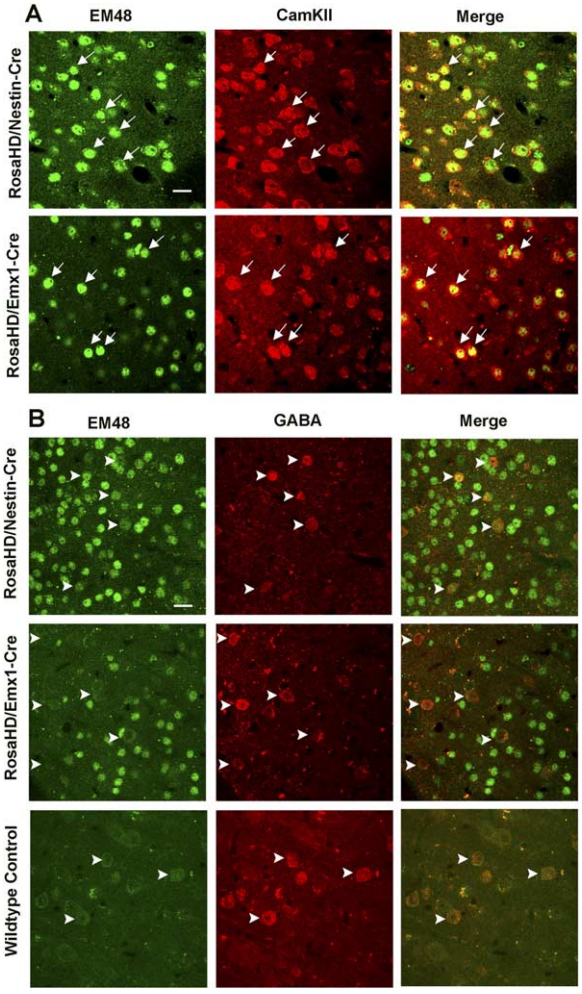

Figure 3. Patterns of Mutant htt Accumulation in the Cortical Pyramidal Neurons and Interneurons in the Conditional HD Mice

(A) Mutant htt accumulation in cortical pyramidal neurons in the conditional HD mice. In 6-month-old brains of both HD models, double immunofluorescent staining shows that the majority of cells with nuclear EM48 staining (green) are also stained with CamKII antibodies (red), a marker for cortical pyramidal neurons. Colocalization of the two staining patterns is shown as yellow (white arrows). (B) Mutant htt accumulation in the cortical interneurons in the conditional HD mice. Double immunofluorescent staining was performed using the EM48 antibody and a murine monoclonal antibody against GABA, a neurotransmitter used by the interneurons. In *RosaHD/Nestin-Cre* cortices, the majority of GABA(+) cortical interneurons (red) have diffuse nuclear EM48 staining (green). In *RosaHD/Emx1-Cre* cortices, GABA(+) cortical interneurons only have faint cytoplasmic EM48 staining comparable to the background staining pattern in the wild-type mice. White arrowheads indicate GABA(+) interneurons in each panel. Scale bars, 20 μm.

in the cortex was approximately 9-fold greater than in wild-type littermate controls ($p = 0.002$, Student's t test; Figures 4A, 4C, and 4G) and 6-fold greater in the striatum ($p = 0.03$, Student's t test; Figures 4D, 4F, and 4H). In *RosaHD/Emx1-Cre* mice, despite mhtt expression and aggregation in about 80%–90% of the cortical neurons, the density of GFAP (+) cells in the cortex and striatum was not significantly different from their wild-type littermate controls (Figures 4B, 4E, 4G and 4H). Since the Rosa26 promoter drives gene expression mostly in the neurons and very little in the glia (Anthony et al., 2004), we interpret the robust reactive gliosis observed in the *RosaHD/Nestin-Cre* mice as a secondary effect due to neuronal dysfunction, rather than due to a primary deficit in the glia. This result suggests that dysfunction of non-pyramidal neurons in the cortex (primarily cortical interneurons) or sub-cortical neurons is required to elicit cortical reactive gliosis in the conditional HD mice.

Since cortical pyramidal neurons are vulnerable to neuronal dysfunction and degeneration in HD (Vonsattel and DiFiglia, 1998) and these neurons express comparable levels of mhtt in our two HD mouse models, we next examined the cellular morphology of these neurons. In an existing HD mouse model, cortical pyramidal neurons were shown to exhibit abnormal wavy apical dendrites called dysmorphic neurites (Laforet et al., 2001). Using Golgi staining, we examined 6-month-old transgenic mice and wild-type littermates for this phenotype ($n = 2$ for each genotype; Figures 5A and 5C). Among the fully impregnated cortical pyramidal neurons, we found that 35 out of 37 neurons in *RosaHD/Nestin-Cre* mice exhibited dysmorphic neurites. This was a significantly higher proportion than in controls (5 out of 40, $p < 0.001$, χ^2 test). We were also able to visualize dysmorphic neurites in *RosaHD/Nestin-Cre* mice by biocytin injections into cortical pyramidal neurons followed by immunostaining (Figure 5D). In *RosaHD/Emx1-Cre* mice, only 8 out of 45 cortical pyramidal neurons displayed kinky apical dendrites (Figure 5B), which was not significantly different from their wild-type littermate controls (4 out of 40 neurons; $p > 0.05$, χ^2 test). These results demonstrate that restricted expression of mhtt to cortical pyramidal neurons is insufficient for pathogenesis of dysmorphic neurites in these neurons, and expression of mhtt in other neuronal types in the brain is required.

We subsequently examined the HD mice for neurodegeneration. Existing HD mouse models expressing mhtt N-terminal fragments exhibit moderate neurodegeneration; these degenerating neurons stain darkly with osmium under electron microscopy (EM) and are thus called dark neurons (Turmaine et al., 2000; Yu et al., 2003). We employed EM to examine brains of 1-year-old HD mice for the presence of dark neurons. In the *RosaHD/Nestin-Cre* cortices (Figures 5E and 5F) and striata (Figure 5G) but not in wild-type controls (data not shown), we detected dark neurons with characteristic ultrastructural features, including enhanced osmium staining, nuclear and cytoplasmic condensation, irregular nuclear border, and a lack of clear morphology of subcellular organelles. The dark neurons in cortical layer V have the characteristic morphology of pyramidal neurons (Figure 5F). Reactive glia with enhanced electron density at the nuclear border is often found adjacent to the dark neurons (Figures 5F and 5G). We did not observe any dark neurons in the 1-year-old *RosaHD/Emx1-Cre* brains. We only observed a few cortical pyramidal neurons with dark lysosome-like cytoplasmic vacuoles suggesting early degenerative changes (Figure 5H; Yu et al., 2003). Striatal neurons from *RosaHD/Emx1-Cre* mice displayed normal ultrastruc-

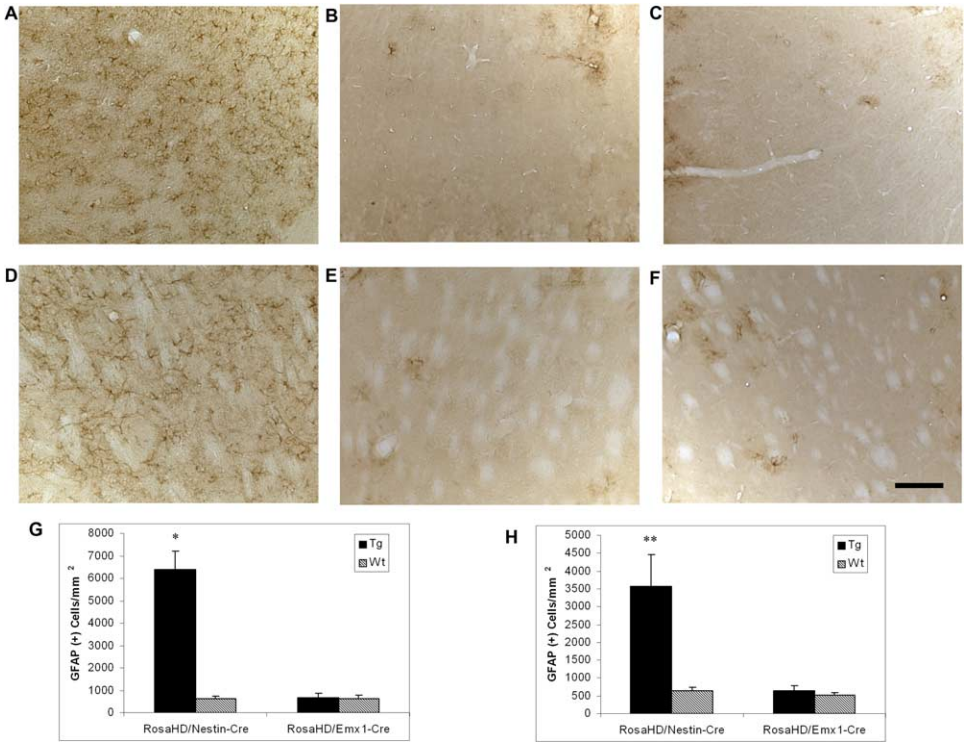

Figure 4. Reactive Gliosis in the Cortex and Striatum of 6-Month-Old *RosaHD/Nestin-Cre* Mice but Not *RosaHD/Emx1-Cre* Mice

Six-month-old HD mouse brains were stained with anti-GFAP antibodies to label the reactive glia. Robust reactive gliosis, as indicated by increase in GFAP staining, is detected only in the cortices and striata of the *RosaHD/Nestin-Cre* mice and not in the *RosaHD/Emx1-Cre* mice. (A) *RosaHD/Nestin-Cre* cortex; (B) *RosaHD/Emx1-Cre* cortex; (C) wild-type cortex; (D) *RosaHD/Nestin-Cre* striatum; (E) *RosaHD/Emx1-Cre* striatum; (F) wild-type striatum. (G and H) Quantitation of GFAP(+) reative glia density in the HD mice is shown for the cortex (G) and striatum (H). Student's t test is used for statistics; values are mean ± SEM, *p = 0.002, **p = 0.03. Scale bar, 100 μm.

tural features (Figure 5I). These results demonstrate that pathogenesis of dark neuron degeneration in the cortical pyramidal neurons also requires mhtt expression in other types of neurons in the brain, and cell-autonomous toxicities of mhtt can only elicit very mild degenerative changes (i.e., degenerating vacuoles) in cortical pyramidal neurons.

In summary, our neuropathological studies show that restricting mhtt expression to cortical pyramidal neurons produced very limited pathology in these neurons, hence our results do not support the cell-autonomy model. Furthermore, since mhtt expression in multiple neuronal types in *RosaHD/Nestin-Cre* mice reproduced several characteristic cortical phenotypes seen in other HD mice and HD patients, these results support the cell-cell interaction model for cortical pathogenesis in the conditional HD mice.

Presynaptic Changes in GABAergic Neurotransmission Suggesting Cortical Interneuron Dysfunction in the Conditional HD Mice

Our findings suggest that neuronal dysfunction in cortical interneurons or subcortical neurons are critical to the pathogenesis of the cortical pyramidal neurons. To gain further insights into which neuronal types may be dysfunctional early in the *RosaHD/Nestin-Cre* mice, we employed whole-cell patch-clamp recordings in brain slices to examine both excitatory and inhibitory synaptic inputs to cortical pyramidal neurons in 6-month-old HD mice. This is the earliest time point at which we observed motor deficits and neuropathology in these mice. We first examined excitatory input by recording spontaneous excitatory postsynaptic currents (sEPSCs), which result from spontaneous glutamate release by other cortical and thalamocortical excitatory neurons. We observed no significant differences in the properties of sEPSCs between the *RosaHD/Nestin-Cre* mice and controls (Figure 6A; Table 1). We then recorded spontaneous inhibitory postsynaptic currents (sIPSCs), which originate from the spontaneous, GABAergic input of cortical interneurons onto the pyramidal neurons. In *RosaHD/Nestin-Cre* mice, we found that the average peak amplitude of sIPSCs was similar to controls, but that sIPSC frequency was significantly reduced compared to the controls (15.1 ± 1.9 versus 20.9 ±1.4 Hz, p = 0.017, Student's t test) (Figures 6B and 6C; Table 1). Since sIPSC frequency is a function of presynaptic GABA release secondary to action potential firing by cortical interneurons, this result suggests that cortical interneurons become dysfunctional early in the disease process in *RosaHD/Nestin-Cre* mice. In *RosaHD/Emx1-Cre* mice, we did not observe any significant differences in the properties of sIPSCs, including frequency and amplitude, from those recorded in wild-type control

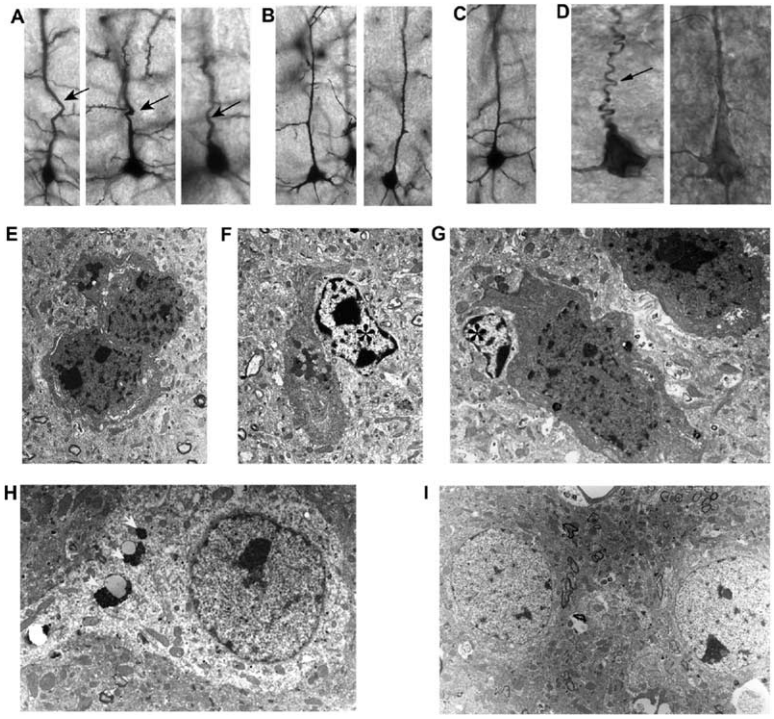

Figure 5. Pathogenesis of Dysmorphic Neurites and Dark Neuron Degeneration in the Conditional HD Mice

Golgi staining was used to reveal the morphology of the apical dendrites in the cortical pyramidal neurons. (A) In *RosaHD/Nestin-Cre* mice, numerous cortical pyramidal neurons exhibit dysmorphic neurites with torturous and wavy apical dendrites. (B) In *RosaHD/Emx1-Cre* mice, the majority of the pyramidal neurons exhibit normal apical dendrite morphology. (C) In wild-type mice, cortical pyramidal neurons also exhibit normal apical dendrite morphology. (D) Biocytin injection followed by immunostaining revealed dysmophic neurites in cortical pyramidal neurons of 6-month-old *RosaHD/Nestin-Cre* transgenic brains (left panel) but not in those of the wild-type littermate controls (right panel). (E–I) Electron microscopy (EM) studies of brains from 1-year-old conditional HD mice and wild-type control mice. (E–G) Degenerating dark neurons in the *RosaHD/Nestin-Cre* brains. (E) Two dark neurons in cortical layer II and III; (F) one dark pyramidal neuron in cortical layer V; an adjacent reactive glia with enhanced nuclear border staining (asterisk); (G) two striatal dark neurons with an adjacent reactive glia (asterisk). (H and I) In *RosaHD/Emx1-Cre* mice, we did not observe any dark neurons at 1 year of age. We only observed a few cortical pyramidal neurons with dark lysosome-like cytoplasmic vacuoles (white arrows) suggesting early degenerative changes (H), and striatal neurons from *RosaHD/Emx1-Cre* mice displayed normal ultrastructural features (I).

mice (Figures 6B and 6C; Table 1). Since the presence of sIPSC abnormalities is correlated with the expression of mhtt in cortical interneurons in these mice (Figure 3B), this result suggests that mhtt expression in cortical interneurons may lead to reduced GABAergic input to the cortical pyramidal neurons in the *RosaHD/Nestin-Cre* mice. Together, our electrophysiological studies show that the reduction of GABAergic inhibitory input onto cortical pyramidal cells is an early phenotype in *RosaHD/Nestin-Cre* mice but not in *RosaHD/Emx1-Cre* mice (Figure 6D). Thus, pathological interactions between cortical interneurons and pyramidal neurons may be critical for cortical pathogenesis in HD.

Discussion

In this study, we have created Cre/LoxP conditional mouse models to test whether a neuropathogenic form of mhtt (mhtt-exon1) can elicit significant pathological cell-cell interactions that critically contribute to cortical pathogenesis in vivo. We found that restricting mhtt expression to cortical pyramidal neurons is sufficient to produce mhtt nuclear accumulation and aggregation in these neurons, but is insufficient to produce other cortical neuropathology or motor deficits. However, expression of mhtt in all the neurons in the brain elicits both progressive motor deficits and certain characteristics of HD cortical pathology including mhtt nuclear accumulation and aggregation, reactive gliosis, dysmorphic neurites, and dark neuron degeneration (Vonsattel and DiFiglia, 1998; Menalled and Chesselet, 2002; Yu et al., 2003). These results provide genetic evidence that expression of neuropathogenic forms of mhtt fragments outside the cortical pyramidal neurons can critically contribute to the pathogenesis of these neurons; therefore, we conclude that pathological cell-cell interac-

Table 1. Properties of sIPSCs and sEPSCs in Cortical Pyramidal Neurons of *RosaHD/Nestin-Cre* Mice and *RosaHD/Emx1-Cre* Mice

	Amplitude (pA)	Frequency (Hz)	$RT_{10\%-90\%}$ (μs)	τ_w (ms)	Number of Cells
RN, sIPSCs					
Control	−34.6 ± 3.3	20.9 ± 1.4	428 ± 21	4.12 ± 0.2	15
RN	−33.7 ± 2.8	15.1 ± 1.9*	453 ± 22	4.81 ± 0.4	13
RE, sIPSCs					
Control	−35.1 ± 1.8	20.0 ± 2.5	400 ± 21	4.24 ± 0.3	9
RE	−30.3 ± 2.2	17.4 ± 2.3	409 ± 15	4.27 ± 0.3	8
RN, sEPSCs					
Control	−12.5 ± 1.2	10.1 ± 1.2	563 ± 29	2.56 ± 0.2	8
RN	−12.8 ± 1.1	10.2 ± 1.2	565 ± 62	2.51 ± 0.3	8

Values are mean ± SE. *p = 0.017. RN, *RosaHD/Nestin-Cre*; RE, *RosaHD/Emx1-Cre*.

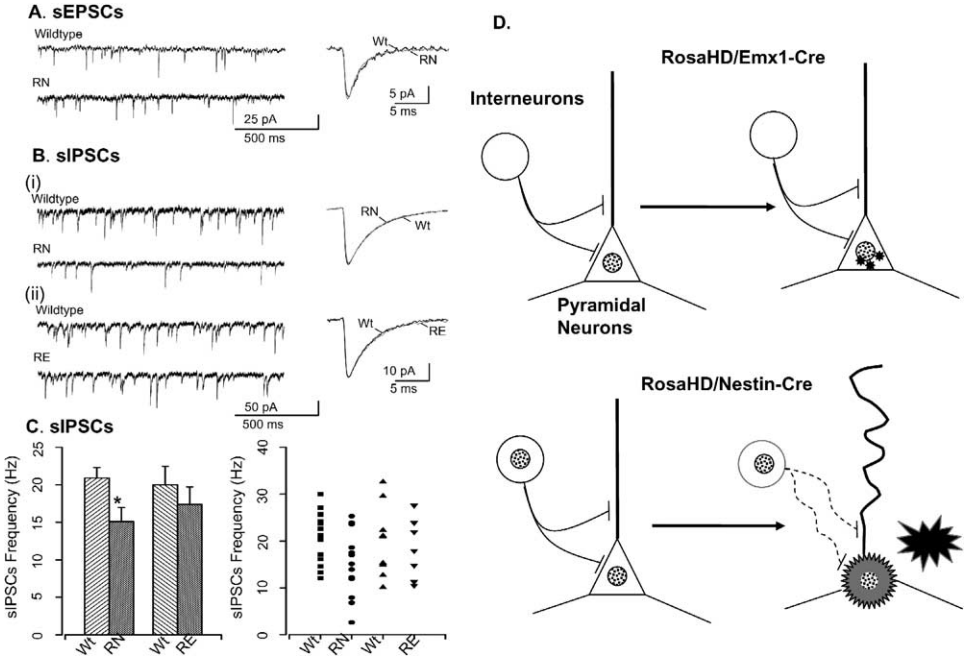

Figure 6. Presynaptic Changes in GABAergic Neurotransmission in 6-Month-Old *RosaHD/Nestin-Cre* Mouse Cortex, but Not in *RosaHD/Emx-Cre* Mouse Cortex, while Glutamatergic Transmission Remains Unaltered

All recordings were performed in layer II/III pyramidal neurons in the motor cortex. (A) AMPA receptor-mediated spontaneous EPSCs (sEPSCs) recorded in wild-type (wt; n = 8 cells) and *RosaHD/Nestin-Cre* (RN; n = 8 cells) transgenic mice show similar frequencies, rise times, amplitudes and decay time constants. Sample traces depict raw EPSCs, with superimposed average sEPSCs traces from >100 events in wild-type controls (black trace) and *RosaHD/Nestin-Cre* mice (gray trace). (B) $GABA_A$ receptor-mediated spontaneous IPSCs (sIPSCs) in the *RosaHD/Emx1-Cre* (RE; n = 8 cells) mice are comparable to those in wild-type (n = 9 cells) mice, but the frequency of IPSCs in the *RosaHD/Nestin-Cre* (RN; n = 13 cells) mice is significantly reduced compared to those in wild-type (n = 15 cells) mice. Raw traces and superimposed events are shown. (C) The comparison of sIPSC frequencies is expressed in the left panel. Data are presented as mean ± SEM. Error bars denote SEM, and asterisks indicate significance ($p < 0.01$, two-tailed t test). Scatter plots (right panel) indicate the mean frequencies recorded in each cell. (D) Schematic models to summarize the neuropathological and electrophysiological analyses of the two conditional HD models. Our results suggest that early cortical interneuron dysfunction in *RosaHD/Nestin-Cre* mice leads to reduced cortical inhibition which in turn contributes to overall cortical pathogenesis. Mutant htt expression (dotted black circle); dark degenerating neurons (spiked gray circle); degenerating vacuoles (small black stars); reduced GABAergic neurotransmission (dotted lines); reactive glia (large black star).

tions can significantly contribute to neuronal toxicity in vivo in a mouse model of HD.

Another significant finding of this study is the demonstration of cortical interneuron dysfunction, primarily a reduction of GABAergic neurotransmission, in the conditional HD mice. Several observations support the interpretation that cortical interneuron dysfunction is important for HD pathogenesis. First, our results suggest that expression of mhtt outside cortical pyramidal neurons, including cortical interneurons or subcortical neurons, is critical for cortical pathogenesis. Second, the presence of interneuron dysfunction is correlated with the expression and accumulation of aggregated forms of mhtt in the cortical interneurons in the conditional HD mice. Third, interneuron dysfunction appears very early in the disease process, at 6 months of age, which is the earliest time point we could detect locomotor deficits and neuropathology in these mice. These results strongly argue that the reduction of GABAergic neurotransmission may be a critical early pathogenic event in the conditional HD mice. Our observation of cortical interneuron dysfunction is also consistent with alterations of interneuron gene expression in the brains of HD patients (Ferrer et al., 1994) and with previous reports on mhtt aggregation in subsets of cortical interneurons and on alterations of interneuron gene expression in the R6/2 mouse model of HD, which also expresses mhtt-exon1 (Kosinski et al., 1999; Meade et al., 2002; Gourfinkel-An et al., 2003). Cortical interneurons are critical for regulating the excitability of cortical pyramidal neurons (Mody and Pearce, 2004). Enhanced cortical excitability has been observed in HD patients (i.e., seizures in juvenile HD patients; Nance and Myers, 2001) and HD mice (Cepeda et al., 2003). In turn, such enhanced cortical excitability has been postulated to promote excitotoxicity in striatal medium spiny neurons and contribute to their degeneration in HD (Grunewald and Beal, 1999). Taking everything into account, we would like to propose a pathogenic model for HD that involves dysfunctional cortical and striatal neuronal circuitry. That is, the early cortical interneuron dysfunction in HD leads to enhanced excitability of cortical pyramidal neurons, which in turn contributes to neuropathogenesis in the cortex and striatum.

Our study has clearly demonstrated that pathological cell-cell interactions are critically important for cortical pathogenesis in a conditional mouse model expressing a neuropathogenic form of mhtt (mhtt-exon1). Our

study, however, did not directly address the relative contribution of pathological cell-cell interactions in HD pathogenesis elicited by the full-length mhtt. Since full-length mhtt was shown to be cleaved into toxic mhtt N-terminal fragments similar to the size of mhtt-exon1 (Lunkes et al., 2002) and accumulation of such mhtt fragments in aggregates precedes the onset of motor deficits and neurodegeneration in HD patients and HD mice (DiFiglia et al., 1997; Gutekunst et al., 1999; Wellington et al., 2002; Zhou et al., 2003), we therefore would like to suggest that pathological cell-cell interactions also contribute to cortical pathogenesis elicited by the full-length mhtt, particularly after accumulation of small mhtt N-terminal fragments in the cortical neurons. Future genetic studies using conditional mouse models expressing full-length mhtt are clearly needed to directly address this issue.

Our study demonstrates that expression of mhtt in cortical pyramidal neurons is *insufficient* to cause significant cortical disease in our mouse model. Nevertheless, we cannot rule out the possibility that mhtt expression in cortical pyramidal neurons is *necessary* for disease pathogenesis. There are two possible scenarios. One is that expression of mhtt in the pyramidal neurons is necessary but not sufficient for cortical pathogenesis. Another scenario is that expression of mhtt outside the cortical pyramidal neurons, such as cortical interneurons or subcortical neurons, is both necessary and sufficient to cause full-scale cortical disease, even in the absence of mhtt expression in the cortical pyramidal neurons. Since restricted expression of mhtt to the cortical pyramidal neurons can elicit mild neurodegenerative changes in these neurons, we believe that the first scenario is likely to be true. Expression of mhtt restricted to the vulnerable neurons alone can elicit a very mild and slowly progressing cell-autonomous disease process, but a combination of this cell-autonomous process with pathological cell-cell interactions will cause the full-scale disease affecting the vulnerable neurons. Further studies using mouse models that selectively turn on mhtt expression in the cortical interneurons or striatal medium spiny neurons will help to address these questions.

Our study also provides insights into the role of mhtt aggregation in HD pathogenesis. The conditional HD mice revealed that progressive mhtt nuclear accumulation and aggregation is a cell-autonomous process. However, the presence of mhtt aggregates in cortical pyramidal neurons alone can only elicit very mild neurodegenerative changes (i.e., degenerating vacuoles), but cannot elicit full-scale neuropathology in these neurons (including dysmorphic neurites and dark neuron degeneration). These results demonstrate that the aggregates themselves cannot be the only pathogenic factor in cortical pyramidal neurons. This result is consistent with studies on SCA1 mouse models demonstrating that large nuclear inclusions are not pathogenic and may even be neuroprotective (Klement et al., 1998). However, we cannot rule out the possibility that mhtt aggregates may be *necessary* for cortical pathogenesis, since the appearance of these aggregates in the *RosaHD/Nestin-Cre* mice is concurrent with the onset of motor deficits, interneuron dysfunction, and cortical pathology.

Finally, our study has important implications for understanding the pathogenesis of other neurodegenerative disorders, including other polyQ disorders, familial Alzheimer's disease, and familial Parkinson's disease. All of these disorders are characterized by selective neurodegeneration caused by widely expressed disease proteins, but the molecular and cellular mechanisms underlying selective neruodegeneration in these disorders are poorly understood. A recent study of amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder affecting the motor neurons, demonstrated that pathological cell-cell interactions elicited by mutant *superoxide dismutase 1* is critical to selective motor neuron degeneration seen in ALS chimeric mice (Clement et al., 2003). Together, our study as well as the ALS study suggests that pathological cell-cell interactions may be a surprisingly common pathogenic mechanism that warrants further studies in all neurodegenerative disorders.

Experimental Procedures

Generation and Breeding of the Conditional HD Mice

Human *mhtt-exon1* with 103 mixed CAA-CAG repeat encoding a glutamine repeat (Kazantsev et al., 1999) was targeted to the Rosa26 locus in *129Sv* ES cells as described (Srinivas et al., 2001). Five correctly targeted ES cell clones were identified by Southern blot analyses (data not shown), and these ES cell clones were microinjected into *C57BL/6J* mouse blastocysts to obtain chimera mice. Two chimera mice produced germline transmission of the correctly targeted allele.

Rosa HD mice were maintained as *129* and *C57BL/6J* hybrids. *Nestin-Cre* mice (Tronche et al., 1999) were maintained on a *C57BL/6J* background, and *Emx1-Cre* mice (Iwasato et al., 2000) were generated in the *129Sv* background and backcrossed onto the *C57BL/6J* background. The F1 and F2 mice from crosses between *RosaHD* mice and the Cre mice (all in a mixed 129Sv and C57BL6J hybrid background) were used in this study. Wild-type littemates were used as controls. *RosaHD*, *Nestin-Cre*, and *Emx-Cre* alleles were genotyped using the established PCR protocols (Srinivas et al., 2001; Tronche et al., 1999; Iwasato et al., 2000). Mice were maintained and bred under standard conditions, consistent with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committees.

Western Blot Analyses

Cortical and striatal tissues from 6-month-old mouse brains were dissected out separately and resuspended in lysis buffer (0.1 M Tris [pH 7.4], 1 mM EDTA, and 50 mM NaCl) with a protease inhibitor cocktail (Roche, Palo Alto, CA), homogenized for 30 s, then centrifuged for 10 min at 12,000 × g at 4°C. The supernatant was collected and stored at −80°C. Protein samples (100 μg) were loaded onto a 7.5% mini-gel and separated by electrophoresis. After transfer to PVDF membrane (Perkin Elmer), mhtt was detected with the mouse monoclonal 1C2 antibody (1:2000 dilution; Chemicon) and enhanced chemiluminesce (Amersham). The membrane was striped and reprobed with a β-actin antibody (1:2000 dilution; Sigma) to confirm the equal loading of samples.

Open Field Test

Naive animals were tested in an open field test at 2, 6, 8, and 10 months of age. For *RosaHD/Nestin-Cre* mice, the following number of transgenic (Tg) and wild-type (wt) mice were used: 2 months, 16 Tg and 17 wt; 6 months, 20 Tg and 28 wt; 8 months, 9 Tg and 14 wt; 10 months, 14 Tg and 18 wt. For the *RosaHD/Emx1-Cre* mice, the following numbers of mice were used: 2 months, 11 Tg and 12 wt; 6 months, 16 Tg and 14 wt; 8 months, 14 Tg and 19 wt; and 10 months, 9 Tg and 13 wt. They were tested and videotaped over a 10 min period according to an established protocol (Menalled et al., 2002). All tests were performed at the same time of day. The

open field consisted of a clear Plexiglass box (15 × 30 × 45cm) in which the floor was divided into two rows of three squares each. Each mouse was initially placed in the middle square of the back row. Horizontal locomotive activity of a mouse was defined as the number of lines crossed by all four limbs over the testing period. Videotapes were reviewed by two examiners who were blind to the genotypes of the mice, and the average crossing number between the two examiners was used for each mouse. A Student's t test with a critical value of $p < 0.05$ was used for all statistical analyses.

Immunohistochemical and Double-Immunofluorescent Staining of Mouse Brain Sections with the EM48 Antibodies

Mutant htt was detected by using an established immunohistochemical staining protocol with the rabbit polyclonal EM48 antibody (Menalled et al., 2002). Sections were imaged using a Zeiss Axioskop 2 microscope.

For double immunofluorescent studies, 35 μm brain sections were prepared as above. Floating sections were blocked 30 min with 2% normal goat serum and 3% bovine serum albumin (BSA). After incubation with rabbit polyclonal EM48 antibodies (1:300 dilution) at room temperature for 18 hr, sections were stained overnight at 4°C with a second primary antibody, either mouse monoclonal anti-CaMKII antibody (1:200 dilution, Chemicon) or mouse monoclonal anti-GABA antibody (1:1000 dilution, Sigma). Alexa Fluor 488-conjugated goat anti-rabbit IgG antibodies and Alexa Fluor 568-conjugated goat anti-mouse IgG antibodies (Molecular Probes) were then applied to the sections at 1:200 dilution and incubated 2 hr in the dark at room temperature. Stained sections were washed three times for 10 min each in 0.1 M Tris buffer (pH 7.6), mounted in Vectashield (Vector), and images were taken using a Leica TCS-SP confocal microscope.

Immunohistochemistry Detection and Quantitation of Reactive Gliosis

We used established protocols for GFAP staining (Yu et al., 2003). To quantify gliosis, we used three 6-month-old transgenic mice and three respective wild-type littermates. Mice were transcardially perfused, and serial 40 μm coronal sections were prepared. The first section was chosen at approximately Bregma 0 mm (Paxinos and Franklin, 2001); this section and three successive sections 240 μm apart (every sixth section) were stained with anti-GFAP antibody. To quantify the severity of reactive gliosis, we counted the number of GFAP(+) cells on each section in a fixed rectangular area (138 μm × 110 μm) under 20× objective on a Zeiss Axioskop 2 microscope. Six nonoverlapping images from each cortical brain section and four images from each striatal brain section were obtained for the quantitation. We calculated the average number of GFAP(+) astrocytes per unit area (mm^2) per mouse for both cortex and striatum for each genotype. The Student's t test was used for statistical analysis.

Golgi Staining

Modified Golgi-Cox impregnation of neurons was performed using FD Rapid Golgi Stain Kit (FD NeuroTechnologies). In brief, 6-month-old nonperfused mouse brains were immersed in impregnation solution for 2 weeks, transferred to Solution C for 2 days, and cut into 100 μm sections with cryostat. The sections were mounted on 3% gelatin-coated slides, air-dried for 2 weeks, then stained with Solution D and E followed by a serial alcohol dehydration prior to mounting with Permount.

Electron Microscopy

We used an established protocol for EM study (Yu et al., 2003). Two 1-year-old *RosaHD/Nestin-Cre* and *RosaHD/Emx1-Cre* transgenic mice and two respective wild-type littermates were used for EM study.

Electrophysiological Recordings

Brain slices were prepared by standard techniques as previously described (Dalby and Mody, 2003). The ACSF contained 126 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.3). The slices were perfused continuously with 32°C–34°C ACSF saturated with 95% O_2 and 5% CO_2 at 3 ml/min containing either 3 mM kynurenic acid to block ionotropic glutamate receptors or 50 μM picrotoxin and 25 μM D-APV to block $GABA_A$ and NMDA receptors, respectively. Whole-cell recordings were made in visually identified cortical layers 3-5 pyramidal neurons with an Axopatch 200 amplifier (Axon Instruments). The intracellular solutions of sIPSCs recordings contained 125 mM CsCl, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.1 mM EGTA, 2 mM Na_2ATP, 0.5 mM Na-GTP, and 5 mM QX-314, while the pipette solution of sEPSCs was as follows: 130 mM $CsMeSO_4$, 5 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM MgATP, and 0.2 mM Na-GTP (pH 7.25; 280–290 mOsm). Voltage-clamp recordings were made at $V_h = -70$ mV. The sIPSCs and sEPSCs were detected and analyzed as described previously (Dalby and Mody, 2003). The means were considered to be significantly different if $p < 0.05$ (t test).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/46/3/433/DC1/.

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