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The Group 2 Metabotropic Glutamate Receptor Agonist LY379268 Rescues Neuronal, Neurochemical and Motor Abnormalities in R6/2 Huntington's Disease Mice

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Abstract

Excitotoxic injury to striatum by dysfunctional cortical input or aberrant glutamate uptake may contribute to Huntington's Disease (HD) pathogenesis. Since corticostriatal terminals possess mGluR2/3 autoreceptors, whose activation dampens glutamate release, we tested the ability of the mGluR2/3 agonist LY379268 to improve the phenotype in R6/2 HD mice with 120–125 CAG repeats. Daily subcutaneous injection of a maximum tolerated dose (MTD) of LY379268 (20mg/kg) had no evident adverse effects in WT mice, and diverse benefits in R6/2 mice, both in a cohort of mice tested behaviorally until the end of R6/2 lifespan and in a cohort sacrificed at 10 weeks of age for blinded histological analysis. MTD LY379268 yielded a significant 11% increase in R6/2 survival, an improvement on rotarod, normalization and/or improvement in locomotor parameters measured in open field (activity, speed, acceleration, endurance, and gait), a rescue of a 15–20% cortical and striatal neuron loss, normalization of SP striatal neuron neurochemistry, and to a lesser extent enkephalinergic striatal neuron neurochemistry. Deficits were greater in male than female R6/2 mice, and drug benefit tended to be greater in males. The improvements in SP striatal neurons, which facilitate movement, are consistent with the improved movement in LY379268-treated R6/2 mice. Our data indicate that mGluR2/3 agonists may be particularly useful for ameliorating the morphological, neurochemical and motor defects observed in HD.

Keywords

Huntington's Disease; Therapy; mGluR2/3; Striatum; Akinesia

Introduction

Several lines of evidence suggest that NMDA receptor excitotoxicity contributes to striatal projection neuron loss in HD (Cowan and Raymond, 2006). Notably, striatal NMDA receptor-mediated responses are enhanced in HD transgenic mice prior to onset of motor deficits and neuronal loss (Cepeda et al., 2001; Li et al., 2004; Van Raamsdonk et al., 2005; Andre et al., 2006; Milnerwood et al., 2006; Milnerwood and Raymond, 2007), and

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consistent with this intrastriatal injection of the NMDA receptor agonist quinolinic acid produces a significantly larger lesion in YAC128 and R6/1 than WT mice prior to symptom onset (Hansson et al., 2001; Graham et al., 2006). The enhanced NMDA receptor responses appear to be mediated by NR2B-containing extrasynaptic NMDA receptors, which show increased surface localization and mediate striatal neuronal loss in HD mice (Shehadeh et al., 2006; Fan and Raymond, 2007; Heng et al., 2009). Activation of extrasynaptic NMDA receptors requires glutamate spillover from synaptic sites to extrasynaptic sites (Milnerwood et al., 2010), which appears to occur in HD mice due to increased glutamate release from corticostriatal terminals (Rebec et al., 2006; Cepeda et al., 2007; Joshi et al., 2009), and deficient glial glutamate uptake via Glt1 (Arzberger et al., 1997; Lievens et al., 2001; Behrens et al., 2002; Shin et al., 2005; Hassel et al., 2008). In this regard, the reduced striatal sensitivity to quinolinic acid in older R6 and YAC128 HD mice may reflect a protective adaptation against an ongoing excitotoxic insult caused by the HD mutation (Hansson et al., 1999, 2001; Graham et al., 2009).

The balance between synaptic and extrasynaptic NMDA receptor activation appears to determine whether receptor activation is beneficial or detrimental (Hardingham et al., 2002; Leveille et al., 2008). Of clinical importance, (Okamoto et al. 2009) showed that treatment of YAC128 mice with low but not high dose memantine, which preferentially blocks extrasynaptic NMDA receptors, was ameliorative. High-dose memantine, however, worsened the YAC128 phenotype, presumably due to the blockade of synaptic as well as extrasynaptic NMDA receptors (Okamoto et al. 2009). The adverse effects of synaptic NMDA receptor blockade may, in part, explain why human trials involving blockade of NMDA receptors have demonstrated little therapeutic value (Fan and Raymond, 2007). While the YAC128 mouse study demonstrating benefit of low dose memantine is encouraging for targeting of extrasynaptic NMDA receptors as an HD therapy, the adverse effects of high dose memantine via blockade of synaptic NMDA receptors suggest that management of a therapeutic dose may be difficult.

As an alternative approach for opposing excitotoxic striatal injury in HD, we have studied the benefit of targeting mGluR2/3 glutamate receptors, which are negatively coupled to adenylate cyclase (Schoepp, 2001), with a maximum tolerated dose (20mg/kg) of the group II metabotropic glutamate receptor agonist LY379268 in R6/2 mice. We targeted this class of glutamate receptors, because systemic administration of mGluR2/3 agonists is neuroprotective in animal models of ischemic injury to hippocampus (Kingston et al., 1999) (which is thought to be mediated by excitotoxicity), and against *in vitro* excitotoxic injury to cortical neurons (Bond et al., 1999, 2000; Cai et al., 1999; Kingston et al., 1999). Given the enrichment of these receptors on cortical terminals and the demonstration that mGluR2/3 agonists reduce cortical glutamate release (Lovinger and McCool, 1995; Lorrain et al., 2003), we believed LY379268 offered a means for reducing release of glutamate from corticostriatal and corticocortical terminals, which would diminish injurious activation of extrasynaptic NMDA receptors in striatum, and presumably cortex as well. A prior study examining the benefit of a low dose of LY379268 in R6/2 mice (Schiefer et al., 2004) showed a slight benefit in open field. Our present results show that a higher dose of LY379268 in R6/2 mice provided a significant cortical and striatal neuron rescue, neurochemical normalization of direct pathway striatal neurons, and reversal of the akinesia that stems from the defect in this pathway.

Materials and Methods

R6/2 HD transgenic mice were maintained from JAX founders (Bar Harbor, ME). The repeat length in the mutant transgene had shortened during successive matings at JAX from its original 150 CAG to about 120 CAG, unbeknown to JAX at that time. Our colony was

maintained by breeding R6/2 mice with CBA $\times$ C57BL/6 F1 (B6CBAF₁) mice, thereby obtaining heterozygous mutants and wild-type (WT) offspring. Genotype and CAG-repeat length were determined by PCR-based amplification using genomic DNA extracted from tail biopsies (Dragatsis et al., 2009). The genotype analysis was carried out by the Laragen Corporation (Culver City, CA). Five sets of mice were studied: 1) R6/2 and WT mice to determine the maximum tolerated dose (MTD) of LY379268 for the treatment efficacy studies (n=23); 2) R6/2 and WT mice treated with vehicle or LY379268 to behaviorally assess LY379268 efficacy (survival, rotarod, and open field) (n=102); 3) R6/2 and WT mice treated with vehicle or LY379268 to produce fixed tissue for histological and immunohistochemical assessment of LY379268 efficacy (volumetric analysis, stereological neuron counts, and immunohistochemical assessment of aggregate formation and striatal projection systems) (n=64); 4) R6/2 and WT mice treated with vehicle or LY379268 to produce unfixed tissue for *in situ* hybridization histochemical (ISHH) assessment of LY379268 efficacy (striatal SP and ENK expression) (n=51); and 5) ten-week old R6/2 mice treated once with 20mg/kg subcutaneous LY379268 to test the acute behavioral effects of MTD LY379268 three hours after the injection (n=4). Animal numbers per group in the behavioral and histological studies are provided in the Results, but typically about 15–20 mice were studied per group in behavioral studies, 15 per group in histological studies on fixed tissue, and 12 per group in ISHH studies. Equal numbers of males and females were used, except in the MTD studies, in which only males were used. Repeat length was 120–125 CAG, and mean repeat length per R6/2 group is provided in the Results.

Maximum Tolerated Dose (MTD)

The treatment dose of LY379268 was determined in an MTD study carried out by us. In phase 1, several increasing doses were tested in two 6-week old WT male mice. The animals were observed 30 minutes before drug administration, and then again at 20, 40, 60 and 90 min after each dose, and again after 20 h. A 10 mg/kg dose was tested first, since the literature indicated this as effective and safe (Bond et al., 1999), followed on subsequent days by 20, 30, 40, and 60mg/kg. The observations included body temperature and neurological evaluation (as per protocol provided by PsychoGenics Inc. Tarrytown, New York), and the procedure yielded a rough MTD estimate of 20mg/kg for more detailed phase 2 testing. In phase 2, five different dosage groups of 8-week old WT mice (three per treatment group) were treated daily with LY379268 (0, 10, 20, 30, 40mg/kg) for 2 weeks. R6/2 groups of three mice each were also tested at the two highest doses (30 and 40mg/kg) to determine their tolerance of these doses. Mice were weighed and evaluated twice a week for signs of toxicity. We found that 20mg/kg was the MTD, as higher doses yielded hypoactivity and stereotyped grooming, particularly in R6/2 mice. Mice used for behavioral or histological assessment of LY379268 thus received either 20mg/kg LY379268 or vehicle (physiological saline) daily at about noon by subcutaneous injection on their rump, beginning during the fourth week of life after genotyping and group assignment. A small subset used in survival and rotarod testing received 10mg/kg LY379268 daily. Subcutaneous delivery was used, since Melior Discovery (Melior Discovery Inc., Exton, PA) had ascertained this route effective in achieving high blood and brain levels of drug. Prior published studies had shown brain and plasma retention of LY379268 of about 10% by 24 hours after 10mg/kg subcutaneous delivery, with the brain concentrations (30nM) adequate for receptor activation (Bond et al., 1999; Imre, 2006). Analyses conducted by Melior Discovery for the currently used batch of LY379268 at a subcutaneous dose of 10mg/kg showed a similar result for brain concentration 24 hours after injection. Consistent with this, systemic delivery of 3–15 mg/kg LY379268 has been shown to modulate neuronal activity *in vivo* (Zhai et al., 2003; Zhao et al., 2006). Thus, the daily MTD used here is expected to have achieved sustained concentrations above those needed for receptor activation. Note that

we found that the brain concentration achieved with 20mg/kg but not 10mg/kg provided survival and motor benefit.

Behavioral Studies

Male and female mice were run in behavioral studies, until death in the case of R6/2 mice or the 18th week in the case of WT mice (by which time all R6/2 mice had died). All mice were weighed daily, and maintained according to best practice standards for mouse care, which involve feed and environmental enrichment, and group housing (Carter et al., 2000; Hockly et al., 2003). Mutant mice were fed Purina Lab Diet 5001 (as a wet mash) placed on the cage bottom as symptoms developed, which allows them to more easily feed as they become neurologically impaired. An environmentally enriched cage contains a transparent MouseTunnel (BioServ # K3323), a Petite Gumabone (BioServ # K3214), and shredded paper.

Survival, Weight and Rotarod—We studied lifespan in 32 R6/2 mice treated daily with 20mg/kg LY379268 (16 males, 16 females), 17 R6/2 mice treated daily with vehicle (9 males, 8 females), and 13 R6/2 mice treated daily with 10mg/kg LY379268 (5 males, 8 females). Rotarod analysis was carried out on these same mice using a San Diego Instruments rodent rotarod (San Diego, CA). For rotarod, RPM increased from 0 to 30 over a four-minute period, and 30 RPM was then maintained for another 2 minutes. Weekly rotarod testing commenced in the fourth week of life. The first rotarod session was a 3-trial training session, and all subsequent rotarod tests were 2-trial sessions. Time to fall was the performance measure. WT mice were treated daily with vehicle (7 males, 8 females), 10mg/kg LY379268 (3 males, 2 females) or 20mg/kg LY379268 (8 males, 11 females). Mice were weighed daily, and a weekly average calculated.

Open Field—We conducted weekly 30-minute open field tests, using a Noldus EthoVision video tracking system (Noldus Information Technology, The Netherlands), and the SEE analysis software of (Drai and Golani 2001). The circular arena had a 200 cm diameter, with a non-porous gray floor and a 50 cm gray wall, which provided contrast for video tracking of the mice. SEE dichotomizes mouse movements into lingering episodes and progression segments, and also distinguishes movements or lingering near the wall from those in the arena center (center defined as >15 cm from wall). SEE provides about 30 endpoints related to locomotion, anxiety, and navigation, and is robust in identifying differences among mouse strains (Drai et al., 2000; Drai and Golani, 2001; Kafkafi et al., 2001, 2003; Lipkind et al., 2004). Four groups were analyzed: 1) 15 R6/2 mice treated daily with 20mg/kg LY379268 (7 males, 8 females); 2) 13 R6/2 mice treated daily with vehicle (7 males, 6 females); 3) eighteen WT mice treated daily with 20mg/kg LY379268 (7 males, 11 females); and 4) 15 WT mice treated daily with vehicle (7 males, 8 females). These mice were also used in survival and rotarod studies. We report here on those open field parameters showing a clear R6/2 – WT difference.

Statistical Analysis—Body weight, rotarod and open field data were analyzed using SAS software and a mixed-model repeated measures ANOVA over weeks 5 to 17, considering genotype, drug, gender and their interactions. In some cases, effects were not seen over this entire period, but seemed evident for part of the period. In those cases, ANOVA was separately carried out for the test period over which the graph suggested benefit might be present.

Histological Studies

Fixed Tissue and Immunohistochemical Studies—Histological analysis was carried out on fixed tissue to determine the effects of LY379268 on: 1) telencephalic, cortical,

striatal and lateral ventricular volumes; 2) cortical and striatal neuron abundance; 3) neuronal intranuclear inclusion (NII) size and abundance in cortex and striatum; and 4) the neurochemical integrity of striatal projection systems. For these studies, 10-week old R6/2 mice and WT mice that had been injected daily with either vehicle or MTD LY379268 since the fourth week of life were deeply anesthetized (avertin; 0.2 ml/g body weight), and perfused transcardially with 40 ml of 0.9% NaCl in 0.1 M sodium phosphate buffer at pH 7.4 (PB), followed by 200 ml of 4% paraformaldehyde, 0.1 M lysine-0.1 M sodium periodate in 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed and stored in a 20% sucrose/10% glycerol solution at 4°C until sectioned frozen on a sliding microtome in the transverse plane at 35 µm. Each brain was collected as 12 separate series in 0.1 M PB 0.02% sodium azide, and stored until processed for immunohistochemistry. A one in six series from each mouse was mounted as sectioned, and stained for cresyl violet.

The peroxidase-antiperoxidase (PAP) procedure was employed to visualize a variety of neurochemical features in R6/2 and WT brains (Meade et al., 2002; Reiner et al., 2007; Dragatsis et al., 2009). Immunolabeling for NeuN, recently identified as Fox-3, a new member of the Fox-1 gene family (Kim et al., 2009), was used to detect neuronal perikarya for counting, using a mouse monoclonal anti-NeuN. Immunolabeling was employed to visualize mutant transgene protein aggregated in ubiquitinated neuronal intranuclear inclusions (NIIs) in R6/2 mice (Davies et al., 1997), using a mouse monoclonal antibody against ubiquitin (Chemicon) or a mouse monoclonal antibody against the N-terminus of huntingtin (EM48, Chemicon). The specificity and efficacy of these antibodies has been shown previously (Askanas et al., 1992; Mullen et al., 1992; Wolf et al., 1996; Spillantini et al., 1998; Li et al., 1999; Tekkok and Goldberg, 2001). Substance P (SP) immunolabeling was used to study SP+ striatal projection systems, and enkephalin (ENK) immunolabeling to study ENK+ striatal projection systems. The anti-SP was a rabbit polyclonal antibody (ImmunoStar, Hudson, WI), whose specificity has been documented previously (Figueredo-Cardenas et al., 1994). The anti-ENK used was a rabbit polyclonal antibody against leucine-enkephalin (ImmunoStar, Hudson, WI), whose specificity has also been shown previously (Reiner, 1987; Reiner et al., 2007).

ISHH Methods—We also analyzed brain tissue from vehicle-treated and MTD LY379268-treated 10-week old R6/2 and WT mice (injected daily since the fourth week of life) that had been fresh-frozen processed for ENK and SP mRNA detection by ISHH (Sun et al., 2002; Wang et al., 2006). ISHH was performed on 20µm thick fresh frozen cryostat sections through the striatum. The sections were collected onto pre-cleaned Superfrost®/Plus microscope slides as sectioned, dried on a slide warmer, and stored at –80°C until used for ISHH. To process tissue for ISHH, the slides were removed from –80°C, and quickly thawed and dried using a hair dryer. After fixation with 2% paraformaldehyde in saline sodium citrate (2x SSC) for 5 minutes, the sections were acetylated with 0.25% acetic anhydride/0.1M triethanolamine hydrochloride (pH 8.0) for 10 minutes, dehydrated through a graded ethanol series, and air-dried. Digoxigenin-UTP labeled cRNA probes (i.e. riboprobes) for preproenkephalin (PPE) and preprotachykinin (PPT) were transcribed from plasmids with PPE cDNA or PPT cDNA inserts (817bp and 900bp in size, respectively), generated by us using RT-PCR. Primers for PPE PCR were: Sense: 5'-TTCCTGAGGCTTTGCACC-3', and Antisense: 5'-TCACTGCTGGAAAAGGGC-3'. Primers for PPT PCR were: Sense: 5'-TCGAACATGAAAATCCTCGTGGCC-3', and Antisense: 5'-CACATCATACAATGACTGAAGACC-3'. The PPE riboprobe was directed against nucleotides 312–1128 (GenBank accession number NM_001002927), while the PPT riboprobe was directed against nucleotides 95–994 (GenBank accession number D17584). The sections were incubated with digoxigenin (DIG)-labeled riboprobe in hybridization buffer containing 50% formamide, 4x SSC, 1x Denhardt's solution, 200µg/ml denatured salmon sperm DNA, 250µg/ml yeast tRNA, 10% dextran sulfate, and 20mM dithiothreitol

(DTT) at 63°C overnight. After hybridization, the slices were washed at 58°C in 4x SSC, 50% formamide with 2x SSC (twice), and then 2x SSC, followed by treatment with RNase A (20 µg/ml) for 30 min at 37°C. Sections were then washed at 55°C in 1xSSC, 0.5xSSC, 0.25xSSC, ethanol dehydrated, and air-dried. Digoxigenin labeling was detected using anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (AP), as visualized with nitroblue tetrazolium (NBT) (Roche, Indianapolis, IN). Sections were coverslipped with a 1% gelatin-based aqueous solution.

Microscopic Analysis

Volume Measurements—To evaluate the effect of the R6/2 genotype and MTD LY379268 treatment on the volume of telencephalon, cerebral cortex, striatum and lateral ventricle, blinded image analysis was carried out (Reiner et al., 2007). An image of each section in the one-in-six cresyl violet series mounted for each mouse at the time of sectioning, from therostral telencephalic pole to just behind the anterior commissure, was captured at 4800 dpi using an Epson scanner. The public-domain software NIH ImageJ (<http://rsb.info.nih.gov/ni-image>) was used to measure the areas occupied by the telencephalon from the end of the olfactory bulb to the level just behind the anterior commissure, the cerebral cortex at these same levels, the striatum at these same levels, and the lateral ventricles. The cerebral cortex was measured from the midline to the rhinal fissure, from pial surface to external capsule. The striatum was defined by the contours of the external capsule and globus pallidus, while the boundaries of the lateral ventricles were readily evident. Section thickness and spacing were used to calculate volume from the area measurements. Values for each side of the brain were averaged per animal. Because brain size scales to body size, the smaller size of R6/2 than WT mice and the possible effect of drug on body size complicate interpretation of the impact of the mutation or drug on brain volumes. We therefore present brain size scaled allometrically to body size, achieved by dividing the measured volumes of telencephalon, cortex, striatum, and lateral ventricle by body weight to its 2/3 power, brain size scaling to the 2/3 power of body mass (Lande, 1979).

Stereological Neuron Counts—Blinded neuron counts were carried out for vehicle-treated and MTD LY379268-treated R6/2 and WT mice. A one-in-twelve series of coronal sections immunolabeled for NeuN from the rostral end of striatum to the anterior commissure was used for striatal and cortical neuron counts in each mouse. The same landmarks as used for volumetric analysis were used to define the limits of cerebral cortex and striatum. Unbiased stereological counts of striatal and cortical neurons were obtained using a NeuroLucida Stereo Investigator system (Micro-Brightfield, Colchester, VT). The dissector counting method was used, in which neurons are counted in frames randomly assigned by the software throughout the areas predefined as striatum and cortex.

NII size and abundance—Sections from vehicle-treated and MTD LY379268-treated R6/2 mice that had been immunolabeled for ubiquitin or N-terminal huntingtin were blindly analyzed for NII size and frequency. For each mouse, images were captured using a 40X objective and analyzed using ImageJ. For cortex, NII size and spatial abundance were determined from images through rostral, mid and caudal primary motor cortex (M1), with measurements conducted separately on layers II/III, V, and VI. For NII spatial abundance and size in striatum, measurements were made for dorsomedial, ventromedial, dorsolateral and ventrolateral striatum at rostral, mid-, and caudal striatal levels. To determine NII diameter from the images of anti-ubiquitin or EM48 immunolabeled sections, NIH ImageJ was used to threshold NIIs. Artifacts in the images were removed prior to thresholding, and the size of the thresholded NIIs determined using the Particle Measurement function. The same thresholding method was also used to determine the spatial abundance of NIIs (i.e.

percent of field occupied by NIIs). Note that we have previously shown that anti-ubiquitin and EM48 anti-huntingtin do not differ in their ability to detect NIIs (Meade et al., 2002). Although anti-ubiquitin was used in the majority of cases, some cases yielded better labeling with EM48, and so we used whichever yielded the optimal NII outcome. Because we observed no regional differences in cortex or striatum, the results for each were pooled.

Image analysis of immunolabeling in striatal target areas—To assess injury to striatal projection systems, blinded analysis using ImageJ was carried to determine the extent and immunolabeling intensity of the striatally derived ENK fiber plexus in globus pallidus externus (GPe), and the striatally derived SP fiber plexus in substantia nigra (SN) (Figueredo-Cardenas et al., 1994; Meade et al., 2000; Sun et al., 2002; Deng et al., 2004; Reiner et al., 2007). Images of the entire GPe or SN were captured bilaterally using a 4X objective from the 4–6 sections spanning their rostrocaudal extent in a one-in-twelve series. Immunolabeled fibers in GPe or SN were highlighted, and their area and intensity determined using the automatic thresholding and optical density measuring capabilities of ImageJ. Autothreshold uses the iterative isodata algorithm of (Ridler and Calvard 1978) to set threshold at halfway between foreground and background. To correct for slight variations in image intensity stemming from variations in lighting during image capture and/or from variations in the staining protocol, the background intensity of the captured images was standardized using the editing capabilities of ImageJ to a value of 100 on the 0–255 white-to-black gray scale employed by Image. The region used to set the background intensity was an area of unlabeled white matter within the field of view - the external capsule for GPe, and the cerebral peduncle for substantia nigra. Per mouse means were calculated for each structure and parameter.

ISHH—Densitometric analysis of striatal signal for PPT and PPE mRNA was carried out on the ISHH tissue using ImageJ. For each mouse, an image of a section just anterior to the level of GPe was captured at 4800 dpi using an Epson scanner. Using ImageJ, the area and signal intensity of striatum were measured, and their product taken to reflect striatal message. Both sides of the brain were analyzed and averaged, and used for analysis of group effects.

Statistical Analysis—Data from the histological, immunohistochemical and ISHH studies were analyzed statistically by 3-way or 2-way ANOVA followed by Fisher PLSD individual group comparisons, reciprocal transforming data as need to normalize distributions.

Results

Behavioral Studies

LY379268 at 20mg/kg increased lifespan in R6/2 mice—The 13 R6/2 mice treated with 10mg/kg LY379268 showed no survival benefit compared to the 17 R6/2 mice treated with vehicle (78.7 days for low dose LY379268 vs 80.5 days for vehicle). We thus combined these two sets of mice (and refer to them as control-treated) for analysis of the effect of 20mg/kg LY379268 on survival in R6/2 mice. The mean survival was significantly longer in the 20mg/kg LY379268-treated than the control-treated R6/2 mice (88.5 vs 79.7 days), by the Mantel-Cox logrank test ($p=0.0276$) (Fig. 1A). The effects of daily 20mg/kg LY379268 were similar for males and females (a 9-day benefit), although male lifespan was on average about 4 days less than for females in both the control and 20mg/kg groups. This difference in male versus-female R6/2 lifespan was, however, not significant.

LY379268 at 20mg/kg had no evident effect on body weight—ANOVA did not detect a significant effect of 20mg/kg LY379268 on body weight ($p=0.5353$), or of the drug-genotype interaction ($p=0.4255$) over weeks 4–18 (i.e. the entire test period) (Fig. 1B). ANOVA did, however, detect highly significant effects of gender ($p<0.0001$) on body weight (males heavier than females for both genotypes), and genotype ($p<0.0001$) on body weight (WT heavier than R6/2 for both treatment groups), and a gender-genotype interaction ($p=0.0004$), which appears to reflect a greater weight loss in R6/2 males than females beyond 10 weeks of age. We did detect an effect of drug on body weight for the 14–16 week period ($p=0.009$), but not of the drug-genotype interaction ($p=0.078$). Thus, prolonged LY379268 treatment caused some weight loss for both genotypes. This may reflect a slight dampening effect of LY379268 on feeding, as reported previously (Peters and Kalivas, 2006).

LY379268 at 20mg/kg improved rotarod performance in R6/2 males—WT mice in both treatment groups typically stayed on the rotarod for nearly the entire 360-second session. By contrast, the 17 vehicle-treated R6/2 mice and the 13 R6/2 mice treated with 10mg/kg LY379268 showed the same pattern of steady decline in rotarod performance, and are thus combined as R6/2 control. LY379268 did not notably improve overall rotarod performance in the 32 R6/2 mice treated with a daily 20mg/kg dose (Fig. 2A), with males and females combined. Although ANOVA confirmed a significant effect of genotype on rotarod for the entire testing period ($p < 0.0001$), the combined male-female ANOVA yielded suggestive indication of an effect of drug ($p=0.0699$) and the drug-genotype interaction ($p=0.0656$). We thus examined the LY379268 effect by gender and weeks of life. We noted that in both male and female R6/2 mice rotarod performance was poorer in 20mg/kg LY379268-treated than control R6/2 mice for the initial 5–7 weeks of testing. ANOVA confirmed this observation, showing a significant effect of genotype ($p=0.0001$) and drug ($p=0.0331$) for these weeks. For weeks 9–14, rotarod performance was poorer in control R6/2 males than control R6/2 females. LY379268-treatment did not improve performance in the R6/2 females but did improve performance in R6/2 males (Fig. 2B). ANOVA for weeks 9–14 confirmed these observations, showing a significant effect of genotype ($p=0.0001$), of the genotype-gender interaction ($p=0.0226$), and of the drug-gender interaction ($p=0.0401$). Thus, R6/2 males performed more poorly than females on rotarod, but improved with 20mg/kg LY379268 beyond 8 weeks of life. Female R6/2 mice, however, did not show rotarod improvement with 20mg/kg LY379268.

LY379268 at 20mg/kg improved motor endpoints in open field—As detailed in the following paragraphs, we found a benefit of LY379268 in R6/2 mice on numerous motor parameters in open field. In general, vehicle-treated males were more impaired than vehicle-treated females, and the LY379268 improvement tended to be greater in males than females. Note that the improvements we observed were long-term benefits of LY379268, since acute treatment of four ten-week old R6/2 mice (2 males, 2 females), yielded a severe blunting effect on motor performance 3 hours after injection, with total distance traveled and the motor parameters described below greatly reduced compared to pre-drug performance.

LY379268 normalized total distance traveled in R6/2 mice until 12 weeks: Vehicle-treated R6/2 mice traveled progressively less than WT mice, with 12-week old vehicle-treated R6/2 mice traversing only 35% of WT distance (Fig. 3A). LY379268 significantly increased distance traveled in R6/2 mice above that in vehicle-treated R6/2 mice, and normalized total locomotor activity until about 12 weeks of age. Although both R6/2 males and females showed a decline in distance traveled, and both benefited from LY379268, the decline in vehicle-treated males was slightly more rapid than in vehicle-treated females, and the drug benefit endured slightly longer into the male lifespan. ANOVA confirmed

significant effects of genotype ($p=0.003$), drug ($p=0.0094$) and the drug-genotype interaction ($p=0.0019$) on distance traveled.

LY379268 improves the ability of R6/2 mice to sustain locomotion: One reason that vehicle-treated R6/2 mice showed a decrease in distance traveled is that they traversed shorter distances during their bursts of locomotion (called progression segments) than did WT mice (Fig. 3B). As in the case of total distance traveled, LY379268-treated R6/2 mice performed better than did vehicle-treated R6/2 mice in progression segment length, and were largely normalized until about 12 weeks of age. ANOVA confirmed significant effects of genotype ($p=0.0057$), drug ($p=0.0404$) and the drug-genotype interaction ($p=0.0026$) on progression segment length. The decline in progression segment length appeared to occur slightly more rapidly in vehicle-treated R6/2 males than females. Related to the effects of genotype and drug on progression segment length, we also found that LY379268 largely normalized progression segment duration until the last few weeks, and increased progression segment duration above that in vehicle-treated R6/2 mice at all time points. ANOVA confirmed significant effects of genotype ($p=0.0015$), and the drug-genotype interaction ($p=0.0240$) on progression segment duration.

LY379268 diminishes the tendency of R6/2 mice to pause: Vehicle-treated R6/2 mice paused more frequently per unit distance than did WT mice (Fig. 4A), and spent a greater proportion of the time between progression segments in such pauses (called lingering episodes) (Fig. 4B). Moreover, the total number of stops (i.e., pauses) per unit distance and the proportion of time spent lingering by vehicle-treated R6/2 increased as disease progressed. These pauses contributed to the overall decrease in activity in vehicle-treated R6/2 mice. LY379268 rendered the R6/2 mice near normal on both number of stops and time spent lingering until about 9–10 weeks of age, and even thereafter the LY379268-treated R6/2 mice paused less than did vehicle-treated R6/2 mice. ANOVA confirmed significant effects of genotype ($p=0.0009$), drug ($p=0.0040$) and the genotype-drug interaction ($p=0.0018$) on stops per unit distance for 5–17 weeks. Similarly, ANOVA confirmed an impact LY379268 on lingering time, since significant effects of genotype ($p=0.001$), drug ($p=0.0173$) and the genotype-drug interaction ($p=0.0116$) were seen for 9–17 weeks. A suggestion of a gender effect was observed ($p=0.0526$), with vehicle-treated R6/2 males tending to linger more than females, and tending to show a slightly greater drug benefit.

LY379268 normalizes locomotor speed of R6/2 mice for much of the lifespan: Maximum locomotor speed during progression segments was much less in vehicle-treated R6/2 mice than in WT mice, and progressively worsened (Fig 5A). LY379268 strikingly improved R6/2 mice, with ANOVA showing significant effects of genotype ($p=0.0001$), drug ($p=0.0240$) and the genotype-drug interaction ($p=0.0003$). Similar results were found for median speed, as well. Acceleration to maximum speed in the vehicle-treated R6/2 mice was also persistently less than in WT mice (Fig. 5B). LY379268 exerted a similarly beneficial effect on acceleration to maximum speed, normalizing it for much of R6/2 lifespan. ANOVA confirmed these conclusions for acceleration, since significant effects of genotype ($p=0.0044$), and the genotype-drug interaction ($p=0.0387$) were observed.

LY379268 normalizes the ability of R6/2 mice to locomote in a straight line: Vehicle-treated R6/2 mice had increasing difficulty walking in a straight line (i.e. progression segments were more curved), and LY379268 strikingly normalized R6/2 mice on this parameter for much of their lifespan (Fig. 6A). Vehicle-treated male R6/2 mice showed an earlier deficit than vehicle-treated females, and also benefited more clearly from LY379268. Beyond 10 weeks, path curvature in vehicle-treated R6/2 males was diminished, as the

shorter progression segments in the few surviving vehicle-treated males may have masked their difficulty in walking in a straight line. Beyond 10 weeks, vehicle-treated females began to show increasing difficulty in walking a straight path. ANOVA confirmed that the drug was beneficial, since significant effects of drug ($p=0.0202$), and the drug-genotype ($p=0.034$) interaction were seen for the 10–15 week period. Gender effects on the R6/2 defect and on the efficacy of LY379268 were also evident by ANOVA, with significant effects of gender ($p=0.029$), the gender-drug ($p=0.0367$) interaction, and the gender-drug-genotype interaction ($p=0.035$) for the 10–15 week period.

LY379268 has an anxiolytic effect in R6/2 mice: Vehicle-treated R6/2 mice showed an increasing tendency to occupy the arena center (i.e. showed reduced anxiety) as symptoms progressed, as has been noted by others (Bolivar et al., 2003). LY379268 exacerbated this tendency, especially males, for the 10–13 week period, as confirmed by significant ANOVA results for genotype ($p=0.0043$), drug ($p=0.0177$), the genotype-drug interaction ($p=0.0059$), and the gender-drug-genotype interaction ($p=0.0113$). Thus, for anxiety-related parameters, LY379268 made R6/2 mice even less anxious than vehicle-treated R6/2 mice.

Analysis of Gender Effects in Behavioral Studies—We observed persistent gender effects on the impact of the R6/2 genotype and of treatment with LY379268 – progression was slightly more rapid in males, and males seemed to benefit more greatly from drug as well. This seemed true for survival, rotarod, and numerous open field parameters. To assess this for our overall data, we scored a variety of parameters as to whether vehicle-treated R6/2 males performed more poorly than R6/2 females at 9–10 weeks, and carried out chi-square analysis to determine if the observed frequency of male worse than female significantly deviated from that expected by the assumption of equal genotype impact on males and females. The parameters were survival, weight, rotarod at 9 and 10 weeks, and several motor-related open field parameters at 9 and 10 weeks (distance, progression segment length, progression segment duration, speed, acceleration, time lingering, spatial spread of progression segments, stops per distance, and progression segment curvature). For the 22 resulting comparisons, the vehicle-treated R6/2 males performed significantly more poorly than the vehicle-treated R6/2 females ($p=0.00283$). We similarly scored if performance was improved in LY379268-treated males and females over that seen in the gender-matched vehicle-treated R6/2 mice. Chi-square found that the instances of improved performance with drug for both male and female R6/2 mice significantly exceeded that expected by chance (male, $p=0.00002$; female, $p=0.0105$). The instances in which the male benefit was greater than the female benefit also significantly exceeded that predicted by the assumption of equal benefit in males and females ($p=0.0105$).

Histological Studies

Regional Brain Volume—As expected, the measured telencephalic, cortical and striatal volumes in vehicle-treated R6/2 mice were smaller than in WT mice. Because brain size scales to body size, however, the invariably smaller body size of R6/2 than WT mice complicates interpretation of the impact of the mutation or drug treatment on regional brain volumes. We therefore scaled brain size allometrically to body size, as noted in the Methods section. When expressed this way (Table 1), we found that LY379268 significantly increased overall telencephalic size in male WT mice compared to vehicle-treated male WT mice ($p=0.0095$). The increase in overall telencephalic size in male WT mice with LY379268 treatment appeared to mainly reflect increased cortical volume, since a similarly significant increase in cortical volume was seen in male WT mice with LY379268 treatment ($p=0.0392$), but not female WT mice with LY379268 ($p=0.5119$). LY379268 treatment had no significant effect on striatal or ventricular volume in WT mice. In the case of R6/2 mice, neither cortical, striatal, nor telencephalic volume were significantly different than expected

for mice of that body size (i.e. not different from WT), irrespective of drug treatment and irrespective of gender. Similar results were found when male R6/2 were separately examined, and for female R6/2 in the case of cortical and striatal volumes. LY379268 treatment did, however, significantly increase telencephalic volume ($p=0.0392$) in female R6/2 mice compared to female vehicle-treated R6/2 mice (although neither were significantly different from vehicle-treated WT females). Finally and notably, lateral ventricle size was significantly increased in R6/2 mice to about 200% of WT mice, irrespective of gender or drug treatment (e.g. male vehicle-treated WT versus male vehicle-treated R6/2, $p=0.0067$; male vehicle-treated WT versus male LY379268-treated R6/2, $p=0.00126$). While we did not see significant evidence of striatal shrinkage from the allometrically scaled striatal volumes, it may be that the possibly small magnitude of shrinkage at 10 weeks mitigated our ability to directly detect any such change. The increase in lateral ventricle volume suggests some slight striatal shrinkage did occur in the R6/2 mice, but was not notably abated by LY379268.

Striatal Neuron Counts—We found no significant differences between vehicle-treated and LY379268-treated WT mice in the abundance of striatal neurons at 10 weeks, and there were also no significant WT gender differences (Table 2). In vehicle-treated R6/2 males, striatal neurons were significantly reduced in abundance compared to vehicle-treated WT males (27.8% loss) at ten weeks ($p=0.0239$), but vehicle-treated R6/2 females did not show a significant striatal neuron loss compared to vehicle-treated WT females ($p=0.7958$). Daily treatment with MTD LY379268 completely rescued the striatal neuron loss in the male R6/2 mice at ten weeks, rendering R6/2 males not significantly different from vehicle-treated WT males in striatal neuron abundance ($p=0.6806$). Moreover, striatal neuron abundance in LY379268-treated R6/2 males was significantly greater than in vehicle-treated R6/2 males ($p=0.0042$). LY379268 treatment did not affect the number of striatal neurons in female R6/2 mice.

Cortical Neuron Counts—We found no significant difference between vehicle-treated and LY379268-treated WT mice in cortical neuron abundance at 10 weeks, and there were also no significant WT gender differences. In the case of cortical neuron abundance among R6/2 mice, the results were more complex (Table 2). For example, vehicle-treated R6/2 females possessed significantly fewer (17.3% fewer) cortical neurons than did vehicle-treated WT females ($p=0.0370$). Although vehicle-treated R6/2 males similarly possessed fewer cortical neurons (13.3% fewer) than did vehicle-treated WT males, statistical analysis did not find this comparison significant ($p=0.4226$). Note, however, that vehicle-treated male R6/2 mice actually had fewer cortical neurons than did female vehicle-treated R6/2 mice, but female vehicle-treated WT mice had more cortical neurons than did male vehicle-treated WT mice. Although the difference in WT cortical values between genders was not statistically significant, it nonetheless distorts comparison of mutation and drug effects between genders. Thus, we pooled all WT cortical neuron counts (since they did not significantly differ between treatment groups and genders) to obtain a single estimate of WT cortical neuron abundance for statistical purposes (Fig. 7). Under these circumstances, we found that vehicle-treated male R6/2 mice had significantly fewer cortical neurons (18.4% less) than the pooled WT mice ($p=0.0109$), and vehicle-treated female R6/2 mice had only 12.3% fewer cortical neurons than the pooled WT mice (nearly significant at $p=0.052$). The abundance of cortical neurons in LY379268-treated female R6/2 mice was also significantly less ($p=0.0172$) than in the pooled WT mice (15.5% less), which represented no significant change from the vehicle-treated female R6/2 mice ($p=0.6969$). Thus, cortical neurons trended toward being diminished in vehicle-treated female R6/2 mice, but daily MTD LY379268 did not improve survival of cortical neurons in female R6/2 mice. By contrast, cortical neuron abundance was improved in the LY379268-treated male R6/2 mice, since it

was not significantly different than in the pooled WT mice ($p=0.0989$), but was significantly more than in the vehicle-treated male R6/2 mice ($p=0.0011$). Thus, in the case of cortical neurons as well, neuron loss was greater in vehicle-treated male than female R6/2 mice, and LY379268 rescued the neuron loss in males.

Neuronal Intranuclear Inclusions (NIIs)—One of the hallmarks of R6/2 mice is the presence of NIIs in nearly all neurons of the brain, which is prominent by 10 weeks (Davies et al., 1997; Meade et al., 2002). Since some data suggest that increased sequestration of mutant protein is associated with a beneficial outcome in animal and culture models of HD (Arrasate et al., 2004; Okamoto et al., 2009), we examined if LY379268 treatment altered the size or frequency of NIIs in R6/2 mice. We found that neither the size of NIIs nor their abundance per mm^2 differed between vehicle-treated and LY379268-treated R6/2 mice for either cortex or striatum. Similar results were observed in males and females (Table 3). Thus, we found no evidence that daily MTD LY379268 altered NII formation. NIIs were, however, significantly larger in cortex than striatum across genders and treatment groups by ANOVA ($p=0.0007$), as seen previously (Meade et al., 2002).

ENK Immunolabeling in GPe—We found that striato-GPe fibers appeared slightly more intensely ENK-immunolabeled in LY379268-treated WT mice than in vehicle-treated WT mice (by about 10%) (Table 4), but the difference was not significant for either males ($p=0.2005$) or females ($p=0.0948$). Male vehicle-treated R6/2 mice showed a significant elevation (24.4%) in ENK+ immunolabeling intensity in GPe fibers ($p=0.0050$) compared to male vehicle-treated WT mice. As considered in the Discussion, it is likely that the increased ENK immunolabeling intensity in striato-GPe fibers reflects increased ENK protein in striato-GPe terminals due to diminished release. A comparable increase in ENK intensity in GPe fibers was not seen in the vehicle-treated R6/2 females compared to vehicle-treated WT females. LY379268-treatment did not have a significant impact on ENK immunolabeling intensity in the R6/2 males, since the ENK immunolabeling intensity of striato-GPe fibers was not significantly different in the LY379268-treated R6/2 males than in the vehicle-treated R6/2 males ($p=0.5914$) and remained significantly higher than in the vehicle-treated WT males ($p=0.0207$). Similarly, in females the ENK immunolabeling intensity of striato-GPe fibers in LY379268-treated R6/2 mice did not differ significantly from that in either vehicle-treated R6/2 females ($p=0.3189$) or vehicle-treated WT females ($p=0.7762$). Note that as ANOVA detected a significant drug-genotype interaction ($p=0.0272$), it appears that LY379268 treatment did enhance ENK levels in striato-GPe terminals in WT mice, although this was not evident for the individual gender-matched WT comparisons.

In the case of ENK+ fiber abundance (Table 4), a significant reduction was observed in vehicle-treated R6/2 males (27.8%) compared to vehicle-treated WT males ($p=0.0126$), but not in vehicle-treated R6/2 females compared to vehicle-treated WT females ($p=0.5722$). LY379268 had a beneficial effect on the abundance of ENK striato-GPe fibers in the LY379268-treated R6/2 males, as their abundance was not significantly different than in vehicle-treated WT males ($p=0.1917$). In contrast, ENK fiber area was significantly less (27.9% less) in the LY379268-treated R6/2 females than in vehicle-treated R6/2 females ($p=0.0235$) and WT females ($p=0.0066$). Thus, LY379268 did not benefit and instead diminished ENK striato-GPe fiber abundance in female R6/2 mice. Finally, significantly increased ENK fiber area was observed in WT females treated with LY379268 ($p=0.0406$), perhaps again as a part of the enhancing effect of LY379268 on ENK levels in striato-GPe terminals in WT mice.

SP Immunolabeling in Substantia Nigra—LY379268 increased immunolabeling intensity for SP in substantia nigra (SN) fibers in WT mice by about 10%, with the increased

intensity in females ($p=0.0020$) but not males ($p=0.2886$) significant (Table 5). A more striking increase compared to vehicle-treated WT mice (16.6% overall increase) was seen in the SP immunolabeling intensity of SN in both male and female vehicle-treated R6/2 mice (male, $p=0.0002$; female, $p=0.0177$). We interpret this effect to reflect reduced neuropeptide release from terminals due to diminished striatal projection neuron activity, for reasons presented in the Discussion. In LY379268-treated R6/2 mice (Table 5), intensity of thresholded striato-nigral fibers remained significantly higher than in vehicle-treated WT for both males ($p=0.0004$) and females ($p=0.0243$), and was not different than in gender-matched vehicle-treated R6/2 mice ($p=0.9456$ male and $p=0.7963$ female). Thus, the SP immunolabeling intensity of thresholded fibers was elevated in R6/2 substantia nigra, and not altered by LY379268 treatment.

A surprising aspect of the SP immunolabeling in the WT versus R6/2 nigra needs to be noted before describing the impact of LY379268 on the abundance of SP+ fibers in the striato-nigral projection system (Fig. 8). Note that an SP-poor area is present in the middle of WT nigra, representing a region in which SP levels in striatal terminals are low. By contrast, no such pale area is seen in the vehicle-treated male R6/2 nigra. As a result (Table 5), a striking increase was seen in the areal extent of immunodetectible SP fibers in the nigra of vehicle-treated male R6/2 mice compared to vehicle-treated WT males (a 48.1% increase, $p=0.0443$). As further considered in the Discussion, we interpret the increased thresholdable nigral fiber area to be due to diminished SP release. Vehicle-treated female R6/2 mice, by contrast, did not show a significant increase compared to vehicle-treated WT females ($p=0.3671$). LY379268 treatment had a significant impact on the immunodetectible fiber abundance in nigra in R6/2 males, lowering it to levels comparable to those in WT mice. For example, nigral fiber abundance in LY379268-treated males was significantly less than in vehicle-treated R6/2 males ($p<0.0001$), and comparable to that in LY379268-treated WT males ($p=0.9877$). In fact, the nigral fiber abundance in LY379268-treated males was reduced to a level significantly less than in vehicle-treated WT males ($p=0.0211$). Note that in figure 7, a pale area is present in the nigra in LY379268-treated male R6/2 mice, indicating rescue of striatonigral SP fiber area. Thus, LY379268 restored SP in striato-nigral fibers in male R6/2 mice nearer to WT levels, resulting in fewer detectible SP fibers than in vehicle-treated R6/2 males.

Striatal ENK ISSH—LY379268 treatment significantly enhanced ENK message in WT mice with genders combined ($p=0.0116$), with the increase being significant in females ($p=0.0230$) and suggestive in males ($p=0.0731$) (Table 6). In the case of the vehicle-treated R6/2 mice, the ENK signal intensity was significantly reduced (42.8% of vehicle-treated WT) compared to vehicle-treated WT mice ($p<0.0001$), with the effect highly significant for both genders. The ENK signal intensity in the LY379268-treated R6/2 was equally reduced (41.5% of WT-vehicle) compared to vehicle-treated WT mice ($p<0.0001$), and no different than in the vehicle-treated R6/2 mice ($p=0.8335$). This too was true for both genders. Thus, striatal ENK expression was substantially reduced in 10-week old R6/2 mice, and LY379268 treatment did not enhance ENK expression in R6/2 striatum.

Striatal SP ISSH—The striatal SP signal intensity in LY379268-treated WT mice was not significantly different than in vehicle-treated WT mice for either gender (males: $p=0.9551$; females: $p=0.1169$). The SP signal intensity was, however, significantly reduced in the vehicle-treated R6/2 mice (74.0% of WT-vehicle) compared to vehicle-treated WT mice ($p=0.0113$), and restored by LY379268 treatment to a level not significantly different (95.1% of WT-vehicle) from vehicle-treated WT ($p=0.5262$), but significantly greater than in vehicle-treated R6/2 mice ($p=0.0067$). Thus, striatal SP expression was reduced in 10-week old R6/2 mice, and LY379268 normalized SP expression by direct pathway striatal

neurons in R6/2 mice. The trends were similar for males and females, but in this case more pronounced in females.

Discussion

We found that daily subcutaneous injection of 20mg/kg LY379268 had a number of beneficial effects in R6/2 mice, and no evident adverse effects in WT mice. An MTD yielded a 9-day improvement in survival (11% lifespan increase), normalization and/or improvement in various locomotor parameters in open field, rescue of cortical and striatal neurons at 10 weeks, and rescue of striatal projection neuron neurochemistry. The morphological and neurochemical benefits with LY379268 are likely to be the cause of the improved motor function. The basis of the survival benefit is uncertain, but the improved motor function may be a significant contributor, as may be the slowed neural decline reflected in the neuronal rescue. In general, the phenotype for the investigated parameters progressed more rapidly in male R6/2 mice, and the LY379268 benefit was generally greater in males. The mechanism of LY379268 benefit is uncertain, as is the basis of the gender difference in the R6/2 deficit and the LY379268 benefit. These issues are discussed in greater detail below.

Neuropathology and Behavior in R6/2 Mice

ENK striato-GPe indirect pathway neurons and their fibers in GPe are lost sooner than are SP direct pathway striatal neurons projecting to globus pallidus internus (GPi) and their fibers in GPi in human HD (Reiner et al., 1988; Richfield et al., 1991, 1995; Richfield and Herkenham, 1994; Sapp et al., 1995; Glass et al., 2000; Deng et al., 2004). Similarly, ISHH and immunolabeling studies indicate that striatal ENK neurons are more affected in R6/2 mice than are striatal SP neurons (Cha et al., 1998; Luthi-Carter et al., 2000; Menalled et al., 2000; Sun et al., 2002), as confirmed in the present study. Prior studies of peri-morbid R6/2 mice at 12 weeks of age also showed a decrease in the abundance of ENK-immunolabeled fibers in GPe, as did we in the present study. Although prior studies reported no significant change in SP-immunolabeled fibers in R6/2 nigra (Menalled et al., 2000; Sun et al., 2002), the current study found substantially elevated SP-immunoreactive fiber abundance in nigra in 10 week-old vehicle-treated R6/2 males, which has been illustrated but not discussed in prior studies (Menalled et al., 2000; Sun et al., 2002). Given the decreased striatal SP expression in R6/2 mice, the increase in SP in nigra cannot stem from increased SP production. Rather, it seems likely that the increase in SP-immunostained striato-nigral terminals reflects a failure of SP release due to striatal neuron dysfunction. Consistent with this interpretation, diminished striatal projection neuron activation following ablation of cortex results in increased SP levels in rat SN (Somers and Beckstead 1990; Bouras et al., 1991), and medium spiny projection neurons in awake - behaving R6/2 mice fail to show the burst activity that ensues from cortical activation (Rebec et al., 2002; Miller et al., 2008). Such dysregulation could alter downstream neurotransmitter release by SP striatal neurons and impair communication with target areas (Charpier and Deniau 1997; Lisman 1997).

Because studies of the direct striatal pathways in human HD have revealed a reduction in the abundance of SP fibers in striatal target areas, the present findings showing elevation of striatally derived SP in nigra in R6/2 mice are surprising. It may, however, be that R6/2 mice reveal an early stage of SP neuron neurochemistry and dysfunction not readily amenable to detection in humans – one that occurs well before the typical HD death and neuropathological examination. Note that hypofunction and attendant retention of ENK in striato-GPe terminals is also likely in R6/2 mice, since peptide levels were slightly elevated in GPe in our vehicle-treated R6/2 mice yet striatal ENK message was significantly reduced. The substantial loss of striatal ENK message (and possibly striatal ENK neurons) that we

observed in 6/2 males at 10 weeks may be the basis of the concomitant loss of ENK terminals in GPe.

R6/2 mice typically show enlarged lateral ventricles at 13 weeks, as well as reduced cortical and striatal volumes (Mangiarini et al., 1996; Stack et al., 2005; Reiner et al., 2007). While we too saw that the absolute volumes of cortex and striatum were reduced in vehicle-treated 10-week old R6/2 mice, when body size was taken into consideration by allometric scaling, we found that cortical and striatal volumes in our vehicle-treated R6/2 mice were not significantly smaller than expected for that body size. The lateral ventricles were, however, significantly enlarged even when scaled, providing evidence for slight cortical and/or striatal shrinkage that was not reflected in the volume measurements for those structures. In one prior study, a 25% decrease in striatal neuron abundance in 12-week old R6/2 mice was reported (Stack et al., 2005), while others have suggested that neuron loss does not occur in R6/2 mice (Li and Li 2004b; Reddy et al., 1999). Our current stereological results indicate an overall cortical and striatal neuron loss of about 15–25% in 10-week old vehicle-treated R6/2 mice, with the loss more prominent in males than females. It seems likely that neuron loss in females lags behind that in males, and would eventually be evident. Our ISHH data indicating much greater loss of ENK than SP message suggest that the striatal neuron loss at 10 weeks found by stereology is predominantly from the ENK population. We cannot exclude the possibility, however, that some SP striatal neurons are lost as well. The loss of both cortical and striatal neurons in our vehicle-treated R6/2 mice is, of course, reminiscent of human HD (Hedreen et al., 1991; Vonsattel and DiFiglia, 1998).

Coupled with these morphological and neurochemical abnormalities, we observed progressive motor decline in the R6/2 mice, as evidenced in rotarod and open field, as have many other authors (Gil and Rego, 2009; Menalled et al., 2009). Since direct pathway neurons facilitate movement initiation and execution (Reiner et al., 1988; Alexander and Crutcher, 1990; DeLong, 1990; Kravitz et al., 2010), the hypofunction suggested by their aberrant neurochemistry may explain the diminished movement and impaired motor control that we observed in open field. The ENK neurons of the striatal indirect pathway, however, also play a role in motor control by suppressing unwanted movements, and their loss is thus likely to also contribute to the motor impairments seen in R6/2 mice, especially in more complex movements involving an interaction of movement facilitation and suppression, for example in rotarod.

The precise interplay between neural and systemic defects in causing demise in R6/2 mice is uncertain, but it is evident that manipulations affecting the nervous system can increase longevity in R6/2 mice (Dragatsis et al., 2009; Gil and Rego, 2009). In this regard, environmental enrichment has been reported to improve the overall well-being and slightly improve the survival of R6/2 mice (Carter et al., 2000; Hockly et al., 2002), and as a result has become the standard of R6/2 care over and above which benefits of specific therapies are judged. Despite the environmental enrichment here, the mean longevity of our vehicle-treated R6/2 mice is somewhat less than the 12–13 weeks reported for enriched R6/2 mice in some prior studies. A major factor in R6/2 mouse longevity and symptom severity is CAG repeat length (Dragatsis et al., 2009), which was not reported in these prior studies. For a larger cohort than presented here, we found that symptoms were greater in R6/2 mice with CAG repeats in the 120–125 range of the present studies than in mice with 130–145 repeats. Thus, CAG repeat may explain the slightly shorter lifespan of our vehicle-treated R6/2 mice than in other studies assessing treatment impact on longevity of R6/2 mice.

Our results for vehicle-treated R6/2 mice in the 120–125 CAG repeat range also show a gender difference in the progression of the motor impairment, and in life span to a lesser extent, with males showing accelerated disease progression and severity. We also observed

greater neuron loss in cortex and striatum and greater neurochemical abnormalities in the striatal projection systems in males by 10 weeks. Although a gender difference in disease progression has not commonly been emphasized for R6/2 mice (Menalled et al., 2009), at least two studies have reported gender differences in HD progression in R6/2 mice and one in Q140 knock-in mice, with males more severely affected (Dorner et al., 2007; Ma et al., 2007; Tallaksen-Greene et al., 2010). Similarly, while gender differences have not commonly been noted for HD onset or course in humans, gender differences in the effects of genetic modifiers on onset have been reported (Kehoe et al., 1999; Arning et al., 2007), one older epidemiological study suggested that age of onset is earlier in males than females (Roos et al., 1991), and a more recent one suggested longer survival in females than males with age of onset and CAG length controlled (Pekmezovic et al., 2007). Gender differences are observed in many neurodegenerative conditions, including Parkinson's disease and ALS, with females less vulnerable, and are generally attributed to a neuroprotective effect of estrogen (Kenchappa et al., 2004; Czlonskowska et al., 2005; Rodríguez-Navarro et al., 2008; McCombe and Henderson, 2010).

LY379268 Benefit in R6/2 Mice

Daily treatment with MTD LY379268 yielded prominent motor improvement in R6/2 mice. The open field benefit was substantial, yielding normalization out to 12 weeks of age typically, an age at which the vehicle-treated R6/2 mice were greatly impaired. The behavioral improvements were associated with a benefit of LY379268 on cortical and striatal neuron survival, with the drug yielding normalization in males at 10 weeks of age. We also observed that LY379268 normalized SP message in striatum, which was reduced by 25% in vehicle-treated R6/2 mice. Additionally, SP striatal terminals in nigra were notably restored to normal abundance by LY379268 in male R6/2 mice, possibly meaning that their burst firing, transmitter release and communication with nigra were normalized. There may also have been some benefit for ENK striato-GPe neurons, since fiber abundance was returned to normal by LY379268 in R6/2 males. LY379268 treatment did not, however, attenuate the ventricular enlargement seen in R6/2 mice. Thus, its benefit did not involve an evident restoration of cortical and/or striatal volume. (Schiefer et al. 2004) previously examined the efficacy of a daily 1.2mg/kg dose of LY379268 in R6/2 mice, and found a similar survival benefit as in our study, but no rotarod benefit. The lack of a rotarod benefit in their study may stem from the low dose used, or from not separately evaluating males and females. Finally, we did not observe any alteration in the size or frequency of NIIs in R6/2 brain with daily MTD LY379268. Thus, unlike with memantine treatment in YAC128 mice, in which the benefit was associated with increased aggregates and presumably reduced soluble toxic mhtt oligomers (Okamoto et al 2009), we have no evidence that reduced abundance of putative toxic oligomers contributed to the LY379268 benefit in R6/2 mice. (Schiefer et al. 2004) had tentatively suggested that cortical and striatal aggregates might be enlarged in R6/2 mice treated daily with a low LY379268 dose. Note, however, that the precise role of mutant huntingtin aggregates in HD pathogenesis remains uncertain (DiFiglia et al., 1997; Dragatsis et al., 2009).

Finally, we observed a gender difference in treatment efficacy, with males typically showing a greater benefit behaviorally and histologically. It is uncertain if this is because the phenotype was more severe in males (and thus any benefit likely to be more evident), or if mGluR2/3 agonist therapy with LY379268 is for some reason more effective in males. In any event, Ma et al. (2007) previously reported that metformin treatment was more effective in R6/2 males than R6/2 females, but few studies have noted inclusion of both males and females, and these typically have not provided gender breakdown of the results.

Pathogenic Mechanisms and Basis of LY379268 Benefit

Several possibilities are likely to explain the beneficial effects of LY379268 in R6/2 mice: 1) LY379268 may have opposed excitotoxic cortical and striatal injury (Kingston et al., 1999; D'Onofrio et al., 2001) by acting on mGluR2/3 receptors on cortical neuron terminals to reduce glutamate release (Battaglia et al., 1997; Cozzi et al., 1997), and/or by acting directly on neurons possessing mGluR3 receptors to diminish their excitability (Testa et al., 1994); 2) LY379268 may have increased TGF- β production by astrocytes and thereby protected against excitotoxic injury (D'Onofrio et al., 2001); and 3) LY379268 could have increased cortical BDNF production and delivery to target neurons in cortex and striatum (Di Liberto et al., 2010; Zuccato et al., 2001; Gauthier et al., 2004; Zuccato and Cattaneo 2007). These possibilities are addressed in more detail below.

Since the finding that NMDA receptor agonists injected into striatum produce a pattern of striatal injury that closely mimics HD (Beal et al., 1991; Figueredo-Cardenas et al., 1994; Shear et al., 1998), a role of NMDA receptor-mediated excitotoxicity in HD pathogenesis has been suspected. Recent evidence suggests that excitotoxic injury to striatal projection neurons in HD is specifically mediated through extrasynaptic NMDA receptors (Andre et al., 2006; Cepeda et al., 2001; Li et al., 2004; Cui et al., 2006; Fan and Raymond, 2007; Fan et al., 2007; Subramaniam et al., 2009; Milnerwood et al., 2010). Several mechanisms may contribute to the excessive activation of extrasynaptic NMDA receptors in HD, including increased glutamate release (Cepeda et al., 2007; Joshi et al., 2009), and reduced glutamate clearance (Lievens et al., 2000; Behrens et al., 2002; Shin et al., 2005; Hassel et al., 2008). Given the enrichment of mGluR2/3 receptors on corticostriatal and corticocortical terminals and the damping effect of mGluR2/3 agonists on glutamate release (Lovinger and McCool, 1995), LY379268 may have produced its benefit in R6/2 mice by reducing release of glutamate from corticostriatal terminals, thereby preventing injurious activation of extrasynaptic NMDA receptors. Consistent with this possibility, one prior study prevented corticostriatal glutamate release in R6/2 mice by ablating cortex, and thereby reversed loss of striatal neurons and striatal shrinkage (Stack et al., 2007). The basis of the seeming greater benefit of LY379268 for SP striatal neurons rather than ENK striatal neurons is uncertain. Since upper layer V cortical neurons project preferentially to striatal SP neurons (Lei et al., 2004; Reiner et al., 2010), it is possible that they are more enriched in mGluR2/3 receptors than are those corticostriatal neurons of deep layer 5 preferentially projecting to striatal ENK neurons.

It is also possible that the striatal LY379268 benefit arises by a direct action on striatal neurons, since about half express mGluR3 (Testa et al., 1994; Allen Brain Atlas). Group II metabotropic glutamate receptors are negatively coupled to adenylyl cyclase (Nakanishi, 1992; Schoepp et al., 1999). Agonist binding to them reduces Ca²⁺ currents via N, L-type Ca²⁺ channels and increases K⁺ currents via IRKC channels and thereby decreases neuronal excitability (Davies et al., 1995; King and Liu, 1996; McCool et al., 1996). If the greater SP neuron benefit of LY379268 was achieved by direct action on mGluR3 receptors on striatal SP neurons, it should be possible to achieve a similar benefit with a dopamine D1 receptor antagonist, since this would also reduce adenylyl cyclase activation (Monsma et al., 1990).

The LY379268 benefit may also have come about by an action on glial mGluR3 receptors (Ohishi et al., 1993; Testa et al., 1994; Liu et al., 1998). Agonists for mGluR3 counteract neuronal NMDA toxicity *in vitro* and *in vivo* via increased astrocytic release of TGF- β 1 (Bruno et al., 1997, 1998; Corti et al., 2007), leading to activation of the MAP kinase and PI-3-kinase anti-apoptotic pathways (Copani et al., 1995; Ren et al., 1997; D'Onofrio et al., 2001). Note, however, that TGF- β 1 levels are reduced in the brains of human HD victims and in HD mice (R6/2 and YAC128), and acute administration of LY379268 does not elevate cortical or striatal TGF- β 1 in R6/2 or YAC128 mice (Battaglia et al., 2011). It is

uncertain thus if the benefit of daily LY379268 in our R6/2 mice could have involved cortical and striatal TGF- β 1.

Finally, LY379268 may have yielded its neuroprotective effect by enhancing cortical production and axonal transport of BDNF to cortical and striatal targets (Altar et al., 1997; Zuccato et al., 2001; Gauthier et al., 2004; Zuccato and Cattaneo, 2007). BDNF promotes development, differentiation, plasticity and survival of neurons in cortex and striatum (Lessmann et al., 2003; Poo 2001; Zuccato and Cattaneo, 2007), and mhtt reduces cortical BDNF expression and protein transport (Zuccato et al., 2001). Placing the R6/1 HD transgene on a hemizygous BDNF knock-out background exacerbates striatal ENK but not SP loss (Canals et al., 2004), and, cortex-specific BDNF knock-out results in cortical and striatal pathology that mimics human HD (Gorski et al., 2003; Baquet et al., 2004; Strand et al., 2007). Conversely, behavioral performance is improved and disease progression slowed in transgenic HD mice overexpressing BDNF (Gharami et al., 2008; Xie et al., 2010; Giralt et al., 2011). Similarly, daily intrastriatal administration of BDNF in R6/1 HD mice increases the number of striatal neurons expressing enkephalin and improves behavior (Canals et al., 2004). Thus, a deficit in BDNF may contribute to the pathogenesis of cortical and striatal injury in HD, and restoration of BDNF in HD mice is therapeutic. A recent study demonstrated that WT mice acutely treated with LY379268 show upregulation of BDNF in cortex and hippocampus (Di Liberto et al., 2010). This finding raises the possibility that our daily LY379268 treatments also boosted cortical BDNF, and thereby benefited cortical and striatal neurons.

HD Therapy and mGluR2/3 Agonists

A variety of therapeutic targets have been tested in HD mice, using diverse delivery approaches and with varying benefits (Gil and Rego, 2009; Crook and Housman, 2011). These treatments have sought to normalize transcription by inhibiting histone deacetylation and methylation, prevent mhtt misfolding and oligomerization, rescue metabolic dysfunction, ameliorate the HD diabetic phenotype, reduce oxidative stress, block excitotoxicity, prevent apoptosis, and knockdown expression of the mutant allele. Of these treatments, silencing the mutant allele requires gene therapy, while the others typically involve pharmacotherapy. Although gene therapy to silence the mutant allele has shown promise in animal studies (Johnson and Davidson, 2010), such therapy may need to be mutant allele-specific, since extensive knockdown of the WT allele is harmful (Dragatsis et al., 2000). It remains unknown, however, if such selectivity can be achieved, and even if it does it is uncertain if the therapy will have prohibitive adverse side effects due to an immune response to the viral delivery construct, or due to the need for repeated administration in the case of antisense oligonucleotides. The benefit obtained in R6/2 mice from pharmacotherapies has varied even for the same therapeutic targets, but comparisons are difficult because many studies did not assess repeat length and repeat length is known to have a great impact on the R6/2 phenotype (Dragatsis et al., 2009; Morton et al., 2009). In any event, prominent benefits for survival and motor performance have been observed in R6/2 mice with bilateral intracerebral NPY administration (which improves feeding and metabolism) (Decressac et al., 2010), oral metformin (which combats diabetes) (Ma et al., 2007), histone deacetylase inhibitors to combat transcriptional suppression (Hockley et al., 2003a, b; Ferrante et al., 2003, 2004; Ryu et al., 2006; Stack et al., 2007), various supplements to improve energy metabolism, and various antioxidants (summarized in Gil and Rego, 2009). Studies to inhibit caspases to prevent apoptosis, to use various small molecules to inhibit aggregation, and to prevent excitotoxicity have produced conflicting results (summarized in Gil and Rego, 2009).

The conflicting results with efforts to combat excitotoxicity are of interest in the case of the present mGluR2/3 agonist therapy. Drugs that act postsynaptically to reduce neuronal

excitability such as riluzole (Schiefer et al., 2002), the NR2B-selective NMDA receptor antagonist ifenprodil (Tallaksen-Greene et al., 2010), or the mGluR5 antagonist MPEP (Schiefer et al., 2004) have had little to no benefit. As noted previously, the NMDA receptor antagonist memantine was effective at low but not high doses in improving YAC128 HD mice (Okamoto et al., 2009). By contrast, ceftriaxone to improve GLT-mediated glutamate reuptake improved the R6/2 phenotype (Miller et al., 2008), as did chronic oral administration of the small molecule pro-drug inhibitor of kynurenine 3-monooxygenase JM6, which increases kynurenic acid and reduces extracellular glutamate in the brain (Zwilling et al., 2011). Thus, the precise process targeted may be critical to the efficacy of anti-excitotoxic therapy for HD.

Group II metabotropic glutamate receptors on the terminals of excitatory neurons have become a target of potential therapeutics for various neurological diseases and conditions, because of the ability of agonists to suppress glutamate release (Kew et al., 2001; Scanziani et al., 1997; Schoepp, 2001). The preferential location of mGluR2/3 receptors at the periphery of the terminals, in particular, mitigates extrasynaptic glutamate spill (Conn and Pin, 1997). The first selective mGluR2/3 agonist developed was LY354740 (Monn et al., 1997) followed by LY379268, which is more effective in suppressing cAMP formation, but preferential for mGluR3 (Monn et al., 1999; Schoepp, 2001). Consistent with their role in opposing glutamate release, LY379268 and other mGluR2/3 agonists have been found to be neuroprotective against NMDA receptor-mediated excitotoxicity in animal models (Bond et al., 1999, 2000; Cai et al., 1999; Kingston et al., 1999; D'Onofrio et al., 2001). This rationale led us to test LY379268 for its efficacy in abating the phenotype of R6/2 mice, which are a good neuropathological HD mimic although not a good genetic HD mimic (Kuhn et al., 2007). As discussed above, however, the benefit of LY379268 may also have arisen entirely or in part by stimulation of cortical and striatal glial TGF- β 1 production or by stimulation of cortical BDNF production. The possible role of increased BDNF in mediating the benefit of LY379268 is of interest in light of evidence that the beneficial effects in R6 mice of environmental enrichment, the selective serotonin reuptake inhibitor sertraline, the positive AMPA receptor modulator ampakine, and nicotinamide may be mediated by enhancing cortical BDNF production (Hannan 2004; Hockly et al., 2002; Peng et al., 2008; Gil and Rego, 2009; Hathorn et al., 2011; Simmons et al., 2011).

The present findings in R6/2 mice, and the evidence that HD can be combated by diminishing extrasynaptic glutamate and enhancing cortical BDNF recommend further consideration of mGluR2/3 agonists for HD therapy. In this light, it is of interest that a yet more potent selective mGluR2/3 agonist, LY404039, has been developed, which can be formulated in an orally bioavailable pro-drug form. Moreover, LY404039 has been tested in a Phase II human schizophrenia clinical trial in its pro-drug form, and found to be well tolerated, safe, and effective in treating schizophrenia symptoms, which are thought to also be mediated by excess glutamate release (Patil et al., 2007). In light of its demonstrated safety in humans, mGluR2/3 therapy via LY404039 could quickly move to Phase III human clinical trials, upon evidence of benefit in a genetically more precise model of HD, such as the YAC128, the BACHD, or Q140 mice (Slow et al., 2003; Gray et al., 2008; Hickey et al., 2008).

Highlights

The mGluR2/3 agonist LY379268 extended lifespan.

LY379268 normalized open field motor parameters for much of the R6/2 lifespan.

LY379268 rescued cortical and striatal neuron loss.

LY379268 normalized Substance P-containing striatal projection neuron neurochemistry.

The R6/2 phenotype is accelerated in males, and LY379268 benefit greater in males.

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Fig. 1.

Graphs illustrating the effects of a daily MTD of LY379268 or of vehicle on survival in R6/2 mice (A) and body weight (B) in R6/2 and WT mice. Note that MTD LY379268 improves survival but does not prevent weight loss in R6/2 mice. The average CAG repeat length was 124.0 for the thirty-two 20mg/kg LY379268-treated R6/2 mice, and 123.6 for the thirty control-treated R6/2 mice.

Fig. 2.

Graphs illustrating the effects of a daily MTD of LY379268 or of vehicle on rotarod performance in R6/2 and WT mice with males and females combined (A) and on rotarod performance in R6/2 and WT mice for males alone (B). Note that MTD LY379268 improves rotarod performance in R6/2 males, but not with R6/2 males and females combined.

Fig. 3.

Graphs illustrating the effects of a daily MTD of LY379268 or of vehicle on total distance traveled in open field in R6/2 and WT mice with males and females combined (A) and on length of progression segments in open field in R6/2 and WT mice with males and females combined (B). Note that LY379268 greatly improves performance on both parameters.

Fig. 4.

Graphs illustrating the effects of a daily MTD of LY379268 or of vehicle on number of stops (pauses) per cm in open field in R6/2 and WT mice with males and females combined (A) and on proportion of time in pauses (called lingering) in open field in R6/2 and WT mice with males and females combined (B). Note that LY379268 greatly reduces stops and lingering.

Fig. 5.

Graphs illustrating the effects of a daily MTD of LY379268 or of vehicle on maximum speed in open field in R6/2 and WT mice with males and females combined (A) and on acceleration in open field in R6/2 and WT mice with males and females combined (B). Note that LY379268 greatly improves maximum speed and acceleration.

Fig. 6.

Graphs illustrating the effects of a daily MTD of LY379268 or of vehicle on progression segment path curvature in open field in R6/2 and WT males (A) and females (B). Note that LY379268 diminishes the tendency of R6/2 mice of both genders to deviate from a straight path. Vehicle-treated R6/2 males show an earlier decline than do females.

Fig. 7.

Graph showing the effect of LY379268 on cortical neuron abundance in male and female R6/2 mice, presented in all cases as a percent of all WT mice ($\pm$ SEM). Cortical neuron abundance is significantly reduced in vehicle-treated R6/2 males compared to WT males (asterisk), and is restored to normal abundance in LY379268-treated R6/2 males. The cortical neuron abundance in LY379268-treated females is significantly less than that in WT mice (asterisk), and nearly so for vehicle-treated R6/2 females (#).

Fig. 8.

Immunolabeling of substantia nigra for SP in WT-vehicle, R6/2-vehicle and R6/2-LY379268 males. Note that in WT nigra, SP immunolabeling is weak in the center of the nigra. By contrast, in vehicle-treated R6/2 nigra it is uniformly high. LY379268 treatment restores SP immunolabeling in R6/2 nigra to a pattern like that in WT-vehicle.

Effect of LY379268 on Allometrically Scaled Regional Volumes in WT and R6/2 Mice

This table presents the effect of LY379268 on the allometrically scaled regional volumes for telencephalon, cortex, striatum and lateral ventricle in WT and R6/2 mice, presented in all cases as a percent of vehicle-treated WT (WTV) ($\pm$ SEM). The results are shown for males and females combined, as well as separately. Statistically noteworthy results are shown in red. Values in bold indicate a significant difference from gender-matched vehicle-treated WT, while an asterisk indicates a significant difference from gender-matched vehicle-treated R6/2. The value in italics indicates a $p=0.0558$ versus gender-matched vehicle-treated WT.

Group	Gender	Number	CAG	telen/wt.66 as % all WTV	ctx/wt.66 as % all WTV	str/wt.66 as % all WTV	vent/wt.66 as % all WTV
WT-V	both	14		100.0 $\pm$ 3.9%	100.0 $\pm$ 4.1%	100.0 $\pm$ 4.8%	100.0 $\pm$ 10.9%
WT-LY	both	14		110.9 $\pm$ 4.8%	113.3 $\pm$ 6.1%	103.0 $\pm$ 5.0%	113.7 $\pm$ 17.3%
R62-V	both	17	119.1 $\pm$ 0.5	99.6 $\pm$ 3.6%	97.0 $\pm$ 4.0%	98.8 $\pm$ 5.3%	212.6$\pm$24.6%
R62-LY	both	16	119.4 $\pm$ 0.6	104.6 $\pm$ 4.7%	99.0 $\pm$ 4.9%	92.3 $\pm$ 4.6%	221.1$\pm$26.7%
WT-V	male	7		94.7 $\pm$ 6.6%	97.6 $\pm$ 8.2%	98.4 $\pm$ 4.8%	97.3 $\pm$ 10.9%
WT-LY	male	8		119.0$\pm$8.2%	120.4$\pm$6.7%	106.7 $\pm$ 11.6%	109.0 $\pm$ 21.5%
R62-V	male	8	119.5 $\pm$ 1.0	104.7 $\pm$ 6.1%	101.4 $\pm$ 7.7%	98.4 $\pm$ 7.3	222.5$\pm$38.5%
R62-LY	male	7	118.7 $\pm$ 1.2	96.8 $\pm$ 6.9%	90.7 $\pm$ 6.0%	86.1 $\pm$ 7.5%	215.7$\pm$39.5%
WT-V	female	7		105.3 $\pm$ 4.2%	102.4 $\pm$ 3.6%	101.6 $\pm$ 6.8%	102.7 $\pm$ 14.8%
WT-LY	female	6		100.2 $\pm$ 3.3%	103.7 $\pm$ 9.6%	98.0 $\pm$ 5.0%	120.0 $\pm$ 26.2%
R62-V	female	9	118.8 $\pm$ 0.6	95.0 $\pm$ 4.4%	93.1 $\pm$ 3.9%	99.3 $\pm$ 8.4%	203.8$\pm$35.4%
R62-LY	female	9	120.0 $\pm$ 0.5	110.7 $\pm$ 6.2%*	105.4 $\pm$ 7.0%	97.1 $\pm$ 6.0%	225.3$\pm$39.7%

Bold = significantly greater than gender-matched vehicle-treated WT

* = significantly greater than gender-matched vehicle-treated R6/2

italics – $p=0.0558$ vs gender-matched vehicle-treated WT

Table 2
Effect of LY379268 on Cortical and Striatal Neuron Abundance in WT and R6/2 Mice

This table presents the effect of LY379268 on cortical and striatal neuron abundance in WT and R6/2 mice, presented in all cases as the neuron counts, as well as a percent of vehicle-treated WT (WTV) ($\pm$ SEM). The results are shown for males and females combined, as well as separately. Statistically noteworthy results are shown in red. Values in bold indicate a significant difference from gender-matched vehicle-treated WT in case of striatum, and all WT in case of cortex. An asterisk indicates a significant difference from gender-matched vehicle-treated R6/2. The value in italics indicates a $p=0.052$ versus all WT in the case of cortex.

Group	Gender	Number	CAG	Striatum	Cortex	Striatum as % all WTV	Cortex as % all WTV
WT-V	both	13		231271.8 $\pm$ 12684.2	437981.7 $\pm$ 29304.5	100.0 $\pm$ 5.5%	100.0 $\pm$ 6.7%
WT-LY	both	13		238841.4 $\pm$ 15211.7	455469.6 $\pm$ 19098.9	103.3 $\pm$ 6.6%	104.0 $\pm$ 4.4%
R62-V	both	16	119.1 $\pm$ 0.6	197833.8 $\pm$ 11929.5	379714.5 $\pm$ 16719.1	85.5 $\pm$ 5.2%	86.7 $\pm$ 3.8%
R62-LY	both	16	119.4 $\pm$ 0.6	231075.6 $\pm$ 11933.6	433587.3 $\pm$ 23733.7	99.9 $\pm$ 5.2%	99.0 $\pm$ 5.4%
WT-V	male	6		237888.0 $\pm$ 9720.0	396836.9 $\pm$ 23390.0	102.9 $\pm$ 4.1%	90.6 $\pm$ 5.9%
WT-LY	male	7		215456.5 $\pm$ 13045.2	443318.5 $\pm$ 24884.4	93.2 $\pm$ 5.5%	101.2 $\pm$ 6.3%
R62-V	male	7	119.6 $\pm$ 1.2	169747.5 $\pm$ 11404.3	364428.9 $\pm$ 21483.7	73.4 $\pm$ 4.8%	83.2 $\pm$ 5.4%
R62-LY	male	7	118.7 $\pm$ 1.2	242364.3 $\pm$ 26221.1 *	505614.4 $\pm$ 38082.4 *	104.8 $\pm$ 11.0% *	115.4 $\pm$ 9.6% *
WT-V	female	7		225600.7 $\pm$ 23756.9	473248.7 $\pm$ 50309.9	97.5 $\pm$ 10.5%	108.1 $\pm$ 10.6%
WT-LY	female	6		266123.7 $\pm$ 27495.9	469645.8 $\pm$ 33463.4	115.1 $\pm$ 12.2%	107.2 $\pm$ 7.1%
R62-V	female	9	118.8 $\pm$ 0.6	219678.6 $\pm$ 16546.2	<i>391603.3</i> $\pm$ 26032.0	95.0 $\pm$ 7.3%	<i>89.4</i> $\pm$ 5.5%
R62-LY	female	9	120.0 $\pm$ 0.5	222295.6 $\pm$ 9441.5	377566.2 $\pm$ 12480.7	96.1 $\pm$ 4.2%	86.2 $\pm$ 2.6%

Bold = significantly different than gender-matched vehicle-treated WT in case of striatum, and all WT in case of cortex

* = significantly different than gender-matched vehicle-treated R6/2

italics – $p=0.052$ vs all WT in case of cortex

Effect of LY379268 on NII in R6/2 Cortex and Striatum

This table presents the effect of LY379268 on NII size and abundance in R6/2 cortex and striatum ($\pm$ SEM). The results are shown for males and females combined, as well as separately. No significant differences were found as a result of LY379268 treatment – that is, NII size and abundance in R6/2 cortex and striatum were no different after treatment with drug as after vehicle treatment.

Groups	Gender	Number	CAG	Ctx nII size (μ m)	Ctx NII per mm ²	Str nII size (μ m)	Str NII per mm ²
R6/2 - V	both	17	119.1 $\pm$ 0.5	1.92 $\pm$ 0.13	4092.5 $\pm$ 197.6	1.63 $\pm$ 0.13	3638.0 $\pm$ 199.0
R6/2 - LY	both	14	119.8 $\pm$ 0.6	1.85 $\pm$ 0.07	3867.2 $\pm$ 274.0	1.48 $\pm$ 0.05	3885.7 $\pm$ 242.0
R6/2 - V	male	8	119.5 $\pm$ 1.0	1.81 $\pm$ 0.26	4068.7 $\pm$ 111.1	1.54 $\pm$ 0.24	3713.1 $\pm$ 191.5
R6/2 - LY	male	5	119.4 $\pm$ 1.5	1.94 $\pm$ 0.14	3812.9 $\pm$ 703.3	1.44 $\pm$ 0.17	3807.5 $\pm$ 428.3
R6/2 - V	female	9	118.8 $\pm$ 0.6	2.03 $\pm$ 0.13	4113.8 $\pm$ 382.1	1.72 $\pm$ 0.12	3571.2 $\pm$ 351.1
R6/2 - LY	female	9	120.0 $\pm$ 0.5	1.80 $\pm$ 0.09	3897.4 $\pm$ 272.3	1.50 $\pm$ 0.02	3929.1 $\pm$ 328.8

Table 4
Effect of LY379268 on ENK in Striato-GPe terminals in WT and R6/2 Mice

This table presents the effect of LY379268 on fiber labeling intensity and abundance of ENK-immunolabeled striato-GPe terminals in WT and R6/2 mice, presented in all cases as a percent of vehicle-treated WT (WTV) ($\pm$ SEM). The results are shown for males and females separately. Statistically noteworthy results are shown in red. Values in bold indicate a significant difference from gender-matched vehicle-treated WT.

Group	Gender	Number	CAG	Intensity as % all WTV	Fiber Area as % all WTV
WT-V	male	7		95.7 $\pm$ 10.5%	106.5 $\pm$ 3.1%
WT-LY	male	8		105.7 $\pm$ 5.2%	95.7 $\pm$ 1.9%
R62-V	male	7	119.0 $\pm$ 1.0	119.0$\pm$4.3%	76.9$\pm$1.4%
R62-LY	male	7	118.7 $\pm$ 1.2	114.7$\pm$7.1%	92.5 $\pm$ 2.1%
WT-V	female	9		104.3 $\pm$ 3.3%	93.5 $\pm$ 3.0%
WT-LY	female	6		117.7 $\pm$ 6.3%	121.0$\pm$4.6%
R62-V	female	10	119.2 $\pm$ 0.7	113.2 $\pm$ 4.0%	88.0 $\pm$ 1.7%
R62-LY	female	9	120.0 $\pm$ 0.5	106.3 $\pm$ 1.9%	67.4$\pm$5.3%

Bold = significantly different than gender-matched vehicle-treated WT

Table 5
Effect of LY379268 on SP in Striato-nigral Terminals in WT and R6/2 Mice

This table presents the effect of LY379268 on fiber labeling intensity and abundance of SP-immunolabeled striato-nigral terminals in WT and R6/2 mice, presented in all cases as a percent of vehicle-treated WT (WTV) ($\pm$ SEM). The results are shown for males and females separately. Statistically noteworthy results are shown in red. Values in bold indicate a significant difference from gender-matched vehicle-treated WT, and an asterisk indicates a significant difference from gender-matched vehicle-treated R6/2.

Group	Gender	Number	CAG	Intensity as % all WTV	Fiber Area as % all WTV
WT-V	male	7		97.5 $\pm$ 5.4%	106.0 $\pm$ 4.4%
WT-LY	male	8		103.4 $\pm$ 4.8%	73.7 $\pm$ 4.3%
R62-V	male	7	119.0 $\pm$ 1.0	120.4 $\pm$ 3.7%	151.4 $\pm$ 5.5%
R62-LY	male	7	118.7 $\pm$ 1.2	118.9 $\pm$ 4.3%	76.4 $\pm$ 7.5% *
WT-V	female	9		102.5 $\pm$ 2.2%	94.0 $\pm$ 5.3%
WT-LY	female	6		120.8 $\pm$ 6.2%	116.4 $\pm$ 3.1%
R62-V	female	10	119.2 $\pm$ 0.7	114.5 $\pm$ 3.5%	110.4 $\pm$ 3.7%
R62-LY	female	9	120.0 $\pm$ 0.5	114.2 $\pm$ 3.3%	107.2 $\pm$ 3.4%

Bold = significantly different than gender-matched vehicle-treated WT

* = significantly different than gender-matched vehicle-treated R6/2

Table 6
Effect of LY379268 on Striatal ENK and SP Message in WT and R6/2 Mice

This table presents the effect of LY379268 on striatal ENK and SP message in WT and R6/2 mice, presented in all cases as a percent of vehicle-treated WT (WTV) ($\pm$ SEM). The results are shown for males and females combined, as well as separately. Statistically noteworthy results are shown in red. Values in bold indicate a significant difference from gender-matched vehicle-treated WT, and an asterisk indicates a significant difference from gender-matched vehicle-treated R6/2.

Group	Gender	Number	CAG repeat	ENK as % all WTV	# of cases	CAG repeat	SP as % all WTV
WT-V	both	12		100.0 $\pm$ 4.2%	12		100.0 $\pm$ 6.4%
WT-LY	both	13		115.9$\pm$6.9%	13		95.9 $\pm$ 5.0%
R62-V	both	12	125.7 $\pm$ 1.2	42.8$\pm$3.2%	13	125.8 $\pm$ 1.1	74.0$\pm$5.5%
R62-LY	both	14	126.4 $\pm$ 1.0	41.5$\pm$1.6%	13	126.5 $\pm$ 1.1	95.1 $\pm$ 5.7% *
WT-V	male	6		91.6 $\pm$ 4.9%	6		90.5 $\pm$ 7.1%
WT-LY	male	7		106.0 $\pm$ 7.1%	7		89.8 $\pm$ 4.2%
R62-V	male	6	127.5 $\pm$ 1.8	42.1$\pm$6.4%	7	125.6 $\pm$ 1.5	74.0 $\pm$ 10.2%
R62-LY	male	8	125.8 $\pm$ 1.2	39.6$\pm$2.3%	7	125.7 $\pm$ 1.4	89.7 $\pm$ 5.6%
WT-V	female	6		108.4 $\pm$ 5.1%	6		109.5 $\pm$ 10.3%
WT-LY	female	6		127.6$\pm$11.8%	6		92.1 $\pm$ 9.6%
R62-V	female	6	125.8 $\pm$ 2.0	43.5$\pm$2.9%	6	126.0 $\pm$ 2.0	74.0 $\pm$ 5.1%
R62-LY	female	6	127.3 $\pm$ 1.8	43.8$\pm$2.3%	6	127.3 $\pm$ 1.8	101.5 $\pm$ 11.0% *

Bold = significantly different than gender-matched vehicle-treated WT

* = significantly different than gender-matched vehicle-treated R6/2