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The mGluR2/3 Agonist LY397268 Improves Morphometric and Behavioral Outcomes in R6/2 Huntington's Disease Mice

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Dennis Craig Lafferty
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ABSTRACT

A prominent theory for the pathology of Huntington's Disease (HD) involves excitotoxic injury to the striatum. Continuous exposure of ionotropic NMDA receptors to glutamate from the cortex may occur and be excitotoxic in HD and leave striatal neurons vulnerable to damage. Activation of presynaptic mGluR2/3 receptors by an agonist dampens glutamate release from corticostriatal terminals. Treatments that target excitoxicity thus may improve symptoms in HD patients, and it is therefore logical to pursue therapies aimed in this direction. LY379268 is an inviting mGluR2/3 receptor agonist that has been shown to be neuroprotective in hypoxic and ischemic injuries to cultured neurons. Daily subcutaneous injection of 20mg/kg LY379268 had a number of beneficial effects in R6/2 mice, including an 11% improvement in lifespan and numerous locomotor parameters in open field. The motor parameters improved included overall activity, speed, acceleration, and endurance, all of which were normalized until 12 weeks of age, and improved over vehicle-treated R6/2 mice at all ages. Histological analysis revealed a 20% loss of cortical and striatal neurons in R6/2 mice, which was rescued with the administration of LY379268. There was no effect of the drug on neuronal intranuclear inclusions (NIIs) or benefit for ENK+ striatal neuron neurochemistry, but SP+ striatal projection neurons were normalized in their neurochemistry. The data indicate that LY379268 is particularly useful in improving the health and functioning of the direct striatal pathway (*i.e.* SP+ neurons), which demonstrably improved the voluntary motor behavior in the R6/2 mice.

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
BDNF	Briain Derived Neurotropic Factor
ENK	Enkephalin
GABA	Gamma-Aminobutyric Acid
GPe	Globus Pallidus Externus
HD	Huntington's Disease
Htt	Huntingtin
Mhtt	Mutant Huntingtin
MSN	Medium Spiny Neurons
NIIs	Neuronal Intranuclear Inclusions
NMDA	N-methyl-D-asparate
SN	Substantia Nigra
SP	Substance P
WT	Wild Type
3-NP	3-Nitroproprionic Acid

CHAPTER 1. INTRODUCTION

Huntington's disease (HD) is an inherited neurodegenerative disorder resulting in motor, cognitive and psychiatric abnormalities. The progressive nature of the disease eventually leads to a disorder of extrapyramidal movement that includes the uncontrolled dance like motion of chorea, and later rigidity and dystonia (Cepeda *et al* 2007; Vonsattel & DiFiglia 1998). Chorea is an early characteristic symptom which can be defined as excessive spontaneous movement with abrupt irregular timing. It is repetitive in nature and all parts of the body can be affected by this symptom. Dystonia is another motor abnormality seen later in HD with sustained muscular contraction. Depression is a frequent psychiatric symptom and often precedes the appearance of motor abnormalities. Over years, the motor symptoms predominantly worsen, such that walking, speaking and eating become more difficult. Average age of onset is usually mid-thirties to mid-forties and the disease lasts 15-20 years. This autosomal dominant disease stems from expanded polyglutamine (CAG) repeats in exon 1of the human HD gene (MacDonald 1993). CAG is the codon for glutamine, and this trinucleotide repeat is responsible for the polyglutamine moiety within the huntingtin protein. Normal range of the expanded glutamine (polyQ) tract is between 25-35 repeats, with HD symptoms appearing with polyQ tracts greater than 35 repeats (Group 1993). There is an inverse correlation between the length of the CAG repeats and the age of onset of HD (Group 1993; Telenius *et al* 1993), and repeat lengths greater than 70 produce an HD phenotype characterized by juvenile onset, and rigidity, dystonia and tremor as presenting symptoms.

Although the HD protein huntingtin (htt) was discovered over a decade ago, its function is still not fully understood. The protein coded by the HD gene is ubiquitously expressed throughout the body and has widespread subcellular localization, thus contributing to the difficulty in determining its function. However, many proteins interact with htt, which can offer clues to elucidate the function of htt and the pathogenesis of HD. These protein-protein interactions are based on the shape of the three-dimensional structure of huntingtin and the poly-Q expansion in the N-terminal region (Poirier *et al* 2005). Based on their known functions, htt-associated proteins can be grouped as proteins that are involved in gene transcription and proteins that are involved with intracellular signaling, trafficking, endocytosis or metabolism (Li & Li 2004a). This involvement means that htt is associated with a variety of cellular structures including the nucleus, endoplasmic reticulum, Golgi complex, synaptic vesicles and mitochondria (DiFiglia *et al* 1995; Gutekunst *et al* 1998; Velier *et al* 1998). Wild type htt undergoes four types of post-translational modifications: sumoylation and ubiquination of lysines at the N-terminal, phosphorylation of serines; and palmitoylation at an unknown amino acid (Huang *et al* 2004; Humbert *et al* 2002; Kalchman *et al* 1996; Steffan *et al* 2004). Huntingtin also contains three protease cleavage sites leading to fragments of both normal and mutant huntingtin (mhtt) (Wellington *et al* 2002). It was found that N-terminal mhtt forms aggregates in the nucleus and the cytoplasm, whereas full-length mhtt is diffuse in the cytoplasm of brain cells (DiFiglia *et al* 1997; Sharp et al 1995). The structure of the N-terminal fragments arises from the inherent protein misfolding conferred by the polyQ expansions and the resulting pathology is still being explored.

Although it has been difficult to define the precise role of huntingtin, its role in embryonic development has been demonstrated. After the discovery of the HD gene it was shown that a complete knockout of the gene is embryonically lethal (Duyao *et al* 1995). Mutant huntingtin is not entirely dysfunctional, since it can rescue the embryonic lethal phenotype seen in the huntingtin-null knockout mice (Dragatsis *et al* 1998).

Pathology of Huntington's Disease

Although wild-type (WT) and mutated huntingtin protein is ubiquitously expressed in many neuronal subtypes, the medium spiny neurons (MSN) of the striatum and to a lesser extent, the pyramidal neurons of the cortex are more vulnerable to degeneration, thus contributing to the clinical symptoms of the disease. Although there is widespread brain atrophy from cell loss, the cortex and striatum are most heavily affected. The striatal atrophy leads to enlargement of the lateral ventricles. Since MSNs encompass 90% of neurons in the striatum, there is a hallmark degeneration of this region of the basal ganglia with accompanying pathological changes in the thalamus and subthalamic nucleus (Hedreen *et al* 1991; Vonsattel & DiFiglia 1998). Gliosis and atrophy are also seen in the substantia nigra pars reticulata. The striatum receives a large glutamatergic and trophic input from the cortex, and this connection can serve as a possible contributor to the resulting pathology. In addition to the changes in the striatum there is atrophy of the cortex and thalamus in late stages of the disease (Vonsattel & DiFiglia 1998).

Within the striatum, HD differentially affects subpopulations of neurons, with projection neurons preferentially being lost rather than interneurons (Albin *et al* 1990; Difiglia 1990). Studies have shown early in the disease process that projection neurons that contain both enkephalin (ENK) and gamma-aminobutyric acid (GABA) are lost more rapidly than those that contain substance P (SP) and GABA (Deng *et al* 2004). The significance of this observation is enkephalin/GABA-containing neurons project to the external globus pallidus (GPe) and constitute the striatal part of the indirect pathway, whose normal activation suppresses unwanted movement. In early HD, thus, the function of the indirect pathway is predominantly disrupted, and basal ganglia circuitry models predict an overall increase in unwanted movement, manifested as chorea. This finding provides an anatomical substrate for the increased adventitious movement that is the hallmark of HD (Albin *et al* 1992; Deng *et al* 2004; Reiner *et al* 1988). The substance P/GABA-containing striatal neurons project to the internal globus pallidus and the substantia nigra, and thus constitute the striatal part of the direct pathway, whose activation promotes planned, appropriate movement. In later stages of adult HD, both populations of striatal projection neurons are affected (Reiner *et al* 1988), and both akinesia predominates.

Huntington's disease is also characterized by the presence of neuronal intranuclear inclusions (NIIs) in striatal and cortical neurons (DiFiglia *et al* 1997). These aggregates consist of the N-terminal fragments of mhtt and appear early in the disease process, often before other phenotypes (Vonsattel & DiFiglia 1998). It is the cleavage of

the mhtt N-terminals that promote this aggregation and it is brought about by activation of proapoptotic caspases (Wellington *et al* 2002). NIIs are also present in several transgenic HD mouse lines with a similar appearance before behavioral phenotypes. It was found that N-terminal htt fragments expressed in transgenic animals or cultured cells are more toxic than full-length htt (Davies *et al* 1997; Hodgson *et al* 1999; Schilling *et al* 1999). It is still unresolved if the aggregates are the cause of clinical symptoms or are the result of a protective mechanism that sequesters mutant protein.

Pathogenesis of Huntington's Disease

Since the gene has been discovered, there has been intense effort to find out why the predominant pathology of HD by mhtt is limited to the brain and particularly affects striatal neurons. One theory is that death in neurons occurs through apoptosis, that is, activation of a programmed cell death pathway triggered by mitochondrial dysfunction. There is evidence that indicates that WT htt triggers molecular events that lead to increased cell survival. Such pro-survival mechanisms include the inhibition of pro-caspase 9 and, and a pro-apoptotic protein, huntingtin-interacting protein 1, by WT htt (Gervais *et al* 2002; Rigamonti *et al* 2000; Zhang *et al* 2006). Mitochondria play a role in the process of caspase activation and apoptosis. 3-nitroproprionic acid (3-NP) is an irreversible inhibitor of mitochondrial complex II, and causes apoptotic neuronal death through the release of cytochrome-c with consequent activation of caspases (Almeida *et al* 2006). Injection of 3-NP or malonate (a reversible mitochondrial complex II inhibitor) into the striatum leads to neuronal loss resembling that in HD (Beal *et al* 1993; Shear *et al* 1998). In human post mortem analysis, DNA fragmentation characteristic of apoptosis has been described in HD brain (Thomas *et al* 1995). Although these findings are promising for understanding HD pathogenesis, it is not yet clear if apoptosis is the principle event responsible for striatal cell death in HD.

Theories of HD pathogenesis also implicate excitotoxicity or energetic dysfunction. Glutamate is responsible for excitatory synaptic transmission in the central nervous system by acting upon three classes of ionotropic receptors: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate receptors. The selective loss of striatal projection neurons could come about due to overactivity of the vast glutamatergic input from the cerebral cortex, while striatal interneurons, which are less affected in HD, have fewer excitatory inputs. MSNs showed an increased NMDA current density and swelling compared to interneurons in striatal slices from HD mutant mice (Cepeda *et al* 2001). This suggests that continuous exposure of ionotropic NMDA receptors to glutamate can be excitotoxic in HD and leave striatal neurons vulnerable to damage. The NMDA receptor is important in synaptic plasticity as well as cell death due to its voltage dependent magnesium block and high calcium permeability (Bliss & Collingridge 1993). Excessive stimulation causes an influx of calcium ions, leading to activation of enzymes that degrade proteins and cause cell death (Berliocchi *et al* 2005). Striatal NMDA receptor function is enhanced in the R6/2 and other transgenic HD models (Cepeda *et al* 2001; Laforet *et al* 2001; Levine *et al* 1999). Presynaptic group II metabotropic glutamate (mGluR2 and mGluR3) receptors located at

the corticostriatal terminals are capable of regulating the release of glutamate (Calabresi *et al* 1996; Cepeda *et al* 2001). The mGluR2/mGluR3 receptors belong to heterogeneous family of glutamate receptors that are linked to GTP-binding proteins, and this particular class is negatively coupled to cyclic AMP production (Nakanishi 1992). It is known that mGluR2 receptors are located presynaptically and can negatively modulate glutamate release (Battaglia *et al* 1997). Activation of these receptors by an agonist reduces excitatory-post-synaptic-potentials (EPSPs) in striatal neurons (Calabresi *et al* 1996), thus dampening the effect of glutamate release from corticostriatal terminals. Any alteration of these receptors could lead to a pathological increase of glutamate release, and it was discovered that there is a decreased expression of mGluR2 and mGluR3 receptors in the transgenic HD mouse R6/2 (Cha *et al* 1998).

Excitotoxicity can also come about due to decreased clearance of extracellular glutamate from the synaptic cleft. This uptake of glutamate is carried out via the EAAT2 transporter by glial cells. There is a decrease of this transporter and the enzyme glutamine synthetase in HD mice (Lievens *et al* 2001). Glutamine synthetase is primarily found in astrocytes in the brain and aids in diminishing excitotoxicity of neurons by uptake of excessive glutamate (Suarez *et al* 2002). Mutant huntingtin also is present in glial cells, and it was shown that there is an increase neuronal vulnerability among neurons cultured with astrocytes expressing mhtt as opposed to neurons cultured with astrocytes expressing wild-type htt (Shin *et al* 2005).

Normal huntingtin has been linked to brain derived neurotrophic factor (BDNF) via different mechanisms involving BDNF transcription and vesicular transport. This protein is important for the survival of striatal neurons (Widmer & Hefti 1994). BDNF is co-localized with huntingtin in cortical neurons that project to the striatum and most striatal BDNF is produced in the cerebral cortex (Altar *et al* 1997; Baquet *et al* 2004; Fusco *et al* 2003). Wild-type huntingtin promotes the vesicular transport of BDNF along microtubules via huntingtin-associated-protein 1(HAP1) (Gauthier *et al* 2004), and BDNF transport to striatum is thus impaired in HD. Evidence for pathogenesis stemming from diminished BDNF production in the cortex and transport to striatum comes from multiple HD mice. Reduced cortical mRNA levels for BDNF and reduced cortical and striatal protein levels are found in YAC mice (Zuccato *et al* 2001), R6/2 mice (Zuccato *et al* 2005), and mutant huntingtin knock-in mice (Gines *et al* 2003). These data indicate that decreased BDNF transcription occurs in HD. It was shown that mhtt reduces the transcription of several exons of BDNF in the cortex due to both loss of wild type huntingtin and a toxicity of the mutant protein (Zuccato *et al* 2001; Zuccato *et al* 2005). Baquet *et al* showed behavioral and striatal changes with a cortical BDNF knock-out mouse model that paralleled the neurological dysfunction seen in R6/2 mice (Baquet *et al* 2004).

Therapies in Huntington's Disease

It is challenging to perform therapeutic trials in HD patients for a variety of reasons. Since there is a low prevalence of the disease, recruitment of a sufficient

number of patients is difficult. The disease has a slow progression so potential drugs that might have a benefit require a long follow up. It is important that all original therapies are tested in cell cultures and animal models before being moving to clinical trials. Although it is not known what causes death of striatal neurons and sickness of cortical neurons in HD as discussed in previous sections, there are novel ideas being tested with various kinds of therapies. The ability to screen for new agents has advanced rapidly with understanding of HD pathogenesis. Among methodologies, yeast and cell culture models are inexpensive and lend themselves to rapid testing. Studies typically begin by screening compounds on cultured neurons for efficacy. High throughput assays screen for molecules that target specific pathways (such as huntingtin aggregation *in vitro*) or that block cell toxicity (Apostol *et al* 2003; Murphy *et al* 2000). Potential therapeutic agents are being identified in studies of excitotoxic and mitochondrial toxin models of HD. A strategy is to move these compounds to a more realistic model than cell cultures and a number of agents have been shown to be effective in transgenic mouse models of HD. In light of the strong evidence of excitotoxic damage in HD as discussed earlier, an *in vivo* model offers a better choice to test ideas about excitotoxicity.

A valid *in vivo* model should show a measureable progression of pathology replicating mid- to late-life onset of motor impairment. The R6/2 HD mouse is a good *in vivo* model because it replicates several phenotypes and neuropathologies seen in humans, including a progressive motor and cognitive impairment (Bolivar *et al* 2003; Carter *et al* 1999; Murphy *et al* 2000), ubiquitinated nuclear and cytoplasmic inclusions of mhtt (Davies *et al* 1997; Li *et al* 1999), and decreased brain and striatum size (Mangiarini *et al* 1996). In a microarray study of postmortem human HD cases, it was found that R6/2 mice reproduce the transcriptomic changes of human HD (Kuhn *et al* 2007). This animal model has been shown to have a decrease in cortical mGluR2/3, which is capable of contributing to excitotoxicity (Cha *et al* 1998). As discussed earlier, an increased NMDA receptor-mediated current could lead to excitotoxic effects and activation of a presynaptic cortical autoreceptor could limit this effect. R6/2 mice were also shown to have a decrease in measured ENK-immunoreactive fiber intensity in GP when compared to wild type mice at 12 weeks of age (Sun *et al* 2002). This may be indicative of an early stage of striatal neuron degeneration in adult onset HD (Reiner *et al* 1988; Sapp *et al* 1995). Sun *et al* found elevated SP-immunoreactive fiber intensity in SN of R6/2 at 12 weeks of age, which counters that found in adult-onset HD. This could reflect a dysfunction in release of SP by striatonigral neurons (Sun *et al* 2002). Volume changes of brain regions are also reflected in the transgenic models. There is a 41% striatal volume reduction and total brain reduction of 44% of R6/2 mice at 90 days (Stack *et al* 2005). This is accompanied by a 26% striatal neuron loss. Preliminary studies with the R6/2 transgenic mouse showed that this model develops several progressive motor pathologies (Mangiarini *et al* 1996). These included progressive hypoactivity, resting tremors, abrupt and shuddering movements, epileptic seizures, foot clasping and dyskinesia of hindlimbs upon tail suspension (Carter *et al* 1999).

Precise and definable measured patterns of movement and locomotion are critical endpoints in assays with HD mice. Open field assays test many motor and behavioral endpoints, and many of these traits are affected in HD. Since the pathology exists in the

basal ganglia, open-field testing is beneficial for evaluating the complexity of exploratory behavior. The Golani and Drai automated open field task allows rapid and automatic measurement of many aspects of locomotion (Drai & Golani 2001). These measured parameters include bursts of locomotion called progression segments and pauses termed lingering episodes. The velocities of the progression segments and the number of stops are calculated for an open field session. The use of SEE (Software for the Exploration of Exploration) allows the parameters to be isolated and quantified. Measured endpoints reflect processes of navigation, motivation, locomotion, spatial memory and learning (Drai & Golani 2001). Rotarod is another assay that measures coordination and balance. R6/2 mice have difficulty maintaining balance on the device at 5-6 weeks of age (Carter *et al* 1999; Luesse *et al* 2001). One caveat of the test is that there is a cerebellar component to completing the task (Crawley 1999). Other endpoints to measure that reflect overall health are weight loss and lifespan. When compared to wild type (WT) littermates, the R6/2 transgenic mice show a decline in body weight after 10 weeks of age. Lifespan for this model is 10-13 weeks (Carter *et al* 1999; Mangiarini *et al* 1996).

CHAPTER 2. RESEARCH OBJECTIVES AND SPECIFIC AIMS

Excessive glutamate release from cortical terminals in striatum can result in a pattern of injury that is similar to the one seen in Huntington's disease. Previous work was shown that the R6/2 Huntington's disease (HD) mouse model has an enhanced striatal NMDA receptor-mediated current (Cepeda *et al* 2001). The increased activation of striatal neurons can result in an excitotoxic loss of medium spiny neurons in the striatum. One method to curtail this increase is the use of an mGluR2/3 receptor agonist. Activation of this receptor, which is localized to cortical terminals in striatum, decreases the release of glutamate and thus dampens excitotoxic effects (Calabresi *et al* 1996).

An inviting drug for HD therapy is an mGluR2/3 agonist. There is evidence that mGluR2/3 agonists have significant neuroprotective potential. Administration of LY379268 (a mGluR2 agonist) was shown to limit ischemic and hypoxic injury (Bond *et al* 1999; Kingston *et al* 1999). Excitotoxic injury involves the same caspases that are involved in HD and thus lends credence to LY379268 being a good choice of experimental therapeutics to limit neuronal death in HD. In a previous study, LY379268 showed a benefit on lifespan but not rotarod performance in R6/2 HD transgenic mice (Schiefer *et al* 2004). While these results are promising, the study was limited in several ways. The drug was dosed at 1.2mg/kg dissolved in water, and then given orally to the subjects via an applicator tube in the upper esophagus. A question can be raised if maximal benefit can be achieved from LY379268 from such a low dose. The neuroprotective effects of LY379268 were seen in other studies with dosings much higher than 1.2mg/kg as used by Schiefer *et al* (Bond *et al* 1999; Cai *et al* 1999; Kingston *et al* 1999). The study used NIIs as a histological endpoint, and found no differences in distribution and size of NIIs among R6/2 mice given LY379268 or a placebo. The authors used NIIs as the only morphological measure of pathological changes in HD mice, while there are other known phenotypical changes that can be considered, such as distinct volume changes and neuronal abundance. A complete study should include measurements of these other morphological changes. Behavioral testing in the Schiefer study included an open field assay which showed a decline in hyperactive spontaneous locomotion with LY379268 administration as compared to control mice. It is possible that more improvement could be observed with a higher dose of the drug and more sophisticated behavioral testing. There are many approaches that measure locomotion and motor skills more robustly than used by Schiefer *et al* (2005). A study is needed showing the efficacy of a higher dose of LY379268 on behavioral endpoints and numerous morphological pathologies. Some of the treatments that target excitoxicity and oxidative stress which have shown to be beneficial in transgenic mouse models may improve some of the symptoms in HD patients and it is therefore logical to pursue therapies aimed in this direction. A more detailed exploration of the ability of LY379268 to reverse the behavioral, motor and histological abnormalities in the R6/2 is carried out with this series of experiments. All of the fixed-tissue histological and morphometric data collection was completed by me, while the behavioral work and *in situ* hybridization (ISH) was carried out by other personnel in the lab, but the behavioral and ISH results are

presented in this thesis because they are a critical adjunct to the histological data in giving a complete picture of potential therapeutic outcomes with LY379268.

The aims of the project were:

- **Aim 1:** To test the hypothesis that LY379268 can normalize known changes in histological endpoints.

- **Aim 2:** To test the hypothesis that LY3979268 can normalize the lifespan, body weight, behavioral and motor abnormalities seen in the R6/2 mouse model.

CHAPTER 3. EXPERIMENTAL PROCEDURES

Subjects

R6/2 mice, which bear a transgene for the 5' end of the human HD gene carrying about 150 CAG repeats and including the promoter region, were obtained from Jackson Laboratories (Bar Harbor, ME). A colony was maintained by breeding R6/2 mice with CBA X C57BL/6 F1 (B6CBAF1) mice, thereby obtaining offspring that were hemizygous for the transgene and wild-type littermates. Mice were genotyped and CAG repeat length determined using a PCR-based amplification method (Sun *et al* 2002). R6/2 and WT mice were randomly assigned to either control treatment with vehicle or treatment with LY379268. Daily subcutaneous injections of LY (20mg/kg) began after genotyping at PND21. Thirty-four mice were used in the four treatment groups for the fixed-tissue histological analysis. Seventy-seven mice were used in the four treatment groups for behavioral analysis.

Tissue Fixation

Under deep anesthesia (avertin; 0.2 cc/g body weight), transgenic R6/2 mice and wild-type mice were perfused transcardially with 60 ml of 6% dextran 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 PB (PLP). The brains were removed and stored for at least 24 hours in a 20% sucrose/10% glycerol solution at 4°C. The fixed brains were 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 series of sections from the brain of each mouse was stained for cresyl violet to study normal brain histology.

Immunohistochemistry

Immunohistochemical labeling was carried out to characterize abnormalities in cortex or striatum. Immunohistochemical single-labeling for ubiquitin, to visualize NIIs in R6/2 mice, was carried out using a peroxidase-antiperoxidase (PAP) procedure described previously (Anderson & Reiner 1990). Immunolabeling was also used for NeuN for neuron labeling, substance P (SP) for SP+ striatal projection systems, and enkephalin (ENK) for ENK+ striatal projection systems. To carry out immunohistochemical labeling, sections were incubated for 24 hours at room temperature in primary antiserum diluted 1:2000–1:5,000 with 0.3% Triton X-100/0.02% sodium azide/0.1 M PB (PBX). Sections were then rinsed and incubated in donkey anti-mouse IgG or anti-rabbit IgG (depending on the primary antiserum) diluted 1:50 in PBX, followed by incubation in the appropriate mouse or rabbit PAP complex diluted 1:200 in PBX, with each incubation at room temperature for 1 hour. The sections were rinsed between secondary and PAP incubations in three 5-minute washes of PB. Subsequent to

the PAP incubation, the sections were rinsed with three to six 10-minute washes in 0.1 M PB, and a peroxidase reaction using diaminobenzidine tetrahydrochloride (DAB) was carried out. Sections were incubated in 5 ml of 0.05 M imidazole/0.05 M cacodylate buffer (pH 7.2) containing 5 mg DAB for 10 minutes and then incubated for an additional 10 minutes after adding 20 µl of 3% hydrogen peroxide, with continuous agitation throughout. Sections were then washed in distilled water, placed in 0.1 M PB, mounted onto gelatin-coated slides, dried, dehydrated, and cover slipped with Permount.

Microscopic Analysis

NII Frequency and Size

Sections immunolabeled for ubiquitin from R6/2 mice and wild-type mice were analyzed for NII size and frequency. This analysis was carried out blinded to the treatment groups. For each mouse, images of primary motor cortex and striatum were captured from each of three sections, one at the level of rostral striatum, one at the level of the midstriatum, and one just behind the anterior commissure using a 40X objective on an Olympus BH-2 microscope, a Spot camera, and a Mac computer. These images were used to determine NII frequency (number of NIIs per field), size, and percent of the field occupied by NIIs for the cortex and striatum. Three adjacent images of primary motor cortex were captured and analyzed for each level, with analysis performed separately on layers II/III, V, and VI. For striatum, images of central, dorsomedial, ventromedial, dorsolateral, and ventrolateral striatum were analyzed for each level. Frequency and percent of the field occupied by NIIs was determined by thresholding NIIs using Image-J software (from U.S. National Institutes of Health, available at http://rsb.info.hih.gov/nih-image). NII size (*i.e.*, diameter) was also measured for cortex and striatum using Image-J. Artifacts in the images were removed prior to thresholding, and the size of the thresholded NIIs was assessed using the Particle Measurement function of NIH Image.

Volume Measurements

Blinded examination of sections through the rostral telencephalon suggested lateral ventricular expansion and striatal shrinkage in the R6/2 brains. To evaluate this quantitatively, image analysis was blindly carried out to determine the volume of the telencephalon, cerebral cortex, lateral ventricle, striatum, and septum for the rostral telencephalon (*i.e.*, from the end of the olfactory bulb to the level just behind the anterior commissure). For each mouse an image of each section in a one-in-six series from the rostral telencephalic pole to the level of just behind the anterior commissure, at which levels the lateral ventricles are narrow in normal mice, was captured at high resolution (4800 dpi) using a Epson scanner. NIH Image was used to measure the areas occupied by the entire telencephalon, the cerebral cortex, the lateral ventricles, the striatum, and the septa on each side of the brain. 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 septum was defined as the region between the corpus callosum and a horizontal line connecting the ventral tips of the ventral horns of the lateral ventricles. Section thickness and spacing were used to determine the volume of each structure from the areal measurements in the cross-section. Because brain size is correlated with brain size, comparisons of absolute brain size of the above individual brain areas between R6/2 mice and WT mice is confounded by the smaller size of R6/2 mice. To correct for this, we present the volume data divided by body weight raised to the 0.66 power (the allometric scaling exponent for the relation of body size to brain size) (Lande, 1979).

Image Analysis of Immunolabeling in Striatal Target Areas

To assess injury to the neurons of origin of the striatal projection systems, quantitative computer-assisted image analysis was carried out on immunolabeled striatal terminals in each of the main striatal projection targets, blindly to treatment group. For these studies, the areal extent and immunolabeling intensity of the ENK+ fiber plexus in the GPe and the SP+ fiber plexuses in the GPi and substantia nigra were quantified. For GPe, its ENK+ plexus in the 2-4 sections spanning its rostrocaudal extent were captured bilaterally. For GPi, images of the entire GPi from 1-2 sections spanning its rostrocaudal extent were bilaterally captured for analysis. For substantia nigra, the entire nigra in 2-4 sections spanning its rostrocaudal extent were captured and analyzed bilaterally. The immunolabeled fibers in these striatal target areas were highlighted and the area occupied by them and their labeling intensity determined using the thresholding and optical density measuring capabilities of Image-J. 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 to a value of 100 on the 0-255 gray scale employed by the Image program using the image editing capabilities of this program. The region of each image used to set the background intensity was an area of unlabeled white matter within the field of view. For GPe and GPi the external capsule was used for optical density standardization, while for images of the substantia nigra the cerebral peduncle was used. Fiber abundance for a given structure in a given case was expressed as the mean percent of the structure occupied by labeled fibers for all measurements for that case. Fiber abundance for each target area for each HD case was then expressed as a percent of control.

Stereology

Stereological neuron counts were carried out blindly to the treatment groups of the mice. A one-in-six series immunolabeled for NeuN from just behind the olfactory bulb to the level of the anterior commissure were used for striatal and cortex neuron analysis. Landmarks were as those noted above for the volume measurements. Unbiased stereological counts of striatal and cortical neurons were obtained by using Neurolucida Stereo Investigator software (Micro-Brightfield, Colchester, VT). The dissector counting method was used where counting frames are taken from a random series of evenly spaced

sections. These counting frames are chosen by the software within predefined areas used for demarcating the volumes of the striatum and cortex.

Behavioral Analysis

Rotarod

Rotarod was performed at the same time of the day to be consistent with the diurnal activity phase. During the training period, each mouse was placed on an accelerating rotarod starting at 0 rpm and accelerating gradually up to 30 rpm at 240 seconds. This maximum velocity was maintained for an additional 120 seconds. Mice received four training trials during the first session, by which time a steady baseline level of performance was achieved. Rotarod sessions were run twice a week, with x trials per session after the first four-trial training session. The trial was considered over when a mouse fell off the rotarod or when it stayed on the rod for a maximum of 360 seconds. The latency to fall off was recorded and used in subsequent analysis.

Open Field

A circular open field arena with a diameter of 180 cm and 60 cm high walls was used to evaluate locomotor activity of the mice. Each mouse was placed in the arena for a 30-minute trial once a week. A mouse was placed near the wall and its behavior in the arena was recorded throughout the session by a stationary video camera whose lens covered the whole cylinder field. The locomotor path traversed by the mouse was recorded by an automated tracking system using Ethovision, and analyzed by SEE software. The SEE software extracted a number of locomotor parameters of interest, as presented here, including length and duration of locomotor bursts called progression segments, time spent locomoting (activity), duration and time spent lingering, speed and acceleration during progression segments, and spatial distribution of progression segments.

Statistical Analysis

The body weight, rotarod and open field data were analyzed statistically using SAS software and a mixed model ANOVA, considering genotype, drug, gender and their interactions in the analysis. Data for the immunohistochemical studies were analyzed statistically by ANOVA followed by Fisher PLSD individual group comparisons, reciprocal transforming data as need to normalize distributions.

CHAPTER 4. RESULTS

Histological Studies of Fixed Tissue

The effects of LY379268 were blindly analyzed on telencephalic, cortical, striatal and lateral ventricular volumes. Other parameters included neuron abundance in cortex and striatum, neurochemical integrity of striatal projections systems, and NII size and frequency. The completed analysis included 8-10 LY-treated R6/2 mice (average CAG = 119.1), 9-10 vehicle-treated R6/2 mice (average CAG = 118.3), 9-10 LY-treated WT mice, and 8-11 vehicle-treated WT mice.

Volumetric Analysis

Volumes were measured blindly from the end of the olfactory bulb to the level behind the anterior commissure. The R6/2 mice had a smaller body mass. To correct for the impact of this on brain size, and determine if the R6/2 genotype and/or the drug treatment affected brain part size, brain volumes were expressed using an allometric correction. Brain and body weights are related by the following allometric equation "brain = κ(body)$^{\alpha}$" where $\alpha = 0.67$ (Lande, 1979). Thus, dividing brain size by body weight raised to the 0.67 power corrects for the impact of body size variation on brain size.

As shown in Table 1, telencephalic volumes differed among the groups, but were similar between the R6/2 vehicle-treated and WT vehicle-treated mice. This data set shows that WT-LY treated group telencephalic volume was greater (69.1mm^2) than in any other group, but the telencephalic size reflected a larger body size. The allometric correction shows that this increase is not significant since corrected telencephalic volume is 104.9% of that in WT-V. The average measured volumes of cortex in R6/2 mice were slightly smaller than in WT mice, but the difference was not significant. Treatment with the LY compound did not produce a significant effect on cortex volume in either the WT or R6/2 mice compared to the vehicle-treated mice. Volume of the striatum in R6/2-LY mice was, however, less than in vehicle-treated WT mice. There was a significant and large increase in ventricular volume in both R6/2 mice groups in contrast to WT mice. Treatment with LY379268 did not cause a significant decrease in ventricular volume in the R6/2 mice compared to the vehicle-treated R6/2 mice. These various results are shown in Table 1.

Neuron Counts

The neuron abundance for cortex and striatum was significantly less in the R6/2 vehicle as compared to the WT vehicle treated mice (85% and 83.7%, respectively). Stereological neuron counts also showed significantly fewer neurons in vehicle-treated R6/2 mice than LY-treated R6/2 mice in cortex and striatum. Cortical and striatal neuron

Table 1. Volumetric Analysis

Mouse Treatment Groups	# of Mice	Telen Volume in mm^2	Body Weight in Grams	Telen Volume / Body Weight	Telen Volume / $(Weight)^{.67}$	Cortex Volume / $(Weight)^{.67}$	Striatum Volume / $(Weight)^{.67}$	Ventricle Volume / $(Weight)^{.67}$	Septum Volume / $(Weight)^{.67}$
WT - Vehicle	8	62.0	21.8	2.92	100%	100%	100%	100%	100%
WT – LY379268	9	69.1	23.8	2.94	104.9%	107.8%	100.7%	114.5%	109.6%
R6/2 – Vehicle	9	62.2	22.3	2.82	99.4%	96.9%	99.6%	250.9%*^	106.5%
R6/2 – LY379268	8	60.6^	20.7	2.95	99.1%	92.8%	87.9%	236.7%*^	99.7%

Note: *significantly different from WT-Vehicle; ^significantly different from WT-LY379268

abundance, however, did not differ between the WT mice and the LY-treated R6/2 mice. Thus, there was about a 20% decrease in neuron abundance in striatum and cortex of R6/2-vehicle mice, which was rescued in the R6/2 mice by daily injections of 20mg/kg of LY379268 (Table 2).

Striatal Projection Systems

Immunohistochemical analysis of striatal projection neurons were measured by thresholding of the terminals projecting to the external pallidal segment (GPe) for enkephalin, internal pallidal segment (GPi) or substantia nigra (SN) for substance P. This labeling technique allows measurement of peptide abundance within these striatal targets. A benefit of the LY drug on R6/2 mice was seen in the SP projection neurons.

Striatonigral

As shown in Table 3, the percentage of nigra covered by SP fibers in R6/2-vehicle mice is significantly larger than in WT-V mice (39%) or WT-LY mice (42.4%). Daily injections of LY379628 reduced the percentage of nigra covered by SP fibers so it was not significantly different from the WT groups, but was less than in R6/2-V mice (21.9% lower). The intensity of SP labeled fibers in nigra is significantly elevated in the LY-WT group and the two R6/2 groups compared to the Vehicle-WT group. The intensity elevation in the WT-LY group indicates that the LY drug did affect SP+ striatonigral neurons in WT mice. Immunolabeling intensity is a reflection of the amount of peptide present, and in this case it is likely it reflects a failure of release, in both the WT-LY mice as well as the R6/2 mice groups, as explained in the Discussion. The pattern of SP immunolabeling in these mouse groups is illustrated in Figure 1. Note that in the R6/2-vehicle mice SP+ fibers cover the entire nigra, but in both the WT-Vehicle and R6/2-LY mice a lateral part of the nigra is notably poor in SP.

Striato-GPi

As shown in Table 4, SP+ fiber coverage was significantly greater in striato-GPi of R6/2-vehicle mice (106% of WT-V) compared to WT-LY mice (95.9% of WT-V), but SP+ fiber coverage was at WT-vehicle levels in R6/2 mice treated with LY379268 (101.6% of WT-V). This is consistent with the trend observed in striatal projection terminals in the nigra, but less dramatic. These data as well suggest that LY379268 normalizes SP+ fiber abundance in GPi in R6/2 mice. The intensity of SP labeling was significantly increased in striatal terminals in GPi of LY-treated WT mice (117.2%) as compared to WT-vehicle treated mice (100%). Thus, mGluR2/3 agonism significantly increased SP+ labeling in striato-GPi terminals in WT mice, which we interpret as diminished SP release due to diminished corticostriatal activation of striato-GPi neurons. The intensity of SP immunolabeling in striato-GPi terminals was also significantly

Table 2. Neuron Counts

Group	Number of Mice	Cortical Neuron Count	Cortical Neuron as % WT	Striatal Neuron Count	Striatal Neuron as % WT
WT – Combined	21	448,700	100%	231,157	100%
R6/2 – Vehicle	10	381,411.3*	85%	193,588.2*	83.7%
R6/2 – LY379268	10	461,906.5#	103.4%	241,399.8#	104.4%

Note: *significantly different from WT; #significantly different from R6/2-Vehicle

Table 3. Effect of LY379268 on Striatal Projection Neurons in Nigra of R6/2 Mice

Mouse Groups	# of Mice	Mean Repeat Length	% of Nigra Covered by SP+ Fibers from Direct Pathway Neurons	Intensity of SP+ Fibers in Nigra Arising from Direct Pathway Neurons
WT – Vehicle	8	N/A	100%	100%
WT – LY379268	8	N/A	96.6%	114.8%*
R6/2 – Vehicle	9	118.3	139.0%*^	115.5%*
R6/2 – LY379268	8	119.1	117.1%#	111.8%*

Note: *significantly different from WT-Vehicle; ^significantly different from WT-LY379268; #significantly different from R62-Vehicle
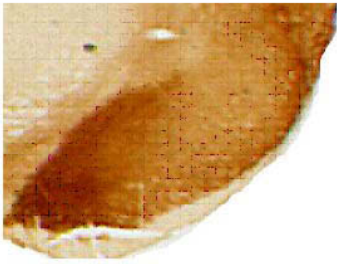
Figure 1. SP in Nigra

Table 4. Effect of LY379268 on Striatal Projection Neurons in GPi of R6/2 Mice

Mouse Groups	# of Mice	Mean Repeat Length	% of GPi Covered by SP+ Fibers from Direct Pathway Neurons	Intensity of SP+ Fibers in GPi Arising from Direct Pathway Neurons
WT – Vehicle	8	N/A	100%	100%
WT – LY379268	9	N/A	95.9%	117.2%*
R6/2 – Vehicle	9	118.3	106.0%^	122.4%*
R6/2 – LY379268	8	119.1	101.6%	120.4%*

Note: *significantly different from WT-Vehicle; ^significantly different from WT-LY379268

increased in both R6/2 mice groups, which is likely to be an effect of the genotype of striato-GPi neuron activity.

Striato-GPe

Coverage of GPe with immunolabeled ENK+ fibers in R6/2 – vehicle treated mice was significantly less than in WT-V mice (92.5% of WT-V). While seemingly similarly reduced in R6/2-LY mice (93.4%), this was not significantly different than WT-V. The fiber coverage in the two WT groups was not significantly different. There were no significant differences among the four groups in ENK+ fiber intensity in GPe. Overall, these results indicate no major effect of genotype on ENK+ fibers in GPe, and no major effect of LY379268 on the ENK+ fibers in GPe in either WT or 6/2 mice. These results are shown in Table 5.

Mutant Protein Aggregation

Measurements were carried out for ubiquitin immunolabeling of NIIs in 6 LY-treated R6/2 and 9 vehicle-treated mice. Measurements of NIIs were only carried out on the R6/2 mice, as NIIs do not form in WT mice. There were no significant differences in frequency (area of field covered by NIIs) or NII size in cerebral cortex or striatum between vehicle-treated and LY379268-treated R6/2 mice. For example, the percent of the field covered by NIIs was 1.27% in R6/2-V mice and 0.82% in R6/2-LY mice. Similarly, the percent of the field covered by NIIs in the striatum was 0.63% for R6/2 mice receiving LY379268 and 0.51% in vehicle treated R6/2 mice. The average NII size in the cortex of R6/2-LY was 2.09μm, and R6/2-Vehicle in 2.06μm, which is not significant. Similarly, NII size in the striatum of R6/2-LY mice was 1.54μm, and in striatum of R6/2-Vehicle mice was 1.61μm. While Schiefer *et al* (2004) reported that NII size in R6/2 mouse cortex was increased 20% using the same agonist, the current study demonstrated no evidence that LY379268 affects nuclear aggregate formation.

Histological Studies of Unfixed Tissue

The *in situ* hybridization (ISH) data was completed by Hong Bing Wang and is presented here for completeness. Tissue was analyzed that had been fresh-frozen ISH processed using digoxigenin detection with riboprobes for ENK and SP ISH from 10 WT-vehicle mice, 11 WT-LY mice, 10 R6/2-vehicle mice, and 11 R6/2-LY mice. All mice were 10 weeks old, and the labeling was analyzed using computer-assisted imaging and the Image-J analysis program. Data were analyzed statistically by ANOVA followed by Fisher PLSD individual group comparisons, reciprocal transforming data as need to normalize distributions.
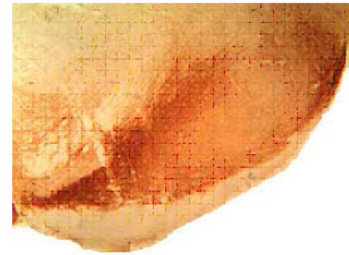
Table 5. Effect of LY379268 on Strial Projection Neurons in GPe of R6/2 Mice

Mouse Groups	# of Mice	Mean Repeat Length	% of GPe Covered by ENK+ Fibers from Indirect Pathway Neurons	Intensity of ENK+ Fibers in GPe Arising from Indirect Pathway Neurons
WT – Vehicle	8	N/A	100%	100%
WT – LY379268	8	N/A	97.1%	109.0%
R6/2 – Vehicle	9	118.3	92.5%*	106.5%
R6/2 – LY379268	8	119.1	93.4%	108.6%

Note: *significantly different from WT-Vehicle

ENK In Situ *Hybridization Analysis*

No significant differences were seen between WT-LY compared to WT-vehicle mice in total striatal ENK signal. Thus, LY379268 did not affect ENK+ striatal neurons in a way that affected ENK message in WT mice. By contrast, in R6/2-V mice overall striatal signal was greatly and significantly reduced (32.4% of WT-V). The overall striatal signal was also similar in the R6/2-LY mice, and was also significantly less than in WT-V (32.5% of WT-V). The ENK signal per perikaryon also did not differ between wildtype groups, but was reduced by about 50% in both R6/2 groups. Thus, as in the case of our ENK immunolabeling of the indirect pathway striatal projection to GPe, there was no benefit of LY379268 treatment for the ENK+ striatal neuronal population. These results are seen in Table 6.

SP In Situ *Hybridization Analysis*

As in the case of ENK message, no significant differences were seen between WT-LY compared to WT-vehicle mice in total striatal SP signal. Thus, LY379268 did not affect SP+ striatal neurons in a way that affected SP message in WT mice (Table 7). Total SP+ signal in the striatum of R6/2-V was 84.4% of WT-V, while in R6/2 mice receiving LY379268 total striatal SP+ signal was elevated to 14.3% above WT-V. Similarly, SP signal per perikaryon was reduced to 81.5% of WT-V in the R6/2-V mice, a significant reduction, but was not significantly different form either WT group in the R6/2-LY mice. Thus, by both immunolabeling and ISH, the data indicate LY379268 to benefit SP+ striatal neurons in R6/2 mice.

Behavioral Analysis

Survival

Figure 2 shows cumulative survival for the thirty-two 20mg/kg LY-treated (LY) and 30 control-treated R6/2 mice. The benefit of daily 20mg/kg LY379268 is evident. Lifespan was studied in 32 R6/2 mice treated with 20mg/kg of LY379268 daily, 17 R6/2 mice with vehicle, and 13 R6/2 mice with 10mg/kg LY379268. There was no significant difference in survival benefit between the R6/2 mice treated with 10mg/kg of LY and the vehicle treated group, thus these two groups were combined and compared with the R6/2 mice treated with 20mg/kg of LY379268. This dose was selected as the maximum tolerated dose (MTD) in tests on R6/2 mice carried out prior to the benefit assessments, using an MTD protocol recommended by the funding agency for this research, the Cure HD Initiative Foundation. The average CAG repeat length was 124.0 for the LY-treated R6/2 mice, and 123.6 for the control-treated R6/2 mice. There was no significant difference between CAG repeat lengths between males and females. The mean survival was significantly longer in the 20mg/kg LY-treated than the control-treated R6/2 mice

Table 6. LY379268 on ENK in Striatal Projection Neurons as a % of WT-Vehicle

Mouse Groups	# of Mice	Mean Repeat Length	Intensity of ENK+ ISH Signal per Perikaryon	Total ENK+ Signal in Striatum
WT – Vehicle	10	N/A	100%	100%
WT – LY379268	9	N/A	103.8%	107.9%
R6/2 – Vehicle	9	123.8	49.6%*^	32.4%*^
R6/2 – LY379268	11	124.7	50.3%*^	32.5%*^

Note: *significantly different from WT-Vehicle; ^significantly different from WT-LY379268

Table 7. LY379268 on SP in Striatal Projection Neurons as a % of WT-Vehicle

Mouse Groups	# of Mice	Mean Repeat Length	Intensity of SP+ ISH Signal per Perikaryon	Total SP+ Signal in Striatum
WT - Vehicle	10	N/A	100%	100%
WT – LY379268	10	N/A	96.7%	90.3%
R6/2 – Vehicle	8	123.9	81.5%*^	84.4%
R6/2 – LY379268	10	124.7	90.2%	114.3%#

Note: *significantly different from WT-Vehicle; ^significantly different from WT-LY379268; #significantly different from R6/2-Vehicle
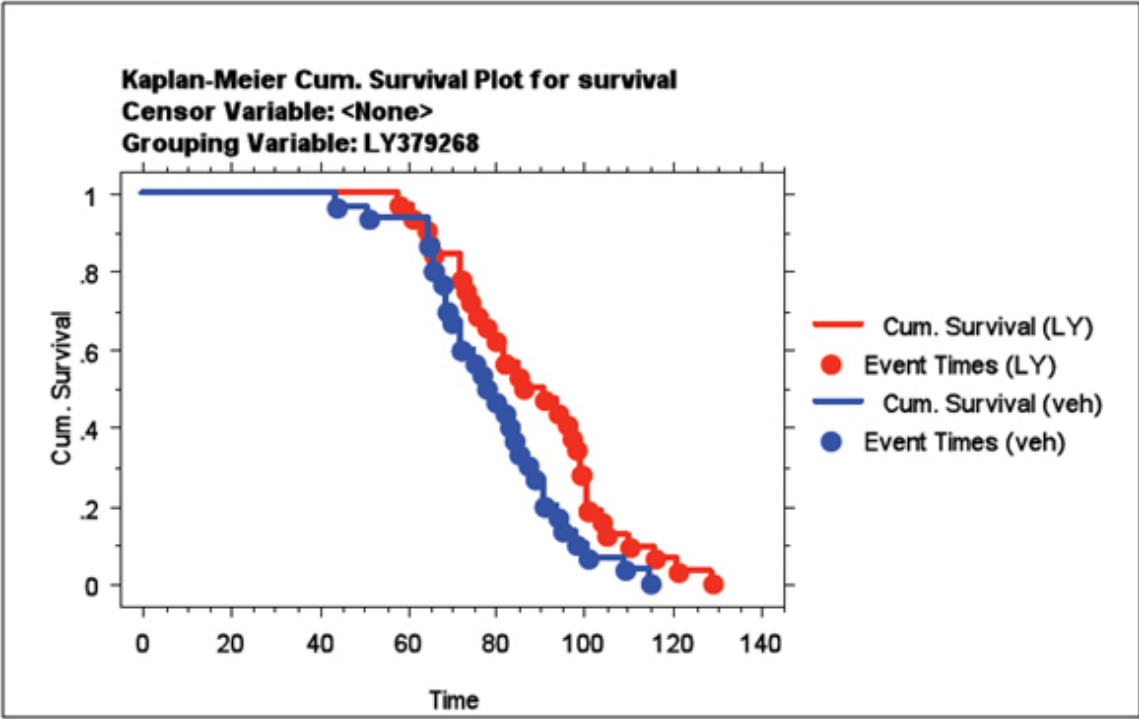
Figure 2. Survival

(88.5 days in drug-treated vs. 79.7 days in control-treated), by Mantel-Cox logrank test (p = 0.0276). There was a 9-day benefit for males and female mice treated with 20mg/kg of LY. Measured lifespan was 4 days less on average with males in both the control group and those receiving 20mg/kg of LY.

Body Weight

Figure 3 shows that mean body weight was persistently slightly lower in the 19 LY-treated WT mice (red) than in the 19 vehicle-treated WT mice (blue). This becomes apparent after 8 weeks. The 32 R6/2 mice treated with LY379268 (green) had a lower body weight than the 30 control R6/2 mice beginning at 13 weeks of life. Statistical analysis, however, did not show a significant effect of drug on body weight throughout the testing period, nor for the R6/2 mice at 14-16 weeks (p = .08). Thus, any effect of drug on body weight is slight and variable. ANOVA analysis did not reveal a significant effect of the drug-genotype interaction, indicating that any effects on body weight of 20mg/kg LY were similar irrespective of gender. There were however, significant effects of gender (males heavier than females in WT, females heavier than males in R6/2) and genotype (WT heavier than R6/2) on body weight.

Rotarod Performance

Figure 4 shows that LY379268 did not improve rotarod performance in 32 R6/2 mouse treated with a daily 20mg/kg dose. There was no discernable benefit of rotarod performance between LY and Vehicle treated R6/2 mice as seen with the blue and red lines in the figure. The R6/2 mice have an inferior performance at all time points when compared to the WT mice. The drug did not hinder rotarod performance in WT mice.

Open Field Measurements

Total Distance Traveled in Centimeters during 30 Minutes

As seen in Figure 5, vehicle-treated R6/2 mice locomote much less than WT mice during the 30-min session (*i.e.* are less active and cover less total distance), and the LY drug yielded better performance in R6/2 mice on this endpoint than observed in the vehicle-treated R6/2 mice at all ages. Of particular note, the LY drug normalized total locomotor activity until about 12 weeks of age. The benefit was comparable in males and females. The drug did not have an obvious deleterious effect on the WT mice in their total distance traveled. These observations were confirmed by ANOVA, which detected significant effects of genotype, drug and the drug-genotype interaction on distance traveled.
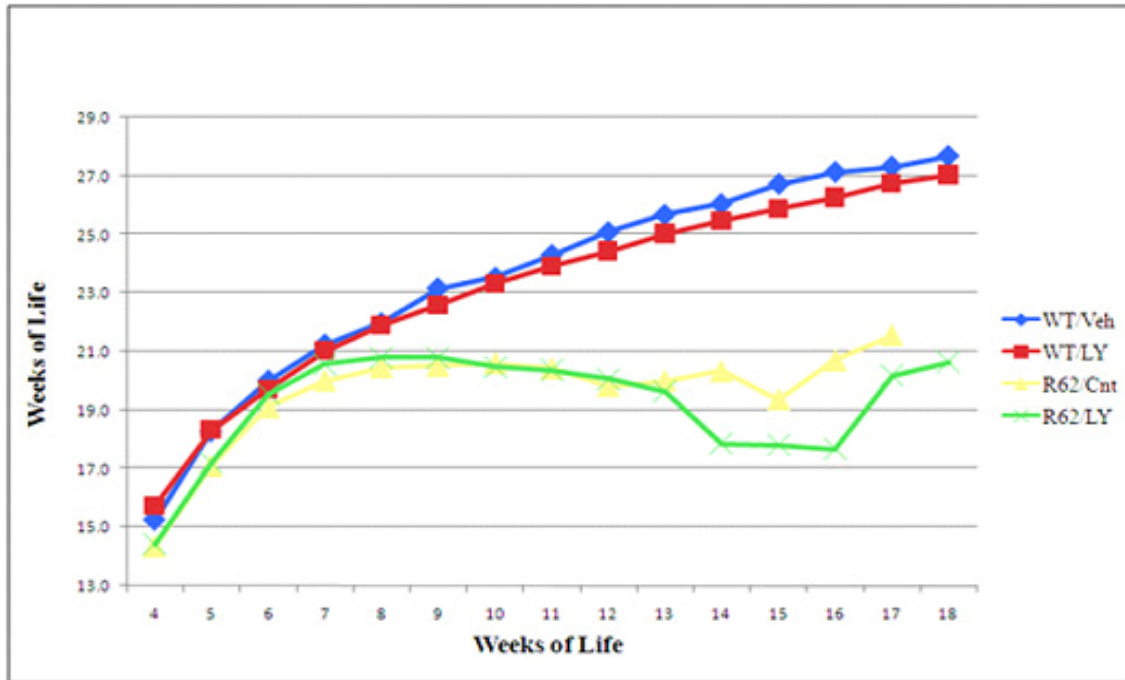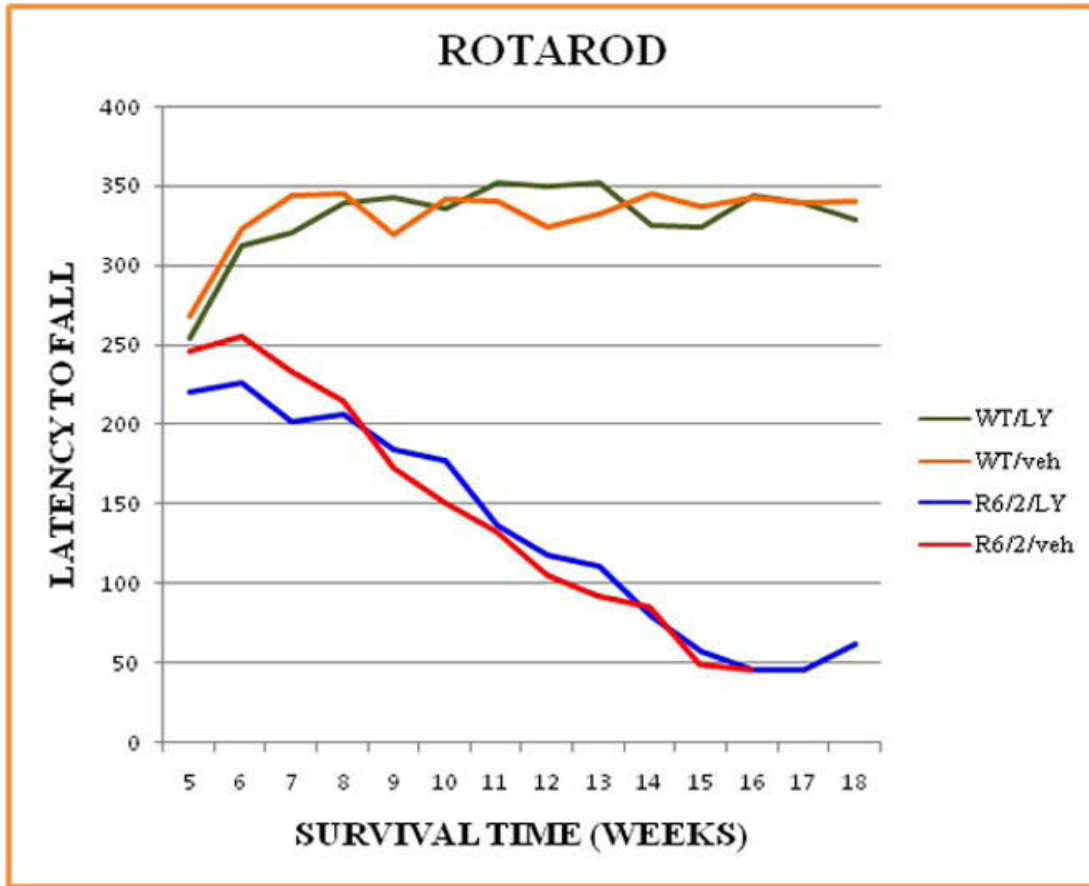
Figure 3. Effect of LY379268 on Body Weight

Figure 4. Rotarod Performance

Figure 5. Total Distance Traveled in Centimeters during 30 Minute Session

Median Progression Segment Length

Figure 6 shows an underpinning of the decreased distance traveled in vehicle-treated R6/2 mice, in that it shows that vehicle-treated R6/2 mice traversed shorter distances during their bursts of locomotion (called progression segments in the Drai and Golani lexicon) than did normal mice. Treatment of LY397268 in R6/2 mice showed an improvement in this parameter and progression segment length increased over those measured in WT mice between weeks 8 and 12 of life. These observations were confirmed by ANOVA, which detected significant effects of genotype, drug and the drug-genotype interaction on progression segment length.

Time Proportion Spent Lingering

Figure 7 shows that vehicle-treated R6/2 mice spent a greater proportion of the time between progression segments in pauses called lingering episodes than did WT mice. These pauses contributed to the overall decrease in activity in the vehicle-treated R6/2 mice. Note that the total proportion of time spent lingering is greater in vehicle-treated R6/2 than WT mice, and increases as the mice age. ANOVA confirmed a benefit of LY379268, with less lingering occurring, since significant effects of genotype, drug and genotype-drug interaction occur for the 9-17 week period (when the defect in vehicle-treated R6/2 mice becomes prominent).

Progression Segment Maximum Speed

Figure 8 shows that maximum locomotor speed during progression segments was much less in vehicle-treated R6/2 mice than in WT mice, and progressively worsened as the R6/2 mice approached the end of their lifespan. The LY drug strikingly improved R6/2 mice on this motor parameter. ANOVA confirmed a benefit of LY379268 in R6/2 mice on maximum locomotor speed, since significant effects of genotype, drug and genotype-drug interaction were observed.

Median Curvature of Progression Segments

Figure 9 shows that vehicle-treated R6/2 mice had increasing difficulty walking in a straight line (*i.e.* the curvature of their progression segments was increased), and also shows that the LY compound strikingly normalized R6/2 mice on this parameter for much of their lifespan. Beyond 15 weeks, path curvature in vehicle-treated R6/2 was diminished, as the shorter progression segments in the few surviving vehicle-treated mice may have made it easier for the mice to walk a straight line. ANOVA confirmed that for the period over which vehicle-treated R6/2 mice showed a progressive impairment, the drug was beneficial in the R6/2 LY-treated mice, since significant effects of drug, and drug-genotype interaction were seen for the 9-15 week period.
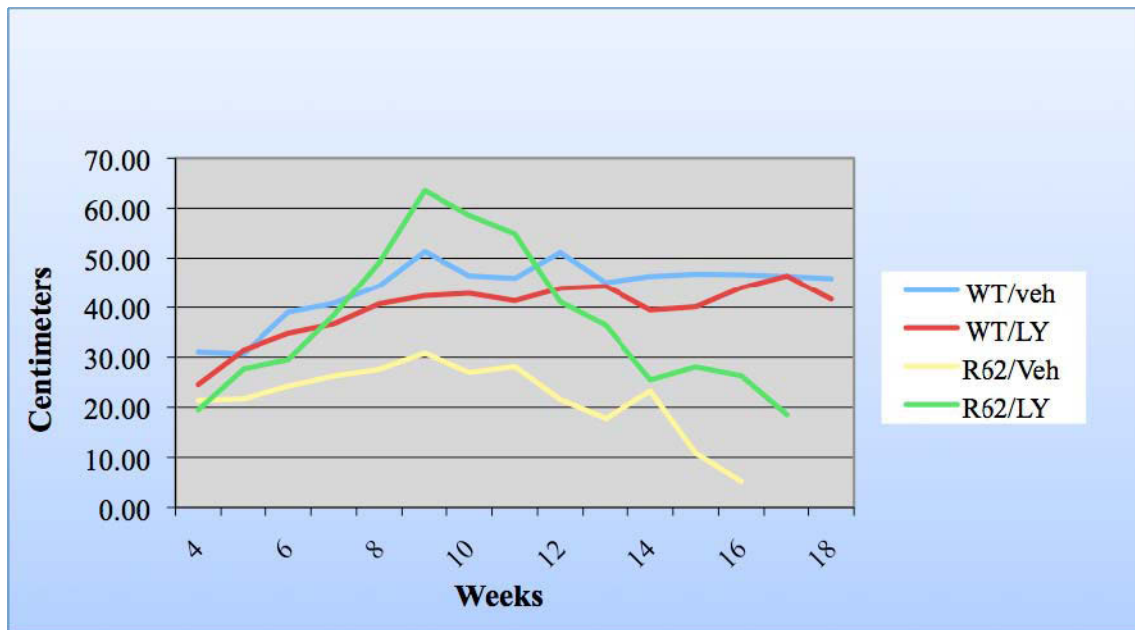
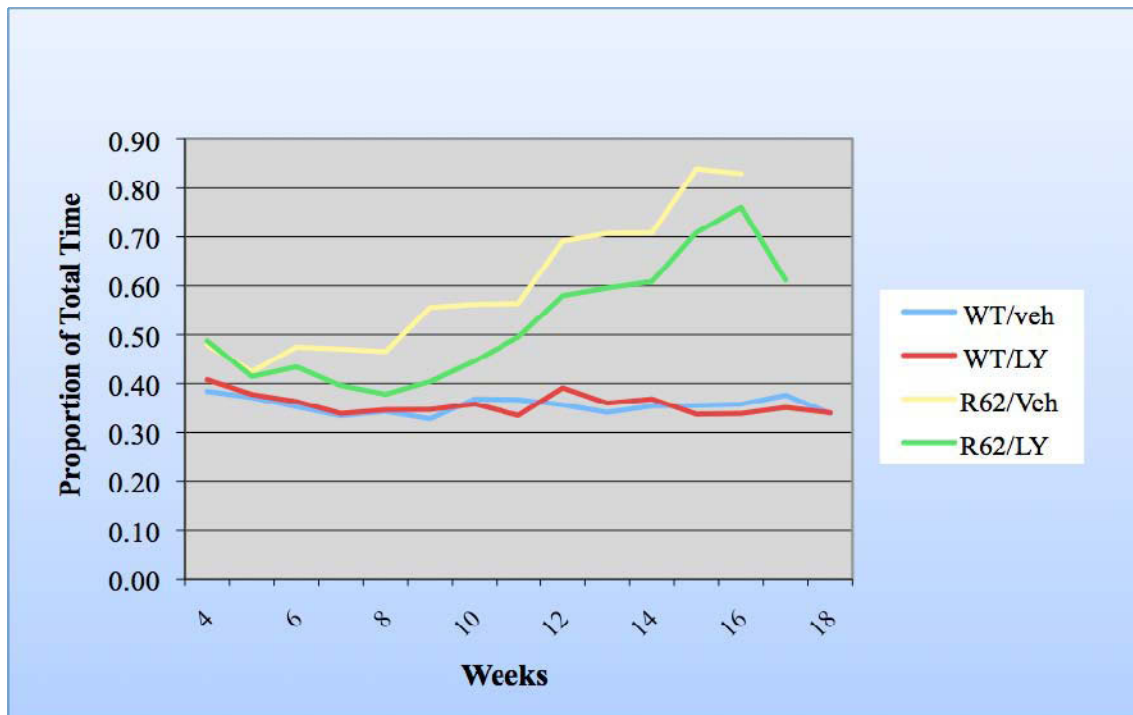Figure 6. Median Progression Length
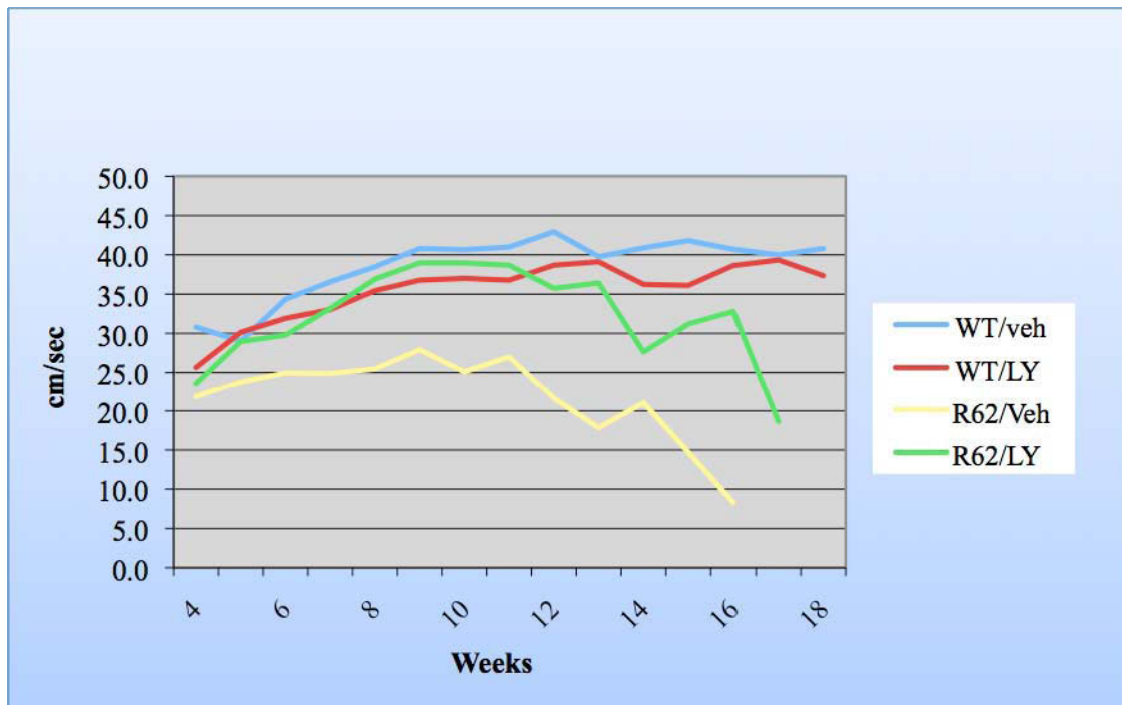
Figure 7. Time Proportion Spent Lingering
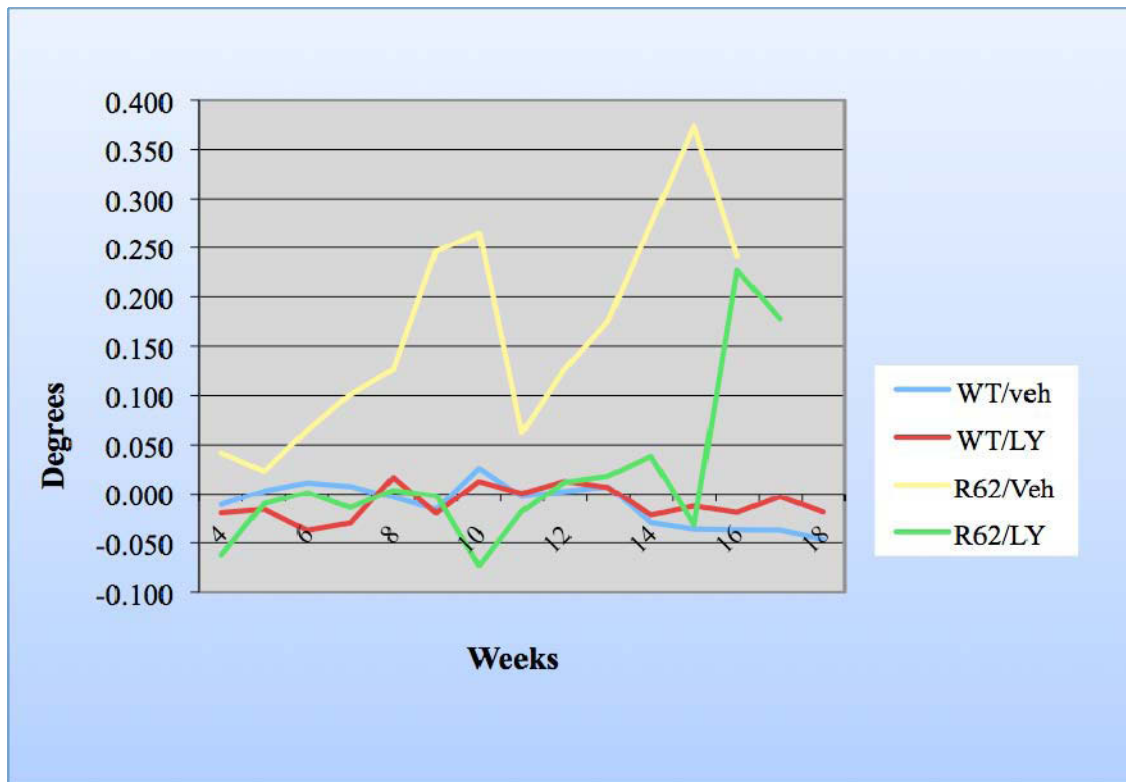
Figure 8. Maximum Progression Speed

Figure 9. Median Curvature of Progression

CHAPTER 5. DISCUSSION

Our results show several benefits of daily injections of an MTD of LY379268 in R6/2 mice. These beneficial effects of this mGluR2/3 agonist include normalized motor parameters at 4-12 weeks in the open field task, including normalized speed, acceleration, endurance and overall activity. Morphological improvement was seen as well in terms of rescue of cortical and striatal neurons, and normalization of the SP+ striatal projection neurons was apparent at 10 weeks. The results for SP neurons are particularly interesting because SP neurons are part of the direct pathway which is responsible for voluntary movement (Albin *et al* 1992; Reiner *et al* 1988). Impairments to this pathway result in hypoactivity (Alexander & Crutcher 1990; DeLong 1990; Reiner *et al* 1988), and it is possible that a defect in this pathway contributed to the decline of open field activity in R6/2 mice that was observed. It is particularly interesting to note, thus, that the akinesia of the R6/2 mice (*e.g.* reduced open field activity) was accompanied by the normalization of the SP pathway. These findings will be discussed in more detail below, as well as the means by which the LY compound could have produced its improvement.

Neuropathology and Behavior Correlations

The phenotype and neuropathology of R6/2 mice replicate several features of the disease observed in humans, including altered levels of neurotransmitters and neuropeptides in the basal ganglia (Cha 2000). As in HD, the striatal projection neurons of R6/2 mice are not uniformly affected over the course of the disease (Cha *et al* 1998; Luthi-Carter *et al* 2000; Menalled *et al* 2000). Immunohistochemistry has shown in human HD that ENK and SP levels differ in their target areas based on the stage of the disease (Reiner *et al* 1988). ENK fibers projecting to the GPe are lost sooner than SP fibers projecting to GPi (Deng *et al* 2004; Reiner *et al* 1988). Analysis of the GPe in R6/2 mice at 12 weeks of age also showed a decrease in ENK labeled fibers in GPe, but no significant change was reported in the measured SP labeled fibers (Sun *et al* 2002). Our study of R6/2 mice at 10 weeks of age showed a slightly elevated SP-immunoreactive fiber intensity for striatal projection fibers to GPi and a substantial elevation for SN. This increase for SP-immunolabeled fibers projecting to SN has been depicted in other studies, but has not been commented upon (Menalled *et al* 2000; Sun *et al* 2002). Our *in situ* hybridization data for 10-week R6/2 mice showed a significantly decreased overall message for ENK (30% of WT) and slight decrease in SP (85% of WT) message in striatum. This matches previous *in situ* hybridization studies on 12-week R6/2 mice that showed enkephalin mRNA is reduced in striatal neurons to a greater extent than substance P mRNA (Menalled *et al* 2000; Sun *et al* 2002). Similarly, a microarray study of R6/2 mice at 12 weeks of age showed a decreased expression of enkephalin mRNA from striatum, but no major changes were seen in Substance P mRNA (Luthi-Carter *et al* 2000). Such results show that the increase in SP in the GPi and SN do not stem from an increase in SP production. An interpretation is that the increase in SP in striato-GPi and striato-SN reflects a failure of peptide release, since less mRNA is being made, yet more peptide is present in the terminals. An argument could be made that the

SP retention in R6/2 striatal projection neuron terminals thus reflects neuronal dysfunction. Previous immunohistochemical studies have shown that ablation of corticostriatal neurons results in increased SP levels of SN in rats (Bouras *et al* 1991; Somers & Beckstead 1990). These authors attribute this effect to a loss of cortical activation of striatal neurons, with a subsequent diminution of SP release (Bouras *et al* 1991). Alterations in the firing activity of neurons have been observed in the striatum of awake- behaving R6/2 mice that may account for the failure of peptide release in striatal target areas. In particular, Rebec and coworkers have noted that MSNs of R6/2 mice are slightly more active than in WT mice, but they fail to show the type of burst activity that ensues from cortical activation (Miller *et al* 2008; Rebec *et al* 2002). Burst activity, which represents spike clusters of varying rates and duration, provide information coding by assemblies of neurons (Sakurai 1999; Salinas & Sejnowski 2001). Burst firing ensures reliable transmission between neurons and induces synaptic plasticity in the striatum (Charpier & Deniau 1997; Lisman 1997). This dysregulation of firing could alter downstream neurotransmitter release of either ENK or SP projection neurons. In the present study, daily injections of LY379268 rendered SP levels in SN down to 130% of WT as opposed to the level of 160% seen with vehicle-treated R6/2. Likewise, SP levels in LY-treated R6/2 mice were down in GPi to 122% compared to the vehicle-treated R6/2 mice measured at 132%. Thus, the LY379268 compound relatively normalized SP levels in GPi and SN.

Our results show that ENK message is greatly reduced but an apparent retention of the peptide results in normal ENK levels in striato-GPe terminals, thus disguising the fact that less ENK is being made. The retention is more obvious with the SP projection neurons because the message is not as greatly diminished, so the peptide retention yields peptide levels above normal. Although SP peptide levels are relatively normalized with LY379268 in GPi and SN, the same effect is not seen with ENK levels in GPe. This is consistent with our *in situ* hybridization data showing the LY compound normalized SP message but did not alter ENK message in R6/2 mice. This implies that the LY379268 benefit is specific to SP neurons. The Direct-Indirect pathway model of basal ganglia function (DeLong 1990) proposes that voluntary movement would be normalized with restored function of SP neurons, which our behavioral data reflects. It would be of interest to know if burst firing is restored in R6/2 mice with administration of LY379268. Research has also shown that a modulator of striatal activity is extracellular levels of ascorbate (Rebec *et al* 2005; Rice 2000). Ascorbate interacts with the glutamate transporter in a heteroexchange mechanism, whereby glutamate is taken up and ascorbate is transported out of astrocytes (Cortright & Rebec 2006; Grunewald & Fillenz 1984). It would be interesting to know if striatal levels of ascorbate increase with administration of the LY379268.

Although R6/2 mice show gross brain and striatal atrophy (Mangiarini *et al* 1996), neuronal loss has only been reported in one study. Stack *et al*, (2005) showed a 25% decrease in total number of striatal neurons in 12-week old R6 /2 mice. Our current results show a 20% loss of striatal neurons in 10-week old R6/2 mice, which was normalized with administration of LY379268. Although it has been suggested that neuron loss does not occur in R6/2 mice (Li & Li 2004b; Reddy *et al* 1999), differences

in methodology might account for the difference in outcomes. Stereology as used in the current study was also used by Stack *et al* (2005), and is a more sensitive approach for detecting neuron loss. A distinction could not be made with stereology, however, as to which population of striatal projection neurons was more decreased, but it would be interesting to know if the benefit seen in neuron rescue by the LY compound affected primarily the SP pathway.

The R6/2 mouse typically shows enlarged lateral ventricles with coinciding striatal atrophy at 13 weeks (Mangiarini *et al* 1996). Volumetric data on striatum, lateral ventricle, and cortex was expressed scaled allometrically to body weight, because of the effect of genotype on animal size. By this standard, telencephalic and cortical volume did not differ among the groups, but ventricular size was doubled in the R6/2 mice. LY379268 treatment did not affect ventricular volume in the R6/2 mice. The ventricular volume increase is suggestive of cortical, septal and/or striatal atrophy, but the only significant effect for any of these seen in the R6/2 mice was for striatal volume, which was about 10% smaller in the R6/2-LY mice. Overall, these data indicate minimal cortical or striatal shrinkage in the R6/2 mice at 10 weeks, and no evident benefit of LY379268 in the R6/2 mice.

Pathogenic Mechanisms

There are a variety of means by which LY379268 could have produced its improvement. The compound may have opposed excitotoxic striatal injury (D'Onofrio *et al* 2001; Kingston *et al* 1999). There are several possible ways it could have done this. Since there are mGluR2/3 receptors on cortical terminals, the drug may have acted by reducing presynaptic release of glutamate (Battaglia *et al* 1997; Cozzi *et al* 1997). This alone would have an effect that would oppose excitotoxicity. In principle, reducing presynaptic glutamate release should reduce excitotoxicity, and reduce striatal pathology in HD. One prior study prevented corticostriatal glutamate release in R6/2 mice by ablating cortical neurons and eliminated loss of striatal neurons and striatal shrinkage (Stack *et al* 2007). LY379268 may have also acted directly on striatal neurons, because mGluR3 is found on some subset of striatal neurons (Testa *et al* 1994), although it has not been established which subset. A third possibility for a mechanism of action is that LY379268 could have increased trophic substance production from striatal astrocytes. In a prior study, TGF-β production was enhanced in striatal glial cells with administration of LY379268 (D'Onofrio *et al* 2001). Another possible mechanism of action is that cortical BDNF was upregulated with the LY compound, as shown to occur in mice (Di Liberto *et al* 2010), which would be beneficial for striatal neurons (Canals *et al* 2004; Zuccato & Cattaneo 2007). Each of these possible mechanisms will be addressed in further detail below.

The notion that excess cortical glutamate release mediates striatal injury presupposes that striatal injury is caused by excitotoxicity, and many lines of evidence suggest this is so. Since NMDA receptor agonists injected into striatum produce a pattern of striatal injury that closely mimics HD pathology (Figueredo-Cardenas *et al*

1994; Shear *et al* 1998), it has widely been thought that excitotoxicity is involved in HD. Several lines of research suggest HD pathology stemming from excitotoxic injury to the striatum is specifically mediated through extrasynaptic NMDA receptors on the striatal projection neurons. Notably, NMDA current is enhanced in HD transgenic mice (Andre *et al* 2006; Cepeda *et al* 2001; Fan & Raymond 2007; Li *et al* 2004), and the enhanced NMDA response stems from increased expression of the extrasynaptic receptors NMDA of striatal projection neurons (Fan *et al* 2007). Activation of these receptors requires glutamate spill from the synaptic sites to the extrasynaptic sites (Milnerwood *et al* 2010). Such activation can enhance mhtt cleavage in toxic fragments, and thus make neurons more vulnerable to death via decreased CREB-PGC-1α activity (Cui *et al* 2006; Milnerwood *et al* 2010; Papadia *et al* 2008; Subramaniam *et al* 2009). Cleaved mhtt is soluble and binds to CREB binding protein, thus interfering with gene transcription (Nucifora *et al* 2001). In mhtt-transfected neurons, CREB function was restored by selectively blocking the extrasynaptic NMDA receptors with low dose memantine (Okamoto *et al* 2009). This same study also showed improved rotarod performance in YAC128 mice with this low dose (Okamoto *et al* 2009). There seems to be an excitatory balance needed for neuroprotection since high dose memantine blocked beneficial synaptic NMDA receptor activity, thus increasing soluble mhtt and worsening the phenotype in this study (Okamoto *et al* 2009). By contrast, low-dose memantine allowed synaptic receptor activation that was beneficial for cell survival, with consequences such as reducing soluble mhtt, increasing mhtt aggregate size and stimulating the CREB pathway (Okamoto *et al* 2009). There is also altered glutamate release from corticostriatal synapses in HD mice prior to motor pathology (Cepeda *et al* 2003), and electrophysiological studies have shown an increase of glutamate release from corticostriatal terminals at an early age (Cepeda *et al* 2007; Joshi *et al* 2009). Glutamate is normally taken up via excitatory amino acid transporters, in particular GLT1, (Shigeri *et al* 2004), and there is decreased expression of this transporter in astrocytes of HD mice and HD humans (Behrens *et al* 2002; Hassel *et al* 2008; Lievens *et al* 2000; Shin *et al* 2005). If LY397268 acts pre-synaptically for its benefit, then excess glutamate release and spillover would be attenuated, and less would reach the extrasynaptic NMDA receptors. If the mechanism of action of the LY compound is post-synaptic (as discussed in the next paragraph), then the effect would dampen the activity of the striatal neurons and produce its benefit in that way. Neither of these mechanisms gives a clear reason why our results showed a greater benefit on SP neurons rather than ENK neurons. It was previously shown that upper layer V cortical neurons project specifically to striatal SP neurons (Lei *et al* 2004), but it is not clear if mGluR2/3 receptors are more abundant on that particular cortical neuron type.

It is possible that the benefit of LY379268 arises by a direct action on striatal projection neurons. An overview of the distribution and mechanism of action of the mGluR2/3 receptors in the striatum offers insight into the basis of potential benefit seen in our results. Within the striatum, mGluR2 receptors are only found in cholinergic neurons and not projection neurons (Testa *et al* 1994). The mGluR3 receptors in the striatum are on astrocytes as well as on neurons (Testa *et al* 1994). It is of interest to note that examination of images in the Allen Brain Atlas indicates approximately 50% of the striatal neurons contain mGluR3, and it is known that approximately 50% of the striatal

projection neurons are SP neurons (Gerfen *et al* 1990; Reiner *et al* 1988). If the SP neurons are the same neurons that contain mGluR3, then the benefit of LY379268 could be achieved by direct striatal action. Activation of mGluR3 is coupled to inhibition of the cyclic AMP pathway (Nakanishi 1992; Schoepp *et al* 1999). This results in decreased membrane excitability stemming from reduced Ca2+ current via N, L-type Ca2+ channels and increased K+ current via IRKC channels (Davies *et al* 1995; King & Liu 1996; McCool *et al* 1996). If the benefit of LY379268 was achieved in this manner, it should be possible to see the same effects if the R6/2 mice are treated with a dopamine D1 receptor antagonist, since this would also yield reduced activation of the cAMP pathway (Monsma *et al* 1990). Further studies are needed to firmly establish the location of mGluR3 receptors on striatal projection neurons.

As mentioned above, the benefit of the LY compound could have come about by an increased production of TGF-β in the striatum. It has been shown that activation of mGluR3 receptors mediates striatal neuroprotection via glial-neuronal interaction (Bruno *et al* 1997). This neuroprotection comes about from TGF-β released from astrocytes, and is effective in preventing NMDA toxicity in *in vitro* and *in vivo* models (Bruno *et al* 1997; Bruno *et al* 1998). Systemic administration of LY379268 in mGluR3 knock-out mice failed to give neuroprotection to striatal neurons against NMDA toxicity (Corti *et al* 2007). This same study showed that the neuroprotection was abolished in cultured neurons with astrocytes lacking mGlur3 receptors (Corti *et al* 2007). These studies suggest that activation of mGluR3 receptors on the striatal astrocytes offer neuroprotection. The neuroprotection from TGF-β could also arise through a different mechanism other than preventing excitotoxicity, including preventing neuronal apoptosis (Copani *et al* 1995; Ren *et al* 1997). Activation of TGF-β receptors leads to phosphorylation of Smad2, a transcription factor, which activates gene expression (Massaous & Hata 1997) involved with inhibiting cyclin-dependent kinases involved with cell cycle check points (Datto *et al* 1995). This mechanism is relevant because inducing an abortive mitotic cycle has been related to the occurrence of neuronal degeneration (Freeman *et al* 1994; Herrup & Busser 1995). A study showed that cyclin D1 and E2F-1 are over-expressed in brains of HD patients (Pelegri *et al* 2008). There is evidence showing these two proteins are involved with abortive cell cycle re-entry in neurons (Camins *et al* 2007; Krantic *et al* 2005), with subsequent neurodegeneration due to oxidative stress and apoptosis (Pelegri *et al* 2008; Woods *et al* 2007; Zhu *et al* 2007).

In addition to TGF-β, BDNF increase by LY379268 administration could play a role in neuroprotection. BDNF is known to promote development, differentiation, plasticity and survival of neurons in several regions of the brain including striatum (Lessmann *et al* 2003; Poo 2001; Zuccato & Cattaneo 2007). It is known that mhtt in HD reduces the transcription of the BDNF gene in the cortex of HD mice and thus decreases protein production (Zuccato *et al* 2001). These authors showed that cortical BDNF mRNA decreased with progression of behavioral symptoms in R6/2 mice (Zuccato *et al* 2005). A study of a cortex specific knock-out model of the BDNF gene showed a progression of the disease that mimicked the pathology of human HD in terms of striatal degeneration (Strand *et al* 2007). Conversely, behavioral performance was improved and disease progression slowed in an HD mouse crossed with mice overexpressing BDNF

(Gharami *et al* 2008). These lines of evidence suggest that a deficit in BDNF contributes to the pathogenesis of striatal injury in HD, thus making it a candidate as a therapeutic agent. Another study showed daily intrastriatal administration of BDNF in R6/1 HD mice increases the number of striatal neurons expressing enkephalin and resulted in improvement in behavioral phenotypes (Canals *et al* 2004). Conversely, in R6/1 on a hemizygous BDNF knock-out background, striatal ENK expression but not SP expression is reduced (Canals *et al* 2004). This suggests that endogenous BDNF might delay the progression of HD in humans. A recent study showed mice acutely treated with LY379268 showed an upregulation of BDNF in cortex and hippocampus (Di Liberto *et al* 2010). The various lines of studies reviewed above give credence to the idea that the benefit from the LY compound stems from a possible increase in cortical BDNF in the mice. Despite this evidence that BDNF must be involved with striatal neuron health, Stack *et al* (2007) showed that decortication in R6/2 mice improves striatal neuropathological phenotypes and restores striatal neuron loss to WT levels. This argues against the idea that striatal neuron loss in R6/2 mice stems from BDNF deprivation because decortication would only exacerbate the BDNF deficit. The study of Canals (2004) showing BDNF benefit for ENK but not SP neurons provides further evidence that BDNF was unlikely to have been the basis for our striatal benefit observed with LY379268 since a large benefit for striatal neurons containing ENK was not seen by us. The benefit of LY379268 in the present study was seen for striatal neurons containing SP. It is likely thus that the striatal benefit of LY379268 does not involve a BDNF mechanism, since our results show little benefit with ENK neurons. It is possible that the cortical BDNF upregulation caused by LY379268 benefits cortex, and explained the rescue of cortical neurons observed here.

mGluR2/3 Therapy

In the past several years, group II metabotropic glutamate receptors have become a target of potential therapeutics. Activation of these mGlur2/3 receptors can suppress presynaptic glutamate release in a negative feedback manner to keep glutamate transmission within a normal physiological range (Kew *et al* 2001; Scanziani *et al* 1997; Schoepp 2001). The location of mGluR2/3 receptors at the periphery of the terminals allows monitoring of excess glutamate spillover from the active zone (Conn & Pin 1997). Developing a ligand with *in vivo* activity is necessary for preventing the hyper-excitability from interfering with normal neuronal function. The first selective agonist developed was LY354740 (Monn *et al* 1997) followed by LY379268, the latter of which has enhanced affinity for mGluR2/3 receptors (Monn *et al* 1999; Schoepp 2001). Metabotropic GluR2/3 are negatively coupled to adenyl cyclase, and thus agonists inhibit the production of cAMP (Monn *et al* 1997). As a more potent agonist, LY379268 suppresses cAMP formation on a magnitude of two orders higher than LY354740 (Monn *et al* 1999). This same study also showed that LY35470 had equal affinity for mGluR2 and mGlur3, while LY379268 is more selective for mGluR3 (Monn *et al* 1999). The early studies of mGlur2/3 agonists looked at efficacy with regards to a benefit for neuroprotection and ischemia. It was shown that LY379268 protects neurons against NMDA toxicity in cell cultures (D'Onofrio *et al* 2001; Kingston *et al* 1999), and this

benefit is linked to a reduced release of glutamate via presynaptic activation of the mGluR2/3 receptors. In contrast to the understanding of the function of presynaptic mGluR2/3 receptors, less is known about the role of metabotropic group II receptors in glial cells. It is known that mGluR3 receptors are expressed in glial cells, but there is no expression of mGluR2 (Liu *et al* 1998; Ohishi *et al* 1993). Since a function of glia is the synthesis and uptake of glutamate (Winder & Conn 1996), it is likely that activation of these receptors by LY379268 results in significant functional benefits. The role of glial cells in neuroprotection was affirmed by Kingston *et al* (1999), where LY379268 was more effective in preventing excitotoxicity in mixed cultures with neuronal and glial cells rather than pure neuronal cultures. The neuroprotection potentially arises from the release of proteins such as TGF-β which stem from the activation of glial mGluR3 (Bruno *et al* 1998). The beneficial effects of mGluR3 agonists come from activating the mitogen-activated protein (MAP) kinase and the PI-3-kinase pathways (D'Onofrio *et al* 2001). Corti *et al* (2007) expanded on these earlier studies with knock-out models of mGlu2/3 receptors and concluded that neuroprotection came about from mGluR3 activation by LY379268. It is probable that neuroprotection from the LY compound arises from a combination of activation of presynaptic mGlu2/3 receptors to inhibit glutamate release and glial mGluR3 release of trophic proteins.

The function of LY379268 at mGlur2/3 receptors at excitatory terminals not only offers neuroprotection as delineated above, but the benefit of the drug is also seen in other diseases where glutamate-mediated transmission plays a role. A mouse model of schizophrenia was developed on the observation that NMDA receptor antagonists such as phencyclidine (PCP) or ketamine can heighten preexisting schizophrenic symptoms, and is associated with excessive glutamate release in limbic regions (Jentsch & Roth 1999; Lorrain *et al* 2003; Marino & Conn 2002). Rodents treated with these NMDA antagonists have stereotyped hyperkinesia and increased forward locomotion (Moghaddam & Adams 1998; Sturgeon *et al* 1979). Hyperlocomotion associated with PCP or ketamine induction is reversed with LY379268 (Clark *et al* 2002; Imre *et al* 2006; Lorrain *et al* 2003). Motor behaviors are associated with an activated dopamine system (Creese & Iversen 1973), and increased dopamine release is a factor in the actions of PCP (Freeman & Bunney 1984). In order to eliminate the possibility of dopamine release as the cause of improved behavioral phenotypes, recent studies with rats that are deficient in serotonin and dopamine shows that the improved locomotor benefit of LY379268 stems from a reduction of evoked glutamate release (Chartoff *et al* 2005; Swanson *et al* 2005). More recently, another mGlur2/3 agonist, LY404039 was used in a double-blind, placebo-controlled Phase 2 clinical trial for schizophrenia. The outcome of the study showed statistically significant improvements of symptoms relative to psychopathology (Patil *et al* 2007). This clinical trial also showed that LY404039 was safe and well tolerated, and it was just as effective as olanzapine with regards to improvement of schizophrenic symptoms (Patil *et al* 2007).

Recent evidence suggests increased glutamate-mediated transmission, in addition to dopamine transmission, might play a role in regulating behavioral actions of drug abuse. Locomotor activity is increased by cocaine under conditions of administration with a conditioned stimulus (Hotsenpiller *et al* 2001). This study also found increased

levels of glutamate in the nucleus accumbens via microdialysis (Hotsenpiller *et al* 2001). Conditioned drug-seeking behaviors brought about by cocaine or heroin are reversed with systemic LY379268 (Baptista *et al* 2004; Bossert *et al* 2004, 2006; Peters & Kalivas 2006). Intraperitoneal injections of the LY compound also reduced hyperlocomotion induced from toluene in rats (Riegel *et al* 2003). Toluene is a solvent found in many inhaled substances that have abuse potential (Miyagawa *et al* 1984; Riegel *et al* 2003). Thus, LY379268 may show benefit in treating drug addiction, since it was effective in reducing drug seeking behavior.

Treatment of R6/2 mice with LY379268 has been shown in the present studies to be beneficial with regards to improvement of motor behavior and neurochemical endpoints. Earlier studies have shown that LY379268 is effective in treating neurodegenerative and psychiatric disorders that are associated with increased glutamate transmission. As a newer mGlur2/3 agonist, it has greater affinity and bioavailability than the previous generation. Moreover, a newer generation of the mGlur2/3 agonists, LY404039 has been through Phase II of a clinical trial. The outcome of the trial showed that the drug is safe and effective. LY404039 could soon move to Phase III clinical trials, if it is also shown effective R6/2 mice as well as in a genetically more precise model of HD as the YAC128 or the Q140. These results give credence to the choice of mGluR2/3 agonists as a therapy in diseases where increased glutamate is the underlying cause of the pathology.

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