Increased calbindin-D_{28k} immunoreactivity in striatal projection neurons of R6/2 Huntington’s disease transgenic mice

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Striatal degeneration in Huntington’s disease (HD) is associated with increases in perikaryal calbindin immunolabeling in yet-surviving striatal projection neurons. Since similar increases have also been observed in surviving striatal projection neurons after intrastrial injection of the excitotoxin quinolinic acid, the increased calbindin in HD striatum has been interpreted to suggest an excitotoxic process in HD. We used immunolabeling to assess if calbindin is elevated in striatal projection neurons of R6/2 HD transgenic mice. These mice bear exon 1 of the human huntingtin gene with 144 CAG repeats and show some of the neuropathological signs (e.g., neuronal intranuclear inclusions) and clinical traits (e.g., wasting prior to early death) of HD. We found an increased frequency of calbindin-immunoreactive neuronal perikarya in the striatum of 6- and 12-week-old R6/2 mice compared to wild-type controls. This increase was most notable in the normally calbindin-poor dorsolateral striatum. We found no significant changes in the total area of striatum occupied by the calbindin-negative striosomes and no consistent changes in striatal calbindin mRNA. The increase in calbindin in R6/2 striatal neurons was thus limited to the matrix compartment, and it may be triggered by increased Ca^{2+} entry due to the demonstrated heightened NMDA sensitivity of these neurons. The data further support the similarity of R6/2 mice to HD, and are consistent with the occurrence of an excitotoxic process in striatum in both.

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Introduction

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disease characterized by extensive loss of neurons in the striatal part of the basal ganglia (Ross et al., 1996; Vonsattel and DiFiglia, 1998). The HD gene contains a polymorphic trinucleotide CAG repeat in exon 1 that is expanded beyond 36 repeats in HD patients (The Huntington’s Disease Collaborative Research Group, 1993). This gene encodes a protein termed huntingtin, which is widely expressed in nervous system and thought to be a membrane-associated scaffolding protein that contributes to protein–protein interactions mediating diverse intracellular functions, most notably vesicular trafficking (DiFiglia et al., 1995; Takano and Gassela, 2002). The means by which the mutant protein causes preferential damage to striatal projection neurons in HD is uncertain, although it is thought that the process involves an adverse gain of function (Ross, 1997; Reiner et al., 2003). The work of DiFiglia et al. (1997) showing that mutant huntingtin accumulates in the brains of HD patients in ubiquinated aggregates in neuronal nuclei and in dystrophic neurites, which are evident by light microscopy in neurons of cerebral cortex and striatum, led to the suggestion that mutant protein aggregation might be involved in the pathogenic process. It is currently uncertain, however, if the light microscopically evident aggregates themselves are pathogenic or whether it is smaller oligomers of mutant huntingtin that are pathogenic (Saudou et al., 1998; Szekenyi et al., 2003; Arrasate et al., 2004).

Calbindin-D_{28k} (CALB) is a calcium-binding protein found in projection neurons of the matrix compartment of striatum (Gerfen and Wilson, 1996). By buffering Ca^{2+}, CALB is thought to spatially and temporally restrict calcium-mediated signaling at sites of Ca^{2+} entry stemming from cell membrane depolarization (Morris et al., 1995; Pickel and Heras, 1996), and some authors have suggested its presence may protect striatal projection neurons against excitotoxic injury (DiFiglia et al., 1989; Figueredo-Cardenas et al., 1998). Increased CALB immunoreactivity has been reported in the soma and dendritic spines of surviving striatal projection neurons in Huntington’s disease (HD) patients (Ferrante et al., 1991; Huang et al., 1995). In an experimental HD model in rats created by intrastrial administration of the NMDA receptor agonist quinolinic acid (QA), a similar finding was observed (Huang et al., 1995). The increase in CALB immunoreactivity in striatal projection neurons in the QA rat model of HD and in HD itself has been suggested to possibly represent a protective response to excess glutamatergic stimulation, from QA in the former and from disturbed glutamatergic corticostriatal transmission in the latter (Ferrante et al., 1991; Huang et al., 1995).

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More recently, transgenic HD models have been introduced and studied to gain insight into HD pathogenesis. The most extensively studied of these is the R6/2 mouse, which bears exon 1 of the HD gene with 144 CAG repeats (Mangiarini et al., 1996). These mice are reported to show HD-like behavioral abnormalities beginning at 9 to 11 weeks, and typically die between 12 and 15 weeks. The gross morphology of the brain in R6/2 mice, however, appears normal, and overt neuronal loss is not evident in cortex or striatum. Ubiquitinated neuronal intranuclear inclusions containing mutant protein are, however, widespread throughout the brain by 12 weeks of age (Davis et al., 1997; Morton and Leavens, 2000b; Meade et al., 2002), and degenerative changes, notably neuronal darkening in histopathologically prepared specimens, occur in the striatum, cingulate cortex, and hippocampus of R6/2 mice (Turmaine et al., 2000; Iannicola et al., 2000). Consistent with the possibility that a subtle degenerative process resembling that in HD affects striatum in R6/2 mice, down-regulation of mRNA and/or protein for various striatal projection neuron markers that are also reduced in HD, such as preproenkephalin (PPE), D1 and D2 dopamine receptors, and cannabinoid receptors (Reiner et al., 2003; Richfield and Herkenham, 1994; Richfield et al., 1995; Glass et al., 2000; Deng et al., 2004), is also seen in R6/2 mice (Cha et al., 1998; Luthi-Carter et al., 2000, 2002; Bibb et al., 2000; Denovan-Wright and Robertson, 2000; Menalled et al., 2000; Sun et al., 2003).

Several studies raise the possibility that an excitotoxic process caused by excess corticostriatal glutamate release and/or increased sensitivity of striatal projection neurons to NMDA receptor activation might contribute to the neurochemical pathologies of striatal projection neurons in R6/2 mice (Cha et al., 1998; Levine et al., 1999; Cepeda et al., 2001, 2003; Klapstein et al., 2001; Rebec et al., 2002; NicNiocaill et al., 2001). Because of the evidence for an excitotoxic process in R6/2 mouse striatum, and the evidence that the striatal injury process is associated with the elevation of CALB in surviving striatal projection neurons in HD and the rat QA model of HD, we used immunohistochemistry and in situ hybridization histochemistry to determine if CALB is elevated in striatal projection neurons in R6/2 mice as well.

**Materials and methods**

**Animals and genotyping**

Breeder R6/2 mice were initially obtained from Jackson Labs (Bar Harbor, ME). A colony was then maintained at the University of Tennessee by breeding R6/2 mice with CBA × C57BL/6 F1 (B6CBAF1) mice, thereby obtaining mice that were either R6/2 heterozygotes or wild-type littermates. Genotyping was carried out using a PCR-based method modified from Mangiarini et al. (1996), as detailed previously (Meade et al., 2002).

**Immunohistochemistry for Calbindin-D28k**

Eight R6/2 mice and eight wild-type mice of 12 weeks of age, and five R6/2 mice and five wild-type mice of 6 weeks of age were perfused through the left ventricle with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) at pH 7.4.Brains were removed and postfixed overnight at 4°C, stored for 24 h in 20% sucrose/10% glycerol at 4°C, and sectioned frozen at 40 μm in the transverse plane using a sliding microtome. The sections from each animal were sequentially distributed into 6 vials so that each vial contained a full series of sections adjacent to that of neighboring vials. Selected sections were incubated with gentle agitation for 72 h at 4°C in a mouse anti-CALB antibody (Sigma Chemicals, Inc.) diluted with 0.1 M PB-0.02% sodium azide-0.3% Triton X-100 (1:500). This antibody has been used by us in previous studies and is specific for CALB (Celio, 1990; Huang et al., 1995; Figueroa-Cardenas et al., 1998). Incubations in secondary antiserum for 2 h and PAP (peroxidase–anti-peroxidase) for 2 h were carried out at room temperature. Sections were rinsed between secondary and PAP incubations in three 5-min rinses with PB. After PAP incubation and PB rinses, sections were incubated for 10 min in a 50-ml solution of 0.05 M imidazole/0.05 M sodium cacodylate (pH 7.2) containing 50 mg of dianinobenzidine tetrahydrochlo rode (DAB). The sections were incubated for an additional 10 min after adding 200 μl of 3% hydrogen peroxide to the DAB incubation solution. Sections were then rinsed several times in distilled water, immersed in 0.1 M PB, mounted onto gelatin-coated slides, dried, dehydrated, and coveredslipped with Permount®. Sections adjacent to those immunolabeled for CALB were stained with cresyl violet to visualize all striatal neurons. The immunolabeling procedures used are described in more detail in Figueroa-Cardenas et al. (1998).

**In situ hybridization histochemistry for Calbindin-D28k mRNA**

Dr. Robert M. Sapolsky of Stanford University kindly provided a plasmid containing a 1117-bp fragment of rat CALB cDNA (Meier et al., 1997), which we used to generate cRNA probes for autoradiographic in situ hybridization histochemistry (ISHH). We linearized the plasmid with EcoRI and transcribed 35S-UTP-labeled cRNA probes with T7 RNA polymerase. The in vitro transcription for CALB cRNA was carried out using the Promega Riboprobe Combination System (Cat. No. P1460) following the manufacturer's protocol. The 35S-labeled cRNA probes were about 830 bases in length. After in vitro transcription, the probes were precipitated in 2.5 μl of 4 M LiCl and 75 μl of 100% ethanol in dry ice for 30 min, and centrifuged at 4°C at 14,000 rpm for 30 min. The pellets were washed with 70% ethanol, and resuspended in 50 μl of nuclease-free H2O. The concentration of labeled probe used for hybridization was 20,000 cpm/μl, diluted in a hybridization buffer that contained 2× SSC (saline sodium citrate), 50% deionized formamide, 10% dextran sulfate, 1× Denhardt’s solution, 250 μg/ml yeast tRNA, 500 μg/ml salmon sperm DNA, and 50 mM DTT.

Four R6/2 mice and four wild-type mice of 12 weeks of age, and three R6/2 mice and three wild-type mice of 6 weeks of age were used for CALB ISHH. The mice were anesthetized with halothane, decapitated, and the brains removed. The brains were then frozen and sectioned at 20 μm using a cryostat, and collected onto Superfrost®/Plus slides. The slide-mounted sections were then fixed in fresh 4% parafomaldehyde in 0.1 M sodium phosphate-buffered saline for 5 min, rinsed, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride for 10 min, briefly rinsed in 70% ethanol followed by 95% ethanol, and air-dried. The sections were covered with the hybridization buffer containing the labeled probes, and kept in a moist slide box in an incubator at 55°C overnight. After hybridization, the sections were briefly washed in 2× SSC/50% formamide. The sections were then digested with 20 μg/ml RNase A in PB at 37°C for 1 h. After RNase A digestion, the sections were rinsed twice for 15 min at 55°C in 2× SSC/50% formamide. The sections were briefly washed in distilled H2O, dehydrated in 70% and 95% ethanol, and
air-dried. The sections were then exposed in the dark to Amersham Hyperfilm for 20 days. Films were then developed in Kodak D-19 developer and fixed in Kodak fixer to visualize hybridization signals. The sections were subsequently coated with Ilford-K5D emulsion and kept in the dark for an additional 20 days. The emulsion-coated sections were then developed in Kodak developer, fixed in Kodak fixer, lightly stained with cresyl violet, air-dried, and coverslipped with Permount®.

Determination of calbindin-D28k immunoreactive neuron frequency in striatum

Striatal CALB-immunoreactive neuron frequency was determined from camera lucida drawings of central and dorsolateral striatum at a level rostral to the anterior commissure (Fig. 1). CALB-immunostained neurons in a representative section for each immunolabeled case were drawn unilaterally for central and dorsolateral striatum at 400× magnification, as were neurons in adjacent cresyl violet-stained sections (Fig. 1). The drawn neurons were counted for a 0.096 mm² area that did not contain striosomes, and the abundance of CALB-immunoreactive and cresyl violet-stained neurons was expressed on a per square millimeter basis. The abundance of CALB-immunoreactive neurons was then expressed as percent of cresyl violet-stained neurons in each individual section.

Size of Calbindin-D28k-negative striosomes in striatum

The striatum is divided into two interwoven but neurochemically and hodologically distinct compartments called the patch and matrix (Gerfen et al., 1985; Graybiel, 1990; Prens et al., 1999). The patch compartment is also known as the striosomal compartment. Among the distinguishing neurochemical traits of the striosomal and matrix compartments is that the neuropil and perikarya of the former largely lack CALB, while the neuropil and perikarya of the latter are rich in CALB. We evaluated the possibility that the differential localization of CALB in the perikarya of these two compartments might be altered in the R6/2 mice. The outline of the striatum and the CALB-negative striosomes within it were drawn using a 10× objective for each immunolabeled R6/2 and wild-type case, at a level rostral to the anterior commissure. The drawings were then scanned using a ScanMaker III (Microtek), and transformed into PhotoShop TIFF files. Using NIH Image 1.6.1, the total CALB-negative striosomal area in each striatal outline was then measured, as was the total striatal area for that section. The percent of striatum occupied by striosome could thereby be determined.

Measurement of ISHH labeling intensity for striatal calbindin-D28k mRNA in film autoradiograms

ARG film images of individual sections were captured using an Olympus BH-2 microscope with a 1× objective, a Newvicon video camera, and the program NIH Image 1.6.1. This magnification allowed capture of the entire striatal region of interest. Image capture was carried out using standardized videocamera settings and light levels, so as to achieve a standard film background pixel value of 100 on a 0 (white) to 255 (black) grayscale. The striatum was divided into dorsolateral, dorsomedial, ventrolateral, and ventromedial quadrants using the marquee tool. The mean optical density value in each quadrant on a 0–255 scale was determined using measurement tools available in NIH Image. One representative film image of rostral striatum was chosen for image analysis for each animal used for ISHH.

Determination of frequency of striatal neurons expressing calbindin-D28k mRNA

The radioactively labeled neurons in the emulsion-coated sections could be readily identified with light microscopy using a 20× objective. Radioactively labeled and cresyl violet-labeled neurons in the central and dorsolateral striatum in emulsion-coated sections labeled by ISHH for CALB mRNA were drawn using camera lucida. The criterion for considering a neuron as specifically labeled with silver grains was a fourfold greater grain abundance over the neuronal perikaryon than over the neuropil adjacent to the perikaryon. This criterion has been commonly employed in prior ISHH studies (Albin et al., 1991; Chesselet et al., 1987; Sun et al., 2003). The drawn neurons in the central and dorsolateral striatum were counted for a 0.38 mm² area, and the abundance of CALB mRNA-expressing and cresyl violet-stained neurons was expressed as the number of neurons per square millimeter. The abundance of CALB mRNA-expressing neurons was then expressed as a percent of cresyl violet-stained neurons in each individual section.

Statistical analysis

Statistical comparisons between age-matched R6/2 and wild-type mice and between genotype-matched mice at 6 and 12 weeks of age were made using ANOVA, followed by Fisher’s PLSD for individual comparisons, for the percent of striatal neurons immunolabeled for CALB, for the striatal labeling intensity for CALB mRNA levels by film ISHH, and for the frequency of CALB mRNA-expressing striatal neurons by emulsion autoradiography. Statistical comparisons between R6/2 and wild-type...
mice at 12 weeks of age for CALB-negative striosomal size were made by a two-group, two-tailed Student’s t test. A $P < 0.05$ was considered statistically significant in all cases. Error bars in graphs are standard errors of the means.

**Results**

**Calbindin-D$_{28k}$-immunoreactive neurons in R6/2 striatum**

More CALB-immunoreactive neuronal perikarya appeared to be present in the R6/2 striatum at 12 weeks of age than in the age-matched wild-type striatum. This was evident upon microscopic examination of the sections and in camera lucida reconstruction of the distribution and abundance of the calbindinergic perikarya (Figs. 2 and 3). The higher apparent abundance of CALB-immunoreactive neurons in R6/2 striatum was especially striking in the dorsolateral somatosensory sector of striatum (Fig. 2A), where CALB-immunoreactive neurons are sparse in wild-type mice (Fig. 2B). Cell counts for the central (square A, Fig. 1) and dorsolateral (square B, Fig. 1) striatum confirmed that CALB-immunoreactive striatal neurons were more common in R6/2 mice than in age-matched wild-type mice (Table 1). At 12 weeks of age, 63.3% of neurons in central striatum in R6/2 mice were immunolabeled for CALB, while in wild-type mice, only 43.5% did. This 45.5% increase in R6/2 mice was statistically significant. Similarly, a significantly higher percentage of neurons in dorsolateral striatum (88.3% more) were CALB-immunoreactive in R6/2 (40.3%) than in wild-type mice (21.4%) at 12 weeks of age (Table 1).

A comparable increase in CALB-immunoreactive neuronal perikarya was already evident in R6/2 mice at 6 weeks of age (Table 1). In central striatum, CALB-immunoreactive neurons represented 43.2% of all striatal neurons in 6-week-old R6/2 mice, and only 27.2% in wild-type mice, a significant 58.8% increase for R6/2 mice. In dorsolateral striatum of 6-week-old R6/2 mice, CALB-immunoreactive perikarya represented 24.1% of all striatal neurons, but only 15.8% in wild-type mice, a 52.3% difference that was not statistically significant for this sample. The results for the 6-week-old mice suggest that the increase in striatal CALB-immunoreactive neurons found in 12-week-old R6/2 mice had largely already occurred by 6 weeks of age and was retained at 12 weeks (Table 1). The overall frequency of CALB-immunoreactive perikarya tended to be greater at 12 weeks than 6 weeks for both striatal regions examined, within genotype, and the difference was significant for the central striatum of wild-type rats and both dorsolateral and central striatum of R6/2 mice. Thus, an age-related increase in the percent of striatal neurons that were CALB-immunoreactive was also evidenced in our data.

**Calbindin-D$_{28k}$ localization in R6/2 striosomes**

To ascertain if the increase in CALB-immunolabeled perikarya in R6/2 striatum included striosomal neurons, which are devoid of CALB-immunoreactive perikarya in wild-type mice, we examined the size of the CALB-negative striosomal compartment in 12-week-old R6/2 mice. We found that the size of the striosomal compartment in R6/2 mice was indistinguishable from that in wild-type mice (Fig. 4). For example, striosomes occupied 7.8% of striatum in R6/2 mice ($n = 8$) and 6.2% in wild-type mice ($n = 8$), which was not significantly different ($P = 0.2$) (Fig. 5). Moreover, CALB-immunoreactive perikarya were absent from the CALB-poor striosomes of R6/2 mice, as they are in wild-type mice.
Thus, striosomal neurons do not show increased CALB immunoreactivity, and the increased CALB immunoreactivity appears limited to the perikarya of the striatal matrix compartment. Similar results were qualitatively evident in the 6-week-old mice.

Calbindin-D28k in situ hybridization histochemistry in R6/2 striatum

We examined striatal CALB mRNA levels in R6/2 mice to see if the increased CALB immunoreactivity in R6/2 striatum was due to increased CALB transcription. The levels of CALB mRNA seen on autoradiographic films, as shown in Fig. 6, were similar in R6/2 (Fig. 6A) and wild-type mice (Fig. 6B). Quantitative analysis confirmed that there was no significant difference in signal intensity for the entire striatum between R6/2 mice and wild-type mice at 12 weeks of age in the film autoradiograms (Fig. 7). The labeling intensity for each striatal quadrant was also statistically indistinguishable between 12-week-old R6/2 and wild-type mice (Fig. 7A), though dorsolateral striatum for both genotypes was lower in CALB mRNA signal and ventromedial striatum higher in CALB mRNA signal than the other quadrants. Similar results were obtained for the striatal CALB mRNA signal in 6-week-old R6/2 and wild-type mice (Fig. 7B).

We then used the emulsion autoradiograms to assess the percent of striatal neurons expressing CALB mRNA in 12-week-old and 6-week-old R6/2 and wild-type mice (Table 1, Fig. 8). In central striatum of 12-week-old mice, the frequency of CALB mRNA-expressing neurons in R6/2 mice (37.9%) was significantly less (24.5% less) than that in wild-type mice (50.2%). The frequency of CALB mRNA-expressing neurons in dorsolateral striatum in R6/2 mice (22.6%) was also less than in wild-type mice (33.2%), although this difference was not statistically significant. In 6-week-old mice, 24.1% of neurons in central striatum in R6/2 mice possessed detectable CALB mRNA, while 27.1% did in wild-type mice. Similarly, 16.9% of striatal neurons possessed detectable message for CALB in dorsolateral striatum in R6/2 mice, and 23.2% did in wild-type mice. There was no significant difference between the 6-week-old R6/2 and wild-type mice for either striatal region (Table 1). These findings with ISHH indicate that the increased CALB immunoreactivity in R6/2 striatum is not due to an increase in CALB mRNA. Striatal CALB mRNA-expressing neuron frequency did, however, tend to exhibit the same overall age-related increase as did CALB perikaryal immunolabeling, within genotype, and the difference was significant for the central striatum of both wild-type and R6/2 mice.

Discussion

Our results show that the proportion of striatal projection neurons that are CALB-immunoreactive is increased in R6/2 mice by 6 weeks of age. The increased frequency of CALB-immunoreactive striatal projection neurons in R6/2 mice was most
prominent in dorsolateral somatosensory striatum, which is normally poor in CALB-containing neurons (Gerfen et al., 1985). The increased CALB in R6/2 striatal neurons was specific to the striatal matrix compartment, since the size of striosomes was unaltered and CALB-immunoreactive perikarya were absent from striosomes. As reported previously by others (Liu and Graybiel, 1992), striatal CALB showed an age-related increase.

The increase in immunodetectable CALB protein in striatal projection neuron perikarya of R6/2 mice was not attributable to increased expression of CALB mRNA. We in fact found that overall striatal CALB mRNA levels, as assessed from the film autoradiograms, were indistinguishable between R6/2 and wild-type mice at either age examined, although the percent of neurons expressing detectable CALB message tended to be slightly lower in R6/2 mice. By contrast, the age-related increase in the frequency of CALB-immunolabeled perikarya was associated with an age-related increase in the percent of neurons expressing CALB mRNA. Microarray analysis confirms no major difference between R6/2 and wild-type striatum in CALB mRNA expression at 6 and 12 weeks (Luthi-Carter et al., 2000, 2002). Increased perikaryal CALB immunoreactivity in surviving striatal projection neurons after QA-mediated excitotoxic striatal injury associated with unaltered CALB mRNA has previously been reported in rats by Huang et al. (1995). To explain the apparent discrepancy between increased CALB immunoreactivity and unaltered message, these authors proposed that dendritic mRNA pools that are not reflected by perikaryal mRNA might contribute to CALB synthesis. This explanation would not apply to our case, since we observed relatively normal (or slightly decreased) CALB mRNA for the striatum as a whole, which would reflect both perikaryal and dendritic CALB mRNA. Huang et al. (1995) also suggested that the increased perikaryal CALB immunoreactivity might reflect a transient prior increase in CALB mRNA. While this could be true in our case, the transient CALB mRNA increase would have had to occur prior to 6 weeks of age in the R6/2 mice. Increased stability of CALB protein or release from an undetectable conformation or intracellular store could also explain the increased perikaryal CALB in R6/2 striatal neurons (Bazzett et al., 1994).

It is unclear whether striatal projection neuron-specific CALB protein itself is increased in R6/2 mice. Previous studies have reported that striatal CALB protein levels in R6/2 mice, by Western blot analysis, are not different or slightly less than in wild-type mice (Hansson et al., 1999; Luthi-Carter et al., 2002). Striatal

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Fig. 5. Histograms comparing the percent of striatal cross-sectional area occupied by striosome in R6/2 mice (A) and wild-type mice (B) at 12 weeks of age for a rostral level of striatum. Analysis was carried out on a level comparable to that shown in Fig. 4.

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Fig. 6. Film images of ISHH labeling for CALB mRNA in transverse sections through rostral striatum, showing that the CALB mRNA level in R6/2 striatum (A) is comparable in its intensity to that in wild-type striatum (B) at 12 weeks of age.
CALB protein, however, is found in both striatal projection neurons and in dopaminergic terminals arising from the substantia nigra (Gerfen et al., 1985; Gerfen et al., 1987; Pickel and Heras, 1996; DiFiglia et al., 1989). Since nigral dopaminergic neurons are abnormal in R6 (R6/1 and R6/2) mice (Hickey et al., 2002; Petersen et al., 2002), projection neuron CALB protein levels could be elevated and masked by a decline in CALB protein of nigral origin.

As noted, increased perikaryal CALB immunoreactivity has been previously reported in striatal projection neurons in QA-injected rats (Huang et al., 1995). They interpreted their findings to indicate that excitotoxic activation of NMDA receptors on striatal projection neurons increases their perikaryal CALB, as shown to be the case for dentate gyrus granule cells activated by perforant path stimulation (Lowenstein et al., 1991). Consistent with the view that heightened excitatory activation of striatal projection neurons increases their perikaryal CALB, mice deficient in D2 dopamine receptor have been found to possess elevated CALB in their cytoplasmic rim (Jung et al., 2000). This effect is likely to be cortically driven, because the enhanced activation of striatal projection neurons upon being freed of the inhibitory effects of D2 receptors depends on cortical input to striatum (Pollack et al., 1999; Campbell and Björklund, 1994). Since a similar increase in CALB in surviving striatal projection neurons was observed by Huang et al. (1995) in HD brains, they suggested that striatal neurons might be subject to excitotoxic injury in HD. The abundance of mutant HD protein in corticostriatal projection neurons and the early abnormalities in corticostriatal axons in HD support the possibility that corticostriatal projection dysfunction could promote excitotoxic injury in HD (Fusco et al., 1999; Sapp et al., 1999).

Several lines of evidence are consistent with the view that the CALB increase we observed in R6/2 mice may also reflect excess cortical activation of striatal projection neurons. First, striatal projection neurons are more sensitive to NMDA receptor agonists in R6/2 mice than they are in wild-type mice (Cepeda et al., 2001, 2003; Klapstein et al., 2001; Levine et al., 1999). Secondly, R6/2 mice at 5–7 weeks of age show enhanced responses to cortical input (Cepeda et al., 2003), and dysregulation of corticostriatal terminal function occurs in R6/1 and R6/2 mice and may favor elevated levels of synaptic glutamate in striatum (Cha et al., 1998; Chen et al., 1999; Behrens et al., 2002; Lievens et al., 2001, 2002; NicNicGallagh et al., 2001; Rebec et al., 2002). Finally, D2 receptor expression is decreased in R6/2 mice (Cha et al., 1998; Chen et al., 1999), and increased perikaryal CALB occurs in striatal projection neurons in mice deficient in D2 receptor expression, however, is found in both striatal projection neurons and in dopaminergic terminals arising from the substantia nigra (Gerfen et al., 1985; Gerfen et al., 1987; Pickel and Heras, 1996; DiFiglia et al., 1989). Since nigral dopaminergic neurons are abnormal in R6 (R6/1 and R6/2) mice (Hickey et al., 2002; Petersen et al., 2002), projection neuron CALB protein levels could be elevated and masked by a decline in CALB protein of nigral origin.

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three.

impact. The similar increase in striatal CALB immunoreactivity in QA excitotoxicity, the available evidence summarized above contributes to this resistance to excitotoxicity, as CALB has been proposed to be protective against excitotoxicity-induced neuron death (Iacopino et al., 1992; Mattson et al., 1991; Rintoul et al., 2001, Tymianski et al., 1993; Figueredo-Cardenas et al., 1998). For example, CALB-positive striatal neurons are more resistant to mild to moderate ischemic insults and to HD than are CALB-negative striatal neurons (Burke and Baimbridge, 1993; Hedreen and Folstein, 1995), and neonatal striatal neurons (which are poorer in CALB) are generally more vulnerable to ischemic injury than adult striatal neurons (Burke and Baimbridge, 1993). In addition, CALB overexpression protects striatal projection neurons against ischemic insults (Phillips et al., 1999; Yenari et al., 2001). Similar results have been obtained for hippocampal neurons (Monje et al., 2001). Moreover, R6/2 striatal neurons have been reported to possess enhanced capacity for buffering cytoplasmic Ca²⁺ (Hansson et al., 2001a). Increased perikaryal CALB, however, may not be the sole basis of the increased resistance of striatum in R6/2 mice to excitotoxic, 3NP, and ischemic injury. The striatal injuries caused by QA injection, mitochondrial toxins, or transient global ischemia are known to depend on the integrity of the cortical input to striatum (Schwarz et al., 1984; Beal et al., 1993; Paschen, 1996), and the corticostriatal projection is known to become impaired in R6/2 mice (Klapstein et al., 2001; Cepeda et al., 2003). Thus, a corticostriatal disconnection might also be a factor in the resistance of R6/2 mice to excitotoxic, 3NP, and ischemic injury.

Irrespective of whether the increase in CALB immunoreactivity in R6/2 striatum contributes to the resistance of these neurons to QA excitotoxicity, the available evidence summarized above suggests that this increase is driven by increased cortical activation of these neurons. The increased activation of striatal neurons may be due either to presynaptic or postsynaptic changes brought about by the HD transgene in these mice, and presumably these events must occur before any corticostriatal disconnection has its putative impact. The similar increase in striatal CALB immunoreactivity in HD patients, rats with intrastriatal QA injection, and R6/2 mice suggests that a similar striatal injury process might occur in all three.

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