THE EFFECTS OF GLYX-13 ON AMINO ACID NEUROTRANSMITTER RELEASE IN THE GLOBUS PALLIDUS OF THE tgHD RAT

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ABSTRACT

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NEUROTRANSMITTER RELEASE IN THE GLOBUS PALLIDUS OF THE tgHD RAT

by Antigone M. Wolfram-Aduan

Huntington's Disease (HD) is a neurodegenerative disease caused by a mutation in the *huntingtin* gene and characterized by pre-symptomatic deficits in memory, executive function, and emotional regulation, and at clinical onset by progressive loss of motor control. There is no cure for HD, available treatments are typically only effective in one symptom domain (cognitive or motor), and at present no interventions are able to arrest the progress of the disease. Drugs able to modulate activity at the N-methyl-D-aspartate receptor (NMDAR) have shown promise in the treatment of HD and the present study aimed to test the efficacy of the drug GLYX-13, a partial agonist at the glycine site on the NMDAR, in the tgHD rat model of HD. The current research was also intended to characterize the underlying physiology of this HD model animal at two different stages of the disease. Extracellular levels of glutamate (GLU), glutamine (GLN), and gamma-aminobutyric acid (GABA) were measured from the globus pallidus of freely-moving rats at baseline (measured post-saline injection) and after administration of GLYX-13. Both transgenic rats and their wild-type littermates were tested at nine months (young) and 24 (aged) months of age, to represent pre-symptomatic and symptomatic stages of HD.

Baseline levels of GLN and GABA were equivalent for all groups. Baseline GLU was significantly different between the wild-type young and aged groups but no other subject groups. GLYX-13 altered extracellular GLN as it decreased over time for all animals, but no significant differences appeared between groups. GLYX-13 appeared to produce a post-drug GABA response interaction with an overall significant between-group difference, but post-hoc analysis of group differences lacked significance. Post-drug GLU responses were equivalent across time

and subject groups. The present study did not reveal physiological differences in tgHD animals compared to their wild-type counterparts at either age. Because results of the present study are limited by treatment groups and a mild HD phenotype, its conclusions regarding the potential of GLYX-13 as an HD treatment or the validity of the tgHD strain as a physiological model of HD are provisional.

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CHAPTER I

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative polyglutamine disease caused by excess, greater than 35, cytosine-adenine-guanine (CAG) nucleotide repeats on the 5' end of the *huntingtin* gene, and resulting in pathogenic glutamine repeats in the protein (The Huntington's Disease Collaborative Research Group, 1993). HD is characterized early on by cognitive dysfunction and emotional disturbances and later the presence of marked motor deficits, most notably chorea (Duff, et al. 2010; Montoya, Price, Menear, & Lepage, 2006; Warby, Graham, & Hayden, 1993). At present, there are no treatments to prevent or delay onset of HD symptoms and symptom management is lacking in viable interventions. Although it is clear that the mutation in the *huntingtin* gene represents the root cause of HD symptoms, the explicit pathogenic downstream effects are difficult to ascertain. The leading hypotheses regarding HD symptom generation are concerned with altered regulation of glutamatergic transmission (Behrens, Franz, Woodman, Lindenberg, & Landwehrmeyer, 2002), N-methyl-D-aspartate (NMDA) receptor activity and excitotoxicity (Milnerwood et al., 2010; Levine, Cepeda, & Andre, 2010; Heng, Detloff, Wang, Tsien, & Albin, 2009), the role of Huntingtin aggregates (Damiano, Galvan, Deglon, & Brouillet, 2010; Michalik & Van Broeckhoven, 2003), metabolic abnormalities (Petersen, Hult, & Kirik, 2009), and explicit striatal neuron loss (Walker, 2007). The present study aimed to characterize the physiological effects of the NMDA receptor modulator Glyxin-13 (GLYX-13) on extracellular levels of glutamate, glutamine, and gamma-aminobutyric acid (GABA) in the globus pallidus of a transgenic rat model of HD using microdialysis. Data available from human and animal research reveals distinct physiological and anatomical changes within the basal ganglia throughout the course of HD. Explorations to

examine methods of attenuating these changes support efforts to produce effective treatments for this progressive disease which are presently deficient.

HD Symptom Progression

In HD, there is characteristic cell loss and atrophy of the medium-spiny neurons (MSNs) within the striatum as well as gross cortical atrophy (Walker, 2007; Rubinsztein, 2002). The loss of MSNs in the striatum leads to reduced GABAergic input to the globus pallidus (GP) and consequent disruption of output to the motor cortex via the thalamus, which produces unwanted motor movements in late stage HD. While striatal cell loss is considered a hallmark of clinically symptomatic HD, it has become evident that dysfunction of both individual neurons and the circuits within the basal ganglia occurs prior to cell loss and the onset of motor symptoms (Dallerac et al., 2011; Mochel, Durant, Durr, & Schiffmann, 2011; Behrens et al., 2002). The earliest symptoms of HD are evident in impaired performance on tasks of working memory and executive function with marked difficulty in shifting strategies during tasks (Wolf, Vasic, Schoenfeldt-Lecuona, Landwehrmeyer, & Ecker, 2007; Montoya et al., 2006). Although the basal ganglia is typically thought of in terms of movement generation it is also involved in the cognitive process of action selection, specifically within cortico-striatal loops, and thus, these cognitive deficits are attributed to impaired communication between and within the cortex and basal ganglia (Beste, Ness, Falkenstein, & Saft, 2011). This is supported by imaging studies in early stage HD patients that indicate altered functional connectivity within cortico-striatal loops (Beste et al., 2011; Wolf, et al., 2007). Correspondingly, rodent models of HD show distinctively different neuronal properties, firing patterns, and release-uptake capabilities when compared to controls which become more pronounced throughout the course of the disease (Traficante et al., 2007; Walker, 2007; Gu et al., 2005; Behrens et al., 2002). Thus, in the pre-symptomatic and

early symptomatic stages of HD, explicit neurodegeneration does not appear to be the root cause of symptoms, rather it seems that altered neuronal properties and neurotransmission may underlie early stage pathophysiology.

Progressive weight loss, the appearance of nuclear and cytoplasmic mutant Huntingtin (mHTT) inclusions, and striatal loss of MSNs are distinctive pathological markers of clinically symptomatic HD. In HD, mHTT forms intraneuronal inclusions, a feature characteristic of all polyglutamine diseases (Michalik & Van Broeckhoven, 2003), and the effects of these aggregates on cellular function are an ongoing subject of interest in HD research. Wild-type Huntingtin (HTT) is known to be involved in regulation of intracellular transport, vesicular transport and release, and control of apoptotic pathways, thus mHTT has the potential to influence these mechanisms (Damiano et al., 2010). There is also evidence to suggest that mHTT inclusions influence gene transcription and alter axonal transport which could in turn affect organelle and mitochondrial localization and subsequently cause deleterious downstream effects in cellular function (Damiano et al., 2010). However, the functional significance of these inclusions in HD is unclear, given that the expression and location of mHTT aggregates do not reliably correlate with neuronal pathology (Eidelberg & Surmeier, 2011).

There is a significant weight reduction associated with the late stages of HD in both human patients and in animal models of the disease that has been correlated with CAG repeat length and this observation has stimulated examination of metabolic function in HD (Petersen et al., 2009). Increased metabolic rate is thought to underlie the decline in body mass in HD via changes in hypothalamic, hypothalamic-pituitary-adrenal (HPA) axis, and neuroendocrine function (Petersen et al., 2009). The presence of altered glutamatergic and dopaminergic transmission is thought to contribute to hypothalamic dysfunction in HD (Petersen et al., 2009).

However, given the complexity of hypothalamic and neuroendocrine pathways it is difficult to tease out the myriad of potential contributing factors in this context. It is clear nevertheless that metabolic malfunction takes a toll as the disease progresses.

Striatal cell loss is a readily observable and consistent feature of late stage HD in human patients (Anitha, Nandhu, Anju, Jes, & Paulose, 2011; Eidelberg & Surmeier, 2011) and in many but not all animal models of the disease (Rubinsztein, 2002; Turmaine, Raza, Mahal, Mangiarini, Bates, & Davies, 2000). A combination of alterations of the NMDA receptor function and aberrant glutamatergic transmission are thought to contribute to neuronal dysfunction and cell death in HD. Excitotoxicity in HD is likely caused by excessive receptor stimulation by endogenous excitatory amino acids (EAAs) and produces pathological cellular changes which can ultimately result in apoptosis (Fernandes & Raymond, 2009). Glutamate is the principle excitatory neurotransmitter in the brain and as such abnormally high glutamate release or diminished uptake is considered to be the major cause of excitotoxicity in the central nervous system. N-methyl-D-aspartate receptors (NMDARs) mediate glutamatergic transmission in the brain and have been extensively studied in the context of excitotoxicity. In a healthy system increased NMDAR activity in the STR would be driven by excitation in the cortex to influence transmission through the basal ganglia in order to process action selection or movement generation. However, in HD it seems that that the amplified activity at NMDARs is largely tonic, rather than phasic, and hence represents an ongoing challenge to homeostasis. It is not clear if these changes in fact represent circuit-wide responses to an altered balance of excitatory and inhibitory transmission within the basal ganglia or if they primarily contribute to the pathogenesis of HD. There appear to be changes in NMDAR sub-type expression, localization, and NMDAR dependent plasticity in HD (Milnerwood et al., 2010; Levine et al., 2010; Heng et

al., 2009). There is also evidence to suggest inappropriate glutamatergic transmission and re-uptake mechanisms in HD (Huang, 2010; Estrada-Sanchez, 2009; Behrens et al., 2002; Lievens et al., 2001). Aberrant activity at striatal NMDARs has been attributed to a decline in glutamate uptake throughout the course of HD, indicated by a reduction in the glutamate transporter, GLT-1, in the cortex of post-mortem HD patients (Hassel, Tessler, Faull, & Emson, 2008) and in animal models of HD (Behrens et al., 2002; Lievens et al., 2001). Up regulation of GLT-1 in the cortex and striatum by administration of the antibiotic ceftriaxone improved motor symptoms in the R6/2 mouse model of HD (Sari, Prieto, Barton, Miller, & Rebec, 2010). Ferrante and colleagues (2002) showed that chronic treatment, beginning at three weeks of age, with the NMDAR antagonist Remacemide significantly prolonged survival, improved motor performance, and decreased cortical atrophy, in the R6/2 strain. These two studies show that modulation of glutamate shows significant treatment effect when administered at both pre-symptomatic and clinically symptomatic stages of HD. Thus, glutamatergic transmission in HD has become a focus of research efforts which will be discussed further in the following sections.

The Basal Ganglia in HD

The basal ganglia was originally thought to exclusively mediate motor movement selection and execution but is now understood to be involved in working memory, habit learning, and goal directed behavior (Chakravarthy, Joseph, & Bapi, 2010). The basal ganglia operates largely on the principle of disinhibition such that there is a tonic level of inhibitory GABAergic transmission within the circuit. The analogy of a brake on a car is often used to describe this mechanism such that when execution of a motor movement is required phasic inhibition is used to release the brake and allow a particular motor pattern to be activated. However, the brake analogy is only useful in a general sense as the release of the brakes on a car only serves one

purpose: to allow the wheels to roll. Action selection and the initiation of movement requires both disinhibition of a distinct set of cells and the continued suppression of potentially competing motor patterns and the pathways of the basal ganglia are organized in such a way to process these complex demands (Benjamin, Staras, & Kemenes, 2010). In HD the communication between and within these pathways is disrupted such that a level of specificity is lost which results in cognitive impairment and difficulty generating desired movements.

A basic explanation of the anatomical connections within the basal ganglia circuitry is necessary in order to properly outline the underlying physiology of HD symptoms. The basal ganglia is a collective term for seven interconnected subcortical nuclei: the caudate nucleus and putamen; together functionally designated as the striatum (STR), the globus pallidus internal (GPi) and external (GPe), the subthalamic nucleus (STN), and the substantia nigra (pars compacta, SNc, and pars reticulata, SNr; Chakravarthy et al., 2010; Parent et al., 2000). Glutamate, GABA, and dopamine are the three major neurotransmitters within the basal ganglia. The STR is the main input region for the basal ganglia, receiving glutamatergic excitatory projections from the cortex and dopaminergic input from the SNc. The STR in turn sends inhibitory GABAergic projections to the GPe and GPi. The GPe interacts with the STN, largely with inhibitory modulation, and the STN also projects into the GPi. The GPi and SNr represent the two output regions of the basal ganglia and exert their influence on the cortex via GABAergic projections to the thalamus (Parent et al., 2000). A simplified pathway within the basal ganglia for motor movement in an intact system is shown in Figure 1. Drive for motor execution arises as excitation in the cortex and subsequent glutamatergic activation of the striatum. Activity in the striatum then induces GABA release into the GPi which inhibits the

tonic inhibition of the thalamus. When inhibition of the thalamus by the GPi is released, the thalamus is able to send excitatory projections into the motor cortex and produce movement.

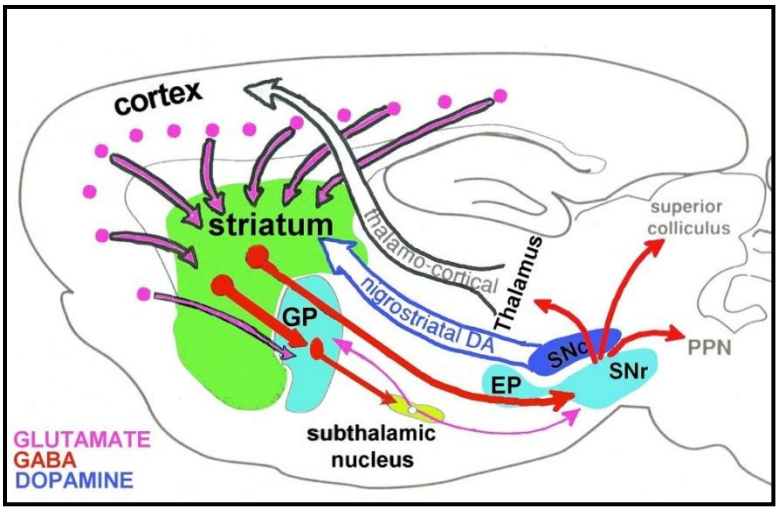


Figure 1. *Schematic of Basal Ganglia Circuitry*

Previous models of basal ganglia circuitry outlined this system as a series of closed loops which mediate motor, cognitive, and limbic processing, but this approach does not address the complexity of symptoms in basal ganglia related diseases (Joel, 2001). Characterization of the functional connectivity of the basal ganglia has evolved in the last decade to emphasize reciprocal and loop circuits within this structure and this model is better suited to address disrupted communication in basal ganglia pathologies (Goldberg & Bergman, 2011). The execution of smooth motor movement requires a precise balance between excitatory and inhibitory transmission within the basal ganglia and consequently disrupted communication between and within basal ganglia structures causes deficits in cognitive function and motor control commensurate with the degree of deviation from normal. The STR and GP represent the key input and output structures of the basal ganglia, respectively. The major role of the STR in

basal ganglia function and cell loss in this region have made the STR a major focus of HD research, but more recently the GP has become a key region of interest.

In HD, it is posited that the deterioration of striatal input to the GP, which regulates activity in this region via disinhibition, leads to hyperactivity in the GP (Ayalon et al., 2003). Given that processing within the GP is thought to involve discrete processing of synaptic input, involving activation of specific populations and suppression of others, hyperactivity could lead to loss of specificity and consequent disruptions in GP output. Interestingly, lesion of the GPe reduced behavioral deficits in a striatal quinolinic acid rat model of HD (Ayalon et al., 2003). Furthermore, inhibitory deep brain stimulation of the GPe was experimentally tested as a treatment and was shown to normalize the connectivity of cortico-basal ganglia circuits in symptomatic human HD patients (Ligot et al., 2011).

Current schematics of the organization of the basal ganglia have placed the GPe in a more dynamic role than simply a 'weigh station' in the indirect pathway for transmission between the striatum and GPi, and at present it is thought that this structure may in fact be a central nucleus of this region (Chan, Surmeier, & Yung, 2005; Goldberg & Bergman, 2011). Striatal input to GP is convergent at a ratio of approximately 1-60, respectively, and almost all GP cells have extensive local axon collaterals which speaks to the complexity and sensitivity of transmission within this region (Chan et al., 2005). GP cells are tonically active and this is thought to correspond functionally to persistent inhibition of motor programs while phasic activity corresponds to removal of tonic inhibition for specific movement sequences via striatal or intrapallidal GABAergic transmission (Benjamin et al., 2010).

It is thought that compensatory changes in receptor distribution and sensitivity within the STR, and further downstream in the GP, are induced to maintain homeostasis within the basal

ganglia circuitry in the early stages of HD (Allen, Waldvogel, Glass, & Faull, 2009). Studies in both human patients and animal models of HD have found up-regulated $GABA_A$ and down-regulated cannabinoid-1 (CB1) receptors in the GP beginning in the pre-symptomatic stages of the disease (Allen et al., 2009). These changes are thought to represent some of the first measurable changes in the course of HD. Examinations of cellular pathology reveal preferential loss of enkephalin containing striatal MSNs which project to the external segment of the GP, corresponding to the indirect pathway, in the early stages of HD (Glass, Dragunow, & Faull, 2009). In later stages, the striatal MSNs enriched with substance P which project to the GPi, representing the direct pathway, also show marked deterioration. This differential loss of input to the GP in the early and late stages of HD may underlie the varied and progressive presentation of symptoms throughout the course of the disease. Furthermore, this loss coincides with a loss of CB1 receptors on the terminals of striatal MSNs and up-regulation of $GABA_A$ and $GABA_B$ receptors in both segments of the GP (Allen et al., 2009). CB1 receptors act pre-synaptically to regulate the activity of striatal MSNs via depolarization inhibition by attenuating the release of neurotransmitters in response to previous stimulation. Thus, the functional consequence of down-regulation of these receptors would be increased GABA release from MSNs and up-regulation of GABA receptors in the GP would increase the influence of GABA release in this region. One current theory is that these changes represent compensatory responses to the loss of MSNs in the striatum and consequent loss of GABAergic input to the GP (Glass et al., 2009). However, if this is the case, it seems that these mechanisms are not sufficient to keep with the progressive deterioration of communication within the basal ganglia. Whether they are inherently pathological or compensatory, neurophysiological alterations in the GP correspond to different

stages in disease progression and, as such, provide markers by which potential therapeutics might be evaluated.

Pharmacological HD Treatments

To date, there are no interventions capable of arresting the progress or fully treating the symptoms of HD. This is due largely to the varied physiological dysfunction inherent in HD as there is no single alteration in neurotransmitter regulation or metabolic process that underlies this pathology and thus no single target for treatment. A wide range of pharmacological treatments have been explored clinically which include dopamine-depleting agents, dopamine antagonists, benzodiazepines, glutamate antagonists, acetylcholinesterase inhibitors, dopamine agonists, anti-seizure medications, cannabinoids (Frank & Jankovic, 2010), and compounds intended to influence energy metabolism (Mestre, Ferreira, Coelho, Rosa, & Sampaio, 2009). The majority of these interventions target the motor symptoms of HD, namely chorea, but some treatments also influence psychiatric features of the disease such as depression, apathy, and irritability (Frank & Jankovic, 2010). Currently Tetrabenazine is the only FDA approved drug for treatment of chorea in HD (Frank, 2010). This drug inhibits release of dopamine and serotonin by binding to the vesicular monoamine transporter (VMAT2) and preferentially depletes vesicular dopamine stores (Huntington Study Group, 2010). Unfortunately, while Tetrabenazine is effective in reducing choreic movements, it also produces detriments in measures of functional outcomes in HD patients (Mestre et al., 2009), which then limits its overall therapeutic value.

Based on evidence to support NMDAR malfunction in HD, several clinical trials have been conducted using agents that influence activity at the NMDAR, which include the low-affinity non-competitive antagonists, Amantadine, Memantine, Rizuole, and Remacemide, as well as the high-affinity non-competitive antagonist Ketamine (Venuto, McGarry, Ma &

Kieburtz, 2012). All four low-affinity non-competitive NMDAR antagonists produced mixed results in the treatment of HD, and though they did have anti-choreic effects overall, with Amantadine showing the most positive results with minor side effects, they did not produce statistically significant results in reduction of chorea (Mestre et al., 2009). Ketamine did not produce positive treatment effects on measures of chorea in HD patients and also showed detrimental effects on tasks of memory as well as psychiatric disturbances (Mestre et al., 2009). Variability in the results of drug therapy trials may be due to the small sample sizes available for this disease and the wide range of symptom severity across patients within studies (Venuto et al., 2012). Additionally, study of patients in early versus late stage symptom presentation in the same experiment could influence results, due to the hypothesized differences in neurophysiology, and potentially different drug responses at different stages of the disease. Although none of the NMDAR modulating drugs have thus far produced an ideal treatment effect, the fact that they do show a benefit in symptom reduction suggests that this receptor remains a viable target for treatment intervention in HD.

NMDAR Activity and GLYX-13

NMDAR activity mediates the process of learning and memory in part by its role in long-term potentiation (LTP) and long-term depression (LTD) which is dependent upon temporally relevant (phasic) input, and if the basal activity of the receptor is altered then the processes of LTP and LTD will be altered as well (Zhang, Sullivan, Moskal, & Stanton, 2008). Hence, altered NMDAR activity may interfere with mechanisms of synaptic plasticity and several lines of evidence suggest that plasticity is indeed impaired in HD (Dallerac et al., 2011). In HD, the activity of NMDARs can be examined at a cellular and system-wide level, and both provide insight into the pathology of symptom generation and degenerative processes. At a cellular level,

dysfunction at the receptor can lead to altered ligand sensitivity, aberrant synaptic plasticity, as well as excitotoxic cell death (Milnerwood et al., 2010; Heng, et al., 2009). On a more global scale, abnormal function of these receptors may interfere with cortico-cortico and cortico-striatal transmission indicated by decreased performance on tasks of working memory and executive function, and altered mood regulation evident in HD patients (Anitha et al., 2011; Duff et al., 2010; Wolf et al., 2007; Thiruvady et al., 2007).

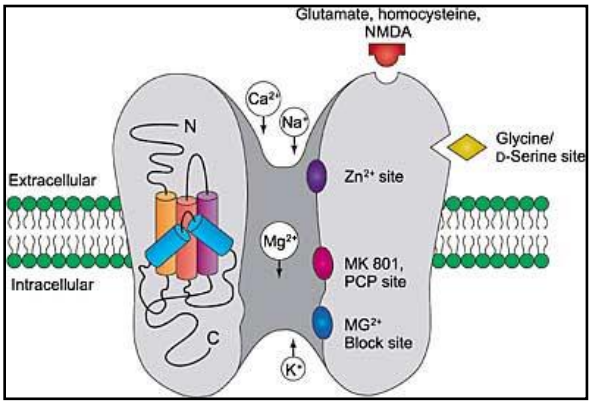


Figure 2. *The NMDA Receptor Complex (Smith, 2002)*

NMDARs have two main binding sites, one for glutamate and one for the co-agonist glycine or D-serine (see Figure 2), and both must be occupied in addition to membrane depolarization for the receptor to be fully activated (Zorumski & Izumi, 2012). The NMDAR complex also contains binding sites for ketamine, phencyclidine (PCP), and dizocilpine (MK-801), which bind with high affinity to non-competitively to block NMDA receptor activation (Szakacs et al. , 2012). Both ketamine and MK-801 show neuroprotective properties but tend to produce psychiatric disturbances which make them unsuitable for clinical applications (Szakacs et al., 2012). As mentioned previously, attempts to treat HD with NMDAR antagonists have shown some benefits, but at the cost of additional deleterious effects. The side effects of agents exerting direct agonism or antagonism at the NMDAR led to the exploration of agents able to act

on the glycine site on the receptor complex (Nilsson, Carlsson, & Carlsson, 1997; Hartmann, Ransmayr, & Riederer, 1994).

In one of the earliest studies to characterize the role of glycine in NMDAR activity, Johnson and Ascher (1987) suggested that glycine-glutamate synergism might influence phasic activity at the NMDAR. They proposed that steady baseline occupation of the glycine site would potentiate the effect of synaptically released glutamate *in vivo* and although at the time it was unclear, it is now evident that the glycine site is not saturated at baseline levels of activity (Nilsson, 1997). However, examination of glycine site agonists is in line with their original assumption that activation of this site directly influences the NMDAR complex (Nilsson et al., 1997; Moskal et al., 2005; Zhang et al., 2008). Glyxin-13 (GLYX-13), a tetrapeptide derived from a monoclonal antibody to act as an NMDAR partial agonist at the glycine site, has been shown to cross the blood-brain barrier and have central nervous system actions (Moskal et al., 2005). This drug binds at the glycine site on the NMDAR complex to produce approximately 87% of the current response induced by saturating glycine levels in the presence of bound glutamate (Moskal et al., 2005). GLYX-13 dose-dependently potentiates activity at the NDMA receptor; it acts as either an agonist or antagonist depending upon the amount of available co-agonist (Moskal et al., 2005). Thus, when GLYX-13 is introduced at a time when physiological activity is at baseline and co-agonist levels are low it serves to potentiate activity the NMDAR and by contrast, when physiological levels of co-agonist are near saturating levels it reduces the current response at NMDARs when stimulated by glutamate (Stanton, Potter, Aguilar, Decandia, & Moskal, 2009). GLYX-13 has been shown to positively affect learning and memory (Burgdorf et al., 2011; Moskal et al., 2005), dose-dependently enhance LTP and depress LTD (Zhang et al., 2008), show neuroprotection in a stroke model (Stanton et al., 2009), and modulate

neuropathic pain (Wood, Mahmood, & Moskal, 2008). Given that NMDAR modulating drugs show some benefit in the treatment of HD and GLYX-13 is able to modulate NMDAR function in a moderate and activity dependent manner, it seemed a prime candidate for exploration in the context of HD treatment. Although GLYX-13 has been studied extensively through *in vitro* cell recordings, behavioral paradigms in rodents, and now clinical trials in humans, its direct influence on neurotransmitter release had not previously been examined *in vivo*.

Animal Model

The CAG 51 rat model of HD was developed by von Horsten and colleagues (2003) in an attempt to produce a model with a course of symptoms close to human subjects, as opposed to aggressive models such as the R6/2, and would also lend itself to *in vivo* imaging procedures, which is more difficult in mice than rats due to their smaller skull size. This transgenic HD (tgHD) strain harbors 51 CAG repeats under the control of the endogenous rat HD promoter within a 1962 base-pair HD cDNA fragment (von Horsten et al., 2003). Imaging studies in this strain at 8 months of age showed enlarged ventricles and focal striatal lesions as well as decreased glucose metabolism (von Horsten et al., 2003). The tgHD strain displays cognitive deficits in a radial maze task at 10 months of age as well as motor deficits in their fore- and hind limbs, which continues to worsen as animals age. Histological examination of these animals at 18 months of age showed the presence of neuropil aggregates in the striatum and lateral GP, and inclusions in the striatum (von Horsten et al., 2003). Further examination of animals at 12, 18, and 24 months of age revealed that labeling for inclusions and aggregates increased in the striatum as the animals aged. Post-mortem tissue processing indicated decreased striatal dopamine, tryptophan, and xanturenic acid (von Horsten et al., 2003). Bauer and colleagues (2006) examined receptor density at 24 months and found decreases in M1 acetylcholine, 5-

$HT2_A$ serotonin, $A2_A$ adenosine, D1 and D2 dopamine, and $GABA_A$ receptors in homozygote tgHD animals, most notably in the caudate putamen (striatum) and nucleus accumbens. Furthermore, a more recent study by Hohn and colleagues (2011) found altered pre-symptomatic frontostriatal processing, using both behavioral measures and *in vivo* electrophysiological recordings as early as four months of age. Taken together, the results of the evaluations of the tgHD rat model suggest that this strain represents a valid model of human HD, in terms of the progression of symptoms, formation of aggregates and inclusion bodies, altered neurotransmitter levels and metabolites, striatal lesions, and altered cerebral metabolism.

There is, however, some controversy regarding the validity of the tgHD model in terms of the presentation of deficits and presence of anatomical changes. Fielding and colleagues (2011) conducted a three year study using the tgHD strain and found inconsistent results on behavioral tasks intended to evaluate frontostriatal processing as well as variable results on tasks of motor function at different ages. This study did not find striatal mHtt aggregates and inclusion bodies at 12 months of age, as indicated by the von Horsten group, and did not see discernible evidence of these until 22 months of age. The Fielding group did use a different staining method to evaluate aggregates and inclusion bodies than the von Horsten group, which may partially explain the difference in their histological results. Additionally, there may have been genetic drift in the CAG repeat length which could have altered the phenotype over time (Fielding et al., 2011). In light of the neurochemical changes observed in the initial evaluation of this model, we believed that this model would provide an appropriate system to evaluate the physiological differences between young (9 months) just prior to the onset of cognitive and early motor symptoms and aged (24 months) when the animals would be expected to show the full symptoms of HD. Thus, exploration of GLYX-13 in this strain was intended not only provide information on the

physiological effects of GLYX-13 *in vivo*, but also to test the validity of the tgHD rat as an HD model system.

Research Goals

Inappropriate activation of striatal NMDARs exerts direct influence on the neurons in GPe, producing aberrant activity in this region and consequently abnormal activation of the motor cortex, via altered thalamic input. Thus, the GP represents a key region of interest in both pre-symptomatic and symptomatic stages of HD. There is evidence to suggest that glutamatergic transmission is excessive in the pre-symptomatic stages of HD and reduced at later stages of the disease (Levine et al., 2010), which necessitates that any treatment would need to modulate glutamatergic transmission in an activity-dependent manner. Given the documented alterations in NMDA receptor activity in HD (Milnerwood et al., 2010; Levine et al., 2010; Heng et al., 2009), the ability of GLYX-13 to influence NMDAR activity GLYX-13 may enhance its therapeutic value as a drug for this disease. The present paradigm aimed to determine both baseline differences in amino acid neurotransmitter release and the effect of GLYX-13 on these neurotransmitters in the GP of the tgHD rat. A factorial design was used to examine the potential interaction effects of GLYX-13 with age and GLYX-13 with genotype. Use of motor monitoring equipment provided information on the influence of GLYX-13 on locomotion, as well as providing basic information on the movement characteristics of each animal group during the experiment, as movement would be expected to influence neurochemical activity within basal ganglia structures (Trytek, White, Schroeder, Heidenreich, and Rebec, 1996). Additionally, given that the current literature suggests distinct neurochemical changes should be evident in the tgHD model at 24 months of age, and potentially at 9 months of age. (Hohn et al., 2011; Nguyen

et al., 2006; von Horsten et al., 2003), another aim of this experiment was to examine the validity of this model in regards to the neurophysiology of HD.

We hypothesized that GLYX-13 would influence activity at the NMDARs in the STR and produce subsequent downstream transmitter release changes measurable in the GP. GABA and glutamate were chosen as target amino acids in this project, based on their intrinisic roles in the function of the basal ganglia, as well as documented changes in these substances and their receptors in HD (Levine, Cepeda, & Andre, 2010; Stack et al., 2007; Lievens et al., 2001). Glutamine levels were also of interest because of the glutamate/GABA-glutamine cycle. After either glutamate or GABA are released at their particular synapse they are taken up by astrocytes and converted to glutamine which is then released into the extracellular space where it is taken up by the pre-synaptic neuron and coverted back into glutamate or GABA, respectively (Bak et al., 2006). Thus, a dramatic change in either glutamate or GABA levels would produce a consequent change in extracellular glutamine levels. GABA was the major concern in this study, given that GABA is the main inhibitory neurotransmitter in the brain, and as such could also be influenced by GLYX-13, due to the necessity for balance between excitatory and inhibitory neurotransmission in the brain. Specifically in the context of HD, GABA is important because it is the major modulatory neurotransmitter in the GP (Jager & Kita, 2011; Ikeda, 2010) and thus GABA release could be modified either locally or via effected afferents. Although there are several studies documenting changes in the GP in HD (Allen et al., 2009; Glass, 2009; Ayalon, et al., 2003), it nonetheless represents a relatively understudied region of interest in the context of HD research.

Given the ability of GLYX-13 to either potentiate the response at the NMDAR when endogenous glycine levels are low or dampen the response when glycine is at saturating levels,

we expected the drug to show biphasic effects in the context of pre-symptomatic and symptomatic stages of HD. Thus, we expected to see a significant difference in percent change response, specifically a decrease in GABA, to GLYX-13 administration under the assumption that younger HD animals would have higher baseline NMDAR activity compared to controls. We expected to see a significant difference in percent change response, presumably an increase, to GLYX-13 administration under the assumption that older HD animals would have lower baseline NMDAR activity compared to controls. We hypothesized that GLYX-13 would reduce NMDAR activity in the young tgHD rat, but increase NMDAR activity in the aged tgHD rats which would result in reduced GABAergic input to the GP in the young group and increased transmission in the aged group. We hypothesized that the drug would not show a pronounced increase or decrease in GABA in the young or aged WT animals because we expect NMDAR activity to be normal and, thus, there would be no deviation to correct. Given that the main input to the GP is GABAergic we did not expect to see a dramatic difference in glutamate or glutamine at baseline or in response to GLYX-13. However, we posited that if the action of GLYX-13 is not specific to the STR it is possible that GLYX-13 could influence glutamate in this region via the glutamatergic projections into the GP from the cortex and STN (see Figure 1).

CHAPTER II

MATERIALS AND METHOD

Animals

This study used four subject groups using the knock-in transgenic rat model tgHD and two different ages and age-matched wild-type littermates. Animals were genotyped using PCR amplification and separation on agarose gel. All animals were given food and water *ad libitum* and housed in pairs until two days prior to surgery, when animals were singly housed to prevent compromise of the guide cannula head stage attachment. To minimize the stress of separation animals were housed next to their cage mates for the remainder or the study.

Obtaining a substantial group size was difficult in the present study, given that it required male homozygote tgHD, animals which had to be bred in-house and tested at two specific ages. Our intention was to have equal *n*'s and closely matched ages for each genotype group, but due to constraints in breeding transgenic animals, rare probe placement misses, and difficulty in obtaining additional GLYX-13, we were unable to fulfill this aim. Of the total seven animals in the wild-type aged group, we used three wild-type Charles-river Sprague Dawley rats from a different in-house colony with slightly-reduced ages. However, as will be discussed in the results section, the wider range of age (see Table 1) within the aged wild-type group did not have significant influence on outcome measures. In total, 15 study animals that underwent implantation surgery were excluded from the study: eight were excluded due to improper probe placement, three animals died of unknown causes (two in recovery prior to dialysis and one post-dialysis), data was lost for two animals due to broken probes during microdialysis, data for one was lost due to experimenter error, and one animal was excluded because HPLC results were well out of normal range.

Table 1. *Animal Age by Group*

Subject Group (total n=26)	Wild Type Aged (n=7)	tgHD Aged (n=7)	Wild Type Young (n=7)	tgHD Young (n=5)
Age Range months	14.1-23.5	21.8-25.2	9.76-10.76	8.73-12.13
Age in months M(SD)	19.25 (4.36)	23.55 (1.27)	10.22 (0.40)	10.22 (1.54)

Surgery

Rats were prepared for surgery by shaving the hair around the incision area using a #40 clipper blade, this area was then disinfected with chlorhexidine, and eye ointment was applied to the animal's eyes before and during surgery as needed to prevent drying. Rats were then be completely anesthetized with a 0.5% isoflurane gas and oxygen mixture and placed into a stereotaxic apparatus. This gas mixture was maintained throughout the surgery to maintain anesthesia, which lasted approximately one and a half hours, and animals were visually monitored for normal breathing patterns throughout the procedure. Body temperature was maintained at 37° C during surgery using a heating pad connected to a rectal thermometer.

A 0.5mL injection of 0.125% Bupivicaine was given beneath the scalp epithelia five minutes prior to incision to provide local analgesia. An incision in the scalp provided direct access to the dorsal skull surrounding the Bregma landmark. Bulldog clips that attach only to the fascia held the scalp open while three holes were drilled to accomodate small stainless steel skull screws inserted such that the screw heads remained above the skull but did not penetrate through the dura. A hole at coordinates 0.90 mm anterior and 4.5 mm lateral to Bregma, was drilled and the guide cannula 21 gauge stainless steel infusion guide cannula (11mm long from plastic base) was inserted at a 20 degree angle lateral to midline down to 4.6 mm from skull surface. The

guide cannula was inserted at an angle to avoid penetration of the lateral ventricles (observed visually from sample tissue from brains of other tgHD rats). A head stage was created using dental cement to adhere the guide cannula to the rat skull. After the head stage was complete, a dummy stylet was inserted into the guide and screwed into place to to prevent debris build-up in the cannula. Finally, the wound edges were pulled free of the head stage and a viscous topical lidocaine solution (2%) was applied around these wound edges to provide local post-operative analgesia.

Following surgery the animal was removed from the stereotaxic apparatus, the isoflurane/oxegen mixture was discontinued, and the rats were given 2-3 mL isotonic saline (intraperitoneal; IP) injections to replenish lost fluids, and allowed to recover in a tub placed on a heated water pad. Recovery of the all experimental animals was monitored 24 and 48 hours post-surgery, using a a score sheet that included an index of behavior, locomotion, food intake, hydration and evaluation of pain or distress (Appendix B). Due to the compromised health status of the aged tgHD, rats we chose to feed them and the aged wild-type controls a mash diet, as well as eight Cheerios per day, in addition to available rat chow pellets following surgery in order to faciliate recovery, prior to the microdialysis experiment. Animals in the young tgHD and young WT groups were maintained on normal diet of rat chow post-surgery.

Microdialysis

The microdialysis experiment took place during the animals' nocturnal phase using the open field apparatus described below. The animal was manually restrained while the microdialysis probe was inserted and artificial cerebral spinal fluid (aCSF) containing 155.0mM Na+, 2.9mM K+, 1.2mM Ca2+, 0.83mM Mg2+, 132.76mM Cl-, 5.9mM D-glucose, and 0.25mM Na-ascorbate (pH between 6.8 and 7.2), was pumped though the probe at 2.0 µl/min throughout

the experiment. After insertion, there was a one-hour discard period to allow the insertion-related biochemical alterations to dissipate, and collections proceeded following the initial saline injection. The saline injection was given IP at a volume to match the GLYX-13 dose which was administered at 5mg/kg (also IP). Four ten-minute collections were taken after the saline injection and six following the GLYX-13 injection (each with a 20 minutes delay post-injection). The 20-minute delay was chosen based on previous explorations of the pharmacokinetics of GLYX-13 as the time when the drug would reach its maximal concentration in the CNS. During the first two dialysis sessions, only four post-drug collections were taken. Following initial analysis of the HPLC, data we decided to add two more collections to the experiment, with the rational that GLYX-13 may take longer than one hour total to show a distinct drug effect. Of the total 26 animals included in the final analysis, 23 had data for all ten microdialysis collections: five wild-type aged, six tgHD aged, seven wild-type young, and five tgHD young. Dialysate collections, captured in plastic (250µl) HPLC vials, were changed out by experimenters at the top of the microdialysis swivel away from the animal's view. After collection, dialysate samples were checked for appropriate output volume by the experimenter to ensure adequate flow was maintained (disruptions indicate broken probes), frozen on dry ice, and subsequently stored in a -80^0 C freezer prior, to analysis.

Motor Monitoring

During the above-described dialysis sessions, animals were set to freely explore 40.64 x 40.64 cm clear polycarbonate boxes, the floor of the box was covered with the same type of bedding used in animals' home cages, and spontaneous locomotion was recorded using a motor monitor system (Kinder Scientific). All subjects were naïve to these chambers until the day of

the dialysis session. Our data were limited to movement within the horizontal zone because the microdialysis probe extended from the holder arm at the top of the box to the animal's head and thus we were unable to measure ambulation in the vertical plane of the apparatus. Monitoring was initiated immediately following the saline injection and continued for the duration of the experiment, with only data corresponding to microdialysis collections used for the statistical analysis.

Histology

Following microdialysis, animals were transcardially perfused with 4% paraformaldehyde. Brains were extracted, post-fixed in 4% paraformaldehyde and allowed to sink in 30% sucrose phosphate buffer, prior to sectioning. Brains were frozen in methyl butane, sliced coronal at 40 µm thickness, and mounted on gelatin-coated slides. Sections were then stained with Cresyl violet and neutral red, and assessed for proper probe placement. Only data from animals with clear placement in the GP were used in the final analysis (see Appendix A for placement images).

HPLC Analysis

Aliquots containing 20 µl of dialysate were analyzed off-line using high performance liquid chromatography with electrochemical detection (HPLC-ED) to assess levels glutamate, glutamine, and GABA. HPLC was performed using an ESA Coulochem III apparatus with a refrigerated auto sampler tray. Dialysate samples were pre-column derivatized using o-phthaldialdehyde and beta-mercaptoethanol using a slightly modified version of the methods optimized by ESA Corporation from Donazanti and Yamamoto (1988) to allow electrochemical detection of these amino acid compounds following separation across a Waters column (Waters

186000598) using a sodium phosphate mobile phase buffer (100Mm Na_2HPO_4, 20% methanol, 3.5% acetonitrile, pH at 6.7) and detection at 600mV.

Statistical Analysis

Median baseline neurotransmitter amounts were compared across genotype using one-way ANOVA. The median value of the baseline (post-saline) collections one through four was used as an internal fixed point against which the last baseline and post-GLYX-13 collections were compared to obtain percent change from baseline for each amino acid. Repeated measures ANOVA on percent change were conducted with time as the within-subjects value and genotype as between-subjects value. Due to the change in number of collections taken post-drug in the microdialysis experiment not all animals from each group had a total of ten collections. Thus, collections five through eight were analyzed to compare drug response between groups for all animals. Collections nine and ten were analyzed separately for the animals with all ten collections.

Open-field parameters were analyzed as total distance traveled in centimeters, basic movements, and fine movements per minute in each ten minute period corresponding to a single microdialysis collection. A basic movement was detected as any change of body position that simultaneously broke two or more laser beams in the monitor. A fine movement was detected as change of body position that broke a single laser beam in the monitor (i.e. if the animal moved their head but not their body). Mean baseline open field measurements one through four were analyzed across groups using a one-way ANOVA. Error variance for post-GLYX-13 open field data was not equal across individual time points, so data was analyzed across time with a repeated-measures ANOVA comparing mean baseline values and the mean value of post-GLYX-13 collections five through eight and Tukey's HSD post-hoc analyses. Movement

parameters comparing mean baseline values and collections nine and ten were analyzed separately using repeated-measures ANOVA and Tukey's HSD post-hoc analyses.

CHAPTER III

RESULTS

Our initial expectation for GLYX-13 was that there should be a significant difference between genotypes on percent change from median baseline amounts across post-GLYX collections for the amino acids studied. However, repeated measures ANOVA showed no main effect of GLYX-13 on percent change from median baseline for last baseline collection and five through eight for glutamate (GLU) [F(2.017, 44.379)=0.214, p=0.930]; glutamine (GLN) [F(1.938, 42.634)=1.903, p=0.163], or GABA [F(1.195, 26.294)=0.887, p=0.373]. No

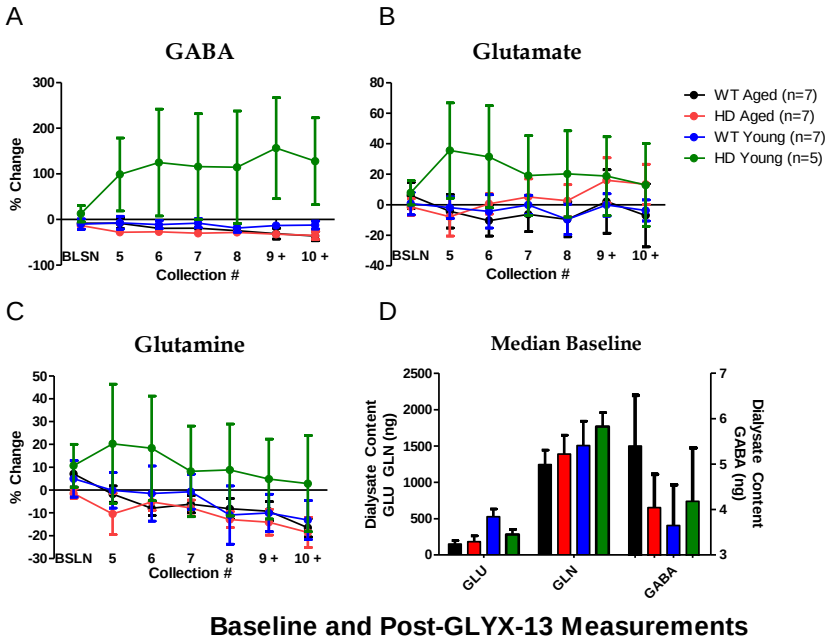


Figure 3. *Microdialysis Results*
Percent Change From Median Baseline Across Post-GLYX Collections (A-C; +WT Aged n=5, HD Aged n=6, WT Young=7, HD Young=5). Median baseline values in nanograms, corrected for probe recovery (D).

interaction effect *time x genotype* was observed for GLU [F(6.052, 44.379)=1.092, p=0.382];GLN [F(5.814, 42.634)=0.482, p=0.813[; or GABA [F(3.586, 26.294)=1.744,

p=0.175]. Values are reported using Greenhouse-Geisser correction for unequal variance. There were no significant group differences in percent change across time for post-drug GLU [F(3,22)=0.983, p=0.419[; GLN[F(3,22)=0.978, p=0.421]; or GABA [F(3,22)=2.177, p=0.119].

Repeated measures ANOVA shows no main effect of GLYX-13 on percent change across collections nine and ten for GLU [F(1.041, 19.770)=0.317, p=0.589] or GABA [F(1.067, 20.27)=1.025, p=0.329]. There was a significant main effect of time for GLN [F(1.073, 20.378)=7.078, p=0.014] which is evident as a progressive decrease in percent change across all collections (Figure 3. C). There was no interaction effect *time x genotype* for GLU [F(3.122, 19.770)=0.728, p=0.424]; GLN [F(3.218, 20.378)=0.268,p=0.860], or GABA [F(3.201, 20.27)=3.016, p=0.051], although this GABA result is borderline (more in Discussion). Values are reported using Greenhouse-Geisser correction for unequal variance. There was no significant difference between groups for GLU [F(3,19)=0.322, p=0.809] or GLN [F(3,19)=0.804, p=0.507] but there was a significant difference for GABA [F(3,19)=3.244, p=0.045]. Pairwise comparisons (Table 2) show GABA percent change was significantly larger in the Young tgHD group compared to all other groups, values not adjusted for multiple comparisons. However, Tukey post-hoc analyses, corrected for multiple comparisons did not reveal any significant group differences, likely a result of between-subject variability.

Table 2. *ANOVA Pairwise Comparisons for GABA*

Genotype		Mean Difference	Std.	p-value
a	**b**	a-b	Error	
HD	**Wild Type Aged**	123.214	48.369	0.020
Young	**HD Aged**	126.258	46.310	0.013
	Wild Type Young	111.847	44.781	0.022

Univariate ANOVA showed no significant difference between groups on median baseline amounts in nanograms for GLN [F(3)22=0.628, p=0.604]; or GABA [F(3)22=0.641, p=0.597] but did show a significant difference for GLU [F(3)22=4.743, p=0.011] (Figure 3. D). Games-Howell post-hoc analysis revealed a significant difference between WT aged and WT young rats (p = 0.047), with younger animals showing higher median baseline values, but no other significant group differences.

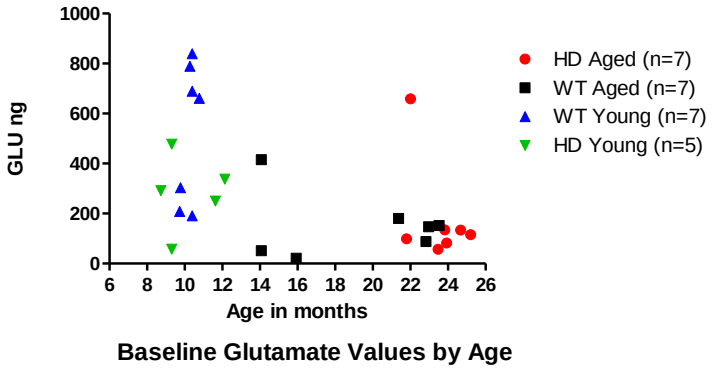


Figure 4. *Median Baseline Glutamate Values*
Corrected for recovery compared to age in months for individual animals.

Repeated measures ANOVA showed a main effect of time for all behavioral parameters: fine movement [F(1)22=13.053, p=0.002]; basic movements [F(1)22=7.585, p=0.012]; and mean distance traveled (cm/min) [F(1)22=7.442, p=0.012]. There was no interaction effect *time x genotype* for fine movement [F(3)22=1.856, p=0.167]; basic movement [F(3)22=1.975, p=0.147]; or total distance traveled [F(3)22=2.179, p=0.119]. There were no significant differences between groups for fine movement [F(3)22=0.642, p=0.596]; basic movements [F(3)22=0.660, p=0.585]; or total distance traveled [F(3)22=1.977, p=0.147]. The main effect of time is evident as an overall decrease in movement. Repeated-measures ANOVA of mean baseline movements and collections nine and ten did not show a significant main effect for fine movement [F(2,19)=0.823, p=0.447]; basic movements [F(2,19)=0.147, p=0.864]; and mean

distance traveled (cm/min) [F(2,19)=0.633, p=0.536]. There was no interaction effect *time x genotype* for fine movement [F(6,38)=0.532, p=0.780]; basic movement [F(6,38)=0.436, p=0.850]; or total distance traveled [F(6,38)=0.343, p=0.910]. There were no significant differences between groups for fine movement [F(3,19)=0.832, p=0.493]; basic movements [F(3,19)=1.531, p=0.239]; or mean distance traveled (cm/min)[F(3,19)=2.982, p=0.057].

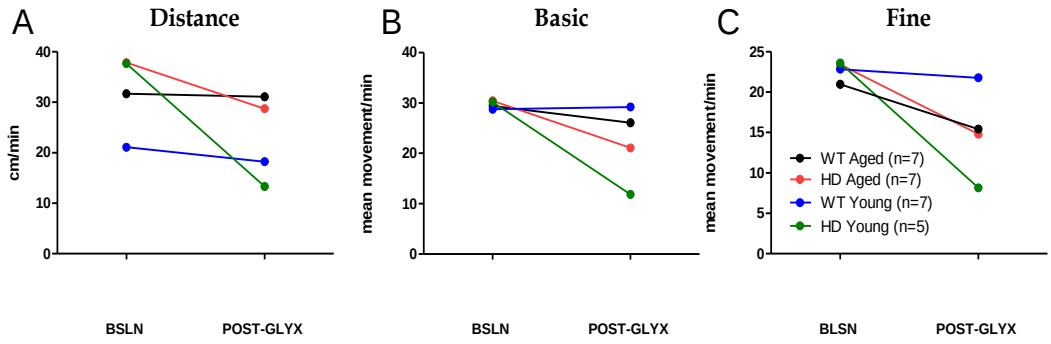


Figure 5. *Open Field Measurements*
Corresponding to mean baseline collections (1-4) and mean post-drug collections (5-8).

Although the range of ages for the aged wild type group is much greater than for the other groups, the animals aged 14.1-15.9 months did not differ significantly from the animals aged 21.4-23.5 months on any of the movement parameters or post-GLYX-13 (data not shown). The Mann-Whitney U-Test showed no significant difference for median baseline values between wild type animals aged 14.1-15.9 and 21.4-23.5: GLU (U=4.0, p=0.480), GLN (U=1.00, p=0.077), GABA (U=8.0, p=0.480). Linear regression showed that age predicts median baseline glutamate values, [b=-0.633, t(24)=4.878 p<0.001]. Age also predicts variance in median baseline glutamate [R^2= 0.234, F(1)24=7.344, p=0.012], which suggests a weak negative correlation between age and median baseline glutamate values. Linear regression comparing age and median baseline values was not significant for GLN [F(1)24=0.166, p=0.687, R^2= 0.083] or GABA [F(1)24=0.426, p=0.520, R^2= 0.132].

CHAPTER IV

DISCUSSION

The aim of the present study was to characterize the levels of the amino acid neurotransmitters glutamate, glutamine, and GABA in the GP of the tgHD rat both at baseline and in response to GLYX-13. We expected the levels of GABA in the GP to differ between the two ages of HD animals, given that each represent a pre-symptomatic and a symptomatic stage of the disease as well as differ in comparison to wild-type controls. In the young group we hypothesized that we would observe higher baseline levels of GABA in the GP compared to WT controls due to an increased glutamatergic drive on the STR (Behrens et al., 2002) and, assuming intact projections from MSNs, consequent increased GABAergic input to the GP (Ayalon et al., 2003). By contrast, in the older animals we expected to see lower baseline levels of GABA compared to WT controls because at this late stage in the disease GABAergic projections from the striatum would be decreased due to decreased function and/or loss of MSNs in the STR (Ligot et al., 2011). Our results show no genotype differences in either baseline levels of the glutamate, glutamine, or GABA and no post-drug genotype differences in percent change for glutamate or glutamine. Analysis of percent change for GABA did reveal a significant genotype difference across collections nine and ten only and the interaction term approached significance ($p=0.051$). However, none of the post-hoc analyses revealed significant differences between genotypes at any age and this is likely due to the high degree of variability (see error bars in Figure 3 A.) within groups which is amplified by the small *n*'s and unequal group sizes for these particular analyses. Nevertheless it is interesting that both these statistical findings, and the apparent dissociation in GABA response in the young tgHD animals (Figure 3A) may suggest either a post-GLYX boost or an elevation of GABA over time within those animals. This finding

in the young tgHD group may have been more dramatic with a larger subject pool, as this group was the smallest (n=5) of all animal groups. If this is considered in fact a GLYX response, it would run contrary to our hypothesis that GLYX would reduce GABA in this group.

The observed main effect of significant decrease in percent change for glutamine across collections was significant only when values from collections nine and ten were analyzed but the decrease is evident over time for all groups when represented graphically (Figure 3. C). At their respective synapses, released GABA or glutamate is taken up into astrocytes, converted to glutamine by glutamine synthetase, released back in the extracellular space and then taken back into the pre-synaptic cell where it can be converted back into GABA or glutamate. According to the stoichiometry of this conversion process, the transfer of glutamine from astrocytes should be roughly equal to the amount of neurotransmitter released at the synapse (Bak et al., 2006). Thus, an increase in glutamine would correspond to an increase in neurotransmitter release and a decrease should correlate with decreased release or impairment in astrocytic recycling. It has been hypothesized that glutamatergic synapses are more dependent upon astrocytic processing of released neurotransmitter than GABA because at the GABAergic synapse released neurotransmitter is also taken up directly into the pre-synaptic terminal for recycling (Bak, et al., 2006). This would suggest that a change in glutamatergic transmission may produce a greater influence on levels of extracellular glutamine than would occur in response to a change in GABA release.

The target region in the present experiment makes it difficult to determine the source of the observed change in glutamine levels because the GP receives both glutamatergic input from the cortex and STN and GABAergic projections from the STR, as well as containing GABAergic interneurons and axon collaterals. Given the direct association of glutamine with reuptake and

recycling of glutamate and GABA, we hypothesized that a significant difference in glutamine levels would correlate with a significant difference in the levels of these neurotransmitters. We observed an overall decrease in glutamine across the experiments for all subjects regardless of age or genotype, but we did not detect any significant differences in percent change for glutamate or GABA. Thus, it does not appear that the decrease in glutamine is directly coupled with a decrease in neuronal release of these neurotransmitters, nor does it appear that the glutamate/GABA-glutamine cycle is impaired, which would result in a pooling of glutamate and/or GABA.

It is possible that subtle differences in drug response between animals groups, percent change for one group increased while another decreased, may have been enough to obscure a main effect, but not dramatic enough to produce an interaction effect or significant group differences for GABA and glutamate. Furthermore, as synaptic release is tightly controlled, microdialysis is most effective in measuring changes in synaptic release when drive is great enough to cause spillover into the extracellular synaptic space, which means that small changes in release would not be as evident. Given that the transfer of glutamine to the pre-synaptic neuron is extrasynaptic, it is possible that changes in glutamine levels might be more easily detected than synaptically-released GABA or glutamate. There is also the issue of the GLYX-13 dose used in the present study which was quite moderate (5 mg/kg), given that doses up to 30mg/kg have been safely tolerated in rats (J. Moskal, personal communication, February 15, 2012). Studies on GLYX-13 have concluded that the drug acts in a dose dependent matter, and it is possible that a significant effect on glutamate and GABA may have been evident with a higher dosage (Moskal, 2005). However, based on both previous and more recent applications of this drug, it seems plausible that its main mechanism of action is represented cortically and within the

hippocampus (Burgdorf et al., 2009; Zhang, et al., 2008; Moskal et al., 2005), rather than within the basal ganglia. This would then depend primarily on the level of cortical activation present during our experiments.

It is interesting that differences in baseline glutamate were observed between the age groups in WT, but not in HD animals. Imaging explorations of glutamate concentration in healthy humans suggest an age-related decline in levels of glutamate, specifically in the motor cortex (Kaiser, Schuff, Cashdollar, & Weiner, 2005), as well as within the basal ganglia (Sailasuta, Ernst, & Chang, 2008), which suggests that this age-related decline in glutamate is an expected observation. It is unclear whether imaging studies in humans would compare directly to *in vivo* measurements in rodents. It is possible that the unequal *n's* in the younger HD rats, five animals compared to seven animals in all other experimental groups, prevented detection of significant differences between the aged and young HD rats. Another possible explanation is that mutant huntingtin influences extracellular glutamate levels in such a way that typical age-related differences are obscured. This could be the case, given that glutamate release and transport are known to be disrupted in human and animal models of HD in both the cortex and striatum (Estrada-Sanchez et al., 2009; Hassel et al., 2008; Behrens et al., 2002) and given that these regions project to the GP, with the cortex having a specific glutamatergic projection, it is possible that these changes also influence pallidal glutamate. In HD, glutamate transport is progressively impaired (Estrada-Sanchez, et al., 2009), which would lead to persistent elevated levels of glutamate across the lifespan rather than an expected age-related decline. If this is occurring, however, it might also be expected to impact the production of glutamine in the HD animals specifically since uptake is necessary for the astrocytes to recycle glutamine, as previously mentioned.

Surprisingly, no difference in baseline or post-GLYX-13 open field movements across genotypes was observed. It was predicted that the aged animals would show less movement than the younger rats overall, and specifically, it was expected that the aged tgHD animals would move less, given that they display significant motor deficits beginning at 15 months of age (von Horsten et al., 2003). However, the lack of observed difference may be attributed to the minimal amount of movement recorded during the experiment, as animals had time to acclimate to the experimental chamber one hour before monitoring was initiated (during the discard period) and, generally ,animals were relatively inactive during the course of the experiment. Furthermore, the lack of movement may have made it more difficult to detect physiological differences at baseline and in response to GLYX-13. The basal ganglia is necessary for the processing and execution of movement, as well as other cognitive demands, but if there is no behavioral demand for activity in this region, it seems that a drug able to modulate physiological activity would not have a target to manipulate. Thus, it is reasonable to expect that differences in activity within the globus pallidus would be more readily apparent when an animal is moving, and consequently, the relative immobility of the animals in the present study may have prevented detection of such differences. There was a significant difference in movement parameters across all animal groups, evident as a decrease between the baseline and post-GLYX-13 collections. However, given that previous explorations of GLYX-13 did not produce ataxia on the rotorod, nor any indication of influence on locomotion in rats (J. Moskal, personal communication, February 15, 2012) it seems the most parsimonious explanation would be to attribute reduced movement to habituation to the environment (Brudzynski & Krol, 1997), rather than a drug effect. Given the previously mentioned connection to movement suggested by the majority of striatal cells becoming activated by movement (Trytek et al., 1996), it also seems parsimonious to presume

that a decreased level of raw materials may have resulted from the decline in movement motivation that may depend more on glutamate than GABA, resulting in decreased recycling and glutamine production over time.

Lack of observable baseline differences between both the young and aged tgHD rats and their wild-type counterparts may have been due to the subtle presentation of symptoms in this strain. This model is supposed to be a mild phenotype, which would make it difficult to see differences, specifically in the younger group as we would not expect extreme deviations in physiology at this age, but based on previous studies of the tgHD model we did expect the aged group to show pronounced differences. Fink and colleagues (2012) found significant differences between nine month old tgHD rats and their wild-type littermates on measures of cognitive function. They did not find significant differences between transgenic and wild-type rats in cortical or striatal cell counts, although there was a trend towards a decrease in striatal cell counts, or the size of the lateral ventricles (Fink et al., 2012). This research supports the utility of this model in producing the cognitive features of pre-symptomatic HD, and at nine months of age, the lack of gross anatomical differences supports the concept that abberant communication likely underlies early stage deficits (Fink et al., 2012). Although the only evaluation done at 24 months comes from the initial accessment of the HDtg rat (vonHorsten, 2003), the presence of striatal degeneration and motor impairments, starting at 8-12 months of age, suggests that, based on the current view of the progressive pathology within the basal ganglia (Miller et al., 2011; Walker et al., 2008) altered physiology should be present and observable at 24 months of age.

Varied onset and subtle presentation of cognitive symptoms, and differences between sexes and homozygote and heterozygote animals, suggests a mild and potentially inconsistent phenotype in the tgHD rat (Fielding, et al., 2011; Faure et al., 2011; Kantor et al., 2006; Baure et

al., 2005). Furthermore, previous examinations of pathophysiology in HD have been conducted in mice with more aggressive phenotypes, like the R6/2, which also harbor different insertion of the transgene. The tgHD rat contains a fragment of mutant huntingtin under control endogenous promotor whereas the R6/2 line contains a fragment, but the promotor is perpetually turned on. Although the tgHD strain has been shown to harbor differences in neurotransmitter receptor concentration, formation of aggregates, and striatal atrophy, it may not produce the same physiological changes in basal ganglia transmission observed in other models. Given that microdialysis is most effective in detecting dramatic changes in physiological levels of activity, this model may not be dramatic enough in terms of disease progression to show distinct physiological changes in release using this method. Additionally, several studies have established that physiological differences in HD are not always evident at baseline levels of activity, that a drive for activation is necessary to reveal disrupted neural processing (Beste et al., 2011; Wolf et al., 2009; Behrens et al., 2002). Thus, the relative lack of movement during testing, and the questionable influence of GLYX-13 on transmission within the GP, may have been insufficient to produce enough activation to elucidate genotype differences.

Although it was relevant to focus on GABA levels in the GP, given the downstream position from the STR, there are additional sources of GABA in this region which could have obscured group differences. The GP contains extensive GABAergic collateral projections both between and within the respective internal and external portions of the structures which modulate local communication (Benjamin et al., 2010). Thus, an activity-dependent release of afferent GABA from the striatum would produce a rise in the extracellular levels of this neurotransmitter in the GP and effectively "quiet" a particular population of globus cells through disinhibition to allow activation of a corresponding set of cells in the thalamus (Mink, 2004). Concurrently,

another population of globus cells involved in competing motor patterns remain under tonic inhibition to prevent interference with the desired motor pattern. The processing of motor movement is highly dependent upon discrete processing of input and local communication between neurons, loss of specificity in transmission, either within networks or locally, can thus have profound effects on basal ganglia output. In HD, transmission from the striatum is disrupted and the appropriate amount of GABA is not released from MSNs, which would suggest lower levels of GABA in the GP at later stages of the disease (Ayalon et al., 2003). However, it is possible that extracellular levels of GABA may remain close to typical levels, due to activity of local inhibitory interneurons, such that what truly affects GP output is the accuracy of GABAergic transmission, rather than total amount of the neurotransmitter available.

The present study was limited by the small *n*'s for each study group, as well as an unequal numbers for one group, which may have made it difficult to see significant group differences in baseline or post-drug measurements due to high variability of the data within groups. Additionally, as it seems that significant differences only arose from the ninth and tenth collection, the fact that these values were not collected for three animals included in the other analyses likely reduced our ability to see a drug effect and/or group differences. The fact that significant differences were not detected on any physiological measures until the later time points in the experiment also suggests that the effect of GLYX-13 may take longer than we originally anticipated. Alternatively, the effects seen may have arisen purely as a function of time, having little or nothing to do with the GLYX-13 given that this drug may well depend on the overall activity of the systems it is influencing. The way to determine this would have been to have a partial, between-subject study, included where some subjects received two injections of

saline rather than the GLYX-13 injection. This is suggested for future studies that may explore GLYX-13 and its capacity in the context of HD.

CHAPTER V

CONCLUSION

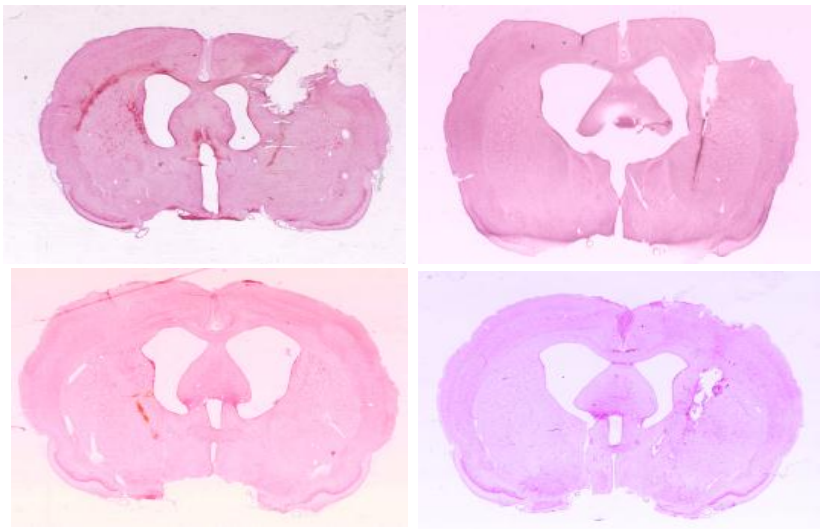
At present these data are not sufficient to support or discredit the tgHD strain as a valid model of the underlying physiology expected to be present in HD. The lack of observable differences between the tgHD and wild type animals on basal amino acid levels could be due, in part, to inherent individual variability coupled with low *n*'s in the present study. Due to multiple confounds, the results of the present study are not sufficient to validate or invalidate the potential of GLYX-13 as an HD treatment. However, given that we did not find evidence of distinct physiological differences between our HD and wild-type animals, it seems there may not have been deficits for the drug to correct. This likely corresponds to low number of CAG repeats and the subtle presentation of symptoms in the tgHD model. The relatively low dose used may have not produced a sufficiently dramatic change in excitatory transmission to be detected by microdialysis. Furthermore, the mild phenotype of the tgHD strain may have occluded a genotype effect. GLYX-13 may also depend more heavily on the activity of the host. It is also possible that a more dramatic drug effect may have been evident in a different target region, such as the cortex. Nonetheless, future studies of these phenomena are encouraged, particularly in the context of a greater behavioral challenge, perhaps one that also exposes challenged mobility as a target for treatment.

APPENDICES

APPENDIX A

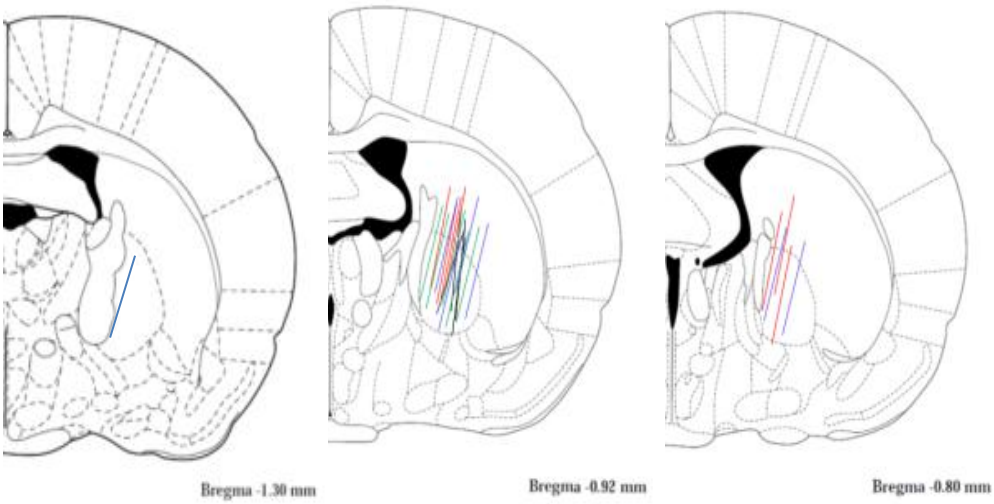
PROBE PLACEMENT

Example probe placement in the globus pallidus.



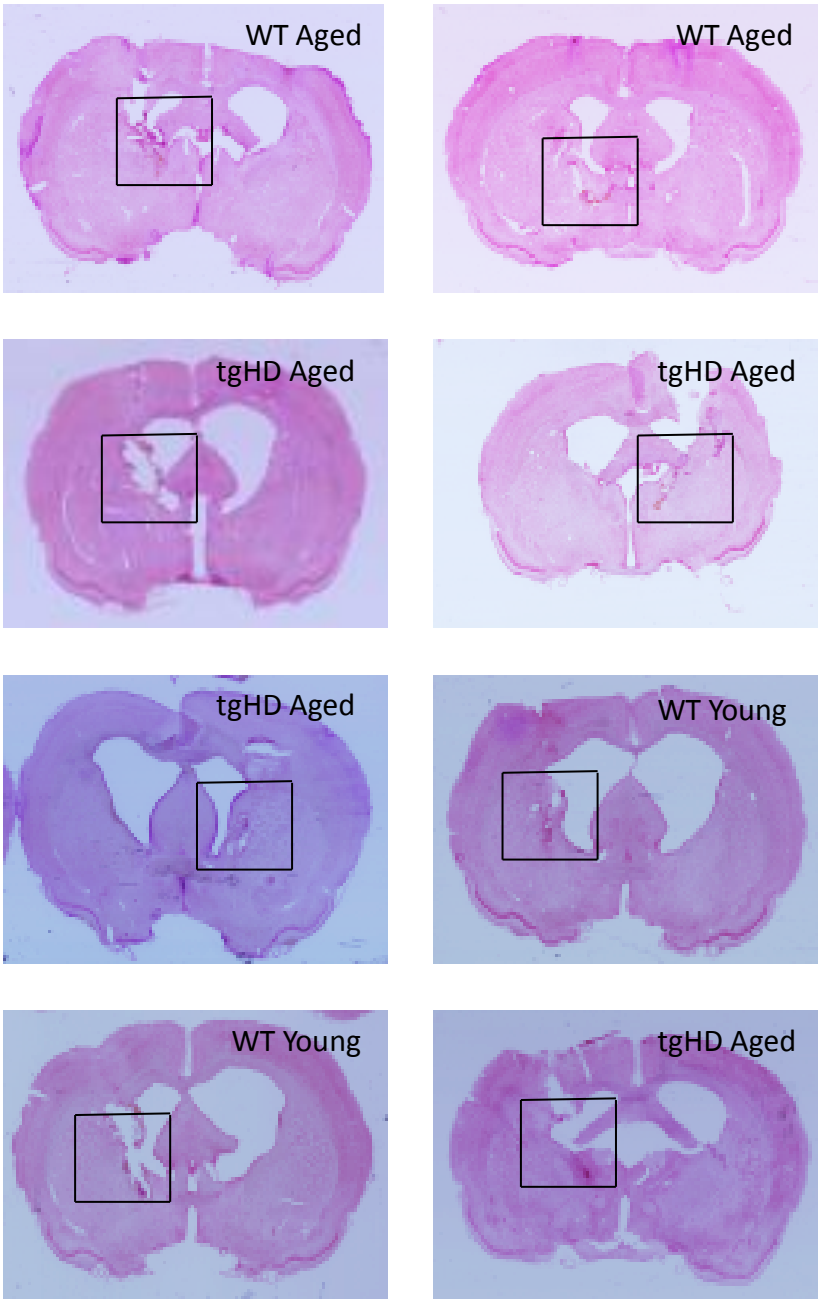
Clockwise from top:
tgHD aged, WT aged, tgHD young, WT young

Representation of probe placement for all animals.



Black= WT aged, Red= tgHD aged, Green= tgHD young, Blue= WT young

Probe placement for all animals excluded from analysis.



APPENDIX B

POST-OPERATIVE MONITORING FORM

Animal Welfare Score Sheet for Rodents

One sheet per animal to record parameters listed below

Species, Animal ID: Rat, #____

Date of Operation: _____

Pre-study Bodyweight:_____

Surgeon:_____

Person Scoring:_____

BODY WEIGHT & B.A.R (bright, alert, responsive) exploration SCORE

(normal is 0, score 1, 2, 3 for ↑in severity) (Yes is normal =0

No is abnormal =1 for approach/exploration responses)

Day	Pre 0	0	1	2

Body wt yesterday				
Body wt today				
Body wt change				
B.A.R				
Exploration: plays with enrichment				

Approach response (inquisitive behavior, investigates your presence)				
Eating: Yes/No Amount:				

GENERAL CLINICAL SIGNS* (score normal animal as 0, score 1,2,3 for ↑ in severity)

Inactive decreased movement or Increased movement				
Hunched posture				
Coat rough-fur on end, matted or dull				
Red eye/nose discharges (porphyria) a				

nonspecific sign of stress (NA in mice)				
Color of ears and feet (should be pink)				
Dilated pupils				
Pink staining of the neck (NA in mice)				
Abnormal breathing Rapid, opened-mouth, shallow, increased salivation				

BEHAVIORAL SIGNS OF PAIN IN RODENTS

(score normal animal as 0, score 1, 2, 3 for ↑ in severity)

Date				
Day	Pre 0	0	1	2

Back arch (hunched up with arched back)				
Belly press (presses belly to cage floor)				
Writhe (twisting of body or flank)				
Stagger (sudden loss of balance/gait)				
Twitch-(spasm of flank muscle), tremor, trembling, seizure				

Muscle Rigidity, Lack of Muscle Tone				
Fall (animal falls over)				
Biting aggression				
Grinding teeth				
Vocalization				

***Observations of any of these above signs are important clinical findings-indicating the animal is in pain and additional analgesia is needed**

-contact the vet for assistance.

WATER BALANCE Normally drink ~10% body wt per day,

eg. 300gm rat should drink 30 mls every 24 hours

eg. 30 gm mouse should drink 3 mls every 24 hours

Day	Pre 0	0	1	2
Start (100 ml rat) or (50ml mouse)of water in the bottle (A)				
Current # of ml in the bottle (B) post 24hours				
Water intake (A-B)				
Dehydration/skin tenting/ sunken eyes				

*Fluid drunk by the animal is one of the best indicators of wellbeing.

Animals in pain do not drink much.

The animal will require fluid therapy and possibly pain medication if the animal is drinking less than 10% of its body weight.

OPERATION SITE (score normal animal as 0, score 1, 2, 3 for ↑ in severity)

Day	Pre 0	0	1	2
Wound OK				
Bleeding				
Redness				
Swelling				
Drainage/discharge				
Sutures/clips OK				

POST-OP SUPPORT- Analgesic Administration

Day	Pre 0	0	1	2
Drug				
Dose				
Fluids by SC injection (amount and frequency)				
Other drugs				

Signature				

Experiment Particulars:

Any particulars pertaining to the experiment may be noted here, eg. Such as that the animals require more frequent daily observations between days 1-3.

Possible interventions/actions:

Fluid therapy, supplemental heat source, isolated/incubator, mash diet, food and water supply will be at the cage floor level if needed,supplemental food source, administration pain relieving agents as noted in approved protocol.

Humane Endpoints: State the combination and degree of severity of clinical signs* which will require euthanasia to be performed.

Other: Indicate what are the criteria are for pulling an animal from the experiment, or what other action might be necessary. Does the principal investigator need to be warned before a seriously sick animal is terminated after hours or during the weekend/holidays.

The P.I. will need to be notified, prior to termination of an animal, as he will transcardial perfuse the animal and collect the tissue.

Scoring

Total the scores

Note if there is a 3 or above in any one category the veterinarian will be contacted for advice

0-5 Normal (as long as no one category has a 3 or above)

6-12 Monitor carefully, consider analgesics contact veterinarian

Greater than 12 Suffering: provide relief, observe regularly, seek veterinarian's advice, consider euthanasia, and reconsider the protocol.

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