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Forebrain striatal-specific expression of mutant huntingtin protein *in vivo* induces cell-autonomous age-dependent alterations in sensitivity to excitotoxicity and mitochondrial function

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ABSTRACT

HD (Huntington's disease) is characterized by dysfunction and death of striatal MSNs (medium-sized spiny neurons). Excitotoxicity, transcriptional dysregulation and mitochondrial abnormalities are among the mechanisms that are proposed to play roles in HD pathogenesis. To determine the extent of cell-autonomous effects of mhtt (mutant huntingtin) protein on vulnerability to excitotoxic insult in MSNs *in vivo*, we measured the number of degenerating neurons in response to intrastriatal injection of QA (quinolinic acid) in presymptomatic and symptomatic transgenic (D9-N171-98Q, also known as DE5) mice that express mhtt in MSNs but not in cortex. After QA, the number of degenerating neurons in presymptomatic DE5 mice was not significantly different from the number in WT (wild-type) controls, suggesting the early, increased vulnerability to excitotoxicity demonstrated in other HD mouse models has a largely non-cell-autonomous component. Conversely, symptomatic DE5 mice showed significantly fewer degenerating neurons relative to WT, implying the resistance to excitotoxicity observed at later ages has a primarily cell-autonomous origin. Interestingly, mitochondrial complex II respiration was enhanced in striatum of symptomatic mice, whereas it was reduced in presymptomatic mice, both relative to their age-matched controls. Consistent with the QA data, MSNs from symptomatic mice showed decreased NMDA (*N*-methyl-D-aspartate) currents compared with age-matched controls, suggesting that in addition to aging, cell-autonomous mechanisms mitigate susceptibility to excitotoxicity in the symptomatic stage. Also, symptomatic DE5 mice did not display some of the electrophysiological alterations present in other HD models, suggesting that blocking the expression of mhtt in cortical neurons may restore corticostriatal function in HD.

Key words: complex II, excitotoxicity, Huntington's disease (HD), mitochondria, *N*-methyl-D-aspartate (NMDA), striatum.

INTRODUCTION

HD (Huntington's disease) is an autosomal-dominant, fatal neurodegenerative disorder characterized by progressive deterioration of cognitive and motor functions. The pathological

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Abbreviations: ACSF, artificial cerebrospinal fluid; EPSC, excitatory postsynaptic current; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FJC, Fluoro-Jade C; $GABA_A$, γ-aminobutyric acid type A; HD, Huntington's disease; htt, huntingtin; IP, intraperitoneally; IPSC, inhibitory postsynaptic current; mEPSC, miniature EPSC; mhtt, mutant htt; MSN, medium-sized spiny neuron; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; PGC-1α, peroxisome-proliferator-activated receptor γ co-activator-1α; QA, quinolinic acid; sEPSC, spontaneous EPSC; sIPSC, spontaneous IPSC; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; TTX, tetrodotoxin; WT, wild-type.

mutation in the HD gene *htt* (huntingtin) is the expansion of a polymorphic CAG (glutamine) repeat to >36, with a longer repeat length being associated with earlier onset and more severe disease progression (Cowan and Raymond, 2006). The functions of normal htt have not been entirely delineated, but it is found in the cytoplasm, soma, dendrites and synaptic terminals of neurons. Htt is necessary for proper mitotic spindle orientation (Godin et al., 2010) and also associates with transcription factors, mitochondria, vesicular membranes and transport machinery. Its association with diverse cellular processes suggests vital functional roles and the mouse htt–null mutation is lethal (Duyao et al., 1995; MacDonald et al., 1996). The mutation in HD may lead to increased oxidative stress, impaired energy metabolism and abnormal interactions with other proteins, which in some cases lead to dysregulation of transcriptional machinery and altered gene expression (Cowan and Raymond, 2006; Zhou and Baudry, 2006; Fan and Raymond, 2007; Brown et al., 2008; Nicholls, 2009). Although mhtt (mutant htt) is ubiquitously expressed and present in all regions and cell types of the brain, striatal GABAergic MSNs (medium-sized spiny neurons) are particularly vulnerable. Other regions, particularly neocortex, are also affected (Vonsattel et al., 1985; Cowan and Raymond, 2006). A challenging issue in the study of HD remains the delineation of mechanisms of neuronal subtype vulnerability.

The 'excitotoxicity hypothesis' of neurodegeneration was first proposed in 1957 (Lucas and Newhouse, 1957) and it persists as a likely pathophysiological mechanism in HD. Excitotoxicity refers to the phenomenon in which over-activated ionotropic glutamate receptors respond to excitatory neurotransmitters via a pathway that leads to neuronal damage. Rapid and prolonged influx of calcium and/or dysregulation of intracellular calcium appear critical to the initiation of degeneration (Collingridge and Lester, 1989; Coyle and Puttfarcken, 1993; Cowan and Raymond, 2006; Zhou and Baudry, 2006; Hardingham, 2009).

Before the creation of transgenic mouse models of HD, chemical insults to the striatum served as useful disease models. Early HD models that recapitulated the behavioural and neuropathological features of HD included intrastriatal injection of NMDAR [NMDA (*N*-methyl-D-aspartate) receptor] agonists, for example QA (quinolinic acid) (DiFiglia, 1990; Guidetti et al., 2004; Sapko et al., 2006; Graham et al., 2009), or injection of an inhibitor of mitochondrial succinate dehydrogenase, 3-nitropropionic acid (Brouillet et al., 1995). The role of excitotoxicity in HD pathophysiology has been investigated in at least six commonly utilized pan-cellular and pan-neuronal HD mouse models (Nicholls, 2009).

In order to examine the contribution of intrinsic, cell-autonomous vulnerability of MSNs to excitotoxicity, we measured the number of degenerating neurons after injecting QA in the striatum of presymptomatic and symptomatic D9-N171-98Q, also known as DE5, transgenic mice (Brown et al., 2008) that, within the forebrain, selectively express mhtt in the MSNs, excluding neocortical and glial expression. Thus, we define 'cell-autonomous' as alterations that occur in MSNs independent of transgene expression in neocortical and striatal interneurons and in glia. Non-cell-autonomous factors include those that require transgene expression in cells other than MSNs, for example, excessive glutamate release and decreased neurotrophic support due to abnormalities of cortical neurons (Altar et al., 1997; Cepeda et al., 2007). Such anatomical inter-dependence compounds the difficulty in assessment of the relative contribution of each region to HD pathogenesis (Gu et al., 2007; Brown et al., 2008). We also examined mitochondrial function and electrophysiological properties of MSNs in the striatum of this model, as abnormalities of both are known to change sensitivity to excitotoxic insult.

MATERIALS AND METHODS

Animals

The creation and characterization of the mice used have been previously described (Brown et al., 2008). Transgenic DE5 mice expressing the first 171 amino acids of htt with 98 CAG repeats (D9-N171-98Q) and their WT (wild-type) littermates were used in this study. This transgenic line was produced, and has remained, on a C57Bl6/J background. Mice were group-housed (4–5/cage) on a 12 h light/12 h dark. Water and food were available *ad libitum*. Mice were either 2–3 or >14 months old at the time of their surgeries. A total of 33 mice received intrastriatal QA injections. The animal protocol was approved by the IACUC of Mount Sinai School of Medicine.

Surgery

QA was dissolved in 0.1 M PBS (pH 7.4). Under pentobarbital anesthesia [50 mg/kg IP (intraperitoneally)], the mice received a unilateral intrastriatal injection of 15 nmol of QA in 1 μl at the following stereotaxic coordinates using a 2.5 μl Hamilton syringe: 0.05 cm rostral to Bregma, 0.2 cm to the right of the midline and 0.3 cm ventral to the dural surface, with the bar set to zero. The injection rate was 0.5 μl/min, and the needle was left in place for an additional 3 min before slowly retracting it to prevent retrograde leakage.

Fluoro-Jade staining

FJC (Fluoro-Jade C; Millipore) stains degenerating neurons in the brain (Schmued et al., 1997). FJC was performed as described in the manufacturer's instruction (Millipore) with some modifications. Briefly, perfusion-fixed brain sections were mounted, dried and immersed in 90% ethanol, followed by basic alcohol solution (1% NaOH in 80% alcohol) for 5 min, followed by 70, 60, 50, 40, 30, 20 and 10% ethanol and Milli-Q water incubation, for 2 min each. Sections were

treated with 0.06% $KMnO_4$ for 10 min. After rinsing in water, sections were immersed in FJC (0.0001% FJC in 0.1% acetic acid) for 10 min in the dark.

Histology

Three days after intrastriatal QA injection, mice were perfused with 4% (w/v) paraformaldehyde in PBS under pentobarbital anesthesia (50 mg/kg IP). The brains were post-fixed overnight in the same solution at 4°C, dehydrated in 30% sucrose/0.1 M PBS, and then mounted using Tissue-TEK O.C.T. compound (Sakura). Mouse brains were cut using a cryostat (Leica CM3050) and every fifth serial coronal section (30 μm) was stained with FJC.

Stereology

For assessment of the number of FJ-positive cells, a 25×25 μm counting frame was randomly placed within the delineated striatal area and then systematically moved through the tissue. The number of FJ-positive cells was then determined by StereoInvestigator software (Microbrightfield). The striatum for each section was delineated rostral-caudal with the anterior commissure marking the caudal boundary of sampling using StereoInvestigator software. Striatal volume was also assessed using the Cavalieri principle (Gundersen and Jensen, 1987).

Mitochondrial respiration assay

After mice were killed without agents that compromise mitochondrial function, brains were quickly removed and striata were isolated in an ice-cold dissection buffer (50 mM Hepes, pH 7.5, 125 mM NaCl, 100 mM sucrose and 2 mM KCl). Striata from two mice of each genotype were pooled for mitochondrial isolation for complex I assay, and striata from one mouse each for complex II and IV assays. Tissue samples were homogenized in 8 ml (complex I) or 4 ml (complexes II and IV) of homogenizing buffer (320 mM sucrose, 5 mM Tris, 2 mM EGTA, final pH 7.4, 4°C) with a Teflon-glass Potter–Elvehjem homogenizer. Samples were centrifuged for 3 min at 2000 ***g*** to remove nuclei and tissue particles. Supernatants were collected and centrifuged for 10 min at 12000 ***g*** to pellet mitochondria and synaptosomes. The crude pellet was resuspended in 3 ml of the homogenizing buffer with the addition of 0.02% (w/v) digitonin to disrupt synaptosomal membranes and release mitochondria. The resuspended pellet was centrifuged for 10 min at 12000 ***g*** to pellet mitochondria, which was resuspended in 30 μl of mitochondria respiration media (70 mM sucrose, 220 mM mannitol, 2 mM Hepes buffer, 5 mM $MgCl_2$, 5 mM $KHPO_4$, 1 mM EDTA and 0.1% fatty acid free BSA, pH 7.4), and protein content was determined by BCA (bicinchoninic acid) assay (Sigma). Isolated mitochondria were resuspended in 2.5 ml mitochondria respiration medium and assayed for state 4 respiration using 8 mM glutamate/8 mM malate (complex I), 4 mM succinate (complex II) or 0.24 mM TMPD (*N,N,N′,N′*-tetramethylphenylenediamine)/2 mM ascorbate (complex IV). ADP was added (40 μM for complex I and 100 μM for complex II) to induce state 3 respiration. After ADP was depleted and respiration returned to state 4, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), a potent uncoupler of oxidative phosphorylation, was added to the reaction chamber (4 μM for complex I and 2 μM for complex II). Mitochondrial respiration was determined using a Clark-type oxygen electrode (Model 5331, YSI) with an oxygen monitor (Model 5300, YSI) and recorded with a potentiometric recorder (LKB 2210, LKB-Produkter AB, Sweden).

Mitochondrial ATP-synthase assay (complex V)

Enzymatic activity of ATPase in the mitochondrial inner membrane was measured by the method described in Taussky and Shorr (1953). The mitochondria samples were incubated at 37°C for 5 min in an enzyme reaction buffer (100 mM NaCl, 10 mM KCl, 50 mM Tris-acetate, pH 7.2, 0.1 mM EDTA, pH 8.0 and 3 mM $MgCl_2$). The reaction was started by adding 3.33 mM ATP, and the mitochondria were further incubated at 37°C for 20 min. The reaction was stopped by adding ice-cold perchloric acid. The mixture was centrifuged at 8000 ***g*** for 10 min at 4°C, and the supernatant was incubated at room temperature (20°C) for 15 min after adding molybdate detection reagent. The absorbance was measured at 660 nm (Sudo et al., 2000).

Slice electrophysiology

Mice were deeply anesthetized with halothane, killed by decapitation and the brains dissected and immediately placed in oxygenated ice-cold low-Ca^{2+} and high-Mg^{2+} ACSF (artificial cerebrospinal fluid) containing 130 mM NaCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM glucose. Coronal slices (350 μm) were cut and transferred to an incubating chamber containing ACSF (with 2 mM $CaCl_2$ and 2 mM $MgCl_2$) oxygenated with 95% O_2, 5% CO_2 (pH 7.2–7.4, 290–310 mOsm/l, 25 ± 2°C). Following recovery, slices were placed on the stage of an upright microscope (Olympus BX51), submerged in continuously flowing ACSF (4 ml/min). All experiments were performed at room temperature. Whole-cell patch clamp recordings in voltage clamp mode were obtained from MSNs in the dorsolateral striatum visualized with the aid of infrared videomicroscopy (Cepeda et al., 1998). MSNs were identified by somatic size and typical basic membrane properties (input resistance, membrane capacitance and time constant). Series resistance was <25 MΩ. The patch pipette (4–6 MΩ) contained the following solution: 125 mM Cs-methanesulfonate, 3 mM KCl, 4 mM NaCl, 1 mM $MgCl_2$, 5 mM MgATP, 9 mM EGTA, 8 mM Hepes, 1 mM GTP, 10 mM phosphocreatine and 0.1 mM leupeptin (pH 7.25–7.3 and osmolarity 280–290 mOsm/l). Passive membrane properties of MSNs were determined by applying a depolarizing step voltage command (10 mV) and using the membrane

test function integrated in the pClamp8 software (Axon Instruments, Foster City, CA, U.S.A.). This function reports membrane capacitance (in pF), input resistance (in MΩ) and time constant (in ms). sEPSCs [spontaneous EPSCs (excitatory postsynaptic currents)] and sIPSCs [spontaneous IPSCs (inhibitory postsynaptic currents)] were first isolated electrophysiologically by holding the membrane at −70 mV and +10 mV respectively. After recording sIPSCs, bicuculline methiodide (BIC, 20 μM) was added to block $GABA_A$ (γ-aminobutyric acid type A) receptors and the membrane was stepped back to −70 mV to record sEPSCs without any possible contribution of $GABA_A$ receptors. mEPSCs (miniature EPSCs) were isolated by blocking Na^+ channels using TTX (tetrodotoxin; 1 μM). The frequency of sEPSCs and mEPSCs was measured with MiniAnalysis software (Synaptosoft Inc, Fort Lee, NJ) using a threshold of 5 pA.

Acutely dissociated neuron preparation

Detailed procedures have been published (Flores-Hernandez et al., 2002; Andre et al., 2010). Coronal slices containing the dorsal striatum were dissected, placed in an oxygenated cell-stir chamber (Wheaton, Millville, NJ, U.S.A.) and enzymatically treated for 15–20 min with papain (0.5 mg/ml, Calbiochem) at 35°C in a Hepes-buffered HBSS (Hanks balanced salt solution; Sigma Chemical) supplemented with 1 mM pyruvic acid, 0.005 mM glutathione, 0.1 mM N^G-nitro-L-arginine and 1 mM kynurenic acid. The tissue was rinsed with Hepes-buffered Na-isethionate solution containing 140 mM Na isethionate, 2 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 23 mM glucose and 15 mM Hepes. Striatal slices were mechanically dissociated with fire-polished Pasteur pipettes. The cell suspension was then plated into a 35-mm Petri dish mounted on the stage of an upright fixed-stage microscope (Zeiss Axioscope, Thornwood, NY, U.S.A.) containing a Hepes-buffered salt solution. The internal solution consisted of 175 mM NMDG (*N*-methyl-D-glucamine), 40 mM Hepes, 2 mM $MgCl_2$, 10 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid (EGTA), 12 mM phosphocreatine, 2 mM Na_2 ATP, 0.2 mM Na_2 GTP and 0.1 mM leupeptin (pH 7.25, 265–270 mOsm/l). The external solution consisted of 135 mM NaCl, 20 mM CsCl, 3 mM $BaCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes and 0.0003 mM TTX. Drugs were applied through a pressure-driven fast perfusion system using application capillaries positioned a few hundred micrometres from the cell. A DC drive system controlled by an SF-77B perfusion system (Warner Instruments, Hamden, CT, U.S.A.) synchronized by pClamp changed solutions by altering the position of the capillary array. NMDA currents were evoked by applying the agonist (100 μM, 3 s duration every 10 s) while holding the cell at −70 mV in the absence or presence of Mg^{2+} (50 μM).

Statistics

Results are presented as means ± S.E.M. and were analysed by unpaired Student's *t* test (two-tailed) between groups of biological relevance (e.g. young DE5 against young WT; old DE5 against old WT; young DE5 against old DE5; young WT against old WT). $P<0.05$ was considered significant, and actual *P* values are noted throughout the text.

RESULTS

Striatal volume is unchanged in mice expressing mhtt only in striatal neurons

Striatal volume loss occurs in HD patients and several pan-neuronal HD mouse models (Rosas et al., 2001; Slow et al., 2003; Chopra et al., 2007; Decressac et al., 2010). We examined possible age- and/or mhtt-dependent changes in striatal volume of DE5 and WT mice since a neuronal and volume loss could impact on conclusions drawn from numbers of degenerating neurons following equal doses of QA. Striatal volume was measured using the Cavalieri principle in all the animals used for QA injection. No significant striatal volume difference was observed between any pair of four cohorts of biological relevance (young DE5 = 17.15 ± 1.13 mm^3, young WT = 15.45 ± 0.88 mm^3, old DE5 = 14.50 ± 0.96 mm^3, old WT = 16.04 ± 0.55 mm^3) (Figure 1).

Striatum of symptomatic DE5 mice is resistant to QA-induced excitotoxicity

Previous studies of excitotoxicity in HD mouse models utilized a wide range of QA doses (4–30 nmol) (Hansson et al., 1999, 2001; MacGibbon et al., 2002; Zeron et al., 2002; Jarabek et al., 2004; Graham et al., 2006; Graham et al., 2009). Within the same study, different doses may be used for younger against older mice due to age-related differences in sensitivity. The DE5 mouse is on a C57BL/6J background that

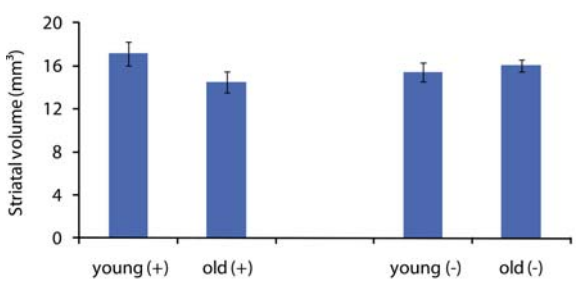

Figure 1 Striatal volume is unchanged in young (presymptomatic) and old (symptomatic) DE5 mice

Perfused and post-fixed brains were coronally sectioned into 30 μm sections extending throughout the striatum. Every 5th section was serially mounted and analysed with StereoInvestigator software, which was used to trace and calculate striatal volume. No significant differences in striatal volume were observed between any pair of cohorts of biological relevance. $n=11$, 10, 5, 6 for young (−), young (+), old (−), old (+) respectively. Results are presented as means ± S.E.M.

is a relatively resistant strain to QA excitotoxicity (McLin et al., 2006). We first injected 4, 6, 15 and 30 nmol of QA into the striatum of young (2–2.5 months old) and old (>1 year old) C57BL/6J mice to identify a single dose that could be applied to both age groups without triggering a seizure, yet induce a measurable number of degenerating neurons. In young C57BL/6J mice, 4 and 6 nmol of QA did not yield a lesion but 30 nmol resulted in a lesion throughout the entire striatum, to which it is hard to make a comparison (results not shown). We therefore utilized a single dose of 15 nmol in all animals, enabling an accurate comparison across ages and genotypes (Figure 2).

FJ-positive neurons were counted after intrastriatal injection of 15 nmol QA in young (presymptomatic) and old (symptomatic) DE5 mice and age-matched controls. There was no difference in the number of degenerating neurons between young DE5 and WT (young DE5 $=27010 \pm 3546$, young WT $=19755 \pm 2563$; $P=0.093$) (Figure 3A), suggesting that non-cell-autonomous factors may lead to increased sensitivity to excitotoxicity observed at the presymptomatic stages in HD pan-cellular and pan-neuronal transgenic models (Hansson et al., 2001; Zeron et al., 2002; Graham et al., 2006, 2009). Importantly, although there was a trend to a greater number of degenerating neurons in young DE5 mice, it was statistically weakened as we increased the number of animals in both groups. Consistent with previous studies in rats and FVB mice (Finn et al., 1991; Cepeda et al., 1996; Graham et al., 2009), both DE5 and WT C57BL/6J mice show a dramatic age-dependent decrease in sensitivity to excitotoxic insult (Figure 3B). DE5 and WT mice exhibited 10-fold and 2-fold decreases in FJ-positive neurons (old DE5 $=2496 \pm 1535$, old WT $=10195 \pm 3046$) respectively compared with their younger counterparts (Figure 3B). Symptomatic DE5 mice showed greater resistance to excitotoxicity relative to presymptomatic DE5 mice ($P<0.0001$) (Figure 3B) and also relative to age-matched WT mice ($P=0.027$) (Figure 3A), similar to data from pan-cellular HD models (Hansson et al., 1999, 2001; Graham et al., 2009). These results imply that striatal-specific expression of mhtt is sufficient to induce resistance to excitotoxicity at the symptomatic stage.

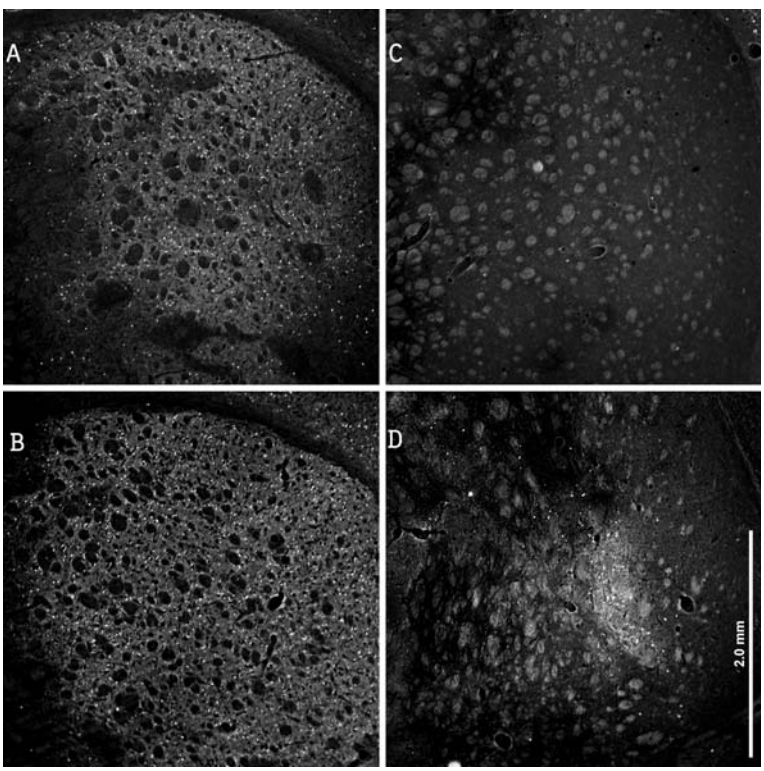

Figure 2 **DE5 symptomatic mice display increased resistance to excitotoxic insult**
Representative images of excitotoxic lesions caused by 15 nmol intrastriatal injection of QA in (**A**) young (presymptomatic) DE5, (**B**) young WT, (**C**) old (symptomatic) DE5 and (**D**) old WT mice. Many FJ-positive neurons (seen here as small, bright white dots) and large lesion areas were observed in the young mice of both genotypes (**A**, **B**). The old mice demonstrated significant resistance to QA excitotoxicity (**C**, **D**) compared with the young mice. The striatum of old DE5 mice (**C**) had obviously fewer FJ-positive cells than their age-matched controls (**D**).

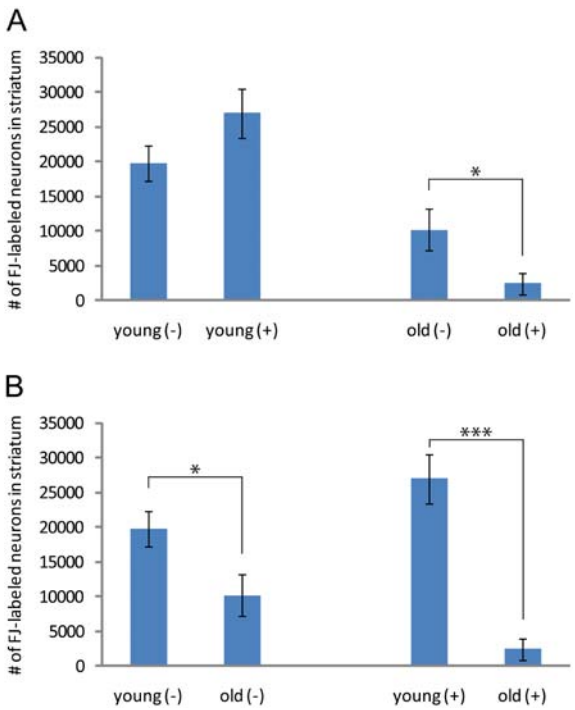

Figure 3 Quantification of FJC-positive degenerating neurons in presymptomatic (young), symptomatic (old) DE5 mice and age-matched controls after 15 nmol intrastriatal QA injection

(**A**) Three days after 15 nmol QA was injected into the striatum, there was no significant difference in the number of degenerating neurons between presymptomatic young DE5-N171-98Q and age-matched WT mice (young DE5=27010±3546, young WT=19755±2563; n=10, 11 respectively; P=0.093). There was a dramatic reduction of FJC-positive neurons in symptomatic, old DE5 against their age-matched controls (old DE5=2496±1535, old WT=10195±3046; n=6, 5 respectively; P=0.027). (**B**) The graph in (**A**) was rearranged to focus on age-dependent changes in response to excitotoxic insult in both genotypes. Young and old WT animals displayed significantly different numbers of FJ-positive neurons, with old WT displaying excitotoxic resistance relative to younger WT animals (P=0.035). Similarly, and even more dramatically, there is a significant reduction of FJ-positive neurons in the symptomatic DE5 against the presymptomatic DE5 (P<0.0001). Results are presented as means±S.E.M. *P<0.05; ***P<0.0001.

Mitochondrial complex II respiration is dysregulated in the striatum of both pre- and symptomatic DE5 mice

In presymptomatic HD, MSN hypersensitivity to excitotoxicity may arise from an increased level of extracellular glutamate (non-cell-autonomous) and/or an imbalance between synaptic and extra-synaptic NMDARs (Milnerwood et al., 2010), mitochondrial dysfunction and altered energy metabolism in the presence of a normal extracellular level of glutamate (cell-autonomous) (Greene and Greenamyre, 1996). Thus, we examined whether mhtt cell-autonomously altered mitochondrial function by measuring striatal mitochondrial respiration using the polarographic method. These assays include (i) state 4 respiration with specific substrates for each complex (glutamate+malate, succinate and ascorbate+TMPD for complex I, II and IV respectively); (ii) state 3 respiration in the presence of ADP; and (iii) maximum rates of respiration by uncoupling mitochondria with FCCP. Complex I activities were not altered in the striatum of young and old DE5 mice relative to WT littermates (Figure 4A), but states 3 and 4 activities of complex II were significantly reduced in presymptomatic DE5 mice compared with their age-matched controls (Figure 4B). Not surprisingly, state 4 respiration of complex II was significantly reduced in old WT mice compared with young WT mice. Conversely, it was significantly enhanced in symptomatic DE5 mice compared with their age-matched controls (Figures 4B and 4E). Increases in state 3 and FCCP-induced maximum respiration were also present in symptomatic DE5 mice compared with presymptomatic DE5 mice (Figure 4B). No alterations in complex IV activities were observed in any of the mice (Figure 4C). ATP-synthase activities of complex V in symptomatic DE5 mice was found to be as efficient as in age-matched controls (Figure 4D). Thus, striatal-specific expression of mhtt affected complex II activities of mitochondrial respiration in an age-dependent manner but did not alter the activities of the other complexes.

MSNs of symptomatic DE5 mice show an increase in input resistance and a decrease in NMDA current

To identify electrophysiological properties potentially associated with resistance to excitotoxicity in the striatum of symptomatic DE5 mice, we examined basic membrane properties and sEPSCs and sIPSCs in MSNs in slices from 18-month-old DE5 mice and their WT littermates (Figure 5A). There was a trend for reduced cell membrane capacitance, but the difference was not statistically significant. The only significant change in membrane properties was an increase in input resistance in the DE5 MSNs relative to WT control slices. This abnormality has been reported in many other HD mouse models, likely arising from a change in inwardly rectifying potassium channels (Cepeda et al., 2003, 2004; Ariano et al., 2005; Cummings et al., 2010). The frequency of sEPSCs and sIPSCs was not affected in DE5 mice. Thus, the transgene did not alter spontaneous synaptic inputs to MSNs, either excitatory inputs from cortex and thalamus or local inhibitory connections. Next, we recorded currents evoked by the application of 100 μM NMDA to acutely isolated MSNs from the mice (Figure 5B). This preparation is used to examine postsynaptic receptors at the cell somatic level. Peak current and current density (a measure that normalizes current by cell size) were reduced significantly, as also previously recorded in older R6/2, YAC128 and BAC103 mice (Graham et al., 2009; Joshi et al., 2009; Cepeda et al., 2010), whereas NMDA currents were increased in the 6-month-old striatal model of Gu et al. (2007). These changes would predict resistance to excitotoxicity and are therefore consistent with our data. A change in Mg^{2+} sensitivity sometimes occurs in MSNs in HD models (Starling et al., 2005; Gu et al., 2007); however, the transgene did not cause any change in Mg^{2+} sensitivity in the DE5 model.

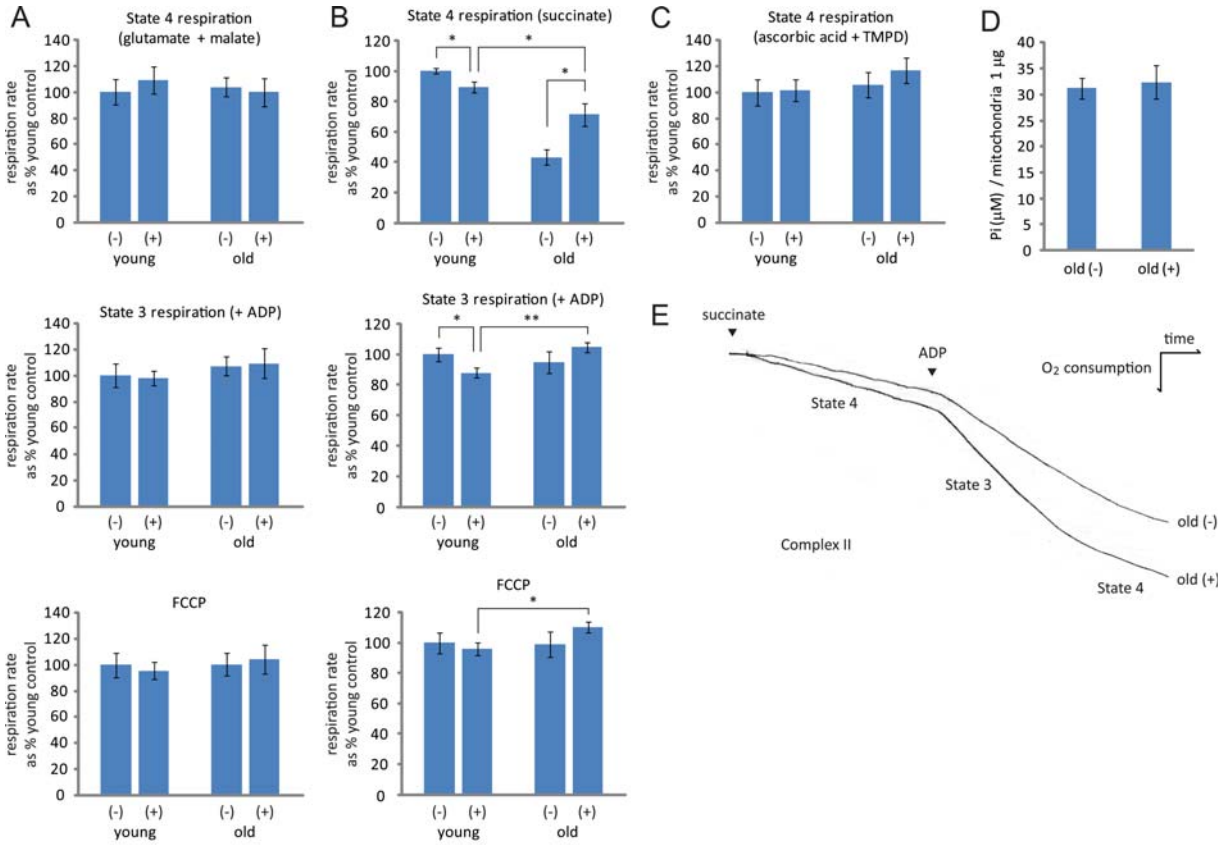

Figure 4 **Dysregulated mitochondrial complex II activities in the striatum of young and old DE5 mice**

(**A**) Complex I activities in the striatum of young (presymptomatic) and old (symptomatic) DE5 mice and their age-matched WT controls (n=4–5, each) are similar. Results are shown as respiration rate as % of young WT in (**A–C**). (**B**) Complex II state 4 respiration is reduced in young DE5 (89.5 ± 3.5%, respiration rate as % of young control; P=0.034) and is increased in old DE5 (old DE5, 71.4 ± 7.4%; old WT, 43.7 ± 5.2%; P=0.012), both compared with their age-matched controls. Comparing old to young DE5, state 4 respiration is decreased with age (P=0.022); the decrease was even dramatic due to normal aging comparing old with young WT (P<0.0001). State 3 respiration is reduced in young DE5 mice compared with young WT (young DE5, 87.9 ± 3.3%; P=0.039), but recovers in old DE5 mice (old DE5, 104 ± 3.5%, P=0.0054). There is no significant difference in state 3 respiration between young WT, old WT and old DE5 (old WT, 94.8 ± 7.4%). There is a significant difference in maximum respiration rates induced by FCCP in young DE5 compared with old DE5 (young DE5, 96.0 ± 4.3%; old DE5, 110.5 ± 3.5%; P=0.026); however, these values are not significantly different from either young or old WT mice (old DE5, 99.0 ± 8.1%). (n=4–6 each). (**C**) Complex IV activities in the striatum of young and old DE5 mice and their age-matched WT controls are similar (n=4–5, each). (**D**) ATP-synthase activities in the striatum of old DE5 and WT mice are similar (n=4 each). (**E**) Representative oxygraphs of striatal mitochondria preparations for complex II from old DE5 and WT mice. After injection of a substrate (succinate), state 4 was measured. After giving a limited amount of ADP, state 3 was measured. After depletion of ADP, O_2 consumption slowed down to state 4 respiration. Results are presented as means ± S.E.M. *P<0.05; **P<0.01.

DISCUSSION

Excitotoxicity appears to be at least partly responsible for the degeneration of MSNs in HD (reviewed in Cowan and Raymond, 2006). Increased levels of glutamate and QA, endogenous NMDAR agonists, have been found in some studies in the striatum of early HD patients (Guidetti et al., 2004), and intrastriatal injections of QA have been used as a model of HD (DiFiglia, 1990; Guidetti et al., 2004; Sapko et al., 2006; Graham et al., 2009). Intrastriatal injections of QA and NMDA have been performed in several pan-cellular and pan-neuronal HD mouse models to determine the effects of mhtt on sensitivity to excitotoxicity. Presymptomatic YAC128 and R6/1 HD mice exhibited increased vulnerability to QA-induced excitotoxicity and enhanced NMDA responses; conversely, symptomatic, older mice became resistant to excitotoxic insult (Hansson et al., 2001; Zeron et al., 2002; Graham et al., 2006, 2009). Utilizing the HD mouse model that expresses mhtt in the MSNs of the striatum but not in the cortex or glia, we demonstrate that increased sensitivity to QA excitotoxicity in young, presymptomatic mice has a largely non-cell autonomous component and the increased resistance to excitotoxic insult observed in older symptomatic HD mice can be primarily attributed to cell-autonomous effects of mhtt. These results support the importance of disease-stage and cell-type-specific therapeutic approaches.

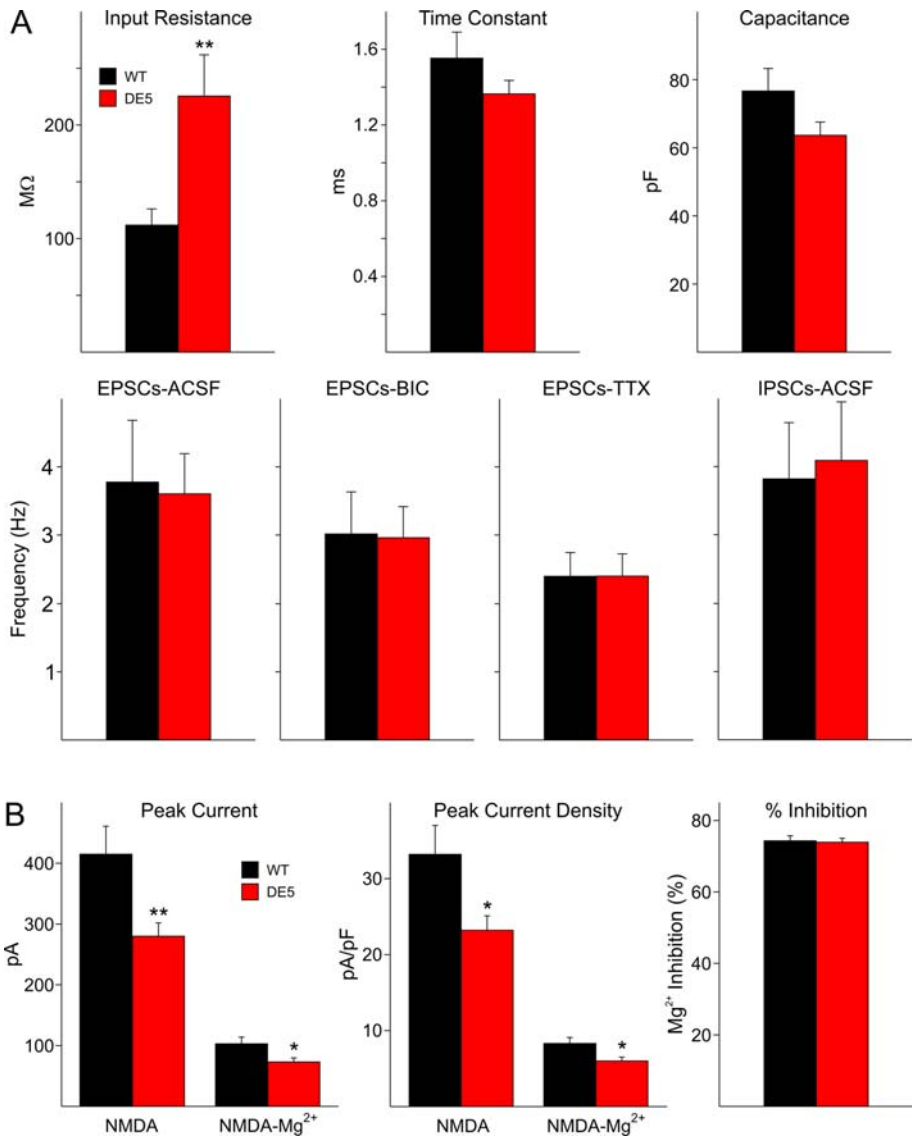

Figure 5 **Electrophysiological properties of MSNs in symptomatic DE5 mice**

(**A**) Top three graphs show differences in basic membrane properties between WT (9 cells, 2 animals) and DE5 MSNs (9 cells, 3 animals). Only the increase in input resistance was statistically significant. Bottom four graphs show that the frequency of sEPSCs and mEPSCs and IPSCs are similar in MSNs from WT and DE5 mice. (**B**) Graphs show peak currents and peak current densities evoked by the application of 100 μM NMDA in MSNs from WT (14 cells, 2 animals) and from symptomatic DE5 mice (21 cells, 3 animals) in the absence or the presence of 50 μM Mg^{2+}. Both peak currents and peak current densities were significantly decreased in DE5 mice, while the percent inhibition by Mg^{2+} was unchanged (right graph). Results are presented as means ± S.E.M. *$P<0.05$ and **$P<0.01$ for comparisons between WT and DE5.

Existing data support the presence of enhanced NMDAR activity in early HD (Cepeda et al., 2001; Fernandes and Raymond, 2009). NMDAR-mediated, and specifically NR2B-dependent, excitotoxicity exacerbates striatal neuronal degeneration in an NR2B-overexpressing HD mouse model (Heng et al., 2009). NMDAR is linked to two opposing signalling pathways leading to either cell survival or death, depending on receptor localization (Hardingham and Bading, 2010; Levine et al., 2010). Mislocalization of NMDAR to the peri- and extra-synaptic membrane (non-PSD fraction) in presymptomatic YAC128 striatum leads to a loss of neuroprotective mechanisms, an increase in activity of cell death pathways and increased neurotoxicity (Okamoto et al., 2009; Milnerwood et al., 2010). In parallel, excitotoxicity triggered by overactivation of NR2B-containing NMDARs results in rapid dissociation of synaptic NMDARs from the postsynaptic scaffolding complex and downstream survival signalling (Gascon et al., 2008). Thus, one possible scenario is that excitotoxic damage occurs in striatal MSNs in the early stage of the disease by dysregulation of NMDAR-linked

signalling pathways, which then leads to a cascade of age-dependent pathophysiological events.

The absence of the typical HD-related presymptomatic sensitivity to excitotoxicity in DE5 mice suggests that these early events are largely regulated in a non-cell-autonomous manner. These mechanisms may include an elevated glutamate level at the corticostriatal synapses due to excessive glutamate release from the cortex (Joshi et al., 2009), abnormal dopaminergic regulation of NMDAR expression (Snyder et al., 1998), decreased trophic support from the cortex and/or dysregulated glutamate uptake by astrocytes (Bradford et al., 2009, 2010; Faideau et al., 2010). The cell-specific expression of mhtt in the DE5 mouse would suggest that cortical and astrocytic functions are unaffected, unless there are secondary changes due to abnormal MSN function. The aetiology of increased extrasynaptic NMDAR in the presence of mhtt is unknown, but may be induced by glutamate spillover from excessive cortical release and/or reduced astrocytic uptake (Potier et al., 2010). Either way, if increased sensitivity to excitotoxicity in presymptomatic HD mice is determined by the synaptic/extrasynaptic NMDAR ratio, our results would imply that this intrinsic MSN abnormality may also be regulated in a non-cell-autonomous manner.

Mitochondrial dysfunction has been identified in HD patients and a variety of HD cell and mouse models (Stahl and Swanson, 1974; Brennan et al., 1985; Mann et al., 1990; Butterworth et al., 1998; Sawa et al., 1999; Tabrizi et al., 2000; Panov et al., 2002; Bae et al., 2005; Benchoua et al., 2006; Solans et al., 2006; Fukui and Moraes, 2007). While no complex I deficiency was found in the frontal and parietal cortices or the cerebellum of HD-patient brains (Browne et al., 1997), other clinical studies showed down-regulated mRNA levels of 12 subunits of complex I in HD brains (Weydt et al., 2006) and decreased complex I activity in platelets and muscle tissues of HD patients (Parker et al., 1990; Arenas et al., 1998). A significant reduction in complexes II and III activity was reported in both HD caudate and putamen, and decreased complex IV activity was also observed in HD putamen (Gu et al., 1996; Browne et al., 1997).

The application of 3-NP (3-nitropropionic acid), an irreversible complex II inhibitor, causes striatal cell loss *in vivo* in rodents, which is ameliorated by decortication or an NMDAR antagonist (Beal et al., 1993). In HD, mitochondrial dysfunction may arise from intrinsic abnormalities of transcription, including of PGC-1α (peroxisome-proliferator-activated receptor γ co-activator-1α), Ca^{2+} overload, NMDAR dysfunction and/or abnormal dopaminergic activity. In primary striatal cultures, dopamine regulates mitochondrial complex II catalytic activity and determines vulnerability of mhtt-expressing neurons to cell death (Benchoua et al., 2008). Our results imply that cell-autonomous abnormalities are sufficient to cause mitochondrial complex II dysfunction in the absence of a decrease in PGC-1α (Thomas et al., 2011), but that this level of decrease in complex II function in presymptomatic mice is not sufficient to cause enhanced sensitivity to excitotoxicity.

As noted above, our results suggest that resistance to excitotoxicity in symptomatic HD mice may be largely mediated by cell-autonomous mechanisms. Aging causes decreased NMDA and AMPA currents and reduced cortical input in MSNs, providing a natural protection from excitotoxicity (Cepeda et al., 1996, 2010; Graham et al., 2009). Indeed, the striatum becomes increasingly resistant to QA excitotoxicity in FVB and C57BL/6J mice (Hansson et al., 2001; Zeron et al., 2002; Graham et al., 2006, 2009). We also showed *in vivo* that striatal neuron-specific expression of mhtt is sufficient to cause an even greater reduction in NMDA current in symptomatic DE5 mice than in non-transgenic aged mice, implying that MSNs expressing mhtt may undergo further cell-autonomous disruption of NMDARs and downstream signalling in an age-dependent manner. It is hypothesized that with HD disease progression, there is a gradual corticostriatal disconnection (Cepeda et al., 2007; Papadia and Hardingham, 2007; Leveille et al., 2008; Joshi et al., 2009), which could contribute to the suppressed excitotoxic response in symptomatic HD mice since induction of an *in vivo* excitotoxic lesion requires the integrity of corticostriatal pathways (McGeer et al., 1978). Our electrophysiological data did not show any decrease of EPSCs in the DE5 mice suggesting that glutamate synaptic transmission is not altered in mice expressing mhtt in MSNs only. IPSCs were not changed either, in contrast to other studies showing an increase of GABA synaptic activity in several models of HD (Cepeda et al., 2004). This indicates that the absence of cortical mhtt expression prevents abnormalities of corticostriatal synaptic function, and thus no true corticostriatal disconnection may occur in symptomatic DE5 mice. In contrast, MSNs showed alterations in some membrane properties (increased input resistance), and postsynaptic currents similar to pan-neuronal models of HD, suggesting those changes are cell-autonomous and might be sufficient to induce motor dysfunction (Brown et al., 2008).

Even without any alteration or loss of glutamatergic input, abnormal postsynaptic receptors are likely sufficient to affect the physiology and excitability of MSNs. Further work is required to determine the number, composition and location of NMDARs in DE5 MSNs. In addition, the enhanced mitochondrial complex II respiration observed in symptomatic DE5 mice may contribute to the paradoxical cell-autonomous resistance to QA-induced excitotoxicity. Sustained over-expression of mitochondrial complex II protein subunits in cultured striatal neurons expressing htt-N171-82Q restores complex II activity and suppresses mitochondrial dysfunction and neuronal cell death (Benchoua et al., 2008). The increased complex II activity in the symptomatic DE5 striatum may be due to up-regulated PGC-1α expression (Thomas et al., 2011), which protects against mitochondrial dysfunction and mhtt-induced striatal toxicity by controlling many mitochondrial genes (Cui et al., 2006). In contrast, the striatum of symptomatic HD N171-82Q mice and HD patients showed decreased PGC-1α and its downstream targets (Cui et al., 2006; Weydt et al., 2006). PGC-1α is regulated by CREB

(cAMP-response-element-binding protein), deactivation of which occurs by extrasynaptic NMDAR stimulation. Thus, imbalance between synaptic and extrasynaptic NMDAR signalling could also contribute to the induction of excitotoxicity via mitochondrial dysfunction. Again, however, in view of opposite effects on PGC-1α level in pan-neuronal and DE5 mice, changes in this molecule alone are unlikely to account for the acquired resistance to excitotoxicity.

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