Investigating Effects of Metformin and Enriched Rehabilitation on Perinatal Hypoxia-Ischemia

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Figure 1 was adapted from ‘New horizons for newborn brain protection: enhancing endogenous neuroprotection’ (Hassell et al., 2015) which is an open access article distributed under the Creative Commons Attribution License.

ABSTRACT

Hypoxia-ischemia (HI) insults can have profound effects on the immature brain, impairing development and leaving survivors with lifelong physical and cognitive deficits. Improvements in neonatal care have resulted in more newborns surviving HI, but effective treatments for the long-term consequences of this disorder have yet to be established. Using the Rice-Vannucci model of hypoxia-ischemia at postnatal day (PND) 7, we investigated the effects of metformin and enriched rehabilitation on short and long-term motor and cognitive outcome in both male and female Sprague-Dawley rats. A battery of behavioural tests was used to assess early development and motor function from PND 8-21, while long-term motor and cognitive function was assessed from PND 49 onwards. Metformin, administered from PND 8-49, improved several aspects of early development that were compromised following HI (weight gain, neurological reflexes). However, it worsened motor impairments in the adhesive strip removal task and Montoya staircase. Enriched rehabilitation, beginning at PND 21, improved motor function in the adhesive strip removal task, open field and Montoya staircase. Additionally, it enhanced cognition in the Barnes maze and Morris water maze. Our results indicated that, despite early beneficial effects on development, metformin was not effective at improving long-term outcome. Enriched rehabilitation led to significant improvements in several aspects of motor and cognitive function, even when administered 2 weeks post-injury. This data suggests that enriched rehabilitation, but not metformin, may be a valuable intervention for treating behavioural impairments resulting from episodes of perinatal hypoxia-ischemia.

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LIST OF ABBREVIATIONS

APKC	atypical protein kinase C
AMPK	5’ adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
CBP	CREB-binding protein
EE	enriched environment
EMM	estimated marginal means
ER	enriched rehabilitation
EAA	excitatory amino acids
GA	gestational age
HI	hypoxia-ischemia
HIE	hypoxic-ischemic encephalopathy
MCAO	middle cerebral artery occlusion
MWM	Morris water maze
NBD	neurobehavioural development
NO	nitric oxide
OFR	oxygen free radical
PND	postnatal day
RT	reach training
SEM	standard error of the mean

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INTRODUCTION

1.1 Hypoxic-Ischemic Encephalopathy (HIE)

Hypoxia-ischemia (HI) is one of the most common causes of mortality and morbidity in children (Alonso-Alconada et al., 2013) and it is estimated that between 1 - 8 per 1000 newborns will suffer from this stroke-like neurological event (Kurinczuk et al., 2010). Survivors are often left with profound physical, cognitive and psychological disabilities, which can persist throughout their lifetime (Calvert & Zhang, 2005; Eunson, 2015) and lead to neurological disorders such as cerebral palsy (Patel et al., 2014; Rice et al., 1981). There are several etiologies through which an HI insult may arise. Premature birth and very low birth weight (<1500g) leave newborns particularly vulnerable to HIE due to the immaturity of their neurovascular system, poor cerebral autoregulation and underdeveloped lungs (Boylan et al., 2000; Hill & Fitch, 2012). In infants born at term, HI can occur as a result of birth complications such as umbilical cord compression, placental insufficiency and asphyxia (Fatemi et al., 2009; Hill & Fitch, 2012). Although advances in obstetrics and neonatal care have increased the chances of survival, effective treatments are still lacking; leaving 25% of term infants (Vannucci & Hagberg, 2004) and 80% of preterm infants who survive an HI insult with permanent disabilities (Hill & Fitch, 2012). To date, the only clinically approved treatment and standard of care for HIE is moderate hypothermia, which has been shown to have modest effects in terms of increasing survival rates and reducing disabilities (Higgins et al., 2011; Jacobs et al., 2007). Although beneficial for some, there are several downsides to this treatment. Hypothermia must be administered within a narrow time window, typically within 6 hours following initial insult (Thoresen, 2000; Thornton et al., 2012). While progress has been made in the detection and diagnosis of HIE, infants may not begin to display overt signs for an extended period of time following injury (Huang & Castillo,

2008), emphasizing the need for treatments that can be administered in a chronic timeframe. Additionally, hypothermia is not currently approved for use in preterm infants due to the risk of exacerbating damage, meaning there is no effective neuroprotective therapy available for these infants (Rumajogee et al., 2016). Lastly, hypothermia has been shown to benefit 1 in 6 newborns; typically those with a moderate degree of encephalopathy respond more favourably (Azzopardi et al., 2009). Gaining a better understanding of the molecular pathways and mechanisms involved in hypoxia-ischemia injury is critical in order to design and implement new therapies capable of helping a wider range of survivors.

1.2 Pathophysiology of Hypoxia-Ischemia

Despite the developing brain's innate ability for neuroplasticity and self-repair, disruptions during critical periods of brain development often lead to life-long sensorimotor and cognitive deficits. HI insults are characterized as multiphasic events **(Figure 1)**, during which injury progresses over time and negatively impacts the functional and structural integrity of the brain (Douglas-Escobar & Weiss, 2015; Hassell et al., 2015; Thornton et al., 2012). During the acute phase of an HI insult, a disruption in normal cerebral blood flow leads to a transient reduction of oxygen and glucose levels. Decreases in the levels of these substrates, which are required to meet normal cellular demands, results in a switch to anaerobic metabolism, acidosis and ultimately the depletion of adenosine triphosphate (ATP) stores (Sukhanova et al., 2016; Vannucci & Hagberg, 2004). As a result, dysfunction of membrane ion pumps, high influx of calcium and sodium, cytotoxic edema, excitotoxicity and inflammation, all of which perpetuate injury, occur. Ultimately, these events lead to necrotic cell death and comprise the *primary phase* of HI injury. The *latent phase* follows, during which reperfusion occurs and oxidative metabolism returns back to baseline levels. The latent phase is typically thought of as the

"therapeutic window" (Hassell et al., 2015), beginning within an hour following initial injury and lasting between 6 to 24 hours. Depending on the duration and severity of the initial insult, secondary injuries may occur following the latent phase. The *secondary phase* of HI injury typically begins 6-24 hours following initial insult and is characterized by a reduction in oxidative metabolism leading to secondary energy failure. Cytotoxic edema, excitotoxicity and an increased production of reactive oxygen species generated by high levels of intracellular calcium are characteristics of this phase. Mitochondria play a central role in injury and neurodegeneration following HI, with reactive oxygen species leading to mitochondrial dysfunction and the release of pro-apoptotic proteins (Huang & Castillo, 2008). Additionally, inflammation plays a prominent role in injury as the inflammatory response is much more pronounced in neonates (Vexler & Yenari, 2009). Microglia are among the first cells to respond to a hypoxic-ischemic insult by increasing activation, proliferation and the release of pro-inflammatory mediators, which have been suggested to exacerbate injury (Biran et al., 2006; Ferrazzano et al., 2013). The *tertiary phase* of HI injury can last weeks to months following initial insult and involves epigenetic changes, astrogliosis and remodeling of the injured brain (Hassell et al., 2015).

Gestational age (GA) at the time of insult is an important determining factor of the subsequent damage profile, as which cell types and brain regions are most vulnerable depends on the stage of brain development (Fernandez-Lopez et al., 2014). In preterm infants (GA 23-32 weeks), HI is a major cause of periventricular leukomalacia, a severe form of cerebral white matter injury and a hallmark of HIE (Carty et al., 2008; Deng et al., 2008). Premyelinating oligodendrocytes are the primary cells present in the immature white matter and are highly susceptible to damage via excitotoxicity, oxidative stress and inflammation (Deng et al., 2008;

Volpe, 2009). Injury caused by HI hinders the cells' ability to differentiate into mature oligodendrocytes, a critical step in the proper myelination and development of the immature brain (Back et al., 2007). Another critical component of the developing brain are subplate neurons; located below the immature cerebral cortex, they are the first neurons to develop and form the initial functional thalamocortical and corticocortical circuits (Deng et al., 2008; McQuillen et al., 2003). Damage to these neurons interferes with the maturation of connections needed for proper motor, visual and cognitive development, leading to deficits typically seen following hypoxia-ischemia (Deng et al., 2008; McQuillen & Ferriero, 2005). In term infants (GA 36-40 weeks), HI is characterized by gray matter damage, particularly within the cortex and hippocampus, and to a lesser extent, white matter damage (Fatemi et al., 2009; Huang & Castillo, 2008; Silbereis et al., 2010).

The notion that HI injury is an evolving process is promising especially when it comes to the development of therapies. Since the window of opportunity to provide life-changing interventions may be extended beyond the few hours immediately following initial insult, research should be aimed at developing chronic treatments capable of supporting long-term recovery and reducing the severity of disabilities in survivors.

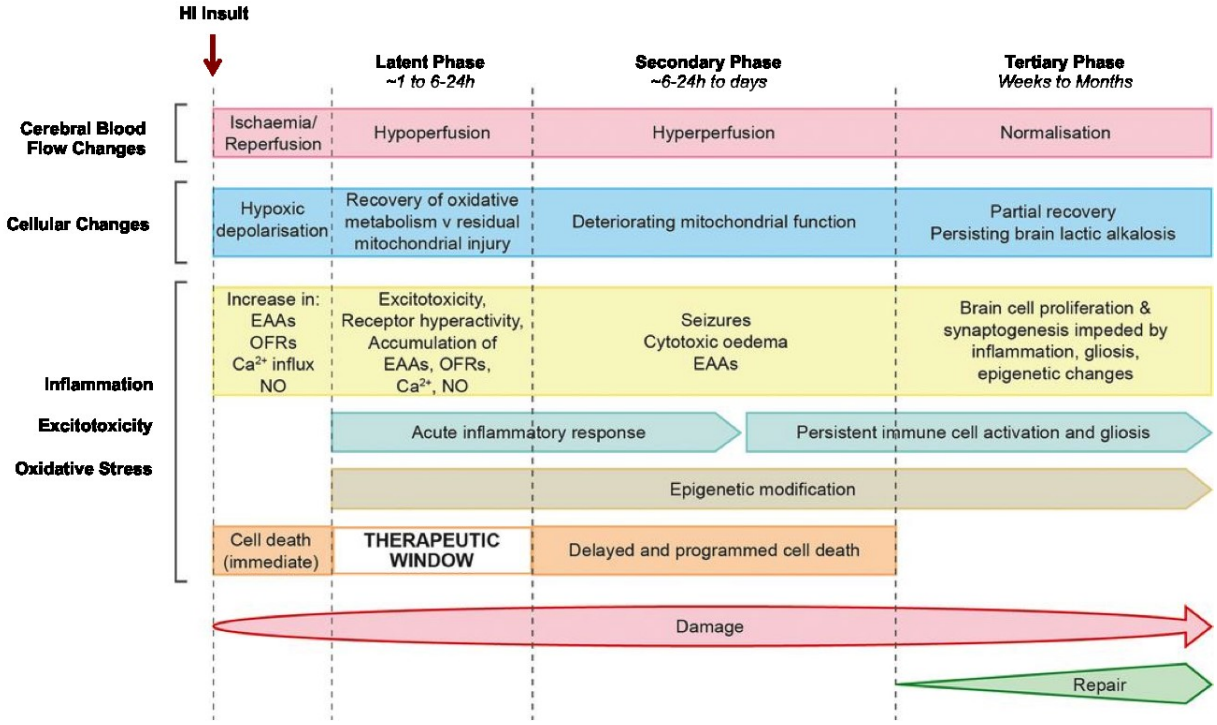

Figure 1. Pathophysiology of hypoxic-ischemic injury. Damage to the immature brain following HI is an evolving process consisting of multiple phases. Following the initial insult, reperfusion occurs followed by the latent, secondary and tertiary phases. The process begins within minutes of initial injury and can continue for weeks to months. (Adapted from Hassell et al., 2015)

1.3 Animal Models of Hypoxia-Ischemia

The use of animal models for research is essential in order to gain a better understanding of the mechanisms underlying injury and how they translate into behavioural outcomes (Mallard & Vexler, 2015). Considerable research has gone into understanding how the timeline of brain development in the newborn human corresponds with that of rodents. Since the vulnerability of specific regions and cells changes depending on the timing of HI injury, it is critical that this is taken into consideration in order to ensure clinical relevance. Although it was originally thought that postnatal day 7 in the rat brain was similar to a human infant at term, recent research has suggested that it may be a better representation of injury in the late preterm infant due to the presence of white matter damage (Balduini et al., 2000; Patel et al., 2014). Various animal models have proven useful for studying HI, however, the Rice-Vannucci model is the most widely studied and used. To produce damage, the common carotid artery is permanently ligated followed by exposure to a hypoxic environment (8% O_2), creating unilateral damage to the cortex, corpus callosum, striatum and hippocampus in the hemisphere ipsilateral to the occlusion (Rice et al., 1981). The injury profile of this model often varies from animal to animal and may include infarction, neuronal death or a combination of the two, typically ranging from mild to severe (Vannucci et al., 1999). One benefit of using the Rice-Vannucci model is that it can be modified to allow researchers to approximate different clinical populations that exist following hypoxia-ischemia. For example, by changing the postnatal day at which injury is induced (PND 1-3), one could examine HI in the context of preterm newborns (Semple et al., 2013) and more closely study periventricular leukomalacia and the associated behavioural outcomes. Additionally, by modifying the amount of time animals are exposed to the hypoxic environment, researchers are able to study subjects with more severe injury profiles (Towfighi et al., 1991).

Behaviourally, animals that undergo the Rice-Vannucci model of HI display deficits similar to those seen clinically. Newborns who have suffered a hypoxic-ischemic episode display diminished reflexes, such as the Moro, plantar, and grasping reflexes, and this disruption is highly predictive of later development of cerebral palsy (Ten et al., 2003). In animals, several neurobehavioural developmental tests are capable of detecting delays in the maturation and appearance of certain neurological reflexes (Lubics et al., 2005). Additionally, there are a number of behavioural tests sensitive to sensorimotor (Bona et al., 1997; Jansen & Low, 1996) and cognitive impairments (Arteni et al., 2003; Ikeda et al., 2001; Pereira et al., 2007) produced by HI.

1.4 Sex Differences in Hypoxia-Ischemia

Data from clinical studies suggests that male infants are more susceptible to brain injury and typically exhibit worse outcomes compared to females with similar injury (Donders & Hoffman, 2002; Hill & Fitch, 2012). Despite this, much of the preclinical data published presents male-only or pooled data. Understanding the differences in vulnerability to HI injury between sexes may reveal critical information necessary for the development of effective treatments. Recently, there has been a trend towards the use of female animals in preclinical research and a number of studies assessing sex differences following HI have emerged. However, inconsistencies surrounding which sex is more negatively affected following HI with regards to behavioural impairment, brain injury, and response to treatment highlights the need for more research in this area (Netto et al., 2017). Research stemming from animal models of adult stroke, as well as hypoxia-ischemia, has led to several hypotheses to explain how sex may influence the brain's response to injury. First, evidence suggests different cell death pathways are activated, with males activating a caspase-*independent* pathway, whereas in females, a caspase-*dependent*

pathway is dominant (Lang & McCullough, 2008; Liu et al., 2009; Renolleau et al., 2008; Zhu et al., 2006). The hormonal environment of the neonatal brain is another factor that could contribute to the increased vulnerability of males. Research in adult models of stroke have demonstrated that testosterone can exacerbate glutamate toxicity following ischemic brain injury (Yang et al., 2002). Thus, elevated testosterone levels during gestation and first year of life could contribute to males being more severely affected following HI compared to females (Hill et al., 2011; Hill & Fitch, 2012). Further research is needed to gain a deeper understanding regarding sex differences following hypoxia-ischemia injury, potentially leading to therapies tailored to be sex-specific.

1.5 Neuroplasticity in the Developing Brain and Enriched Rehabilitation

Neuroplasticity is an important component in brain development and plays a critical role in the recovery process following brain injury. However, without regulation, post-injury plasticity can become maladaptive (Johnston et al., 2009). One way to regulate this is through behavioural interventions aimed at driving experience-dependent plasticity (Johnston, 2009; Kolb et al., 2013). The immature brain has been shown to be heavily influenced by environmental stimuli (Meaney & Aitken, 1985) and both preclinical and clinical research has shown that individuals who are reared in stimulating environments fare better than those who are not (Chou et al., 2001; Salmaso et al., 2014). In a preclinical setting, this can be studied through the use of an enriched environment (EE). This form of rehabilitation, which has been extensively studied in the stroke recovery literature, involves socially housing animals in large cages filled with interactive objects (ramps, toys) (Johansson, 2004; Kolb et al., 1998) and changing the configuration of these cages often to promote sensory, motor and cognitive stimulation (van Praag et al., 2000; Will et al., 2004). Housing HI rats in EE cages 9 hours/day for 9 weeks was

found to promote cognitive recovery by reversing reference and working memory deficits in the Morris water maze (Pereira et al., 2007). Researchers using similar parameters also found EE was capable of improving cognitive function in the novel object recognition task, which they attributed to the preservation of hippocampal dendritic spine density within the CA1 region (Rojas et al., 2013).

Although EE has shown promise in enhancing cognitive function following HI, limited benefit has been observed with respect to motor function (Rojas et al., 2013). It has been suggested that because the enriched environment paradigm is not task-specific, nor complex enough, it is not designed to enhance fine motor function (Biernaskie & Corbett, 2001). Thus, EE can be combined with daily reach training (RT), referred to as enriched rehabilitation (ER), to encourage the use of the affected forelimb and promote sensorimotor function (Biernaskie & Corbett, 2001). Using adult stroke animal models, Biernaskie and Corbett (2001) showed that ER enhanced reaching in the Montoya staircase task following middle cerebral artery occlusion (MCAO) and increased dendritic branching in the contralesional motor cortex. More recently, ER has been tested following neonatal hypoxia-ischemia and successfully led to improvements in motor learning and coordination (Schuch et al., 2016*b*).

A limitation to using ER in preclinical studies of early hypoxia-ischemia is timing, as newborn rats are not capable of fully engaging/participating in ER until they are weaned, typically 1-2 weeks following injury. Stroke research in both humans and animals has demonstrated the importance of initiating training early following stroke (~1 week) (Wahl & Schwab, 2014). Indeed, delaying ER by 2 weeks does not confer the same benefits in functional outcome as when it is initiated 5 days following focal ischemic injury (Biernaskie et al., 2004). Given that many of the mechanisms leading to HIE occur within minutes to days following

initial injury, it is important to administer a treatment early in order to minimize the negative consequences and potentially prime the brain to be more receptive to the effects of rehabilitation. It has been shown that using enriched rehabilitation in combination with a growth factor cocktail promotes recovery sooner following ischemic injury compared to ER alone (Jeffers et al., 2014). Furthermore, research has shown the effectiveness of rehab may depend on the sequence of its administration; recovery of function resulting only when drug administration occurred prior to rehab rather than concurrently (Wahl et al., 2014).

1.6 Combinational Therapy Approach to Recovery: Repurposing Metformin

Repurposing clinically approved drugs for use in different disease models represents an efficient strategy for developing new therapies. In recent years, metformin has been studied for its potential novel application in several fields, including stroke recovery. Metformin, a biguanide antidiabetic drug, has long been the first-line treatment provided to individuals with type II diabetes due to its ability to suppress hepatic gluconeogenesis (Foretz et al., 2014). Its effects in the body are achieved via its action on the mitochondria, where it disrupts the electron transport chain by briefly inhibiting complex I (Owen et al., 2000). This interference leads to a disruption in cellular energy homeostasis and activation of the cell's energy sensor, 5' adenosine monophosphate-activated protein kinase (AMPK) (Pryor & Cabreiro, 2015). Animal studies have shown inhibition of gluconeogenesis in primary hepatocytes via metformin is initiated by this indirect activation of AMPK (Zhou et al., 2001), which then phosphorylates CREB binding protein (CBP), a transcriptional coactivator, via the atypical protein kinase C (aPKC) pathway, ultimately leading to a reduction of glucose production (He et al., 2009).

This aPKC-CBP pathway is also involved in and appears necessary for the differentiation of embryonic neural precursor cells (Wang et al., 2010). Researchers

demonstrated that metformin was capable of activating this pathway within the brain, just as it does in hepatic cells, leading to neural differentiation (Wang et al., 2012). Wang and colleagues (2012) showed that metformin increases the genesis of neurons from cortical precursor cells and human embryonic stem cell derived neural precursors. Additionally, *in vivo* metformin treatment enhanced hippocampal neurogenesis, which subsequently increased the plasticity of spatial memory assessed by a reversal probe in the Morris water maze (MWM). Naïve mice treated with metformin spent more time searching for the platform in the new quadrant, whereas vehicle treated mice spent more time in the original quadrant, suggesting metformin improved their ability to update spatial memory formation (Wang et al., 2012). It has been shown that following HI injury, similar to ischemic injury in adults (Felling et al., 2006), there is an inherent increase in neural precursor cells, some of which migrate to the site of injury (Dixon et al., 2015; Ong et al., 2005). This increase in neural precursor cells is often not sufficient to translate into functional recovery, potentially due to a lack of trophic factors (Ong et al., 2005). Therefore, the idea of using metformin to promote and enhance the survival, function, and possibly the integration of these newly generated cells has been gaining attention.

Using adult animal models, researchers have shown that chronic metformin treatment, given within a clinically relevant time window following experimental stroke, was capable of inducing changes at a cellular level and promoting functional recovery. Metformin has been shown to promote neurogenesis (Jin et al., 2014; Liu et al., 2014), angiogenesis (Jin et al., 2014; Liu et al., 2014; Venna et al., 2014) and improve motor and sensorimotor function following MCAO (Jin et al., 2014; Venna et al., 2014). Additionally, activation of AMPK has been shown to suppress inflammation (Salminen et al., 2011), therefore it has been suggested that metformin may be capable of attenuating inflammatory responses following injury (Hyun et al., 2013; Jin et

al., 2014). Given metformin's multiple mechanisms of action, the idea of repurposing it to promote brain repair and recovery following neonatal hypoxia-ischemia injury is promising. Dadwal and colleagues (2015) have recently tested this idea in a murine model of HI and found that 200mg/kg of metformin, when administered 24 hours following HI, was able to activate endogenous neural precursor cells and promote differentiation. Additionally, they showed that metformin treatment was able to enhance sensorimotor recovery shortly following injury (Dadwal et al., 2015). To date, there have been no studies assessing metformin in a rodent model of hypoxia-ischemia and its impact on development and long-term motor and cognitive function.

1.7 Rationale

Hypoxia-ischemia often leaves survivors with persistent motor and cognitive deficits. Despite this, there are currently no effective treatments capable of supporting long-term recovery. Given the complexity of HI pathophysiology, it is evident that potential therapies should consist of multiple, complementary interventions, each capable of targeting several mechanisms underlying injury and repair. Thus, this thesis aims to determine whether enriched rehabilitation and metformin, alone and in combination, are able to promote normal behavioural development and improve outcome following hypoxia-ischemia injury.

1.7.1 Hypothesis

Enriched rehabilitation and metformin will improve motor and cognitive function following HI injury, with the greatest benefit occurring when the two are administered as a combination therapy.

1.7.2 Objectives

(i) Evaluate injury profile following hypoxia-ischemia injury.

(ii) Assess neurobehavioural development following HI and the effect of early metformin treatment.

(iii) Assess long-term motor and cognitive function following HI and the effect of ER and metformin on outcome.

MATERIALS AND METHODS

2.1 Subjects and Experimental Design

Timed-pregnant Sprague-Dawley rats were acquired from Charles River Laboratories (Montreal, Quebec) and housed on a 12:12 reverse light/dark cycle until parturition. Food and water was available *ad libitum*. Once born (PND 0), both male and female pups (N= 90) were housed with their dams. On PND 7, dams were randomly assigned to the vehicle or metformin treatment group and their pups were randomly assigned to receive sham or hypoxia-ischemia surgery. All sham pups came from dams in the vehicle treatment group. Five HI pups died during the surgical procedure and sixteen died during hypoxia. Additionally, four HI pups from the metformin group were sacrificed for blood work at PND 14. On PND 21, the remaining pups (N= 65) were weaned and randomly assigned to be exposed to standard housing (no rehab) or enriched rehabilitation (rehab); all shams were standard housed. The resulting experimental design consisted of Sham animals (no treatment) (n= 11) and 4 hypoxia-ischemia conditions: No Rehab + Vehicle (n= 12), Rehab + Vehicle (n= 11), No Rehab + Metformin (n= 15), and Rehab + Metformin (n= 16). Behavioural testing occurred at several points throughout the experiment **(Figure 2)**. All procedures and testing occurred during the dark cycle. Animal procedures were conducted with the approval of the University of Ottawa's Animals Care Committee and in accordance with the Guidelines of the Canadian Council of Animal Care.

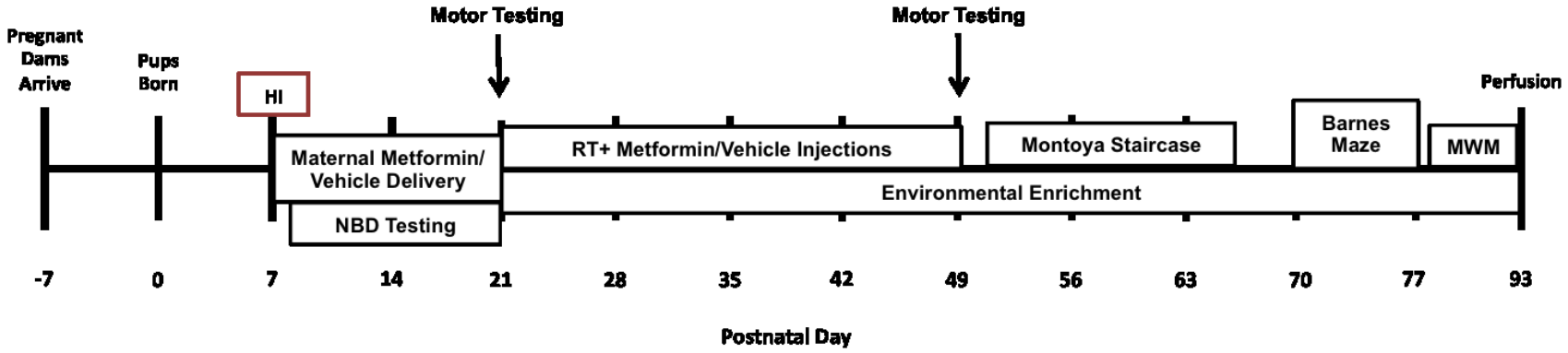

Figure 2. Experimental timeline.

2.2 Hypoxia-Ischemia Injury Model

At PND 7, pups were split into two groups: sham and hypoxia-ischemia. HI animals were anesthetized using isoflurane (3% induction, 1.5% maintenance) and an incision was made along the midline of the neck. The Rice-Vannucci model of injury was used, in which the left common carotid artery was carefully isolated from surrounding nerves and ligated using 4-0 surgical silk (Rice et al., 1981). The skin was then closed using tissue adhesive (3M Vetbond) and topical bupivacaine was applied to the incision. Pups were returned to their dams for 2.5 hours before being placed in a hypoxia chamber for 90 minutes (8% oxygen and 92% nitrogen) at 37°C. Following hypoxia, the pups were returned to their home cage. Sham animals were anesthetized and an incision was made, however, the carotid artery was not ligated, nor did they undergo hypoxia. Dams received two bupivacaine injections (0.05mg/kg) to help with pain control in the pups: pre-surgery and 4-6 hours following the first dose.

2.3 Metformin Administration

Starting on PND 8, metformin (1-1-Dimethylbiguanide hydrochloride, Sigma-Aldrich) (20mg/kg/day) or 0.9% saline was delivered to the pups via the mother's milk by implanting a mini-osmotic pump subcutaneously (Alzet, model 2002, 0.5µl/hour, 14 days). Briefly, dams were anaesthetized using isoflurane (5% induction, 2% maintenance) and an incision was made between the scapulae where the pump was inserted and the skin was then sutured. At PND 21, once weaned, pups began receiving daily subcutaneous injections (BD Allergy Syringe, 1/2ml 27G x 1/2) of metformin (200mg/kg/day) or saline for 4 weeks.

2.4 Enriched Rehabilitation

On PND 21, pups were weaned and separated by sex. Animals assigned to the rehabilitation group were placed into large enrichment cages in groups of 4-6, where they lived for the remainder of the study (24 hours/day). Enriched cages were filled with a variety of objects (toys, ramps, etc.) that were rearranged on a weekly basis **(Figure 3)**. Animals began daily reach training 5 days/week from PND 22-49. Training consisted of 3, 15-minute sessions/day with a 45-minute break between sessions. Rats were placed in individual Plexiglas chambers where they had to reach through a narrow slot for sucrose pellets (TestDiet), which were only accessible by reaching with the forelimb contralateral to the artery occlusion. Animals assigned to the non-rehabilitation groups were pair housed in standard GreenLine cages.

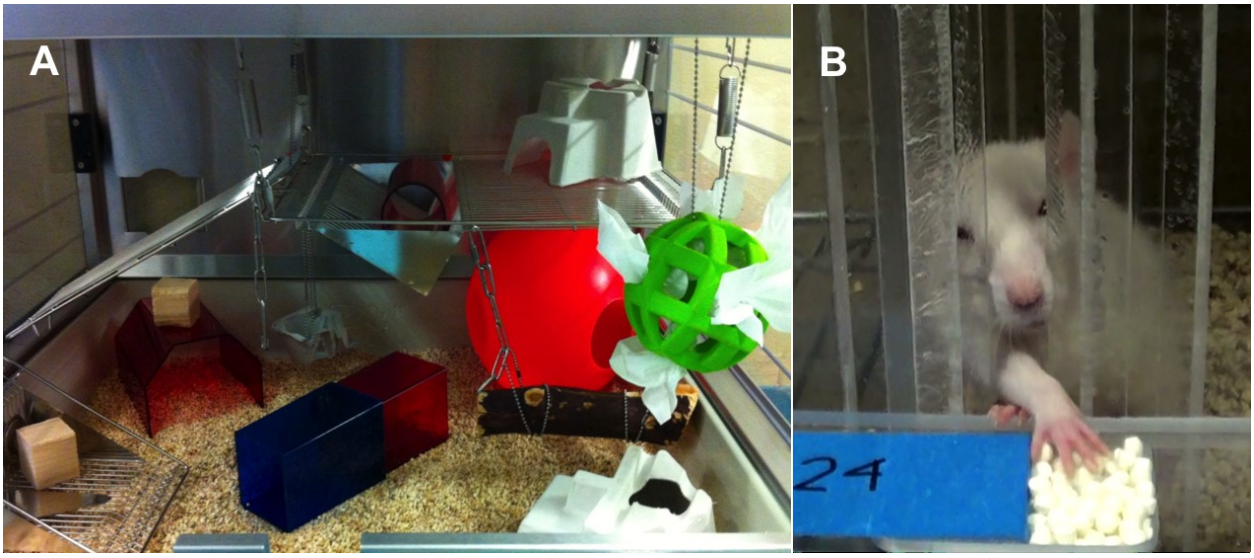

Figure 3. Enriched rehabilitation (ER) paradigm. Animals assigned to the rehab group were socially housed in large enrichment cages filled with objects (toys, ramps) **(A)** and performed daily reach training (RT) sessions **(B)**.

2.5 Behavioural Testing

2.5.1 Neurobehavioural Development (NBD) Testing

Neurobehavioural development assessments began the day after HI surgery (PND 8) and continued until animals were weaned at PND 21. Early differences between sham and hypoxia-ischemia pups were assessed using a battery of tests for neurological reflexes that typically begin to emerge after PND 7 (Altman & Sudarshan, 1975). In addition to monitoring their weight and examining the appearance of physical characteristics such as eye opening, pups were tested daily for the following neurological signs or reflexes (Lubics et al., 2005):

a. *Negative Geotaxis:* pups were placed in the center of a 30 cm inclined board (25°) facing downward. The day the pups began to turn around (180°) and climb up the board was recorded. This task had an allotted time of 30s to be completed otherwise it was considered incomplete.
b. *Limb Placing:* the dorsal surface of the forelimbs was brushed along the edge of a table. The day the animals lifted their forelimbs and placed them on the table was recorded.
c. *Limb Grasp:* forelimbs and hindlimbs were touched with a metal rod. The day the pups were able to grasp onto the rod was recorded.
d. *Gait:* pups were placed in the center of a white paper circle (15 cm diameter). The day they began to move off the circle was recorded. This task had an allotted time of 30s to be completed otherwise it was considered incomplete.
e. *Auditory Startle:* first day of startle reflex to a loud noise (dog clicker) was recorded.

2.5.2 Adhesive Strip Removal Task

Adhesive strip removal testing was conducted at two test points: pre-weaning (PND 18, 19 and 20) and post-weaning (PND 49). Sensorimotor deficits were assessed using this task (Bouet et al., 2009). Two equal-sized pieces of adhesive tape (1cm x 1cm) were placed onto the animal's forepaws. The animal was then placed into a transparent cylinder for 5 minutes or until both pieces were successfully removed. The task was filmed from below and the time to initial contact and time to remove the tape was recorded. Animals were given two trials/day and the average was calculated.

2.5.3 Ladder Walking Test

Ladder walking performance was assessed at two test points: pre-weaning (PND 20) and post-weaning (PND 49). Animals were placed on a horizontal ladder apparatus consisting of Plexiglas walls and metal rungs that were irregularly spaced (1-3 cm). Animals were given two training sessions prior to the first test point in order to acclimate to the ladder. Testing consisted of 4 crossings and the average number of forelimb and hindlimb slips/incorrect placements was analyzed and used as a measure of motor function and coordination (Metz & Whishaw, 2002).

2.5.4 Open Field

Open field testing occurred at two test points: pre-weaning (PND 21) and post-weaning (PND 49). Animals were placed in a large open-topped box and allowed to freely explore for 5 minutes. Exploratory and motor activity parameters were measured using EthoVision tracking software (Noldus) such as total distance travelled and average velocity.

2.5.5 Montoya Staircase Test

Montoya staircase testing was conducted from PND 51-64. Skilled forelimb reaching (Montoya et al., 1991) and motor learning ability was assessed using this task. Animals were placed in a Plexiglas box with a set of 7 steps descending on each side of their body. Each step contained a small well in which 3 sucrose pellets were placed (total of 21 pellets on each side). Animals were given two, 15-minute sessions/day (separated by 4 hours) to reach for pellets. To ensure sufficient motivation, animals were food restricted (~12g/day) for the duration testing. Daily reaching performance was measured by calculating the average number of pellets retrieved across the two sessions.

2.5.6 Barnes Maze

Barnes maze testing was conducted from PND 70-77. Spatial learning and memory was assessed using this task (Barnes, 1979). Animals were placed on an elevated circular platform (100cm diameter) containing 18 holes surrounding the perimeter. The animal had to learn to find a dark escape box located beneath one of the holes in order to escape the maze and an aversive stimulus, consisting of bright surgical lights and radio static. Distal visual cues, consisting of geometric shapes, were placed 30 cm away from the edge of the platform and were meant to aid the animals in navigating the maze. Habituation occurred one day prior to testing, during which animals were shaped to enter the escape hole in the presence of the aversive stimuli. No visual cues were present. For the *acquisition* testing, animals were randomly assigned a distal visual cue and the goal box was placed in the same location relative to that cue. Testing consisted of placing the animal under a holding chamber and activating the aversive stimulus. Lifting the chamber signaled the start of the trial. The trial ended when the animal entered the goal box or 3 minutes had passed. The animal was given 1 minute to rest in the goal box and returned to its

home cage. Acquisition testing consisted of one trial/day for 7 days. Twenty-four hours following the last acquisition trial, the goal box was removed and the animals were tested on a *probe* trial to determine if they remembered its location. Animals were tracked using a ceiling mounted camera and latency to reach the goal box and time spent in the target quadrant were analyzed using EthoVision tracking software (Noldus).

2.5.7 Morris Water Maze (MWM)

Morris water maze testing was conducted from PND 82-92 to assess spatial learning and memory (Morris, 1984). Animals were placed in a circular pool filled with water (185 cm diameter, 24°C) that was made opaque using non-toxic blue tempera paint. A platform (10 cm diameter) was submerged 2 cm below water level. Distal visual cues, consisting of black and white geometric shapes, were placed on the walls of the room surrounding the pool. During the *acquisition* phase, the escape platform was located in one of the four quadrants of the pool. Animals were given four, 60-second trials/day for four days to locate the platform, each trial starting from a pseudorandomly assigned quadrant. If animals were unable to locate the platform within the allotted time, they were gently guided to it. All animals were given 20 seconds to rest on the platform before being returned to their home cage. Inter-trial interval was 20 minutes. Two, 60-second *probe* trials, 24 and 72 hours following the last acquisition trial, were used to assess memory retention. The platform was removed and animals entered the pool from the quadrant opposite to where the platform had been located. *Reversal* testing was performed 24 hours following the last probe trial. The platform was placed in the opposite quadrant and the animals' ability to learn a new platform location was tested. Once performance plateaued, a single reversal probe was given 24 hours later. Animals were tracked using a ceiling mounted

camera and several parameters including latency to escape, distance to platform, velocity and time spent in target quadrant were analyzed using EthoVision tracking software (Noldus).

2.6 Histological Procedures

Following completion of behavioural testing, animals were euthanized using a lethal dose of euthanyl administered by intraperitoneal injection. The animals were then transcardially perfused with heparinized saline (120mL at a rate of 20mL/min), followed by 4% paraformaldehyde (PFA). Brains were carefully removed and post-fixed in 4% PFA at 4°C overnight and transferred into 20% sucrose the following day for cryoprotection. The brains were then frozen using isopentane (-50°C to -30° C) and stored at -20°C. Brains were sectioned using a cryostat (Leica CM1850) at 20μm and 1 in every 10 sections was mounted. Sections were stained with 0.25% cresyl violet and imaged at 20x. Using ImageJ software, both ipsilateral and contralateral hemisphere, cortex and hippocampus volumes were determined. This was achieved by measuring the area of each region of interest across 10 equally spaced sections beginning at *1.20* mm and ending at *-3.80* mm relative to Bregma and calculating the volume (sum of all areas X tissue between samples). The percent of ipsilateral tissue remaining was calculated as: volume of ipsilateral tissue ÷ volume of contralateral tissue X 100.

2.7 Statistical Analysis

Hierarchical cluster analysis using Ward's method (Ward, 1963) was performed using "percent ipsilateral hemisphere remaining" as the target variable in SPSS Statistics Software (v24) in order to determine the number of groups that would best represent the range of injury present. This resulted in HI animals being subdivided into two levels of injury: "HI/mild" and "HI/severe". Data was analyzed using SPSS and graphed using GraphPad Prism (v6). For behavioural tests conducted prior to weaning, data was analyzed using the variables: "Sex"

(female and male), “Cluster” (sham, mild, severe) and “Drug” (vehicle and metformin). For tests conducted following weaning, the variable “Housing” (no rehab and rehab) was analyzed in addition to the variables previously mentioned. Repeated measures analysis of variance (ANOVA) was used to analyze behavioural data that was conducted across multiple days. Univariate analysis was used to analyze behavioural data consisting of a single time point as well as histological data. Estimated marginal means (EMM) were used to determine differences between groups, despite small group sizes, by utilizing a general linear model to predict how a larger population would behave. For all analyses, significance was defined as $p< 0.05$ and values reported are estimated marginal means ± SEM. Due to the number of variables in this study, graphs are only shown for data that yielded significant statistical effects. Data showing non-significant interactions can be found in Appendix B.

RESULTS

3.1 The Rice-Vannucci model of injury resulted in different degrees of damage and metformin exacerbated injury in females, but not males, with severe damage

Univariate analysis found a significant cluster * sex effect on the % of ipsilateral hemisphere tissue remaining ($F= 27.446$, $p<0.001$). Post-hoc analysis found that both female and male HI/severe animals receiving vehicle treatment had significantly less ipsilateral hemispheric **(**$p<0.001$; **Figure 4A**), cortical **(**$p<0.001$; **Figure 4B)** and hippocampal **(**$p<0.001$; **Figure 4C)** tissue remaining compared to sham and HI/mild counterparts. Interestingly, HI/severe females had significantly less hemispheric and cortical tissue remaining, but not hippocampal, compared to males (denoted by *a;* $p<0.001$; **Figure 4A-C**). Additionally, univariate analysis found a significant cluster * sex * drug effect on the % of ipsilateral hemispheric ($F=7.995$, $p=0.005$) and hippocampal ($F=26.391$, $p<0.001$) tissue remaining. HI/severe females receiving metformin had significantly less hemispheric tissue **(**$p<0.001$; **Figure 4D)**, a trend towards less cortical tissue **(Figure 4E)**, and significantly less hippocampal tissue **(**$p<0.024$; **Figure 4F)** compared to those receiving vehicle treatment; a trend not observed in males **(Figure 4D-F)**. Interestingly, HI/severe males had significantly more hippocampal tissue remaining when treated with metformin ($p<0.001$; **Figure 4F)**. Cresyl violet stained images of the three clusters of injury are shown for females **(Figure 5A-C)** and males **(Figure 5D-F)**. Microinfarcts were observed but not quantified within HI animals **(Figure 6)**. This data confirmed that the Rice-Vannucci model resulted in global damage in both sexes, with some animals appearing relatively unaffected and others suffering from large infarctions. Female HI animals with severe damage displayed greater injury compared to their male counterparts and were made worse by metformin treatment, whereas male hippocampal tissue was protected.

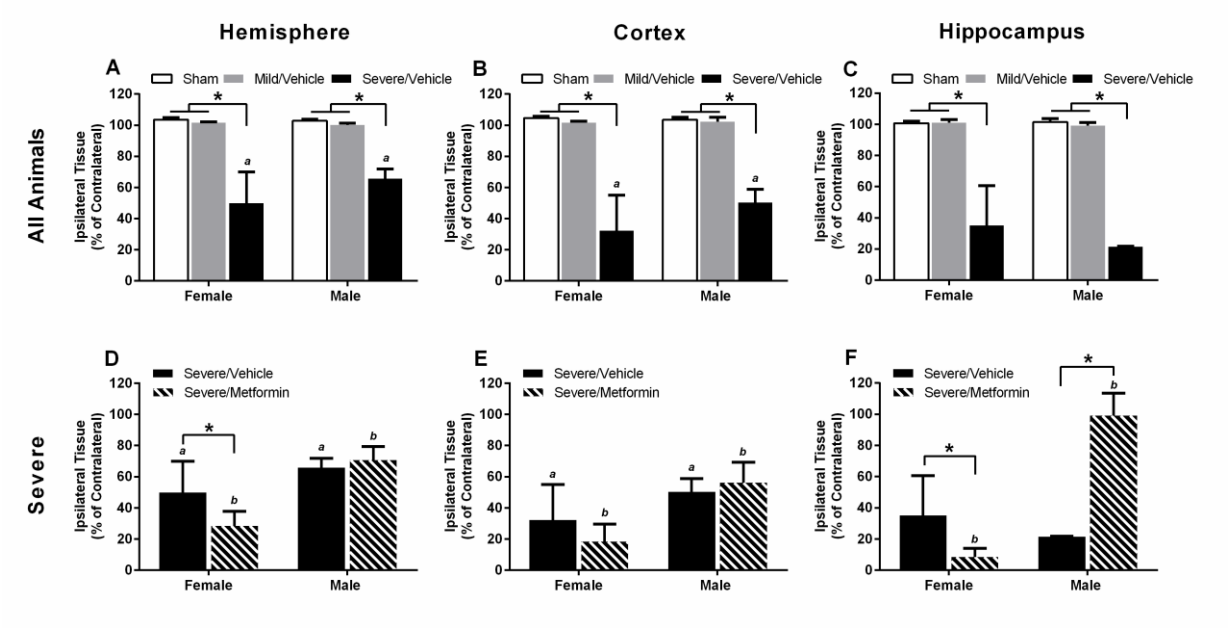

Figure 4. Ipsilateral brain volume remaining (% of contralateral). HI/severe female and male animals receiving vehicle treatment had significantly less ipsilateral hemispheric **(A)**, cortical **(B)**, and hippocampal **(C)** tissue remaining compared to their sham and HI/mild counterparts. Metformin decreased the % of ipsilateral hemispheric **(D)**, cortical **(E)** and hippocampal **(F)** tissue remaining in HI/severe females, while protecting hippocampal tissue in HI/severe males **(F)**. *a* and *b* denote significant differences between females and males within the same group. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild/Vehicle n= 9; Severe/Vehicle n= 2; Severe/Metformin n= 2) (Male: Sham n= 6; Mild/Vehicle n= 10; Severe/Vehicle n= 2; Severe/Metformin n= 2).

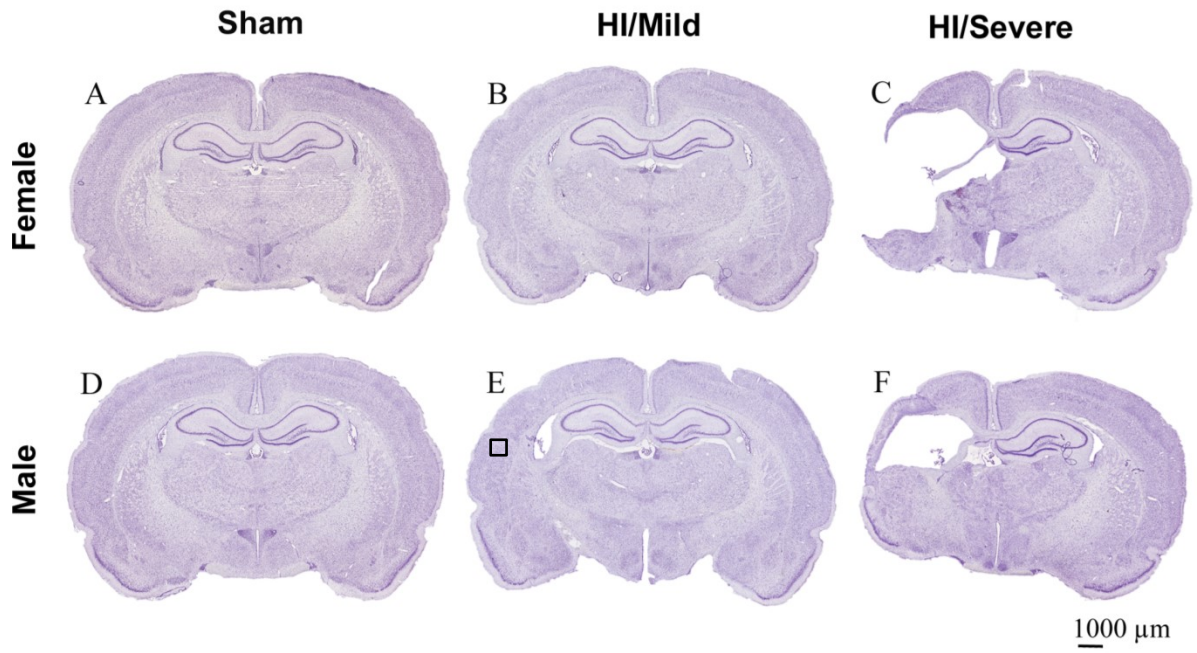

Figure 5. Cresyl violet stained representation of HI injury. Coronal sections from female sham **(A)**, HI/mild **(B)** and HI/severe **(C)** animals. Male sham **(D)** and HI/mild **(E)** animals appear similar to the females, while HI/severe males **(F)** have less damage compared to females. Outline in **(E)** represents location of microinfarct seen below.

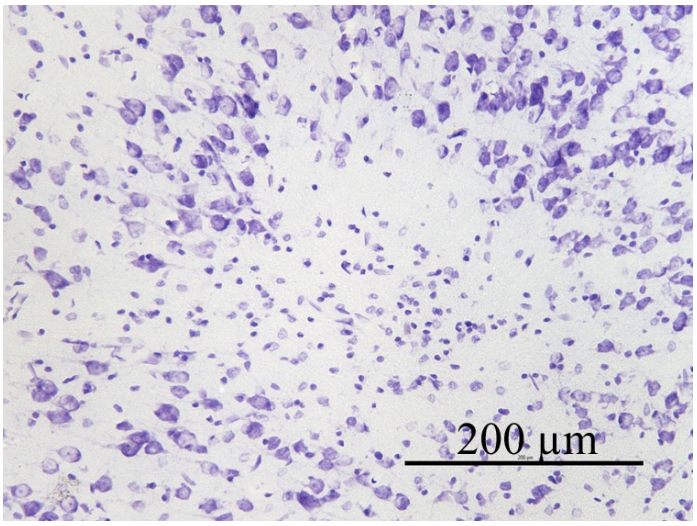

Figure 6. Cresyl violet stained representation of cortical microinfarct.

3.2 Early metformin treatment helped normalize weight gain following HI

Repeated measures ANOVA showed a significant day * cluster * drug (F=4.748, p=0.020) effect on body weight. HI/mild animals receiving vehicle treatment weighed significantly less than shams from PND 8-20. HI/severe animals receiving vehicle treatment weighed significantly less than shams from PND 8-13 and 20 **(Figure 7A)**. Although not significant, there was a greater difference between the trajectories of shams and HI female animals **(Figure 7B)**; this trend was not as distinct in males **(Figure 7C)**. HI/mild animals receiving metformin weighed significantly more than animals receiving vehicle across the 13 days **(Figure 7D)**. A similar trend was observed in females **(Figure 7E)** and males **(Figure 7F)**. HI/severe animals receiving metformin weighed significantly more from PND 14-20 compared to those receiving vehicle treatment **(Figure 7G)**; a similar trend was observed in females **(Figure 7H)** and males **(Figure 7I),** although the effects were more prominent within female animals. Early metformin treatment helped HI animals achieve normal weight gain during development, an effect that appeared more robust in HI/severe females compared to male counterparts.

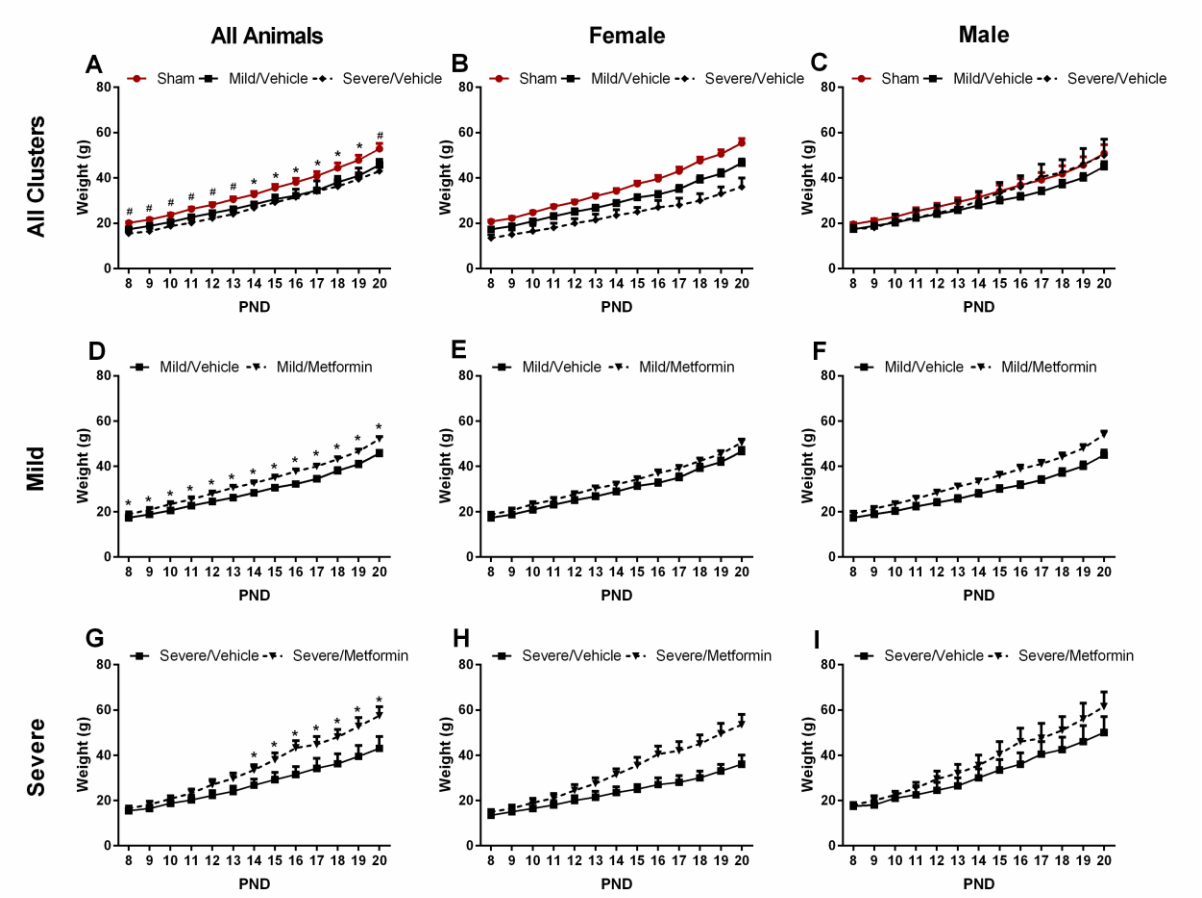

Figure 7. Body weight following HI (pre-weaning). HI/mild and HI/severe vehicle treated animals weighed significantly less than shams **(A)**; a similar trend was observed in females **(B)** and males **(C)**. Within HI/mild and HI/severe animals, those receiving metformin weighed significantly more than those receiving vehicle **(D, G)**; a similar trend was seen in females **(E, H)** and males **(F, I)**, with female HI/severe animals appearing to benefit most from metformin. (# denotes shams being significantly different from mild and severe animals, * denotes difference from mild only). Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild/Vehicle n= 9; Mild/Metformin n= 15; Severe/Vehicle n= 2; Severe/Metformin n= 2) (Male: Sham n= 6; Mild/Vehicle n= 10; Mild/Metformin n= 12; Severe/Vehicle n= 2; Severe/Metformin n= 2).

3.3 Metformin lessened delays in neurobehavioural development in those with severe damage

Univariate analysis revealed a significant cluster effect in three neurological signs **(Appendix A; Table A1)**: gait ($F=3.488$, $p=0.037$), ipsilateral forelimb grasp ($F=7.860$, $p=0.001$) and contralateral forelimb grasp ($F=15.768$, $p<0.000$). Post-hoc analysis revealed that HI/mild animals began moving off the circle significantly earlier than HI/severe animals ($p=0.032$) and began grasping with their ipsilateral ($p=0.001$) and contralateral ($p<0.001$) forelimbs significantly earlier as well. Univariate analysis revealed a significant effect of cluster * drug in two neurological signs **(Appendix A; Table A2)**: ipsilateral hindlimb grasp ($F=4.627$, $p=0.036$) and auditory startle ($F=4.403$, $p=0.040$). Post-hoc analysis showed that HI/mild animals receiving vehicle treatment began grasping with their hindlimb significantly earlier than HI/severe animals receiving vehicle ($p=0.012$). However, HI/severe animals receiving metformin began grasping significantly earlier than their vehicle counterparts ($p=0.009$). A similar trend was seen in contralateral hindlimb grasp, however it was not statistically significant ($F=3.843$, $p=0.055$). Additionally, post-hoc analysis found that metformin delayed the appearance of auditory startle in HI/mild animals ($p=0.044$) compared to those receiving vehicle treatment.

3.4 Early adhesive strip removal task impairments in HI animals were worsened by metformin treatment

Time to Contact

Repeated measures ANOVA showed a significant effect of cluster on time to contact the tape on the *ipsilateral* forelimb (F=4.407, p=0.017). Post-hoc analysis revealed that HI/severe animals took significantly longer, on average, to contact the tape compared to sham animals **(**p=0.014; **Figure 8A)**. Repeated measures ANOVA found a significant day * cluster * sex interaction (F=2.927, p=0.027), where HI/severe females took significantly longer to contact the tape on PND 19 compared to their sham (p<0.001) and HI/mild counterparts (p<0.001; **Figure 8B)**. There were no significant differences between the male clusters **(Figure 8C)**. Repeated measures ANOVA showed a significant effect of cluster on time to contact the tape on the *contralateral* forelimb (F=5.455, p=0.007). Again, HI/severe animals took significantly longer to contact the tape, on average, compared to sham (F=6.608, p=0.002) and HI/mild animals **(**F=6.808, p=0.020; **Figure 8D)**. Although there were no effects of sex, female HI/severe animals appeared to take longer to contact the tape compared to their sham and HI/mild counterparts at PND 19 **(Figure 8E)**. Male animals performed similarly across the three days of testing, although there was a trend towards HI/severe males taking longer **(Figure 8F)**. The average time to contact for both forelimbs is shown in **Figures 8G-I**. There were no significant effects of metformin on time to contact tape. Overall, HI/severe animals had significant sensory impairments and this was most apparent in females.

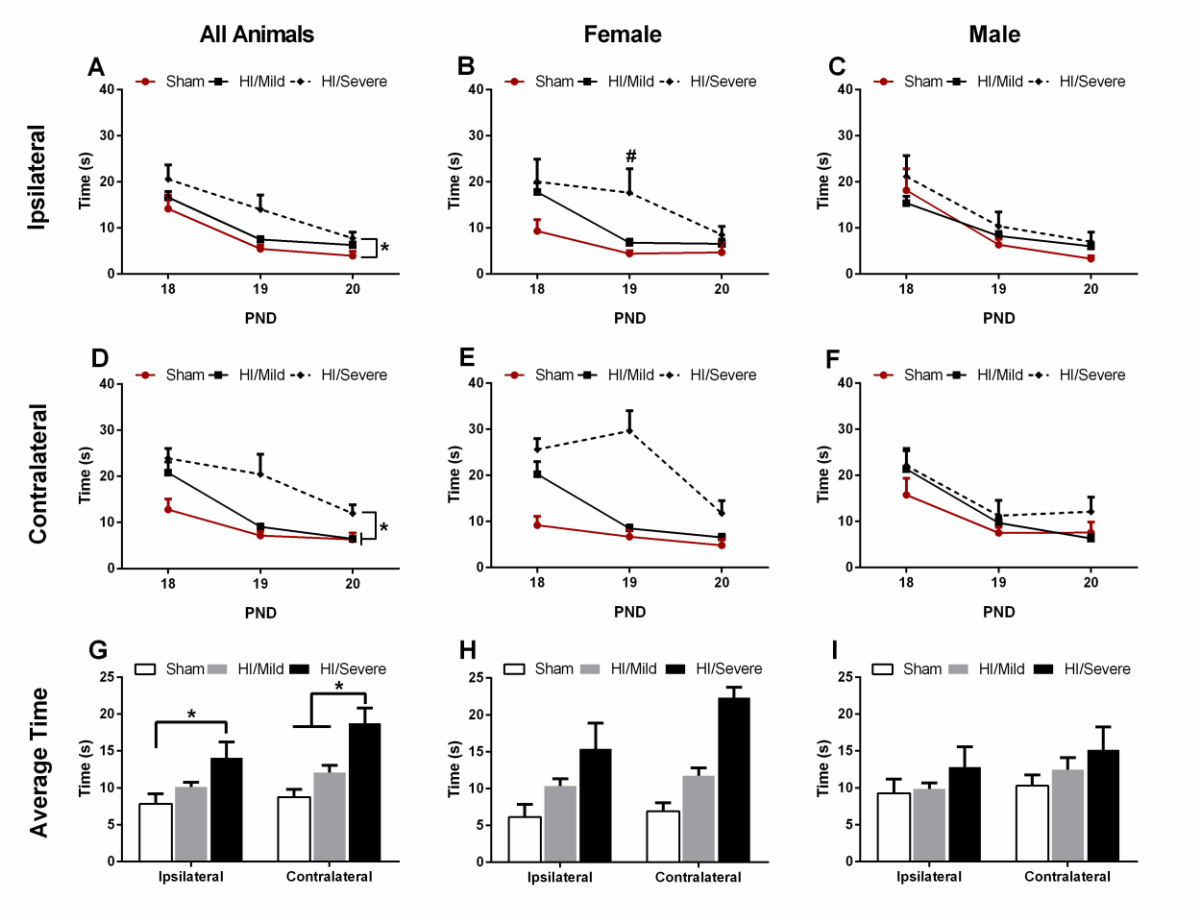

Figure 8. Average time to contact tape in the adhesive strip removal task (pre-weaning). HI/severe animals took significantly longer to contact the tape on their ipsilateral forelimb compared to shams **(A)**; this was seen in females on PND 19 **(B)**, but not in males **(C)**. HI/severe animals took significantly longer to contact the tape on their contralateral forelimb compared to both shams and HI/mild animals **(D)**; a similar trend was seen in females **(E)**, whereas males performed similarly regardless of cluster **(F)**. Average time taken to contact the tape across the three days of testing is shown in **G-I**. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild n= 24; Severe n= 4) (Male: Sham n= 6; Mild n= 22; Severe n= 4).

Time to Remove

Repeated measures ANOVA showed a significant effect of cluster on time to remove the tape from the *ipsilateral* forelimb ($F=7.693$, $p=0.001$). Post-hoc analysis revealed that HI/mild animals took significantly longer, on average, to remove the tape compared to sham animals **(**$p<0.001$; **Figure 9A)**. Although there were no significant effects of sex, there was a trend toward HI/mild and HI/severe females taking longer to remove the tape compared to shams **(Figure 9B)**, while only HI/mild males appeared to take longer **(Figure 9C)**. Repeated measures ANOVA showed a significant effect of cluster on time to remove tape from the *contralateral* forelimb ($F=3.783$, $p= 0.029$). Post-hoc analysis revealed that HI/mild and HI/severe animals **(**$p<0.001$, $p=0.016$, respectively) took significantly longer to remove the tape compared to sham animals **(Figure 9D)**. Although not significant, female HI/mild and HI/severe animals appeared to take longer to remove the tape **(Figure 9E)**. However, in males, only HI/mild animals appeared to take longer compared to their counterparts **(Figure 9F)**. The average time to remove for both forelimbs is shown in **Figures 9G-I**. Repeated measures ANOVA revealed a significant drug effect ($F=8.256$, $p=0.006$) on time to remove the tape, therefore the data was collapsed to assess these effects. HI animals receiving vehicle treatment, regardless of cluster or sex, took significantly less time to remove the tape from their ipsilateral **(**$p=0.006$; **Figure 10A)** and contralateral forelimb **(**$p=0.021$; **Figure 10B)** compared to those receiving metformin. The average time to remove the tape from both forelimbs is shown in **Figure 10C**. HI animals, regardless of cluster, displayed motor impairments in the adhesive strip removal task, which were worsened by metformin treatment. In females, there was a trend toward both HI clusters performing worse than shams, whereas only HI/mild males appeared impaired.

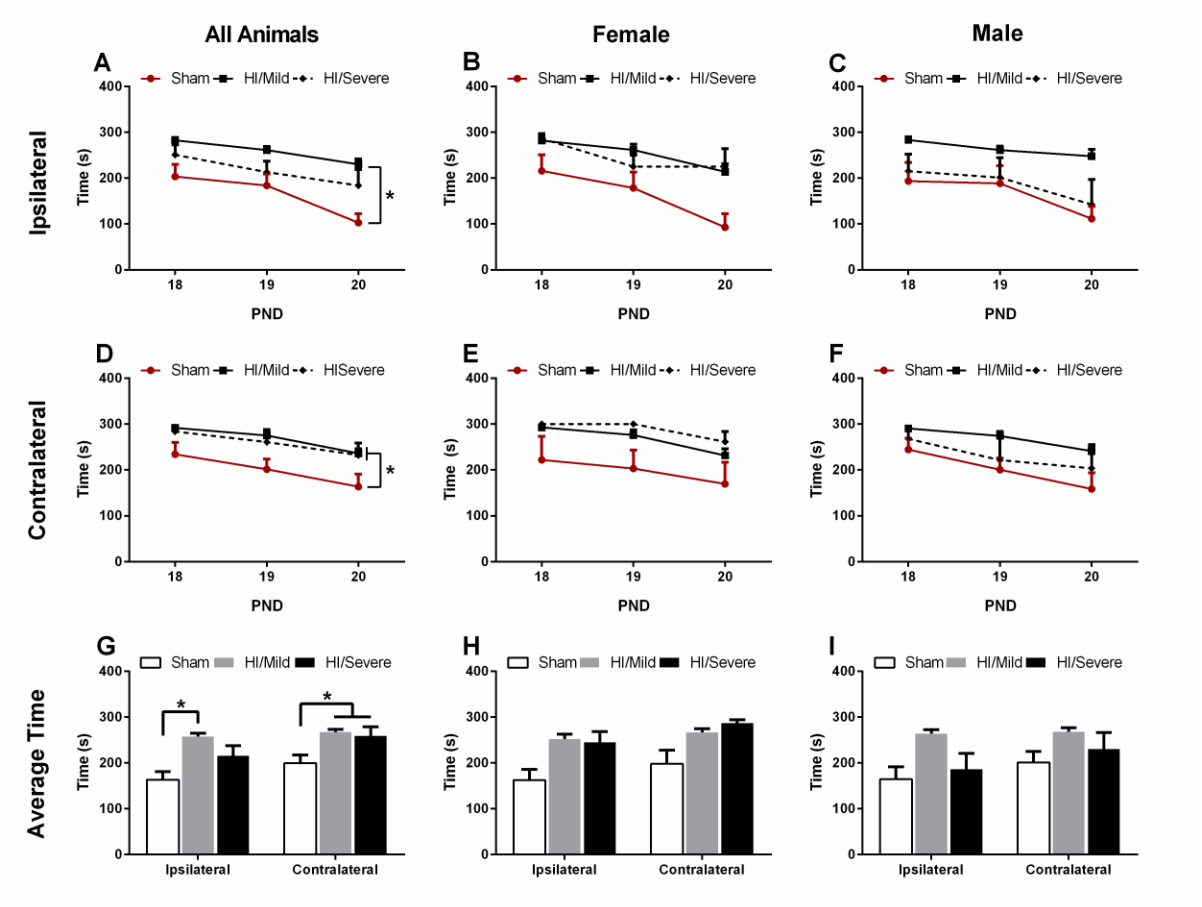

Figure 9. Average time to remove tape in the adhesive strip removal task (pre-weaning). HI/mild animals took significantly longer to remove the tape from their ipsilateral forelimb compared to shams **(A)**. HI/mild and HI/severe females took more time to remove the tape compared to shams **(B)**, whereas only HI/mild males appeared to take longer **(C)**. HI/mild and HI/severe animals took significantly longer to remove the tape from their contralateral forelimb **(D)**. Again, female HI/mild and HI/severe animals took longer compared to shams **(E)**, while only HI/mild males appeared to take longer **(F)**. Average time taken to remove the tape across the three days of testing is shown in **G-I**. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild n= 24; Severe n= 4) (Male: Sham n= 6; Mild n= 22; Severe n= 4).

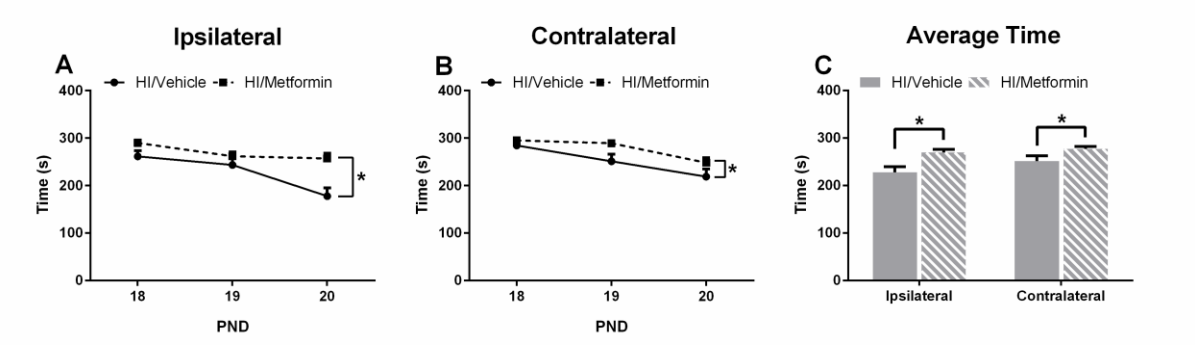

Figure 10. Effect of metformin on average time to remove tape in the adhesive strip removal task (pre-weaning). HI animals receiving vehicle took significantly less time to remove the tape from their ipsilateral **(A)** and contralateral **(B)** forelimbs compared to those that received metformin. Average time taken to remove the tape across the three days of testing is shown in **C**. Values are estimated marginal means ± SEM. (HI/Vehicle n= 23; HI/Metformin n= 31).

3.5 No significant effects of HI or metformin on activity in the open field

Univariate analysis found no significant differences between the groups in the open field. There was a trend toward HI/severe females exhibiting reduced activity levels compared to their sham and HI/mild counterparts. HI/mild and HI/severe males appeared to be less active than shams **(Appendix A; Figure A1)**.

3.6 No significant effects of HI or metformin on performance in the ladder walking test

Univariate analysis showed no significant differences between groups in either forelimb or hindlimb errors **(Appendix A; Figure A2)**.

3.7 Rehab, but not metformin, lessened chronic sensorimotor impairments caused by HI in the adhesive strip removal task

Time to Contact

When sexes were combined, there were no significant differences between clusters **(Figure 11A, D, G)**. Univariate analysis showed no significant effects of cluster or sex on time to contact the tape on the *ipsilateral* forelimb. Univariate analysis found a significant effect of cluster * housing * sex on time to contact the tape on the *contralateral* forelimb ($F=4.780$, $p=0.034$). Post-hoc analysis showed that HI/severe females not receiving rehab took significantly longer to contact the tape compared to their sham counterparts ($p=0.009$); there was also a trend toward HI/mild females taking longer compared to shams **(Figure 11B)**. This was not observed in male animals **(Figure 11C)**. Additionally, within HI/mild females, those receiving rehab took significantly less time to contact the tape on their contralateral forelimb compared to those not receiving rehab ($p=0.014$; **Figure 11E**), a trend not observed in males **(Figure 11F)**. Due to the small group size for the female HI/severe rehab group, no conclusions could be made **(Figure**

11H). No significant differences were found within HI/severe male animals **(Figure 11I)**. Univariate analysis showed a significant main effect of housing on time to contact the tape on the ipsilateral forelimb ($F=5.708$, $p=0.022$). Data was split by surgery to assess the effects of housing within HI animals. Overall, animals receiving rehab took significantly less time to contact the tape on their ipsilateral forelimb ($p=0.021$; **Figure 12**). Regardless of sex or cluster, rehab reduced the amount of time it took to contact the tape on the ipsilateral forelimb, while only significantly improving contralateral forelimb performance in HI/mild females.

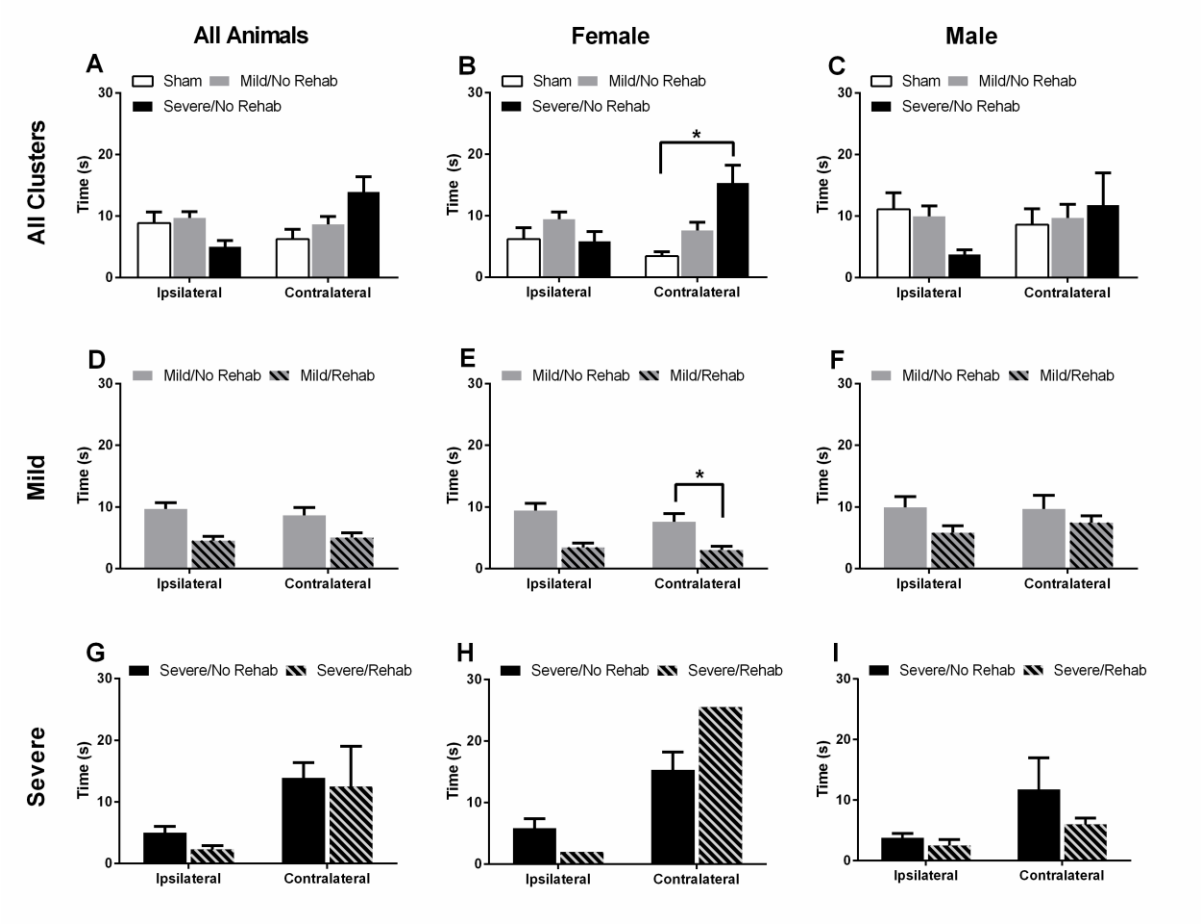

Figure 11. Average time to contact tape in the adhesive strip removal task (post-weaning). No significant effects in time to contact the tape on the ipsilateral or contralateral forelimb when all animals were analyzed together **(A)**. Female HI/severe animals not receiving rehab took significantly longer to contact the tape on their contralateral forelimb compared to shams **(B)**; not observed in males **(C)**. When sexes were combined, no significant effect of housing was found within HI/mild animals **(D)**. Female HI/mild animals receiving rehab took significantly less time to contact the tape on their contralateral forelimb compared to those not receiving rehab **(E)**, this effect was not as pronounced in HI/mild rehab males **(F)**. No significant differences within HI/severe animals **(G-I)**. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild/No Rehab n= 11; Mild/Rehab n= 13; Severe/No Rehab n= 3; Severe/Rehab n= 1) (Male: Sham n= 6; Mild/No Rehab n= 11; Mild/Rehab n= 11; Severe/No Rehab n= 2; Severe/Rehab n= 2).

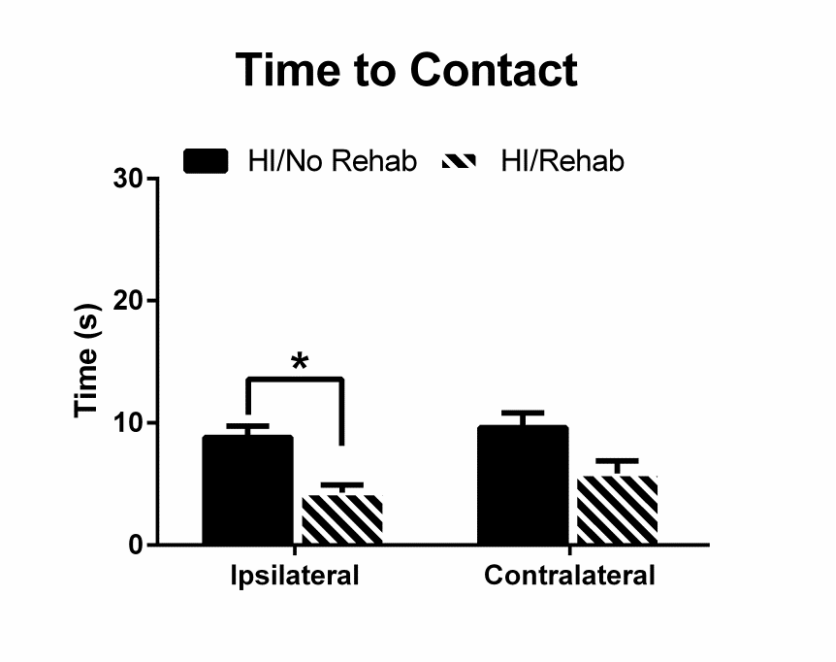

Figure 12. Effect of rehab on average time to contact tape in the adhesive strip removal task (post-weaning). Regardless of cluster or sex, HI animals receiving rehab took significantly less time to contact the tape on their ipsilateral forelimb. Values are estimated marginal means ± SEM. (HI/No Rehab n= 27; HI/Rehab n= 27).

Time to Remove

Univariate analysis showed a significant cluster * housing effect on time to remove the tape from the *ipsilateral* forelimb (F=4.129, p=0.048). Post-hoc analysis revealed that HI/mild animals not receiving rehab took significantly longer to remove the tape compared to shams (p=0.038; **Figure 13A**); a similar trend was observed in both females **(Figure 13B)** and males **(Figure 13C)**. No significant differences were found in time to remove the tape from the *contralateral* forelimb; however, there was a trend toward both HI/mild and HI/severe females **(Figure 13B)** and HI/mild males **(Figure 13C)** taking longer to remove the tape compared to their sham counterparts. Within HI/mild animals, those receiving rehab took significantly less time to remove the tape from their ipsilateral forelimb compared to those that did not receive rehab (p=0.001), a similar trend was observed for the contralateral forelimb **(Figure 13D)**. Similar trends were observed in females **(Figure 13E)** and males **(Figure 13F)**. No significant differences were found within HI/severe animals **(Figure 13G-I)**.

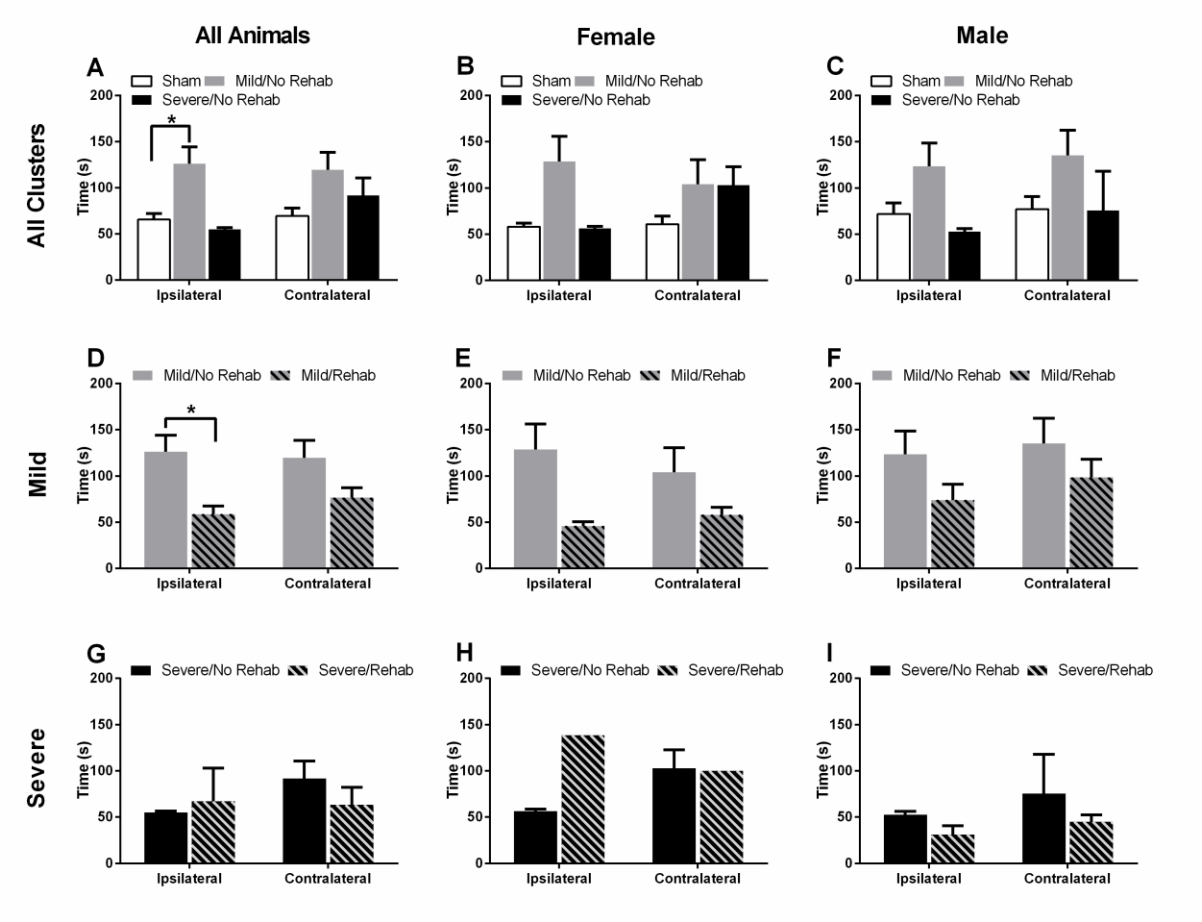

Figure 13. Average time to remove tape in the adhesive strip removal task (post-weaning). Mild/no rehab animals took significantly longer to remove the tape from their ipsilateral forelimb compared to sham animals; a similar trend was observed in the contralateral forelimb **(A)**. Although not significant, a similar trend was seen in females **(B)** and males **(C)**. Within the HI/mild cluster, rehab animals took significantly less time to remove the tape from their ipsilateral forelimb compared to no rehab animals; a similar trend was seen in the contralateral forelimb **(D)**; observed in females **(E)** and males **(F)**. No significant differences within HI/severe animals **(G-I)**. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild/No Rehab n= 11; Mild/Rehab n= 13; Severe/No Rehab n= 3; Severe/Rehab n= 1) (Male: Sham n= 6; Mild/No Rehab n= 11; Mild/Rehab n= 11; Severe/No Rehab n= 2; Severe/Rehab n= 2).

3.8 Rehab enhanced hindlimb performance in the ladder walking test

No significant differences were detected in the number of forelimb foot faults made between HI animals and shams **(Appendix A; Figure A3)**, nor did metformin or rehab affect performance. Similarly, no hindlimb impairments were detected in this task **(Appendix A; Figure A4)**. However, univariate analysis showed a significant cluster * housing effect in the number of foot faults made per step with the *ipsilateral* hindlimb ($F=6.030$, $p=0.018$). Post-hoc analysis revealed that within HI/mild ($p=0.013$) and HI/severe clusters ($p=0.005$), animals receiving rehab made significantly fewer foot faults compared to animals that did not receive rehab **(Appendix A; Figure A4)**. However, this effect in HI/mild animals appeared true only for females. Univariate analysis showed a significant housing effect on the number of foot faults made with the *contralateral* hindlimb ($F=8.744$; $p=0.005$). HI animals receiving rehab made significantly fewer errors with their contralateral hindlimb, regardless of sex, compared to those not receiving rehab ($p=0.003$; **Appendix A; Figure A5**).

3.9 Rehab, but not metformin, increased motor activity in the open field

Univariate analysis found no significant effects of surgery on performance in the open field **(Appendix A; Figure A6)**, however, there was a significant effect of housing on locomotor speed ($F= 15.178$, $p<0.001$) and distance ($F=15.177$, $p<0.001$). Data was split by surgery to assess the effects of housing within HI animals. Post-hoc analysis revealed that HI animals receiving rehab travelled more rapidly ($p<0.001$, **Figure 14A**) and for greater distances ($p<0.001$; **Figure 14B**) within the first minute of open field compared to HI animals that did not receive rehab.

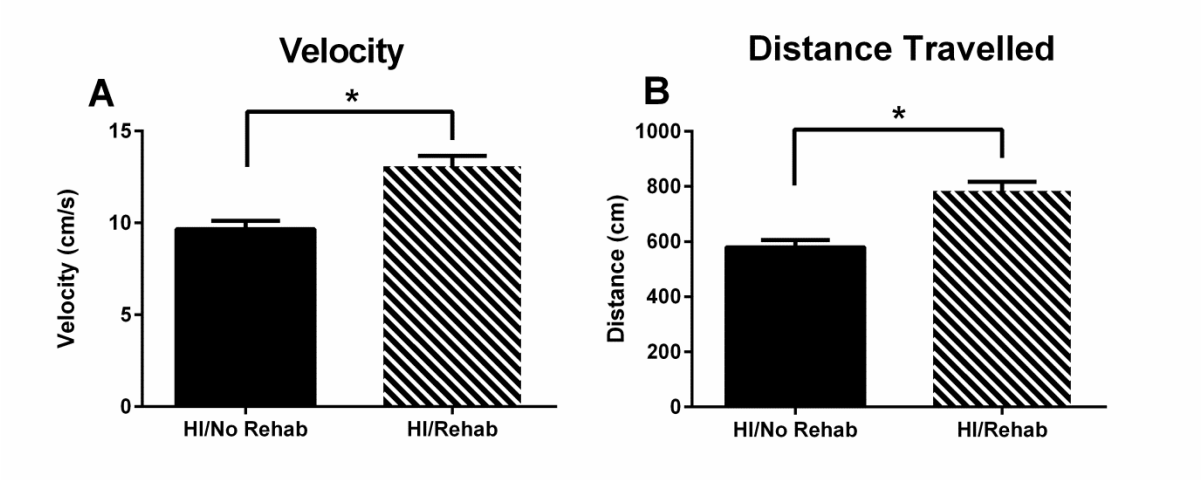

Figure 14. Effect of rehab on motor activity in the open field (post-weaning). HI animals receiving rehab travelled at greater velocities **(A)** and distances **(B)** in the 1st minute compared to those that did not receive rehab. Values are estimated marginal means ± SEM. (HI/No Rehab n= 27; HI/Rehab n= 27).

3.10 Rehab, but not metformin, improved motor learning in the Montoya staircase test

Repeated measures ANOVA found no significant effects of cluster or sex in the number of pellets retrieved with the *ipsilateral* forelimb **(Figure 15A)**. Both female **(Figure 15B)** and male **(Figure 15C)** clusters showed similar learning curves. Repeated measures ANOVA showed a significant effect of cluster on the average number of pellets retrieved across the 14 days with the *contralateral* forelimb ($F=13.231$, $p<0.001$). Post-hoc analysis revealed that HI/severe animals reached significantly fewer pellets, on average, compared to both sham ($p<0.001$) and HI/mild animals ($p<0.001$; **Figure 15D**). Similar trends can be seen within female **(Figure 15E)** and male clusters **(Figure 15F)**, although more pronounced in female animals. The average number of pellets retrieved across the 14 days of testing is shown in **Figures 15G-I**.

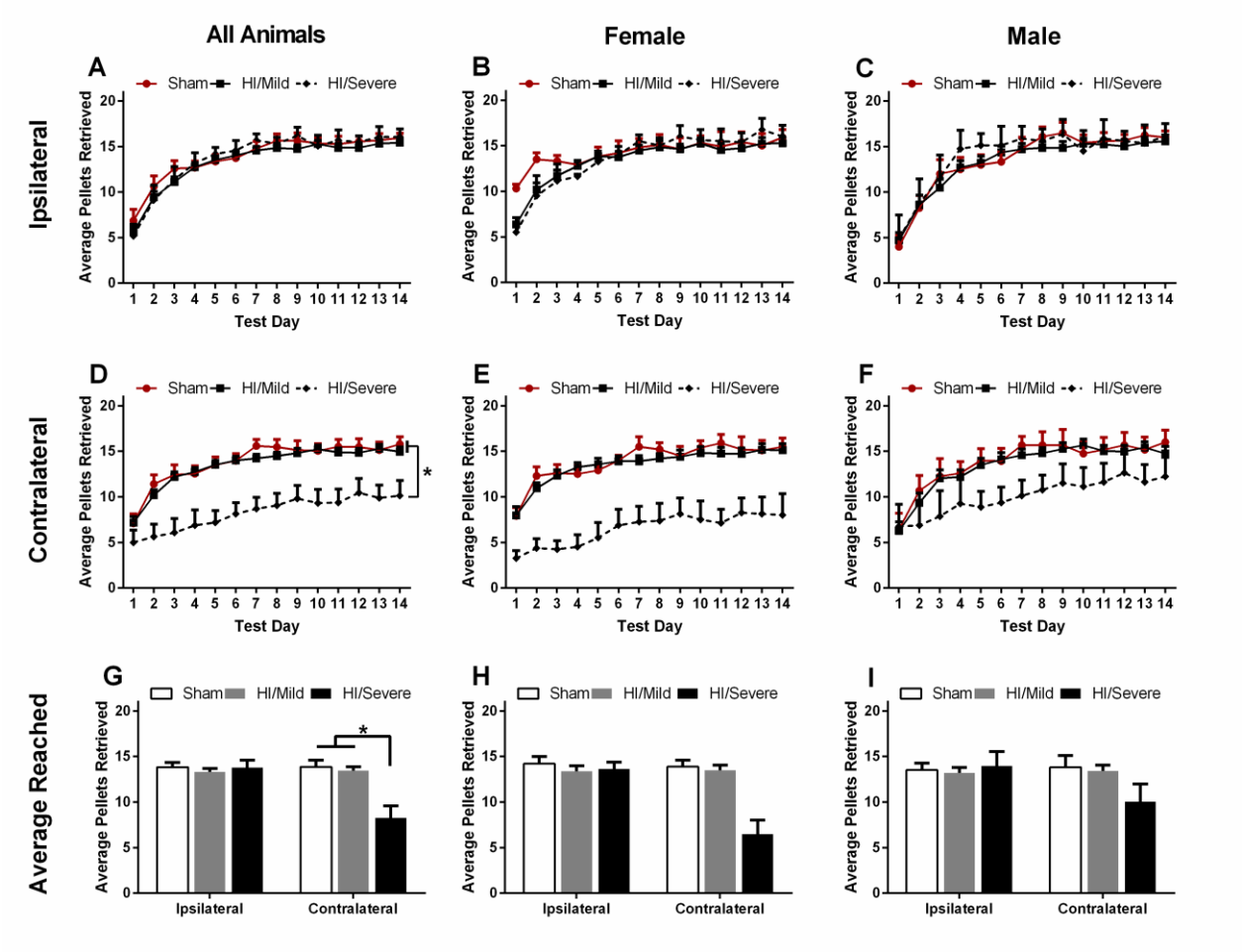

Figure 15. Average number of pellets retrieved in the Montoya staircase test (post-weaning). All clusters displayed similar learning curves with their ipsilateral forelimb **(A)**; this trend was seen in both females **(B)** and males **(C)**. HI/severe animals reached significantly fewer pellets compared to sham and HI/mild animals **(D)**, observed in both females **(E)** and males **(F)**. Average number of pellets retrieved across the 14 days of testing is shown in **G-I**. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild n= 24; Severe n= 4) (Male: Sham n= 6; Mild n= 22; Severe n= 4).

Repeated measures ANOVA showed a significant effect of day * housing on *ipsilateral* forelimb performance (F=4.338, p=0.002). Data was split by surgery to assess the effects of housing within HI animals. Post-hoc analysis revealed that HI animals receiving rehab reached significantly more pellets in the first 3 days of staircase with their ipsilateral forelimb compared to those not receiving rehab **(Figure 16A)**. Repeated measures ANOVA showed a significant overall housing effect on the number of pellets retrieved with the *contralateral* forelimb (F=5.841, p=0.020). Post-hoc analysis showed that HI animals receiving rehab, regardless of cluster, reached significantly more pellets with their contralateral forelimb compared to those not receiving rehab **(Figure 16B)**. Average performance from the 14 days is shown in **Figure 16C**. Repeated measures ANOVA showed a significant overall drug effect on *ipsilateral* forelimb performance (F=8.049, p=0.007). Post-hoc analysis showed that HI animals receiving metformin, regardless of cluster, reached significantly fewer pellets with their ipsilateral forelimb compared to those receiving vehicle treatment **(Figure 16D)**. Although not significant, a similar trend was observed in *contralateral* forelimb reaching performance **(Figure 16E)**. Average number of pellets retrieved across the 14 days is shown in **Figure 16F**. To further investigate the effects of rehab on performance, the number of successful reaches during reach training was correlated to total pellets retrieved. In HI animals, there was a significant positive correlation between the number of successful rehab reaches performed during rehabilitation and the total number of pellets retrieved with their contralateral forelimb (p=0.021, R^2=0.167; **Figure 17**).

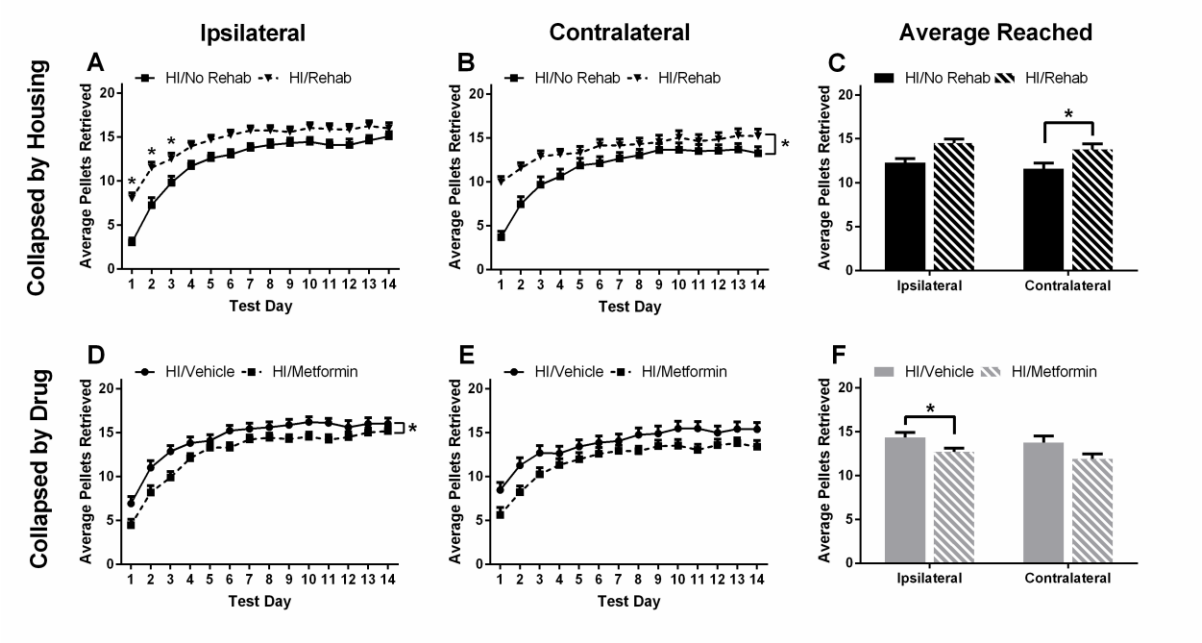

Figure 16. Effect of rehab and metformin on reaching performance in the Montoya staircase test (post-weaning). HI animals receiving rehab reached significantly more pellets in the first three days of testing with their ipsilateral forelimb compared to those that did not receive rehab **(A)**. HI/rehab animals also reached significantly more pellets, on average, with their contralateral forelimb compared to those not receiving rehab **(B, C)**. HI animals receiving metformin reached significantly fewer pellets with their ipsilateral forelimb **(D, F)**, and displayed a similar trend with their contralateral forelimb **(E, F)**. Values are estimated marginal means ± SEM. (HI/No Rehab n= 27; HI/Rehab n= 27; HI/Vehicle n= 23; HI/Metformin n= 31).

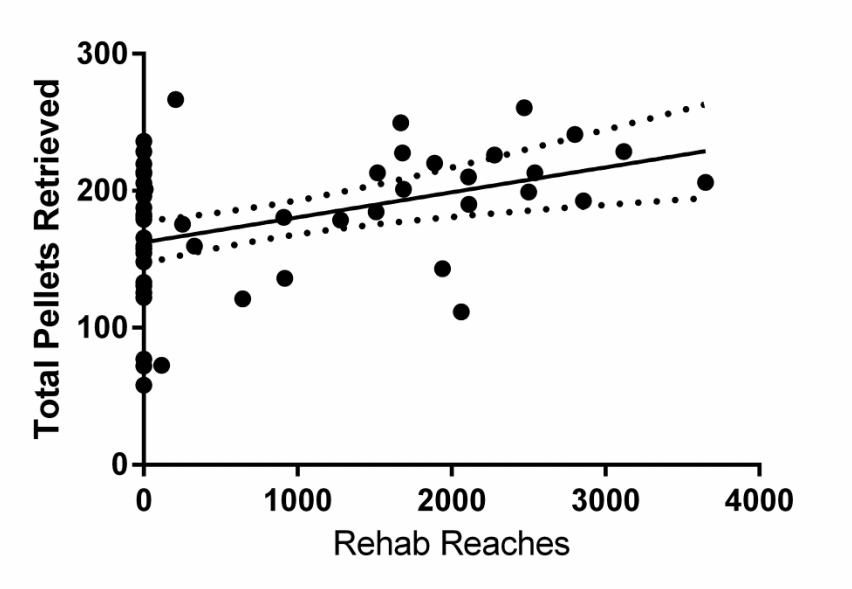

Figure 17. Correlation between RT participation and total number of pellets retrieved in the Montoya staircase test (post-weaning). Within HI animals, there was a significant positive correlation between the number of successful reaches during rehab and the total number of pellets reached with their contralateral forelimb. (HI n= 54).

3.11 Rehab, but not metformin, improved memory retention in the Barnes maze probe

Repeated measures ANOVA found no significant differences in the time it took animals to locate the goal box during the acquisition phase **(Figure 18A)**; this was true for both female **(Figure 18B)** and male animals **(Figure 18C)**. Univariate analysis showed a significant effect of cluster * sex on the latency to first visit where the goal box had been located during the 24-hour probe **(**F=3.424, p=0.041, **Figure 18D)**. Post-hoc analysis could not determine which groups were different; however, it appears that female HI/severe animals took longer to visit the goal box location compared to their sham and HI/mild counterparts **(Figure 18E)**; this was not observed in males **(Figure 18F)**. Univariate analysis showed a significant cluster effect on the time spent in the target quadrant during the 24-hour probe (F=6.334, p=0.004). Post-hoc analysis showed that HI/severe animals spent significantly less time in the target quadrant compared to shams (p=0.005; **Figure 18G)**. A similar trend was observed in both female **(Figure 18H)** and male HI/severe animals **(Figure 18I)**.

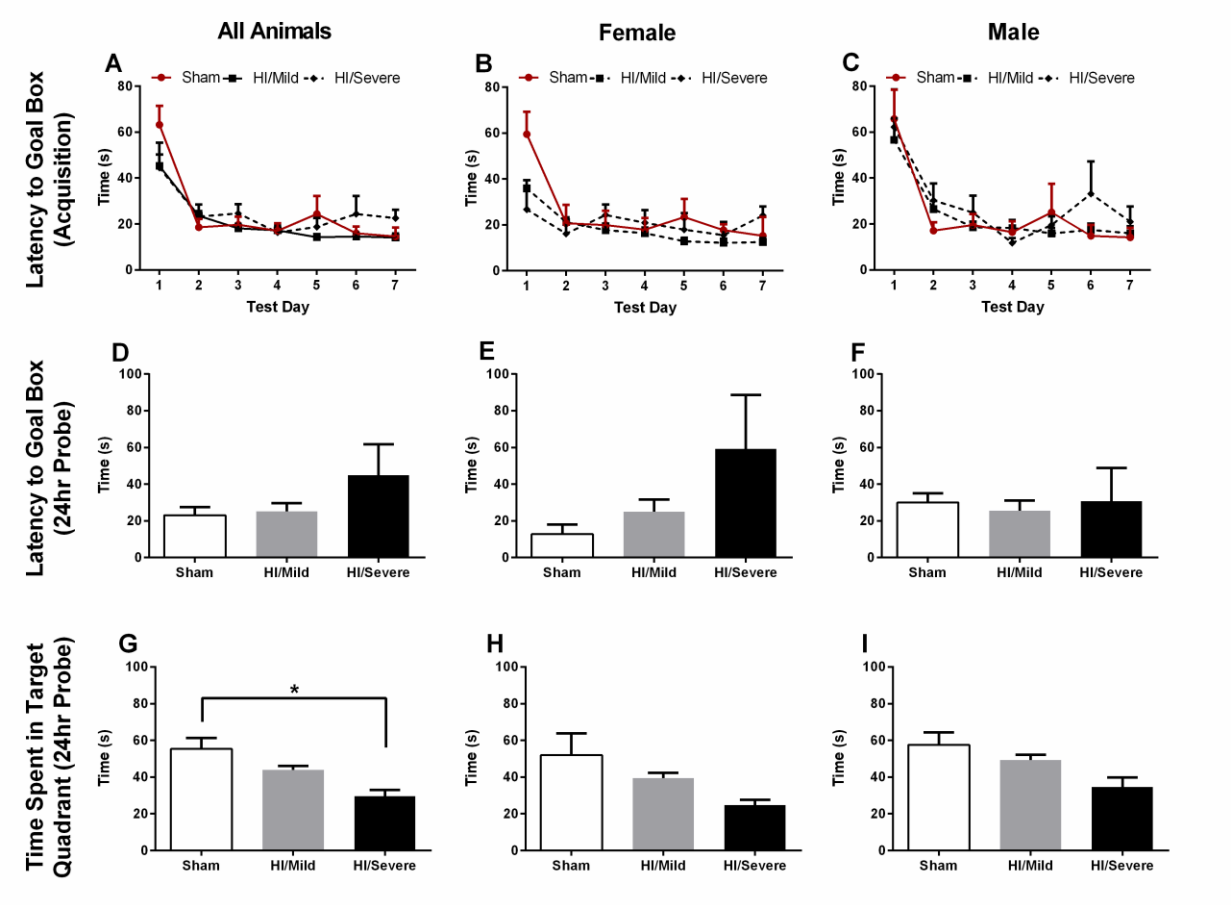

Figure 18. Spatial learning and memory in the Barnes maze (post-weaning). There were no significant differences in the learning curves during the acquisition phase **(A)**; a similar trend was observed in females **(B)** and males **(C)**. In the 24-hour probe, HI/severe animals appeared to take longer to first visit where the goal box was **(D)**; a trend seen in females **(E)** but not males **(F)**. HI/severe animals spent significantly less time in the target quadrant during the probe compared to shams **(G)**; a similar trend was observed in females **(H)** and males **(I)**. Values are estimated marginal means ± SEM. (Female: Sham n= 4; HI/Mild n= 24; HI/Severe n= 4) (Male: Sham n= 6; HI/Mild n= 20; HI/Severe n= 4).

There were no significant differences in latency to locate the goal box during the acquisition phase between HI/no rehab and HI/rehab animals when the data for the sexes was combined **(Figure 19A)**, females alone **(Figure 19B)** or males alone **(Figure 19C)**. However, univariate analysis showed a significant effect of housing on the latency to first visit where the goal box had been located during the probe ($F=6.952$, $p=0.012$). Data was split by surgery to assess the effects of housing within HI animals. Post-hoc analysis showed that HI animals receiving rehab, regardless of cluster or sex, took significantly less time to first visit the goal box compared to HI animals that did not receive rehab ($p=0.002$; **Figure 19D**). A similar trend was observed in both females **(Figure 19E)** and males **(Figure 19F)**. There were no significant differences in the amount of time spent in the target quadrant **(Figure 19G-I)**.

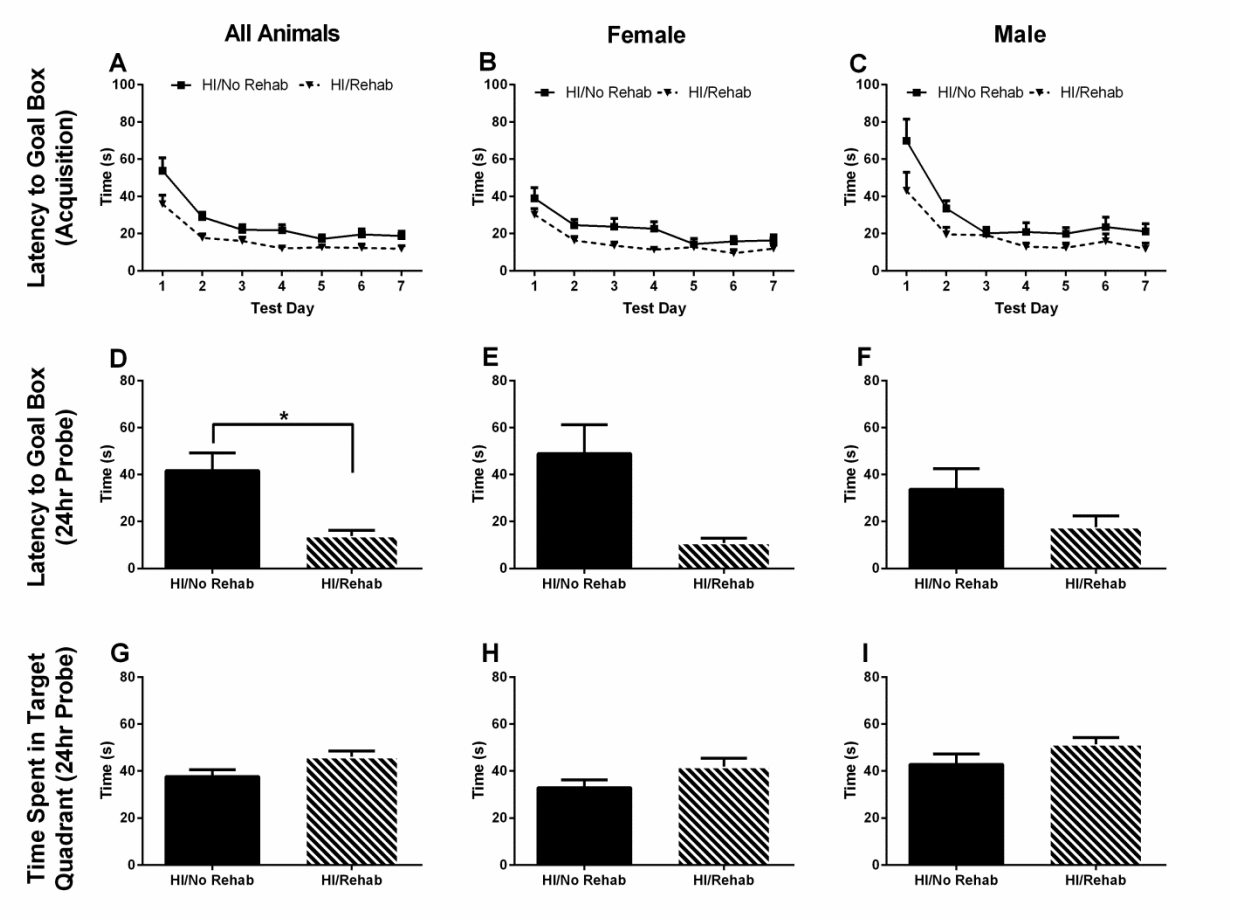

Figure 19. Effect of rehab on spatial learning and memory in the Barnes maze (post-weaning). There were no significant differences in latency to the goal box during the acquisition phase between HI/no rehab and HI/rehab animals **(A)**; similar trend was seen in females **(B)** and males **(C)**. In the 24-hour probe, HI/rehab animals took significantly less time to visit where the goal box had been compared to HI/no rehab animals **(D)**, a similar trend was seen in both females **(E)** and males **(F)**. No significant differences were found in time spent in the target quadrant **(G-I)**. Values are estimated marginal means ± SEM. (Female: HI/No Rehab n= 14; HI/Rehab n= 14) (Male: HI/No Rehab n= 13; HI/Rehab n= 11).

3.12 Rehab, but not metformin, enhanced spatial learning and memory in the Morris water maze

No significant differences were found in any of the parameters measured during the acquisition phase **(Appendix A; Figure A7)** or probes **(Appendix A; Figure A8)** between shams and HI/mild animals not receiving rehab. Repeated measures ANOVA revealed a significant housing effect ($F=5.557$; $p=0.028$). Data was split by surgery to assess the effects of housing within HI animals **(Appendix A; Figure A9**). Post-hoc analysis showed that HI animals receiving rehab took significantly less time, on average, to locate the platform during the acquisition phase ($p=0.017$). Although not significant, it appears that the reduction in time to locate the platform was primarily due to a reduced path length, rather than differences in velocity. No significant effects of rehab were found during the 24 or 72-hour probe following acquisition **(Appendix A; Figure A10)**. Additionally, no significant effects were found during reversal learning or the 24-hour reversal probe **(Appendix B; Table B10)**.

DISCUSSION

Using the Rice-Vannucci PND 7 model of hypoxia-ischemia in the rat, this study assessed the feasibility of using enriched rehabilitation and metformin to promote normal development and improve motor and cognitive function following injury. We found sex affected the severity of damage following HI, which often translated into worse functional outcome. Despite promising effects early on, metformin failed to show long-lasting benefits, whereas enriched rehabilitation enhanced several aspects of both motor and cognitive function, regardless of sex. Our findings suggest a combinational treatment for HI using metformin is no more beneficial than ER alone.

4.1 Injury Profile and Outcome Following Hypoxia-Ischemia

Histological analysis of the brains revealed damage ranging from mild to severe within the hemisphere ipsilateral to the artery occlusion, consistent with previous reports (Rice et al., 1981). We found that the majority of HI animals in this study presented with mild damage, as indicated by little to no change in the volume of ipsilateral brain regions. Interestingly, although these animals appeared largely unaffected, patchy neuronal necrosis was observed within the ipsilateral cortex of some animals, similar to what others have reported (Schuch et al., 2016*b*; Towfighi et al., 1991). The remainder of HI animals, approximately 15%, had severe infarctions, whereby ipsilateral brain regions were significantly atrophied compared to the contralateral side. This was an important distinction to make as treating all HI animals as a homogenous group would have potentially masked impairments on behavioural tasks. Interestingly, within severely injured animals, females had significantly less tissue remaining compared males. Clinical reports indicate that males are more susceptible to HI and often exhibit worse outcome compared to

females (Donders & Hoffman, 2002; Hill & Fitch, 2012). However, the literature regarding sex differences with respect to brain damage is inconsistent. Animal studies have reported no significant differences between females and males (Ashwal et al., 2014; Smith et al., 2014), that males are more vulnerable to cortical and hippocampal tissue loss (Mayoral et al., 2009), or that females have greater hemispheric tissue reductions following HI (Sanches et al., 2013*a*; Sanches et al., 2013*b*). It is difficult to reconcile these results as differences in methodology (e.g. animal model/strain, age at HI injury, duration of hypoxia) could affect histological outcome (Johnston, 1983; Semple et al., 2013; Towfighi et al., 1991). Our results indicate that female brains may be more sensitive to HI compared to males. Interestingly, the severity of brain damage often resulted in worse outcome on several measures including weight gain post-injury, a commonly used measure of well-being. We found that HI animals gained weight at a slower rate compared to shams, similar to previous reports (Carty et al., 2008; Lubics et al., 2005; Yao et al., 2016), and that severely injured animals were most affected. Additionally, several neurological signs were slower to emerge in animals with severe damage compared to mild. In accordance with previous findings (Ten et al., 2003), these developmental milestones proved to be good predictors of later outcome as chronic impairments were detected in a number of behavioural tests. Interestingly, although both female and male animals in the HI/severe group had significantly less tissue remaining compared to their sham and HI/mild counterparts, only females displayed significant bilateral sensorimotor impairments in the adhesive strip removal task. There was a trend toward males in the severely injured group performing worse, however, it was not significant. Considering males had significantly more hemispheric and cortical tissue spared compared to females following HI, it is possible that this was enough to minimize the impairments on the task through compensatory mechanisms. It is important to note that bilateral

impairments are not typically expected in models of unilateral injury such as hypoxia-ischemia. Although the administration of global hypoxia affects both hemispheres, the combination of artery ligation and hypoxia is necessary to produce damage, thus the contralateral hemisphere should not be injured (Rice et al., 1981). However, several animal studies using an adult MCAO model have previously reported bilateral motor impairments following unilateral damage (Grabowski et al., 1993; Sharkey et al., 1996; Virley et al., 2000). These findings suggest that communication between hemispheres may be critical for coordinated forelimb use. It is possible that in severely injured animals, where a large proportion of the hemisphere is missing, the corpus callosum has consequently been affected and the crosstalk between hemispheres has been disrupted. Interestingly, bilateral impairments were no longer evident 6 weeks post-injury when severely injured females only showed sensorimotor deficits with their contralateral forelimb, but not ipsilateral. Spontaneous recovery of sensorimotor function is not uncommon following unilateral injury (DeVries et al., 2001) and this may be the result of reorganization and remapping of circuitry within the uninjured hemisphere, allowing it to adopt the function of the injured hemisphere (Jung et al., 2016). Similarly, Montoya staircase testing 6 weeks following HI revealed that severely injured animals only had contralateral forelimb impairments, with a trend toward females performing worse. Additionally, severely injured females had reduced activity in the open field, which continued 6 weeks post-injury. We believe this further supports motor function impairments rather than anxiety, as differences in activity during the first-minute of open field, when the task is most novel, were not as pronounced. Together, these results demonstrate that the severity of brain injury, defined as the volume of ipsilateral tissue remaining, plays a role in determining motor outcome and function following HI. However, volumetric analysis may not always reliably predict whether an animal will be impaired or not.

We found that mildly injured HI animals, regardless of sex, displayed chronic bilateral motor impairments in the adhesive strip removal task. Given the vulnerability of oligodendrocytes to hypoxia-ischemia, it is possible that reduced myelination of axon tracts compromised inter-hemispheric crosstalk (Back et al., 2007; Reimer et al., 2011; Wellmann et al., 2015) without overtly impacting the volumes of brain regions. However, further examination of white matter atrophy and myelination would be necessary to confirm this hypothesis.

Spatial learning and memory was assessed 9 weeks post-HI injury. We found no significant impairments in HI animals compared to shams during the acquisition phase of either Barnes maze or Morris water maze. These results were unexpected given that animals in the HI/severe group had extensive hippocampal atrophy, a region known to be important in learning and memory (McDonald & White, 1994; O'Brien et al., 2006). Additionally, Ten and colleagues (2004) demonstrated that the extent of cerebral damage in a mouse model of HI was highly correlated with performance in the spatial memory component of MWM (Ten et al., 2004). However, studies have demonstrated that neurons within the uninjured hippocampus have more complex dendritic branching following HI compared to the injured side (Zhao et al., 2013). Considering our cognitive tests were administered 9 weeks after injury, it is possible that there may have been sufficient reorganization within the contralateral hippocampus to allow normal learning. Another possible explanation for the lack of acquisition impairments observed in the Barnes maze is that the task may not have been aversive enough to motivate the animals to escape (Sunyer et al., 2007). Indeed we found that as trials went on, rats would choose to explore the maze rather than enter the escape box once they had located it, potentially skewing the acquisition data. Thus, we decided to add the MWM to our battery of tests during the second wave, as this test is considered more aversive. However, because none of the HI animals in this

wave had severe damage we could not fully explore learning and memory impairments for this cluster. Interestingly, during the 24-hour Barnes maze probe we found that females with severe damage took longer to visit where the goal box had been located. Moreover, severely injured HI animals, regardless of sex, spent significantly less time in the target quadrant during the probe. Thus, the severity of injury does impact memory retention, but is not necessarily sufficient to impair learning.

When assessing the efficacy of therapies, it is important to choose a clinically relevant model that exhibits similar features to the disorder in order to increase the translational potential of results (Northington, 2006). Many HI studies assess acute behavioural outcomes since chronic deficits can be difficult to detect (Jansen & Low, 1996). Here, we have identified a battery of behavioural tests sensitive to short- and long-term motor and cognitive deficits, which is important as HI survivors are often left with persistent disabilities. Together these findings demonstrate that the severity of injury impacts performance on a number of behavioural outcomes and these impairments are often chronic.

4.2 Enriched Rehabilitation

We found that enriched rehabilitation had no effect on brain volumes. Previous studies have shown that neither EE nor ER alter infarct size following HI (Pereira et al., 2007, 2008; Schuch et al., 2016*b*). Perhaps waiting until weaning (~PND 21) to administer rehab is too late to be neuroprotective since by that point injury has entered into the tertiary phase (Douglas-Escobar & Weiss, 2015; Hassell et al., 2015). Indeed, when others have administered EE the day after HI, a reduction in the amount of morphological damage caused by HI was observed (Schuch et al., 2016*a*). Nevertheless, we found that ER improved outcome and enhanced performance on a number of behavioural measures. Sensorimotor impairments observed in the adhesive strip

removal task were only improved in mildly injured animals that received rehab. Due to the small group sizes (n= 1-3) within severely injured animals and the variability of the data, we were unable to confidently assess the impact of rehab on performance within this subgroup. However, other behavioural measures such as open field indicated that rehab improved motor activity regardless of the severity of injury. These beneficial effects are likely the result of the sensory and motor stimulation provided by the EE cages (Will et al., 2004). Additionally, we found rehab significantly improved motor learning during the Montoya staircase task in HI animals, and this was positively correlated with the number of successful reaches made during the daily reach training component of ER. Again, rehab was able to improve performance regardless of the severity of injury as well as initial level of impairment. It is likely that the task-specificity of daily reach training and promoting the use of the affected forelimb was necessary to challenge severely injured HI animals and consequently led to the recruitment and reorganization of non-injured brain tissue, contributing towards improved function (Nudo et al., 1996). These results confirm previous work from our lab indicating that ER improves motor function following HI (Schuch et al., 2016*b*). We found that enriched rehabilitation not only improved motor performance but also enhanced learning and memory and reduced memory retention impairments, regardless of injury severity. Our results are in accordance with previous studies that have found improvements in cognitive function (Griva et al., 2017; Pereira et al., 2007, 2008). This could be explained by ER's ability to augment the brain's endogenous plasticity mechanisms (Livingston-Thomas et al., 2016). Increases in neurogenesis (Wurm et al., 2007), growth factors (Gobbo & O'Mara, 2004), and axonal sprouting/dendritic arborization (Biernaskie & Corbett, 2001; Rojas et al., 2013) have all been observed following rehabilitation in various animal stroke models. Most importantly, many of these changes have also been

observed within the uninjured hemisphere, which is particularly beneficial for HI survivors who have suffered extensive damage to one hemisphere.

Together, these results demonstrate that enriched rehabilitation administered 2 weeks post-HI injury was able to enhance both motor and cognitive function, and in most cases, regardless of sex or severity of brain damage. It is also important to note that administering metformin as a pre-treatment did not influence the effectiveness of enriched rehabilitation, as improvements in ER animals were seen regardless of drug treatment. This is promising for several reasons as treatments currently available to HI survivors must be administered within *hours* of initial insult and are not suitable for those who have experienced a severe hypoxic-ischemic insult (Azzopardi et al., 2009). We show that even late administration of ER had a positive impact on outcome following HI.

4.3 Metformin

We found that metformin exacerbated damage in severely injured females, whereas it appeared neuroprotective within the hippocampus of severely injured males. The effect of metformin on brain injury has been assessed in the context of various animal models of stroke. Despite findings that AMPK activation following stroke worsens damage (Li et al., 2007; McCullough et al., 2005), metformin, a known AMPK activator, has been shown to either decrease brain infarct sizes (Liu et al., 2014) or have no effect (Jin et al., 2014). We found that metformin was able to mitigate difficulties with early weight gain following HI, regardless of injury severity, and had a mild positive influence on the appearance of certain neurological signs in severely injured animals. Despite benefits for early development, metformin either had no effect or worsened motor function at later stages, as seen in the adhesive strip removal task and Montoya staircase. These results are in contrast to previous findings showing that metformin

rescues motor deficits following MCAO in adult animals (Jin et al., 2014; Liu et al., 2014; Venna et al., 2014). Moreover, Dadwal et al. (2015) showed that 7 days of metformin treatment, beginning one day following HI, was able to rescue sensorimotor deficits on the cylinder task two weeks post-injury (PND 22); evidenced by a reduction in the preference to use the uninjured paw for support. Brain injury was not assessed in this study and would've been an important factor to consider, as our data has shown that it can impact performance. Additionally, all of the aforementioned studies utilized mice. A previous study assessing the effects of minocycline therapy following HI found contrasting outcomes when the treatment was administered in mice versus rats (Tsuji et al., 2004). Therefore, it is possible that the beneficial effects of metformin may be dependent on the animal model used. In terms of cognitive function, we found no effects of metformin on performance despite evidence showing it enhanced spatial memory formation during the MWM in uninjured mice (Wang et al., 2012). Interestingly, the neuroprotection observed in male hippocampal tissue did not translate into better performance on cognitive tasks. Future experiments should assess specific regions within the hippocampus, such as the CA1, which is known to be vulnerable to HI and has been linked to spatial cognitive function in order further elucidate metformin's role in cognition.

There are several possible explanations as to why metformin didn't improve outcome but worsened it in some cases. Perhaps waiting two weeks to administer the full dose via injections may have been too late to be effective. Pharmacological studies have shown that for breastfeeding mothers who take metformin (1000-1500 mg/day) the estimated dose ingested by infants is 0.18- 0.28% of the maternal dose (adjusted for weight) (Gardiner et al., 2003; Hale et al., 2002). Thus, although metformin treatment began one day after injury and we observed early improvements compared to vehicle treated pups, it is likely that the dose reaching the pups was

less than 200mg/kg. Similar to the "critical period" that exists following stroke, during which the brain is highly plastic and most receptive to treatment (Murphy & Corbett, 2009), there is thought to be a "therapeutic window" following HI. This window occurs during the *latent phase* of injury and lasts anywhere between 1 to 6-24 hours following initial insult (Hassell et al., 2015). Thus, it is possible that we were not administering the necessary dose during this critical time to minimize or prevent damage from occurring during the *secondary phase*. While this could explain why there were no improvements in function, it does not explain why metformin worsened outcome. Animals were tested on the Montoya staircase test 2 days following the completion of metformin treatment. While speculative, these animals may have been experiencing withdrawal-like effects which could have inadvertently affected performance. However, this is unlikely since metformin treated HI animals also performed significantly worse during the pre-weaning testing period.

Although metformin has shown promise in promoting recovery in various adult animal models of stroke, its potential for use following HI is less convincing. It is possible that during the early stages of injury, when the immature brain is in a repair and reorganizational state, the addition of metformin may be negatively impacting endogenous repair processes. There is precedent for adverse effects of drugs on outcome in adult stroke models, where delivery of an inverse GABA agonist worsened outcome when given less than 3 days after cortical stroke (Clarkson et al., 2010). Future experiments are needed to determine how metformin affects the mechanisms and pathways implicated in recovery following HI in order to better understand the negative behavioural outcomes observed in this study.

CONCLUSION

Using a preclinical model of hypoxia-ischemia, we demonstrated that enriched rehabilitation, but not metformin, resulted in long-lasting improvements in motor and cognitive outcome following injury at PND 7. Additionally, our results highlighted the importance of considering factors such as sex and injury severity when assessing the efficacy of potential therapies. Gaining a better understanding of the mechanisms underlying these treatments may provide insight on how to better complement the actions of enriched rehabilitation and guide the design of future experiments. Identifying safe and clinically relevant therapies is crucial in order to improve the quality of life of survivors of hypoxia-ischemia.

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APPENDIX A

Table A1. Average day of appearance of physical and neurological signs (pre-weaning) (EMM ± SEM).

Neurological Sign	Day of Appearance		
	Sham (n=11)	**HI/Mild (n=39)**	**HI/Severe (n=8)**
Eye Opening (Ipsilateral)	13.62 ± 0.4	14.36 ± 0.2	14.63 ± 0.4
Eye Opening (Contralateral)	13.72 ± 0.3	13.47 ± 0.2	14.13 ± 0.4
Negative Geotaxis	14.90 ± 1.2	16.98 ± 0.6	14.25 ± 1.4
Forelimb Placing (Ipsilateral)	8.18 ± 0.1	8.19 ± 0.1	8.63 ± 0.2
Forelimb Placing (Contralateral)	8.88 ± 0.3	8.83 ± 0.1	8.88 ± 0.3
Gait	10.62 ± 0.5	10.25 ± 0.2^{b}	11.88 ± 0.6^{b}
Forelimb Grasp (Ipsilateral)	9.35 ± 0.3^{a}	8.46 ± 0.1^{a,b}	9.75 ± 0.3^{b}
Forelimb Grasp (Contralateral)	9.33 ± 0.3	8.47 ± 0.1^{b}	10.25 ± 0.3^{b}

a< 0.05 HI/Mild vs. Sham
b< 0.05 HI/Mild vs. HI/Severe

Table A2. Effect of metformin on average day of appearance of neurological signs (pre-weaning) (EMM ± SEM).

Neurological Sign	Day of Appearance				
	Sham (n=11)	**HI/Mild + Veh** (n=19)	**HI/Mild + Met** (n=27)	**HI/Severe + Veh** (n=4)	**HI/Severe + Met** (n=4)
Hindlimb Grasp (Ipsilateral)	13.82 ± 0.2	13.42 ± 0.2^{a}	13.25 ± 0.1	14.50 ± 0.3^{a,b}	13.25 ± 0.3^{b}
Hindlimb Grasp (Contralateral)	14.00 ± 0.3	13.73 ± 0.2	13.39 ± 0.2	15.25 ± 0.5	13.50 ± 0.5
Auditory Startle	12.57 ± 0.3	12.95 ± 0.2^{c}	13.58 ± 0.2^{c}	12.25 ± 0.5	11.25 ± 0.5

a< 0.05 HI/Mild + Veh vs. HI/Severe +Veh
b< 0.05 HI/Severe + Veh vs. HI/Severe vs. Met
c< 0.05 HI/Mild + Veh vs. HI/Mild + Met

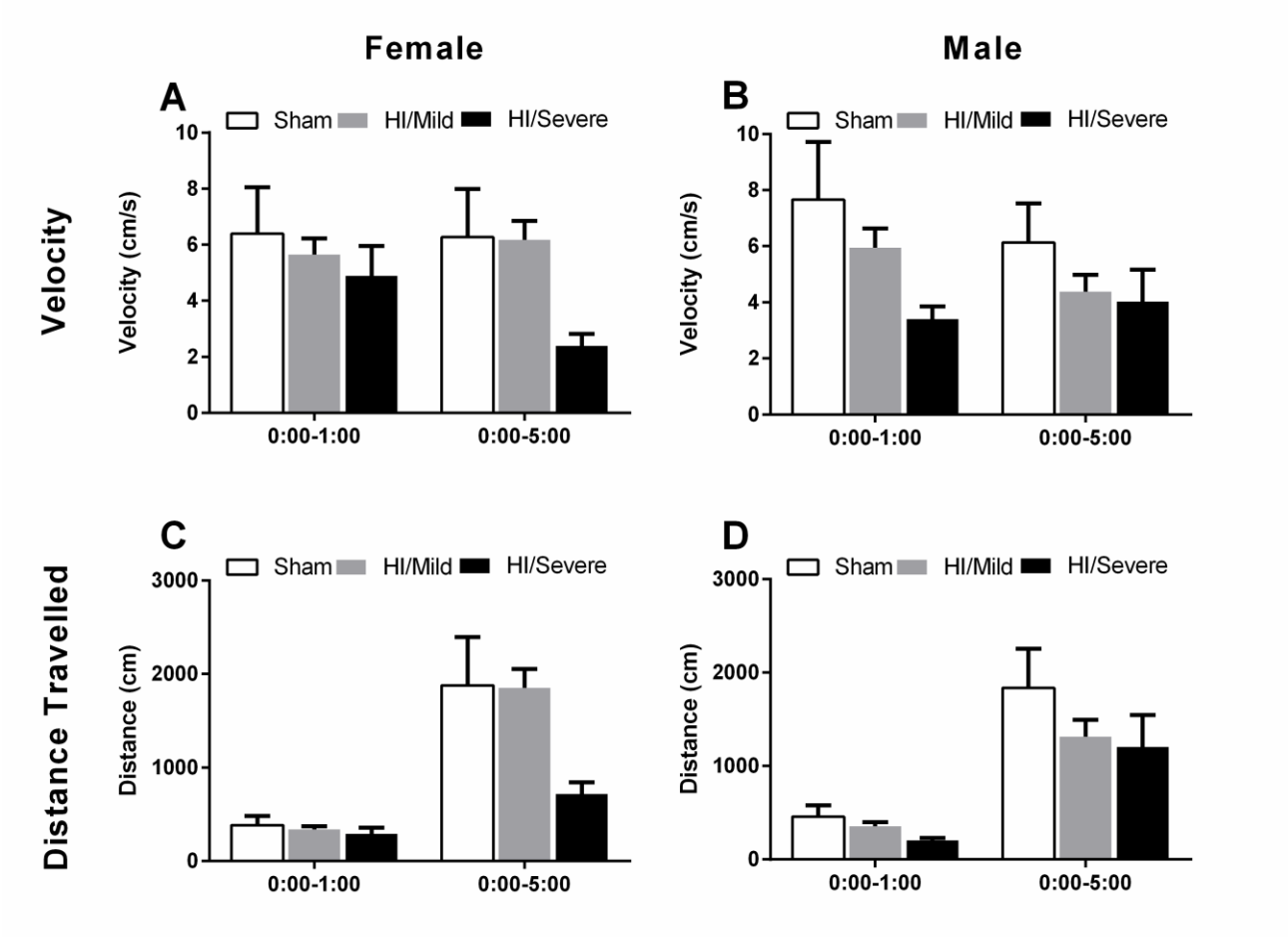

Figure A1. Motor activity in the open field (pre-weaning). There were no significant differences in the velocity **(A, B)** or distance travelled **(C, D)** during the 1st minute or the entire 5-minute open field trial. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild n= 24; Severe n= 4) (Male: Sham n= 6; Mild n= 22; Severe n= 4).

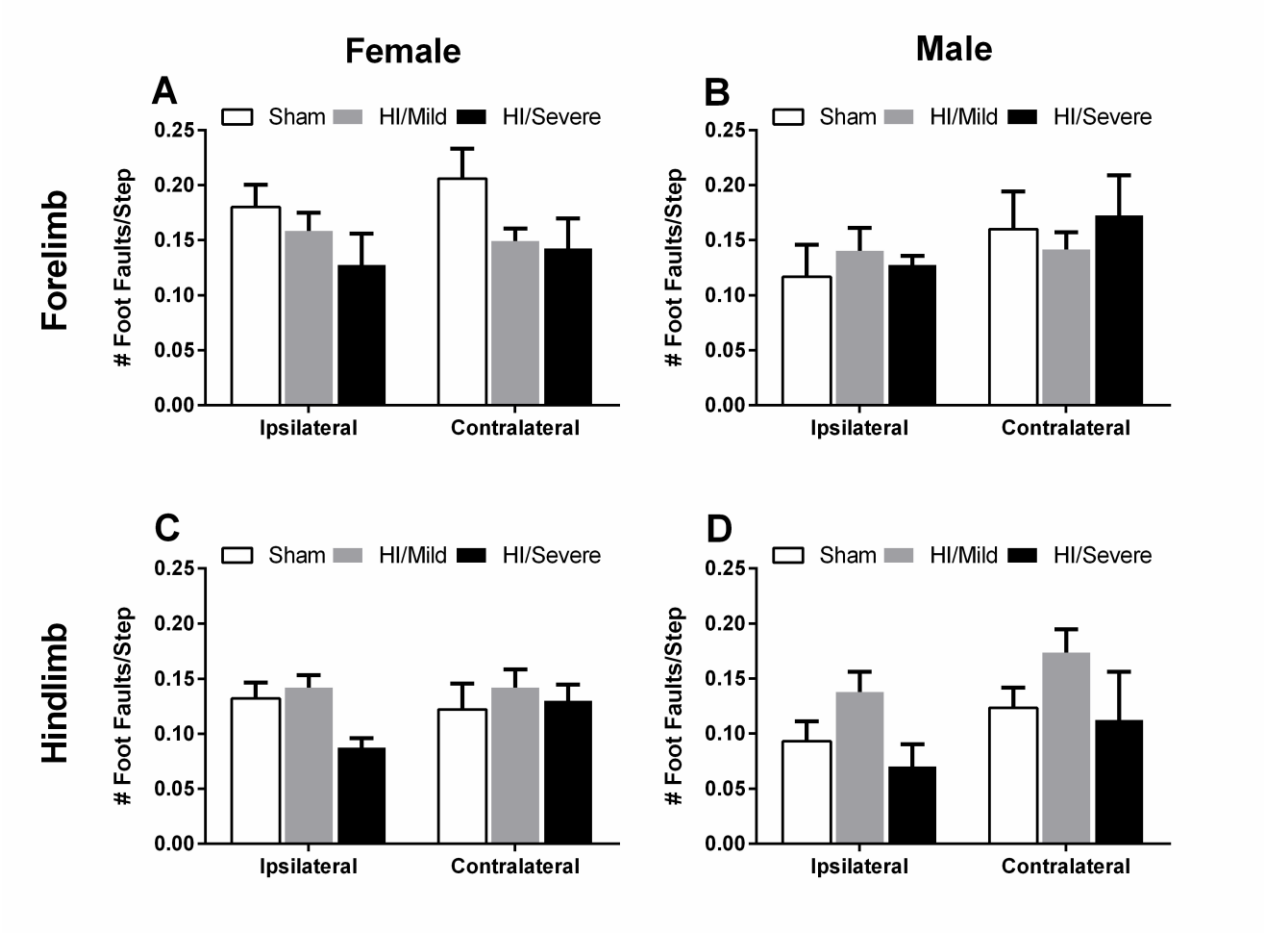

Figure A2. Foot faults in the ladder walking test (pre-weaning). There were no significant differences in the number of foot faults animals made with either of their forelimbs **(A, B)** or hindlimbs **(C, D)**, regardless of cluster or sex. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild n= 24; Severe n= 4) (Male: Sham n= 6; Mild n= 22; Severe n= 4).

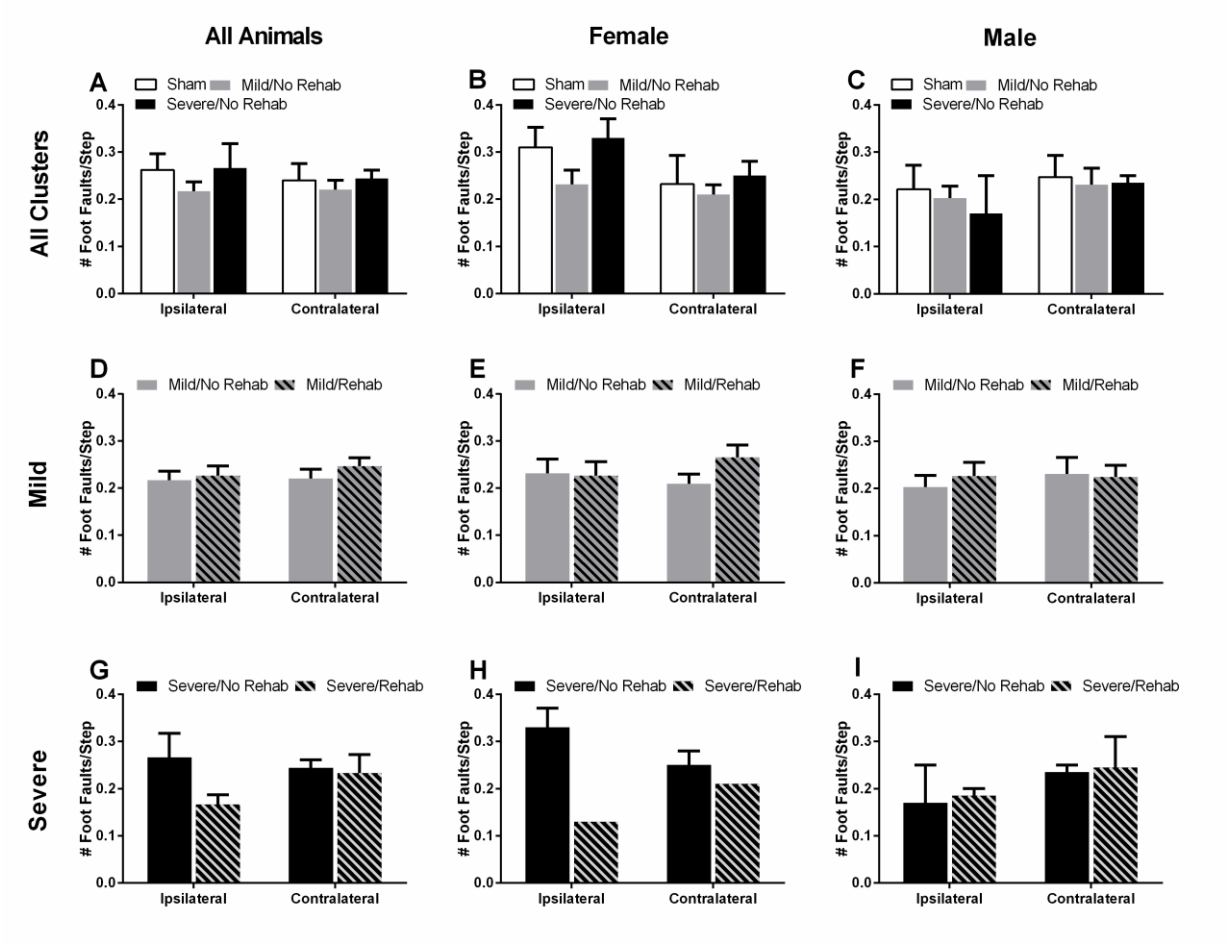

Figure A3. Effect of rehab on forelimb foot faults in the ladder walking test (post-weaning). No significant impairments were detected when all animals were analyzed together **(A)**; similar trends were observed in females **(B)** and males **(C).** HI/mild animals receiving rehab performed similarly compared to those not receiving rehab **(D-F)**. There was a trend toward HI/severe animals receiving rehab making fewer foot faults with their ipsilateral forelimb compared to those not receiving rehab **(G)**; a similar trend was observed in females **(H)** but not males **(I)**. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild/No Rehab n= 11; Mild/Rehab n= 13; Severe/No Rehab n= 3; Severe/Rehab n= 1) (Male: Sham n= 6; Mild/No Rehab n= 11; Mild/Rehab n= 11; Severe/No Rehab n= 2; Severe/Rehab n= 2).

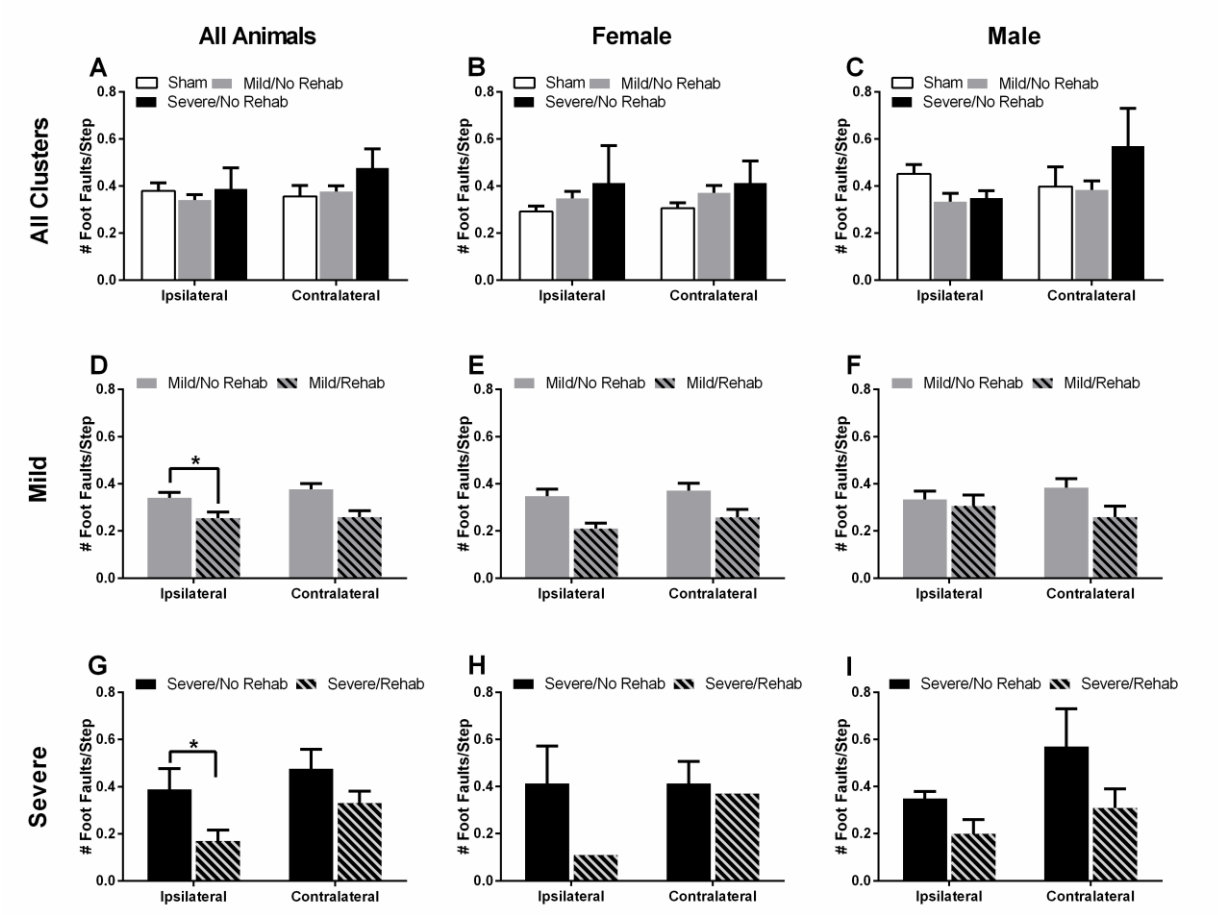

Figure A4. Effect of rehab on hindlimb foot faults in the ladder walking test (post-weaning). No significant impairments were detected when all animals were analyzed together **(A)**; similar trends were observed in females **(B)** and males **(C).** HI/mild animals receiving rehab made significantly fewer foot faults compared to those not receiving rehab **(D)**; a similar trend was seen in females **(E)** but not males **(F).** HI/severe animals receiving rehab made significantly fewer foot faults per step with their ipsilateral hindlimbs **(G)**, a similar trend was seen with the contralateral hindlimb. Although not significant, similar trends were observed in females **(H)** and males **(I)**. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild/No Rehab n= 11; Mild/Rehab n= 13; Severe/No Rehab n= 3; Severe/Rehab n= 1) (Male: Sham n= 6; Mild/No Rehab n= 11; Mild/Rehab n= 11; Severe/No Rehab n= 2; Severe/Rehab n= 2).

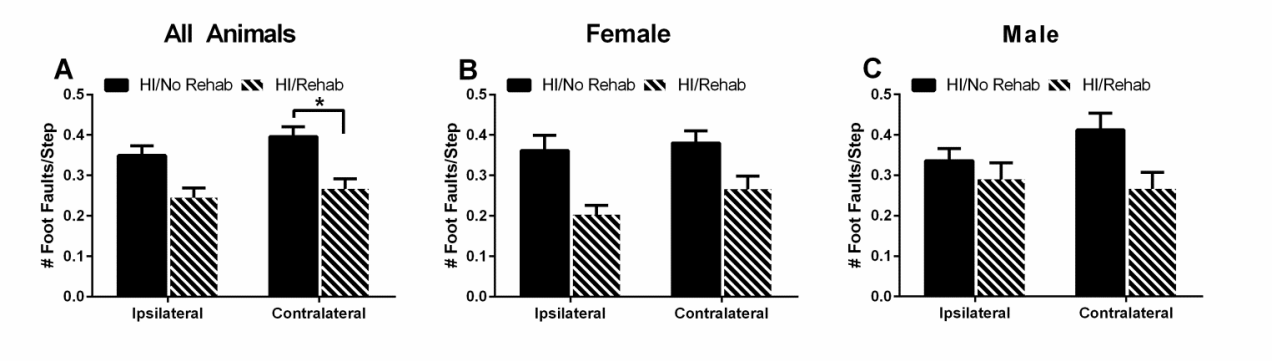

Figure A5. Effect of rehab on contralateral hindlimb foot faults in the ladder walking test (post-weaning). All HI animals receiving rehab, regardless of sex, made significantly fewer foot faults per step with their contralateral hindlimb **(A)**; this was observed in females **(B)** and males **(C)**. Values are estimated marginal means ± SEM. (Female: HI/No Rehab n= 14; HI/Rehab n= 14) (Male: HI/No Rehab n= 13; HI/Rehab n= 13).

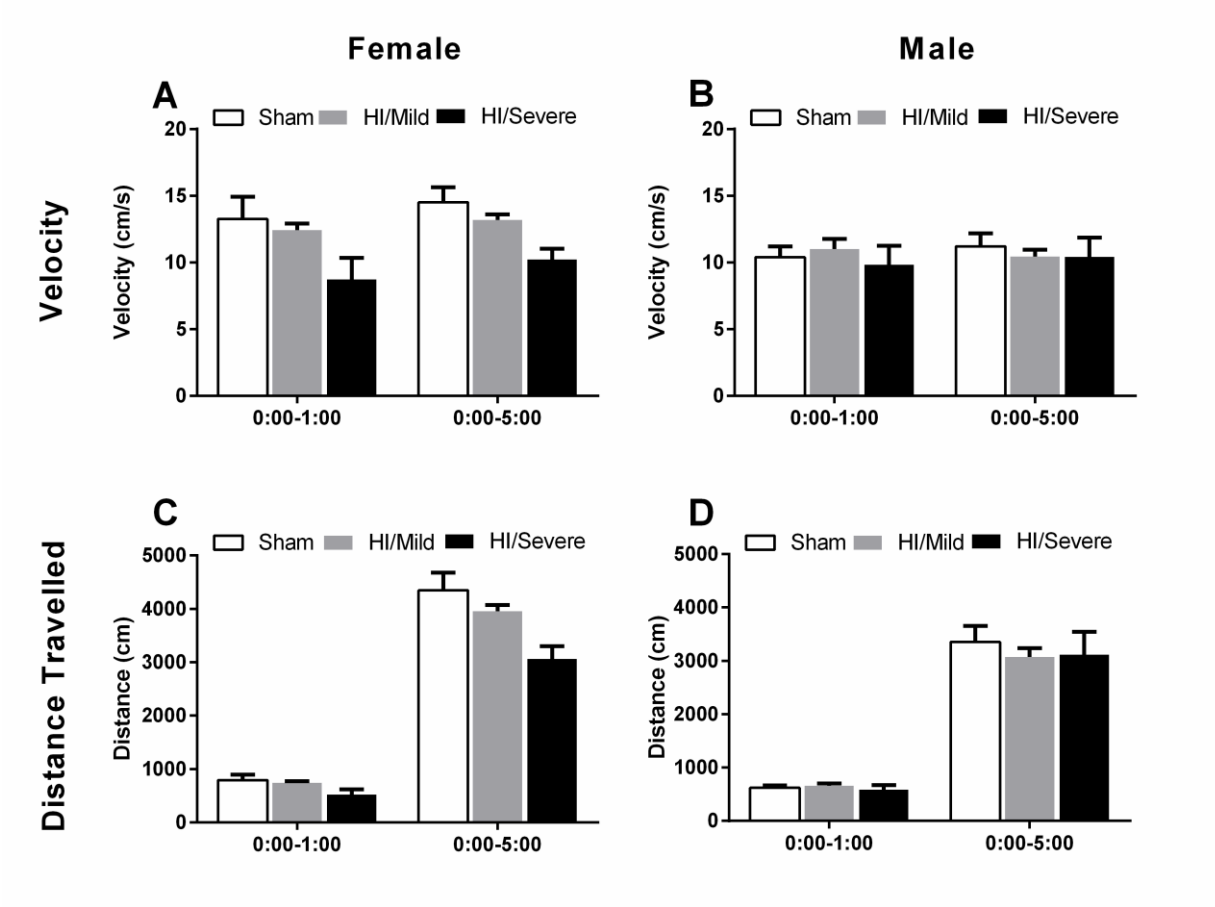

Figure A6. Motor activity in the open field (post-weaning). There were no significant differences in velocity **(A, B)** or distance travelled **(C, D)** during the 1st minute or the entire 5-minute open field trial. Values are estimated marginal means ± SEM. (Female: Sham n= 5; Mild n= 24; Severe n= 4) (Male: Sham n= 6; Mild n= 22; Severe n= 4).

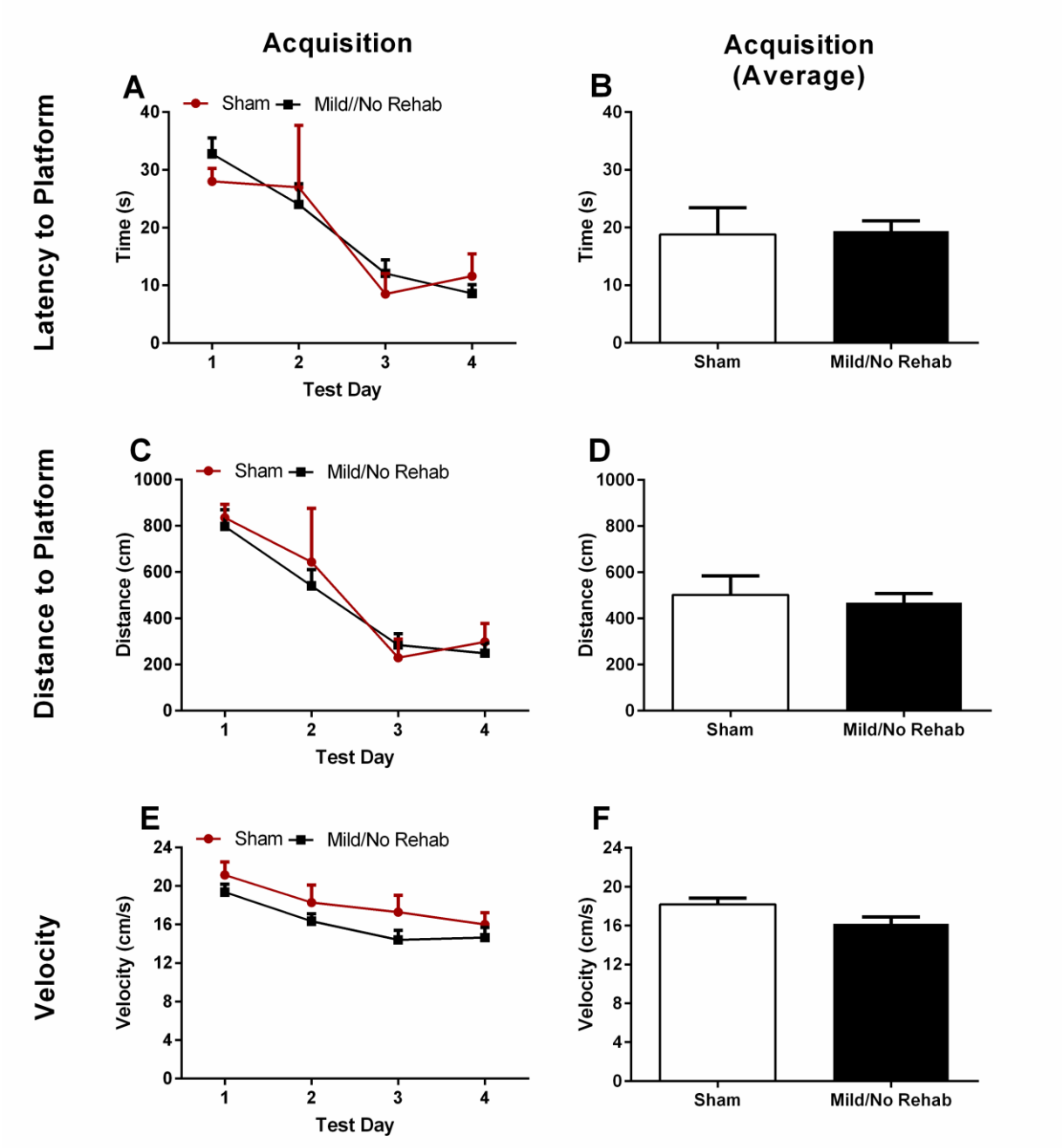

Figure A7. Spatial learning and memory in the Morris water maze (post-weaning). No significant differences were found between sham and HI/mild animals not receiving rehab during acquisition **(A, B)**. Animals travelled similar distances **(C, D)** to find the platform and at similar velocities **(E, F)**. Values are estimated marginal means ± SEM. (Sham n= 4; Mild/No Rehab n= 16).

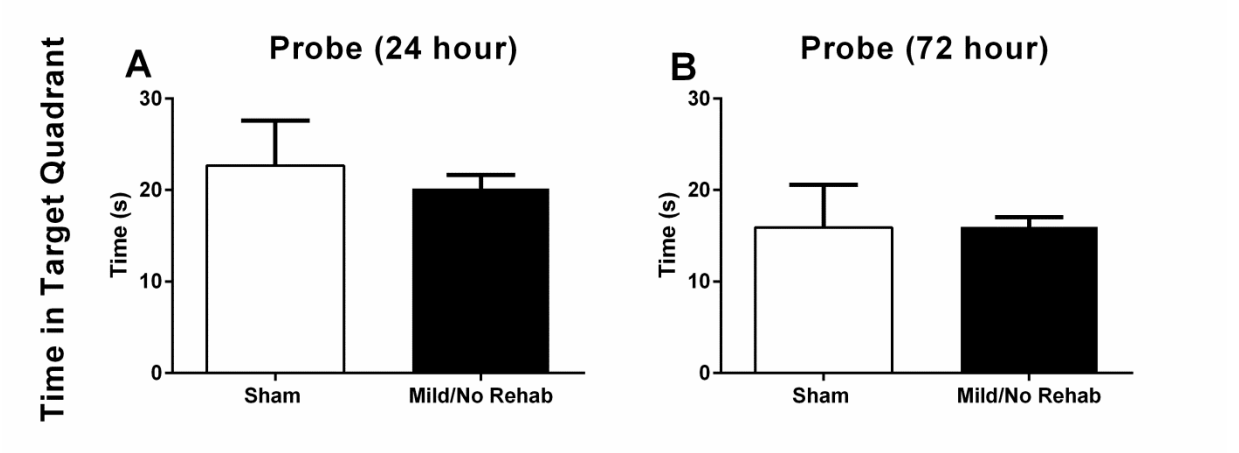

Figure A8. Memory retention during the Morris water maze probe (post-weaning). No significant differences between shams and HI/mild animals not receiving rehab in time spent in the target quadrant during the 24 **(A)** or 72-hour **(B)** probe. Values are estimated marginal means ± SEM. (Sham n= 4; Mild/No Rehab n= 16).

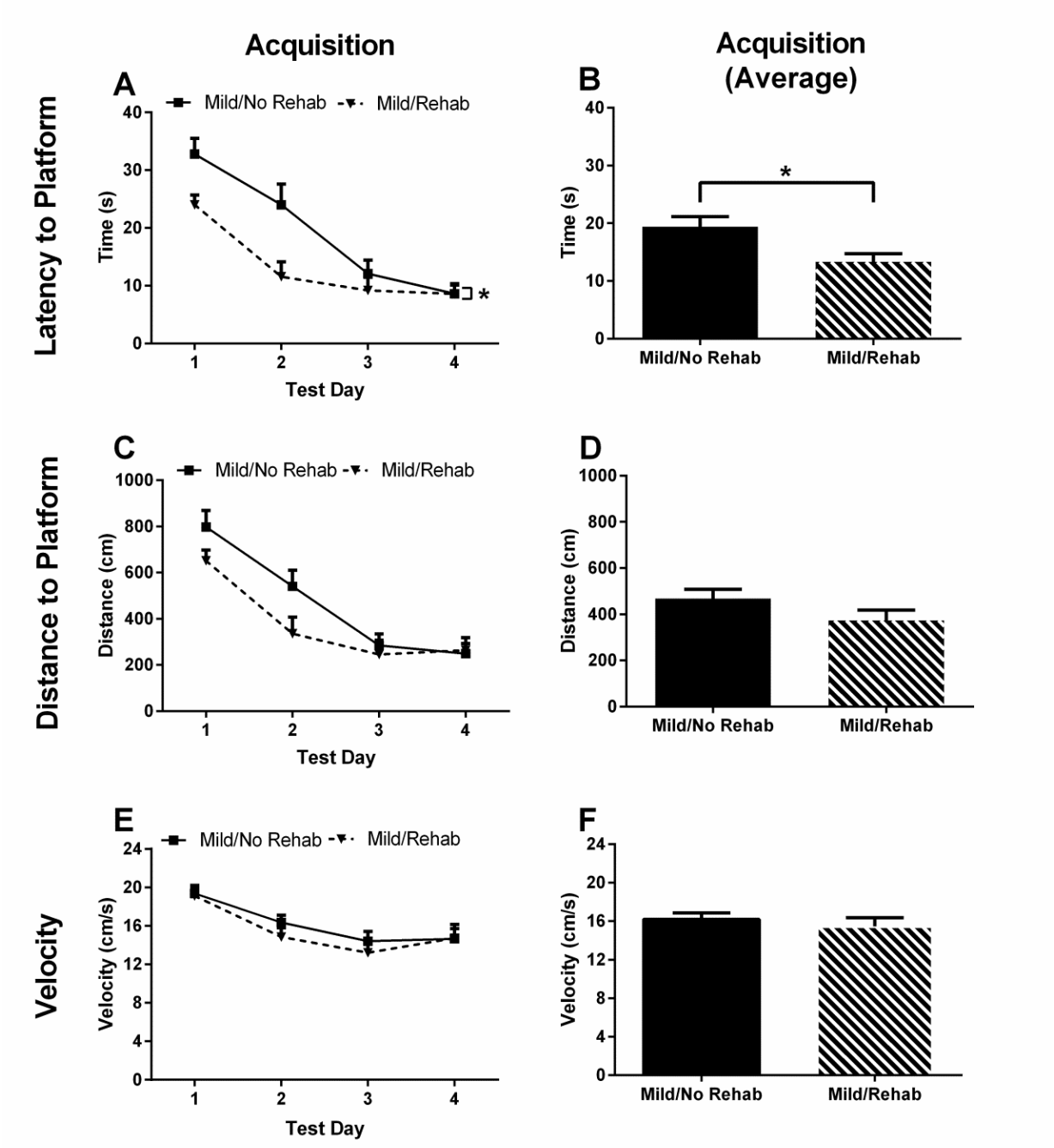

Figure A9. Effect of rehab on spatial learning and memory in the Morris water maze (post-weaning). HI/mild animals receiving rehab took significantly less time to locate the escape platform **(A, B)** and there was a trend towards them travelling less distance to locate the platform compared to those not receiving rehab **(C, D)**. Both groups travelled at similar velocities **(E, F)**. Values are estimated marginal means ± SEM. (HI/No Rehab n= 16; HI/Rehab n= 14).

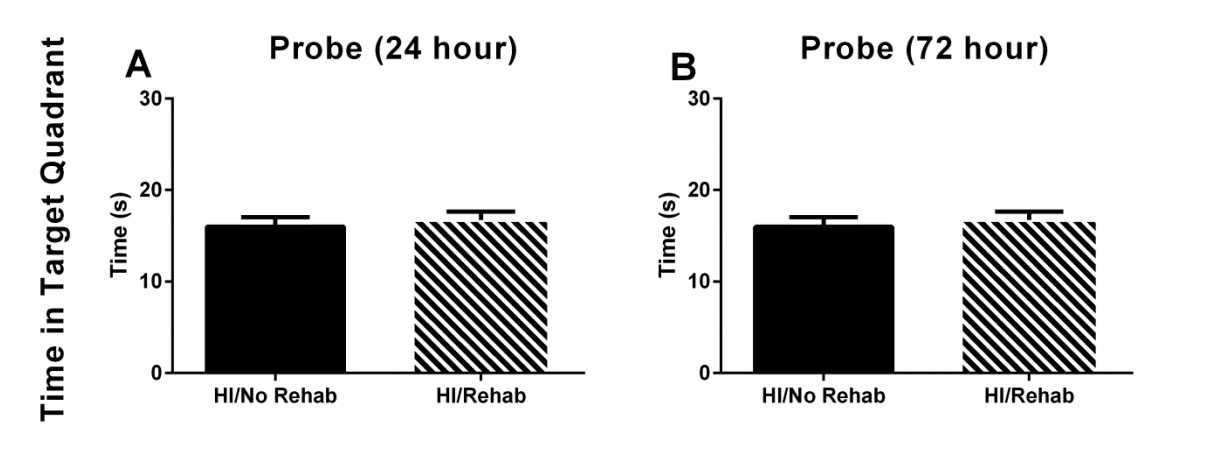

Figure A10. Effect of rehab on memory retention in the Morris water maze probe (post-weaning). No significant differences were found between HI/no rehab and HI/rehab animals in time spent in the target quadrant during the 24 **(A)** or 72-hour **(B)** probe. Values are estimated marginal means ± SEM. (HI/No Rehab n= 16; HI/Rehab n= 14).

APPENDIX B

Table B1. Average time (s) to contact and remove the tape in the adhesive strip removal task (pre-weaning) (EMM ± SEM).

Adhesive Strip Removal Task	Ipsilateral Contact	Contralateral Contact	Ipsilateral Remove	Contralateral Remove
HI Female				
Mild/Veh (n=9)	10.11 ± 1.6	13.31 ± 2.0	230.50 ± 15.9	247.89 ± 14.7
Mild/Met (n=15)	10.52 ± 1.2	10.82 ± 1.5	265.47 ± 12.3	278.22 ± 11.4
Severe/Veh (n=2)	17.75 ± 3.3	21.75 ± 4.2	241.25 ± 33.7	286.17 ± 31.2
Severe/Met (n=2)	13.00 ± 3.3	22.92 ± 4.2	249.67 ± 33.7	288.09 ± 31.2
HI Male				
Mild/Veh (n=10)	9.24 ± 1.5	13.31 ± 2.0	241.95 ± 15.1	261.92 ± 14.0
Mild/Met (n=12)	10.42 ± 1.3	10.82 ± 1.5	282.53 ± 13.7	274.13 ± 12.8
Severe/Veh (n=2)	13.00 ± 3.3	21.75 ± 4.2	130.17 ± 33.7	180.59 ± 31.2
Severe/Met (n=2)	12.67 ± 3.3	22.92 ± 4.2	242.34 ± 33.7	281.34 ± 31.2

Table B2. Average velocity (cm/s) and distance (cm) travelled during the 1st minute and entire 5-minute open field trial (pre-weaning) (EMM ± SEM).

Open Field	Velocity (1st minute)	Distance (1st minute)	Velocity (5 minutes)	Distance (5 minutes)
HI Female				
Mild/Veh (n=9)	5.74 ± 1.1	343.62 ± 65.1	6.06 ± 1.0	1816.51 ± 312.2
Mild/Met (n=15)	5.60 ± 0.8	335.86 ± 50.4	6.25 ± 0.8	1874.34 ± 241.8
Severe/Veh (n=2)	6.52 ± 2.3	389.88 ± 138.1	2.69 ± 2.2	804.93 ± 662.3
Severe/Met (n=2)	3.25 ± 2.3	194.54 ± 138.1	2.10 ± 2.2	628.12 ± 662.3
HI Male				
Mild/Veh (n=10)	5.71 ± 1.0	341.94 ± 61.8	3.87 ± 1.0	1158.40 ± 296.2
Mild/Met (n=12)	6.16 ± 0.9	368.98 ± 56.4	4.81± 0.9	1442.59 ± 270.4
Severe/Veh (n=2)	3.28 ± 2.3	196.34 ± 138.1	5.07 ± 2.2	1518.02 ± 662.3
Severe/Met (n=2)	3.52 ± 2.3	210.29 ± 138.1	3.00 ± 2.2	896.96 ± 662.3

Table B3. Average number of errors per step during the ladder walking test (pre-weaning) (EMM ± SEM).

Ladder Walking Test	Ipsilateral Forelimb	Contralateral Forelimb	Ipsilateral Hindlimb	Contralateral Hindlimb
HI Female				
Mild/Veh (n=9)	0.19 ± 0.03	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.03
Mild/Met (n=15)	0.14 ± 0.02	0.15 ± 0.02	0.14 ± 0.02	0.13 ± 0.02
Severe/Veh (n=2)	0.09 ± 0.06	0.12 ± 0.05	0.09 ± 0.05	0.14 ± 0.06
Severe/Met (n=2)	0.16 ± 0.06	0.17 ± 0.05	0.08 ± 0.05	0.13 ± 0.06
HI Male				
Mild/Veh (n=10)	0.13 ± 0.03	0.14 ± 0.02	0.11 ± 0.02	0.15 ± 0.03
Mild/Met (n=12)	0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.20 ± 0.02
Severe/Veh (n=2)	0.13 ± 0.06	0.19 ± 0.05	0.04 ± 0.05	0.19 ± 0.06
Severe/Met (n=2)	0.12 ± 0.06	0.16 ± 0.05	0.10 ± 0.05	0.04 ± 0.06

Table B4. Average time (s) to contact and remove the tape during the adhesive strip removal task (post-weaning) (EMM ± SEM).

Adhesive Strip Removal Task	Ipsilateral Contact	Contralateral Contact	Ipsilateral Remove	Contralateral Remove
HI Female				
Mild				
No Rehab/Veh (n=4)	9.88 ± 2.1	10.75 ± 2.4	62.88 ± 31.0	70.38 ± 36.1
No Rehab/Met (n=7)	9.14 ± 1.6	5.79 ± 1.8	166.50 ± 23.4	123.29 ± 27.3
Rehab/Veh (n=5)	2.90 ± 1.9	3.20 ± 2.2	43.00 ± 27.7	60.60 ± 32.2
Rehab/Met (n=8)	3.81 ± 1.5	2.88 ± 1.7	47.81 ± 21.9	56.44 ± 25.5
Severe				
No Rehab/Veh (n=2)	4.50 ± 3.0	16.25 ± 3.4	54.00 ± 43.9	113.50 ± 51.0
No Rehab/Met (n=1)	8.50 ± 4.2	13.50 ± 4.9	61.00 ± 62.0	81.50 ± 72.1
Rehab/Veh (n=0)	n/a	n/a	n/a	n/a
Rehab/Met (n=1)	2.00 ± 4.2	25.50 ± 4.9	138.50 ± 62.0	100.00 ± 72.1
HI Male				
Mild				
No Rehab/Veh (n=5)	10.5 ± 1.9	9.30 ± 2.2	135.30 ± 27.7	134.00 ± 32.2
No Rehab/Met (n=6)	9.50 ± 1.7	10.00 ± 2.0	113.92 ± 25.3	136.17 ± 29.4
Rehab/Veh (n=5)	6.60 ± 1.9	7.00 ± 2.2	46.50 ± 27.7	76.60 ± 32.2
Rehab/Met (n=6)	5.17 ± 1.7	7.83 ± 2.0	97.00 ± 25.3	116.58 ± 29.4
Severe				
No Rehab/Veh (n=1)	4.50 ± 4.2	6.50 ± 4.9	56.00 ± 62.0	33.00 ± 72.1
No Rehab/Met (n=1)	3.00 ± 4.2	17.00 ± 4.9	49.50 ± 62.0	118.00 ± 72.1
Rehab/Veh (n=1)	3.50 ± 4.2	5.00 ± 4.9	40.50 ± 62.0	37.50 ± 72.1
Rehab/Met (n=1)	1.50 ± 4.2	7.00 ± 4.9	22.00 ± 62.0	52.50 ± 72.1

Table B5. Average number of errors per step during the ladder walking test (post-weaning) (EMM ± SEM).

Ladder Walking Test	Ipsilateral Forelimb	Contralateral Forelimb	Ipsilateral Hindlimb	Contralateral Hindlimb
HI Female				
Mild				
No Rehab/Veh (n=4)	0.32 ± 0.04	0.23 ± 0.1	0.34 ± 0.1	0.42 ± 0.1
No Rehab/Met (n=7)	0.18 ± 0.03	0.20 ± 0.04	0.35 ± 0.04	0.34 ± 0.1
Rehab/Veh (n=5)	0.15 ± 0.04	0.26 ± 0.04	0.18 ± 0.1	0.27 ± 0.1
Rehab/Met (n=8)	0.27 ± 0.03	0.27 ± 0.03	0.23 ± 0.04	0.25 ± 0.05
Severe				
No Rehab/Veh (n=2)	0.37 ± 0.1	0.28 ± 0.1	0.27 ± 0.1	0.34 ± 0.1
No Rehab/Met (n=1)	0.25 ± 0.1	0.19 ± 0.1	0.70 ± 0.1	0.56 ± 0.1
Rehab/Veh (n=0)	n/a	n/a	n/a	n/a
Rehab/Met (n=1)	0.13 ± 0.1	0.21 ± 0.1	0.70 ± 0.1	0.37 ± 0.1
HI Male				
Mild				
No Rehab/Veh (n=5)	0.19 ± 0.04	0.26 ± 0.04	0.32 ± 0.1	0.37 ± 0.1
No Rehab/Met (n=6)	0.21 ± 0.04	0.21 ± 0.04	0.34 ± 0.1	0.39 ± 0.1
Rehab/Veh (n=5)	0.22 ± 0.04	0.24 ± 0.04	0.25 ± 0.05	0.20 ± 0.1
Rehab/Met (n=6)	0.24 ± 0.04	0.21 ± 0.04	0.35 ± 0.05	0.30 ± 0.1
Severe				
No Rehab/Veh (n=1)	0.25 ± 0.1	0.22 ± 0.1	0.32 ± 0.1	0.41 ± 0.1
No Rehab/Met (n=1)	0.09 ± 0.1	0.25 ± 0.1	0.38 ± 0.1	0.73 ± 0.1
Rehab/Veh (n=1)	0.17 ± 0.1	0.31 ± 0.1	0.14 ± 0.1	0.23 ± 0.1
Rehab/Met (n=1)	0.20 ± 0.1	0.18 ± 0.1	0.26 ± 0.1	0.40 ± 0.1

Table B6. Average velocity (cm/s) and distance travelled (cm) during the 1st minute and entire 5-minute open field trial (post-weaning) (EMM ± SEM).

Open Field	Velocity (1st minute)	Distance (1st minute)	Velocity (5 minutes)	Distance (5 minutes)
HI Female				
Mild				
No Rehab/Veh (n=4)	10.52 ± 1.3	630.36 ± 74.5	11.84 ± 1.1	3549.75 ± 335.5
No Rehab/Met (n=7)	11.27 ± 0.9	675.68 ± 56.3	12.94 ± 0.8	3879.31 ± 253.6
Rehab/Veh (n=5)	13.96 ± 1.1	835. 34 ± 66.7	15.03 ± 1.0	4503.78 ± 300.1
Rehab/Met (n=8)	13.41 ± 0.9	804.05 ± 52.7	12.95 ± 0.8	3884.64 ± 237.2
Severe				
No Rehab/Veh (n=2)	7.85 ± 1.8	469.35 ± 105.4	10.58 ± 1.5	3166.38 ± 474.5
No Rehab/Met (n=1)	5.83 ± 2.5	348.52 ± 149.0	7.97 ± 2.2	2386.61 ± 671.0
Rehab/Veh (n=0)	n/a	n/a	n/a	n/a
Rehab/Met (n=1)	13.36 ± 2.5	798.83 ± 149.0	11.77 ± 2.2	3523.08 ± 671.0
HI Male				
Mild				
No Rehab/Veh (n=5)	7.90 ± 1.1	473.03 ± 66.7	9.96 ± 1.0	2729.79 ± 300.1
No Rehab/Met (n=6)	10.41 ± 1.0	624.14 ± 60.8	10.68 ± 0.9	3203.84 ± 273.9
Rehab/Veh (n=5)	10.99 ± 1.1	658.80 ± 66.6	10.42 ± 1.0	3122. 67 ± 300.1
Rehab/Met (n=6)	14.20 ± 1.0	850.80 ± 60.8	10.61 ± 0.9	3179.26 ± 273.9
Severe				
No Rehab/Veh (n=1)	7.95 ± 2.5	475.53 ± 149.0	11.73 ± 2.2	3508.66 ± 671.0
No Rehab/Met (n=1)	8.80 ± 2.5	526.33 ± 149.0	10.03 ± 2.2	3002.23 ± 671.0
Rehab/Veh (n=1)	14.06 ± 2.5	840.50 ± 149.0	13.31 ± 2.2	3984.77 ± 671.0
Rehab/Met (n=1)	8.46 ± 2.5	505.92 ± 149.0	6.64 ± 2.2	1987.20 ± 671.0

Table B7. Average number of pellets retrieved during the Montoya staircase test (post-weaning) (EMM ± SEM).

Montoya Staircase Test	Ipsilateral Reaching	Contralateral Reaching
HI Female		
Mild		
No Rehab/Veh (n=4)	11.56 ± 1.2	13.80 ± 1.3
No Rehab/Met (n=7)	12.31 ± 0.9	12.20 ± 1.0
Rehab/Veh (n=5)	15.29 ± 1.0	16.34 ± 1.2
Rehab/Met (n=8)	14.07 ± 0.8	12.71 ± 0.9
Severe		
No Rehab/Veh (n=2)	14.88 ± 1.7	7.59 ± 1.9
No Rehab/Met (n=1)	12.86 ± 2.3	5.50 ± 2.7
Rehab/Veh (n=0)	n/a	n/a
Rehab/Met (n=1)	11.89 ± 2.3	5.18 ± 2.7
HI Male		
Mild		
No Rehab/Veh (n=5)	13.39 ± 1.0	14.09 ± 1.2
No Rehab/Met (n=6)	10.28 ± 1.0	11.26 ± 1.1
Rehab/Veh (n=5)	15.47 ± 1.0	15.20 ± 1.2
Rehab/Met (n=6)	14.14 ± 1.0	13.51 ± 1.1
Severe		
No Rehab/Veh (n=1)	15.29 ± 2.3	5.14 ± 2.7
No Rehab/Met (n=1)	12.36 ± 2.3	8.71 ± 2.7
Rehab/Veh (n=1)	17.64 ± 2.3	12.75 ± 2.7
Rehab/Met (n=1)	10.61 ± 2.3	13.57 ± 2.7

Table B8. Average latency to goal box (s) and time spent in target quadrant (s) during the acquisition and probe trials in the Barnes maze (post-weaning) (EMM ± SEM).

Barnes Maze	Latency to Goal Box (Acquisition)	Latency to Goal Box (24hr Probe)	Time in Target Quadrant (24hr Probe)
HI Female			
Mild			
No Rehab/Veh (n=4)	21.18 ± 3.4	62.61 ± 14.8	34.78 ± 6.9
No Rehab/Met (n=7)	22.74 ± 2.5	29.29 ± 11.2	36.01 ± 5.2
Rehab/Veh (n=5)	14.19 ± 3.0	16.66 ± 13.3	42.36 ± 6.1
Rehab/Met (n=8)	15.75 ± 2.4	7.71 ± 10.5	43.07 ± 4.8
Severe			
No Rehab/Veh (n=2)	20.41 ± 4.7	80.68 ± 21.0	23.02 ± 9.7
No Rehab/Met (n=1)	28.24 ± 6.7	67.87 ± 29.7	23.02 ± 13.7
Rehab/Veh (n=0)	n/a	n/a	n/a
Rehab/Met (n=1)	14.11 ± 6.7	7.61 ± 29.7	29.63 ± 13.7
HI Male			
Mild			
No Rehab/Veh (n=5)	35.28 ± 3.0	20.86 ± 13.3	48.13 ± 6.1
No Rehab/Met (n=6)	24.40 ± 2.7	38.20 ± 12.1	43.74 ± 5.6
Rehab/Veh (n=5)	15.50 ± 3.0	22.94 ± 13.3	50.97 ± 6.1
Rehab/Met (n=4)	20.94 ± 3.4	15.47 ± 14.8	56.81 ± 6.8
Severe			
No Rehab/Veh (n=1)	33.19 ± 6.7	21.22 ± 29.7	23.22 ± 13.7
No Rehab/Met (n=1)	32.30 ± 6.7	84.28 ± 29.7	31.03 ± 13.7
Rehab/Veh (n=1)	20.94 ± 6.7	13.41 ± 29.7	47.85 ± 13.7
Rehab/Met (n=1)	29.64 ± 6.7	3.80 ± 29.7	36.24 ± 13.7

Table B9. Spatial learning and memory during the initial acquisition and probe trials in the Morris water maze (post-weaning) (EMM ± SEM).

MWM (Acquisition)	Latency to Platform (s)	Distance to Platform (cm)	Velocity (cm/s)	Time in Target Quadrant (s) (24hr Probe)	Time in Target Quadrant (s) (72hr Probe)
HI Female					
Mild					
No Rehab/Veh (n=3)	21.96 ± 4.2	559.37 ± 96.5	18.29 ± 1.4	18.32 ± 2.9	15.75 ± 2.4
No Rehab/Met (n=6)	20.80 ± 3.0	491.26 ± 68.2	16.85 ± 1.0	18.19 ± 2.0	14.41 ± 1.7
Rehab/Veh (n=2)	12.82 ± 5.1	341.18 ± 118.1	15.17 ± 1.8	20.42 ± 3.5	15.97 ± 2.9
Rehab/Met (n=7)	14.13 ± 2.7	446.81 ± 63.2	17.59 ± 0.9	20.31 ± 1.9	15.89 ± 1.6
HI Male					
Mild					
No Rehab/Veh (n=2)	17.95 ± 5.1	453.82 ± 118.1	16.32 ± 1.8	19.77 ± 3.5	15.12 ± 2.9
No Rehab/Met (n=5)	16.70 ± 3.2	390.44 ± 74.7	14.11 ± 1.1	23.82 ± 2.2	18.34 ± 1.9
Rehab/Veh (n=3)	13.21 ± 4.2	308.37 ± 96.5	13.21 ± 1.4	23.62 ± 2.9	20.32 ± 2.4
Rehab/Met (n=2)	11.23 ± 5.1	251.09 ± 118.1	11.74 ± 1.8	23.77 ± 3.5	15.12 ± 2.9

Table B10. Spatial learning and memory during the reversal acquisition and probe trials in the Morris water maze (post-weaning) (EMM ± SEM).

MWM (Reversal)	Latency to Platform (s)	Distance to Platform (cm)	Velocity (cm/s)	Time in Target Quadrant (s) (24hr Probe)	Time in Original Quadrant (s) (24hr Probe)
HI Female					
Sham					
	13.38 ± 2.6	325.68 ± 63.4	21.56 ± 1.8	22.52 ± 2.7	7.84 ± 1.8
Mild					
No Rehab/Veh (n=3)	15.43 ± 2.6	394.49 ± 63.4	21.58 ± 1.8	19.35 ± 2.7	12.98 ± 1.8
No Rehab/Met (n=6)	8.33 ± 1.8	239.17 ± 44.8	21.54 ± 1.3	23.16 ± 1.9	9.63 ± 1.3
Rehab/Veh (n=2)	12.51 ± 3.1	349.93 ± 77.6	23.13 ± 2.2	28.98 ± 3.2	9.61 ± 2.3
Rehab/Met (n=7)	8.74 ± 1.7	283.51 ± 41.5	21.23 ± 1.2	21.75 ± 1.7	10.51 ± 1.2
HI Male					
Sham					
	7.36 ± 4.4	193.40 ± 109.8	13.58 ± 3.1	36.24 ± 4.6	6.21 ± 3.2
Mild					
No Rehab/Veh (n=2)	9.27 ± 3.1	218.83 ± 77.6	12.84 ± 2.2	26.68 ± 3.2	8.01 ± 2.3
No Rehab/Met (n=5)	9.02 ± 2.0	221.21 ± 49.1	13.15 ± 1.4	27.15 ± 2.1	8.53 ± 1.4
Rehab/Veh (n=3)	8.68 ± 2.6	216.88 ± 63.4	13.69 ± 1.8	25.06 ± 2.7	10.01 ± 1.8
Rehab/Met (n=2)	6.62 ± 3.1	166.95 ± 77.6	12.55 ± 2.2	26.13 ± 3.2	10.16 ± 2.3