Understanding the Effects of Sildenafil on Neurons, Cell Death, and Cell Signalling Pathways after Neonatal Hypoxia-Ischemia

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TABLE OF CONTENTS

Abbreviations/Acronyms

Akt	Protein Kinase B
ATP	Adenosine Triphosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
DCX	Doublecortin
EAE	Experimental Autoimmune encephalomyelitis
EGR	Early growth response
GAP-43	Growth associated protein 43
GluR1	Glutamate receptor 1
GSK-3	Glycogen synthase 3
HI	Hypoxia-Ischemia
HIE	Hypoxic Ischemic Encephalopathy
HRP	Horse radish peroxidase
HT-22	Immortalized mouse hippocampal cell line
IL-1β	Interleukin-1 beta
LPS	Lipopolysaccharide
MCM-2$^+$	Minichromosome maintenance protein-2-positive cells
MLKL	Mixed lineage kinase domain-like protein
mTOR	Mammalian target of rapamycin
NeuN	Neuronal nuclear protein
PBS	Phosphate buffered saline
PDE-5	Phosphodiesterase-5
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Px	Postnatal day x
RIP3	Receptor-interacting protein kinase 3
RIPA buffer	Radioimmunoprecipitation assay buffer
RPM	Rotations per minute
TBS	Tri-phosphate buffered saline
Wnt	Wingless-related integration

Abstract (English)

Introduction: Birth asphyxia and the resulting hypoxic-ischemic encephalopathy (HIE) is a life-threatening complication in newborns, often leading to death or neurological impairment. The current treatment for HIE is therapeutic hypothermia, offering a neuroprotective effect by decreasing oxygen demand leading to less energy depletion in the injured brain. Currently, there are no treatments that can repair the brain after hypoxia-ischemia (HI). Sildenafil has been suggested as a promising neurorestorative agent in a rat model of neonatal HI for the improvement of neurological deficits after injury and reduction of infarct size in the cortex and hippocampus. However, its effects on neurons, cell death and cell signalling pathways remain to be further elucidated.

Objective: To investigate the effects of HI and/or sildenafil on neurons, cell death, and important cell signalling pathways in the cortex and hippocampus.

Methods: We used the Vannucci rat model of term HI (left carotid artery ligation followed by a two-hour exposure to 8% oxygen) to induce injury in rat pups on postnatal day 10 (P10). Rats were randomly assigned to one of three experimental groups: Sham surgery with vehicle (placebo) treatment served as the control group, HI surgery with vehicle treatment, or HI surgery with 50 mg/kg sildenafil. Placebo or sildenafil were given by oral gavage every 12 hours for 7 days (total of 14 doses) starting 12 hours after HI. Cortex and hippocampus tissue were collected at P12, P17 and P30 for Western Blot analyses. Markers for neurons, autophagy and necroptosis, and mammalian target of rapamycin (mTOR) and

wingless-related integration (Wnt) signalling pathways were tested by Western Blot.

Results: In the cortex, HI decreased neuronal nuclear protein (NeuN) (p = .02) and early growth response 1 (Egr1) (p = .04) expression compared to sham. HI also increased the expression of mixed lineage kinase domain-like protein (MLKL) compared to sildenafil treated rats (p = .03). In the hippocampus, HI decreased the expression of phosphatase and tensin homolog (PTEN) (p = .01), growth associated protein 43 (GAP-43) (p = .01), Beclin-1 (p = .01), phosphorylated protein kinase B (pAkt) (p < .01), mTOR complex 1 (mTORC1) (p = .02), mTORC2 (p < .01), and Wnt3 (p = .04) expression. HI increased levels of receptor-interacting protein kinase 3 (RIP3) compared to sham (p = .01). Sildenafil treatment restored these markers to levels no longer significantly different than sham levels.

Conclusion: HI led to impairments in neurons, increased cell death via necroptosis and decreased autophagy in the cortex and hippocampus, and decreased mTOR and Wnt pathway activity in the hippocampus. Sildenafil may reverse these effects.

Résumé (Français)

Introduction : L'asphyxie à la naissance et l'encéphalopathie hypoxique-ischémique (EHI) qui en résulte constituent une complication potentiellement mortelle chez les nouveau-nés, entraînant souvent la mort ou une déficience neurologique. Le traitement actuel de l'EHI est l'hypothermie thérapeutique, qui offre un effet neuroprotecteur en diminuant la demande en oxygène, ce qui entraîne une diminution de l'épuisement énergétique dans le cerveau lésé. Actuellement, il n'existe aucun traitement capable de réparer le cerveau après une insulte anoxo-ischémique (HI). Le sildénafil a été suggéré comme un agent neurorestaurateur prometteur dans un modèle de rat d'IH néonatale pour l'amélioration des déficits neurologiques après lésion et la réduction de la taille d'infarctus dans le cortex et l'hippocampe. Cependant, ses effets sur les neurones, la mort cellulaire et les voies de signalisation cellulaire doivent encore être élucidés.

Objectif : Étudier les effets de l'HI et/ou du sildénafil sur les neurones, la mort cellulaire et les voies de signalisation cellulaire importantes dans le cortex et l'hippocampe.

Méthodes : Nous avons utilisé le modèle de rat Vannucci à terme d'HI (ligature de l'artère carotide gauche suivie d'une exposition de deux heures à 8 % d'oxygène) pour induire des lésions chez les ratons au jour postnatal 10 (P10). Les rats ont été répartis au hasard dans l'un des trois groupes expérimentaux : la chirurgie sham avec traitement par véhicule (placebo) a servi de groupe témoin, la chirurgie HI avec traitement par véhicule, ou la chirurgie HI avec 50 mg/kg de sildénafil. Le

placebo ou le sildénafil ont été administrés par gavage oral toutes les 12 heures pendant 7 jours (14 doses au total), en commençant 12 heures après l'HI. Les tissus du cortex et de l'hippocampe ont été prélevés à P12, P17 et P30 pour des analyses Western Blot. Les marqueurs des neurones, de l'autophagie et de la nécroptose, ainsi que de la cible mammalienne de la rapamycine (mTOR) et des voies de signalisation Wnt ont été testés par Western Blot.

Résultats : Dans le cortex, l'HI a diminué l'expression de la protéine nucléaire neuronale (NeuN) (p = 0.02) et la protéine 1 d'expression précoce pour la croissance (Egr1) (p = 0.04) par rapport au traitement sham. L'HI a également augmenté l'expression de la protéine de type domaine kinase à lignée mixte (MLKL) par rapport aux rats traités au sildénafil (p = 0.03). Dans l'hippocampe, l'HI a réduit l'expression de la phosphatase et de l'homologue de la tensine (PTEN) (p = 0.01), de la protéine associée à la croissance neuronale 43 (GAP-43) (p = 0.01), de la Beclin-1 (p = 0.01), de la protéine kinase B phosphorylée (pAkt) (p < 0.01), du complexe mTOR 1 (mTORC1) (p = 0.02), du mTORC2 (p < 0.01) et de la Wnt-3 (p = 0.04). L'HI a augmenté les niveaux de la protéine kinase 3 interagissant avec les récepteurs (RIP3) par rapport au traitement sham (p = 0.01). Le traitement au sildénafil a rétabli ces marqueurs à des niveaux qui n'étaient plus significativement différents de ceux de l'essai sham.

Conclusion : L'HI a entraîné des altérations des neurones, une augmentation de la mort cellulaire par nécroptose et une diminution de l'autophagie dans le cortex et l'hippocampe, ainsi qu'une diminution de l'activité des voies mTOR et Wnt dans l'hippocampe. Le sildénafil peut inverser ces effets.

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Contribution of Authors

Principal Investigator Dr. Pia Wintermark conceptualized and designed the study. She assisted with planning the experiments, data analysis, interpretation of the results, and reviewing and editing the thesis paper.

Research Assistant Ms. Zehra Khoja performed surgeries and the manipulation needed to induce hypoxic-ischemic injury in the rats. Ms. Khoja also sacrificed the rat pups and performed tissue extractions to obtain the tissue needed for western blot analysis.

Ms. Maiuri Maheswaran performed the western blot experiments for the markers related to cell death. Ms. Joy Song performed the western blot experiment for glutamate receptor 1 (GluR1) expression.

The author of this thesis, Mr. Belal Howidi, conducted the remainder of the experiments, and was responsible for the analysis and interpretation of the results.

Understanding the Effects of Sildenafil on Neurons, Cell Death, and Cell Signalling Pathways after Neonatal Hypoxia-Ischemia

Introduction

Birth asphyxia and the resulting hypoxic-ischemic encephalopathy (HIE) is a life-threatening complication in newborns, appearing in 1 to 26 per 1000 live births (Douglas-Escobar & Weiss, 2015). HIE is responsible for 8.5% of the deaths of children under the age of five (Lawn et al., 2007). Infants who survive HIE are likely to suffer long-term neurological impairments and disabilities. HIE is caused by birth asphyxia, as a result of the impediment of blood and/or oxygen to the brain and other organs around the time of birth. Birth asphyxia often results from sentinel events, such as placental abruption, prolapse of the umbilical cord, or uterine rupture. Therapeutic hypothermia is the current standard treatment offered to newborns with HIE, providing neuroprotection to the brain and preventing brain damage from occurring. However, the improvements seen with therapeutic hypothermia are modest; damage still occurs in the brain despite the neuroprotective effects of hypothermia treatment. As such, there is an urgent need to develop neurorestorative treatments that could repair the hypoxic-ischemic brain injury seen in the affected newborns. The pathogenesis of HIE occurs in phases, with the first phase starting right after hypoxia-ischemia (HI), and the last phase (chronic brain injury phase) occurring days to months after the initial insult (Li et al., 2017). An ideal treatment for HIE would target the different phases of injury, by preventing and/or repairing damage following the initial insult.

Background

Pathophysiology of Hypoxic-Ischemic Encephalopathy

Birth asphyxia can occur from a multitude of conditions, such as cord prolapse, cord knotting, chronic maternal hypoxia (e.g. pulmonary embolism, vascular disease, or acute hypotension), and placental abruption, all of which lead to a sudden or prolonged hypoxic-ischemic state in the fetus or newborn (Edwards et al., 2018; Novak et al., 2018). Brain injury from perinatal HI is one of the most prevalent types of neonatal brain injury. Among those developing brain injury, around 40% fail to survive the neonatal period. Of those that survive, 30% develop chronic neurological impairments, such as epilepsy, cerebral palsy, mental retardation, visual impairments, and learning disabilities (Rocha-Ferreira & Hristova, 2015). Neonatal HIE is a global health predicament, affecting 0.3-0.7% of newborns in developed countries, and up to 2.6% of newborns in developing countries.

Neonatal HIE occurs around the time of birth and is an ongoing process leading to neuronal cell death and increased neuroinflammation after the initial insult (Greco et al., 2020). The impairment in the flow of oxygenated blood to the brain can have severe cellular and systemic consequences on the newborn. The neonatal brain requires a constant supply of energy in the form of adenosine triphosphate (ATP) to promote cell survival and function. HIE causes the supply of ATP to suddenly deplete. Since neonatal HIE is an ongoing disorder, it can be divided into three distinct phases. The initial phase, called the primary energy failure phase, lasts approximately six hours, and is characterized by the lack of

constant ATP, failure of the ATP-dependent Na/K pump, and anaerobic respiration. These malfunctions increase lactate production and intracellular sodium and calcium levels. The resultant increase in positively charged ions inside the cell causes the neurons to depolarize, releasing the excitatory neurotransmitter glutamate. Glutamate release further impairs cell integrity by allowing a greater influx of sodium and calcium ions into the cell. This influx can have severe detrimental effects on the cell, such as intracellular calcium toxicity, microvascular impairment, ischemia, and cerebral edema (Li et al., 2017). Free fatty acids also accumulate as a result of increased phospholipid turnover in the membrane, eventually leading to lipid peroxidation, significantly damaging the cell membrane (Perlman, 2007). Cellular energy failure, increases in intracellular calcium and sodium ions, increases in glutamate release, and lipid peroxidation all significantly impair cell integrity and function, leading to early neuronal death and necrosis, which concludes the acute or primary energy failure phase of HIE.

After the primary energy failure, there is a short period known as the latent phase. During this period, many cells in the brain show a partial, or sometimes complete recovery from the damage caused by the insult. This is characterized by the recovery of oxidative metabolism in cells to almost normal levels, residual mitochondrial injury, secondary inflammation, and receptor hyperactivity (Gunn & Thoresen, 2019). The latent phase lasts from one to six hours after the initial event, and is the therapeutic window for applying hypothermia treatment to prevent HIE.

If the severity and duration of injury are intense enough, the brain may enter a delayed brain injury phase, known as secondary energy failure. This phase can

last from a few hours to a couple of days, and is characterized by a decrease in the ratio of cerebral phosphocreatine:inorganic phosphate concentration (an index of oxidative metabolism), which occurs as a result of mitochondrial dysregulation. This dysregulation is directly correlated to the extent of energy depletion (Lorek et al., 1994). The end result of the secondary phase is seizures, cytotoxic edema, and eventually cell death by apoptosis as a result of the increase in free radicals and oxidative stress (Gunn & Thoresen, 2019; Buonocore & Groenendaal, 2007)

The third phase of HIE, the chronic brain injury phase, occurs days to months after the initial insult. This is usually characterized by chronic inflammation, astrogliosis, and complex epigenetic changes that contribute to abnormal neuron growth, synaptogenesis, and delayed cerebral atrophy, leading to further damage (Li et al., 2017). However, many of the physiological and mechanistic properties of this phase are not well understood. It is imperative that the long-term pathophysiology of HIE is better studied to ensure that new therapeutic strategies are able to act on the chronic pathological changes observed in HIE.

Therapeutic Treatments for Hypoxic-Ischemic Encephalopathy

The therapeutic window for preventing brain injury after HIE is extremely narrow, lasting only a few hours after the initial event, which explains the necessity for applying preventative therapies, such as therapeutic hypothermia, within the first six hours after birth (Lemyre & Chau, 2018; Vannucci & Perlman, 1997). In order to prevent the most damage, treatment must be started as early as possible within the latent phase (Gunn & Thoresen, 2019).

The current standard of care for treating HIE is therapeutic hypothermia. In addition to reducing oxygen demand, hypothermia therapy was found to decrease energy depletion, reduce glutamate levels, and decrease apoptosis (Roka & Azzopardi, 2010). In animal models of HIE, hypothermia therapy was found to reduce neuronal cell loss in the cerebral cortex, hippocampus, and thalamus, compared to animals that did not receive cooling (Gunn et al., 1997). Hypothermia therapy involves cooling either the head or the entire body of the infant to a temperature of around 33.5°C for a duration of 72 hours, followed by steady rewarming (Allen & Brandon, 2011). Meta-analyses looking at randomized controlled trials of up to 3592 newborns showed that treating neonates suffering from HIE with hypothermia within the first six hours of life significantly reduced risks of mortality, neurodevelopmental disability, cerebral palsy, cognitive impairments, and psychomotor impairments (Abate et al., 2021; Jacobs et al., 2013). Despite the beneficial effects of hypothermia therapy, approximately 30% of neonates with HIE that undergo hypothermia therapy still develop neurodevelopmental impairments by 18 months of age. In addition, treatment with hypothermia did not significantly reduce the rates of death or disability in neonates with severe cases of HIE (Edwards et al., 2010). Hence, the need for new treatments to improve survival rates and the neurodevelopmental outcome is still crucial for asphyxiated neonates. It is also critical that new treatment candidates provide neurorestoration, in addition to neuroprotection, to help repair any damage that has already occurred before neuroprotective therapies are initiated.

Effect of Sildenafil in Adult Stroke

Sildenafil citrate is a phosphodiesterase-5 (PDE-5) inhibitor that works by inhibiting cyclic guanosine monophosphate (cGMP) and promotes repair mechanisms in adult stroke animal models. Specifically, sildenafil enhances neurogenesis, oligodendrogenesis, and reduces neurological impairments (Zhang et al., 2012; Zhang et al., 2002). Eighteen-month-old rats treated with 3 mg/kg/dose of sildenafil subcutaneously for 7 consecutive days, starting a week after focal cerebral ischemia was induced, significantly improved functional recovery compared to saline-treated rats (Zhang et al., 2006). Sildenafil also increased the number of minichromosome maintenance protein-2-positive (MCM-2^+) cells and $Ki67^+$ cells (markers of proliferating cells), compared to saline-treated rats. Doublecortin (i.e., a marker of migrating neuroblasts) expression was also increased in sildenafil-treated rats compared to control, suggesting neurogenic effects of sildenafil through PDE-5 inhibition in adult ischemic rats (Zhang et al., 2006).

Sildenafil treatment also promotes angiogenesis and increases cerebral blood flow in a rat model of adult stroke. Li et al. (2007) demonstrated this by subjecting three to four-month-old-male Wistar rats to embolic stroke. Twenty-four hours after stroke, rats were given either a subcutaneous dose of 10 mg/kg sildenafil or saline daily for six days to study whether sildenafil can repair the brain after injury. Rats were followed up to six weeks post-stroke. Sildenafil treatment enhanced angiogenesis a week after starting treatment, and an increase in the cerebral blood flow level was found in the ischemic boundary, improving

neurological functional recovery compared to the control group (Li et al., 2007). Functional performance was also significantly increased after sildenafil administration compared to saline-treated rats. Sildenafil also significantly increased angiogenesis in rats with stroke up to six weeks after the insult (Ding et al., 2008). This suggests that sildenafil administration in adult rats can repair brain damage secondary to adult stroke through neurogenesis and angiogenesis. Regarding the safety profile of sildenafil, treatment with sildenafil in both animal models and adult patients with mild to moderate severity of stroke is safe (Silver et al., 2009).

Effect of Sildenafil on the PI3K/Akt/mTOR and Wnt Signalling Pathways

The PI3K/Akt/mTOR pathway is involved in cell survival, cell growth, motility, and proliferation, making it necessary for the survival of neurons (Crowder & Freeman, 1998). After cerebral infarction, neuronal stem cells play a crucial role in the repair process of brain tissue, where endogenous neural stem cells in the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampus differentiate into neurons, oligodendrocytes, and astrocytes to repair damaged tissue (Temple, 1989). In cases where ischemia and reperfusion are present, the PI3K/Akt/mTOR pathway is significantly affected by the ischemic insult, inhibiting the pathway and inducing apoptosis (Koh & Lo, 2015). Since sildenafil increases cGMP levels through PDE-5 inhibition, the increase in neurogenesis seen after sildenafil treatment in adult stroke may be due to its action on the PI3K/Akt/mTOR pathway. Previous research studied the effects of sildenafil

administration on neurogenesis in the cGMP-mediated PI3K/Akt pathway in adult rat progenitor cells from the subventricular zone and showed that incubation of neurospheres (cultures of neuronal stem cells) with sildenafil led to a significant increase in the level of phosphorylation of Akt, and downstream target glycogen synthase 3 (GSK-3) (Zhang et al., 2005). This was associated with an increase in cGMP levels and higher levels of neurogenesis. This suggests that the PI3K/Akt pathway could be important in the neurogenesis seen with sildenafil administration. Furthermore, previous research found that blocking the PI3K/Akt pathway with LY 294002 (PI3K blocker) nullified the sildenafil-induced phosphorylation of Akt and blocked the cell proliferation in the subventricular zone seen after sildenafil administration (Wang et al., 2005). Although sildenafil may lead to neurogenesis through Akt activation, sildenafil's effects on downstream targets, such as mTOR, are poorly understood. Additionally, it is crucial to determine whether these effects can also be seen in the neonatal HI model. The activation of mTOR by Akt has been proven to play an essential role in the regulation of cell proliferation, apoptosis, cell metabolism, and angiogenesis in the brain (LiCausi & Hartman, 2018). mTORC1 and mTORC2 subunits are involved in various functions of metabolism and cell survival, and also play an important role in differentiation and the development of neurons (Szwed et al., 2021). mTORC1 is also a downstream target of Akt, which stimulates growth and proliferation in the cell (Rai et al., 2019).

Another important pathway that is involved in many stages of brain development is the Wnt signalling pathway, responsible for regulating cell proliferation, and differentiation (Jia et al., 2019). Previous research studying the

effects of sildenafil on the Wnt pathway suggested that sildenafil may alter Wnt pathway members and promote a neurogenic response to HI injury (Engels et al., 2017). It is known that the Wnt pathway is involved in the regulation of neuroplasticity following ischemia (Zhong et al., 2019). However, the role the Wnt pathway plays in neonatal HI remains unclear. The Wnt signalling pathway is especially important in early development and is involved in regulating transcriptional and post-transcriptional processes, as well as influencing multiple aspects of hippocampal neurogenesis (Arredondo et al., 2020). Understanding how Wnt is affected by sildenafil in HI rat pups is essential to further our understanding of sildenafil's mechanisms of repair.

Treatment of Hypoxic-Ischemic Encephalopathy with Sildenafil

Sildenafil is already being used in neonates and was proven to be an effective and safe treatment for persistent pulmonary hypertension (Shah & Ohlsson, 2007; Simonca & Tulloh, 2017). However, only a few studies have investigated the effects of sildenafil on the injured neonatal brain after hypoxia-ischemia. One study investigated the effects of sildenafil on P7 Sprague-Dawley rats after inducing hypoxia-ischemia by unilateral carotid ligation and hypoxia (Charriaut Marlangue et al., 2014). Rats were given either an intraperitoneal injection of 10 mg/kg sildenafil treatment or phosphate-buffered saline (PBS) just after HI. Compared to the control group, rats treated with sildenafil showed reduced inflammation, reduced tissue loss, increased cerebral blood flow, and

improved motor coordination from 72 hours and up to 7 days after the hypoxic-ischemic insult.

In our lab, we have previously demonstrated the potential neurorestorative effects of sildenafil after inducing HI (i.e., ligation of the left carotid artery followed by two hours of 8% hypoxia) in P10 Long-Evans rats (Yazdani et al., 2016). Rats were then randomized to receive sildenafil or vehicle orally twice daily, started 12 hours after HI and continued for seven days. HI led to significant impairment in gait on P27 and reduced left hemisphere size on P30. Compared to the sham group, HI rats treated with sildenafil showed reduced neurological deficits, a reduction of damage in the left hemisphere, reduced neuroinflammation, and an increase in the number of neurons near the infarct zone.

A recent review on the use of sildenafil for neonatal brain injury suggested that sildenafil treatment in response to brain injury can modulate and reverse a variety of behavioural impairments, leading to improvements in motor coordination, higher neurological scores, and improved general activity based on pre-clinical studies utilizing animal models of neonatal encephalopathy (Zinni et al., 2021). The authors provided pre-clinical evidence that sildenafil reduced microvessel damage and protected the integrity of the blood-brain barrier in the early stages after neonatal HI (Charriaut Marlangue et al., 2014). The authors also mention the clinical trial in our lab as the only ongoing clinical trial to be studying the use of sildenafil to treat neonatal encephalopathy, with other ongoing or completed clinical trials mainly focusing on using sildenafil to treat adult stroke, strongly suggesting a neuroprotective role for sildenafil in adults (Adelson et al., 2011;

Webb et al., 2021; Kenney et al., 2019; Zinni et al., 2021). The most recent emerging research on sildenafil treatment for neonatal encephalopathy comes from our lab, suggesting neuroprotective roles of sildenafil on cell death, as well as increases in neurogenesis in the subventricular zone to promote the migration of neurons to injured areas in the brain up to three weeks after sildenafil administration, albeit little investigation has been done in this area (Yazdani et al., 2021, Yazdani et al., 2016).

Experiments conducted in our lab were able to demonstrate changes in the expression patterns of various immature and mature neuronal markers in the hippocampus tissue of rats exposed to HI. Using the same Vannucci rat model of HI and method of sildenafil treatment implemented in Yazdani et al. (2016), rats were sacrificed on either P12, P17, or P30, where the left hippocampus was extracted and prepared for analysis. After HI, there was a significant reduction in the expression patterns of both early and late neuronal markers in the ipsilateral hippocampus compared to rats that received the sham surgery (Yazdani et al., 2021). After sildenafil administration, expression levels of these markers were reverted to levels no longer significantly different than the sham rats (Yazdani et al., 2021). Additionally, the effects of sildenafil on early neuronal markers Sox2, Nestin, and doublecortin were seen as early as P12. Since sildenafil was found to also decrease apoptosis after HI insult, it is possible that sildenafil may be playing a neuroprotective role (Yazdani et al., 2021). Similarly, sildenafil's effects on late neuronal markers suggest that sildenafil reverts the effects of HI by promoting normal neural development from neural stem cells to mature neurons back to sham

levels. These findings suggest that sildenafil may be involved in the repair process in the hippocampus after HI injury. The focus now is to gain a better understanding of what other characteristics of repair sildenafil may be leading to in the hypoxic-ischemic brain.

Rationale for Study

It is clear from current research that sildenafil promotes neurogenesis and neurorestoration in adult stroke models, with some evidence for similar effects in neonatal HI. Further studying the role of sildenafil treatment on neurons allows for a more comprehensive understanding of the neurorestorative and neurogenic effects that sildenafil may be causing in neonatal HIE. By investigating how sildenafil affects different characteristics of neurons, its involvement in cell death, and its involvement in cellular pathways such as the PI3K/Akt/mTOR and Wnt signalling pathways, we may improve our understanding of the repair processes after HI in the neonatal brain. This could pave the way for new therapeutic strategies that take advantage of our understanding of the characteristics behind sildenafil's neurorestoration, and hence improve the outcomes of those newborns who suffer from HIE.

Based on this, it was **hypothesized** that **HI insult impairs neurons, decreases cell survival through autophagy and necroptosis in injured areas, and decreases PI3K/Akt/mTOR and Wnt pathway expression, while sildenafil treatment promotes recovery by reverting neuron impairment, decreasing**

the amount of cell death by autophagy and necroptosis, and increasing PI3K/Akt/mTOR and Wnt pathway expression.

To test this hypothesis, the following aims were planned:

Aim 1. Investigate the effects of sildenafil on the expression of markers for neurons, axons, and synapses in HI injured areas. **Aim 2.** Investigate the effects of sildenafil on important markers of necroptosis and autophagy. **Aim 3.** Investigate the effects of sildenafil on the expression levels of the PI3K/Akt/mTOR and Wnt pathways in HI injured areas**.** By studying the effects of sildenafil on these different markers in HI brain tissue, this allows us to focus future research on areas that are most involved in the repair and neurorestoration seen after sildenafil administration in neonatal HI.

Methods

Induction of Term Neonatal Hypoxic-Ischemic Encephalopathy (HIE)

The well-established Vannucci rat model of term neonatal HIE was used. This paradigm combines left common carotid artery ligation followed by placing the rats in a hypoxia chamber for 2 hours at 8% oxygen on postnatal day 10 (P10) to induce HI. Rats were randomly divided into three groups to evaluate the effects of the HI surgery and the effects of sildenafil: sham surgery-vehicle treatment, HI surgery-vehicle treatment, and HI surgery-sildenafil treatment.

Rat pups were initially weighed and were given an intraperitoneal injection of a cocktail consisting of fentanyl (0.2 mg/kg) and midazolam (1 mg/kg). This deeply anesthetized the rat pups and made them unresponsive to noxious stimulation. The rats were then placed on a heating pad set to 42°C. Lidocaine-prilocaine cream (EMLA®; AstraZeneca Inc.) was then applied to the left ventral neck where the incision was made. This was done around 15 minutes before surgery to ensure that the rat pups did not feel the incision when it was being made. After the 15-minute period, a surgical blade was used to make a vertical incision along the left ventral surface of the neck. The left common carotid artery was then located, isolated from nerves and connective tissue around it, and then ligated with a triple-knot suture. Once the unilateral ligation was completed, a drop of splash block (Lidocaine 2%/Bupivacaine 0.5%; 1:1 mix) was added to the incision area. The skin was then closed with an adhesive (n-butyl cyanoacrylate; 3M VetBond Tissue Adhesive®; 3 M USA). The rat pups were then given 1.5 hours to recover from the surgery, where they were placed on a heating pad set to 38°C. The rats,

while still on the heating pad, were then placed in a sealed hypoxia chamber (Plastic Concepts, North Billerica, USA). The sealed chamber was then infused with nitrogen gas until a level of 8% oxygen was reached inside the chamber, as detected by an oxygen analyzer (analyzer model 600, Engineered Systems & Designs, Newark, DE). This was maintained for two hours. Throughout the procedure, the core temperature of the rats was recorded to ensure the rat pups were normothermic. This was done using a rectal probe before the surgery, immediately after occlusion of the left carotid artery, immediately before hypoxia, and right after hypoxia exposure was completed. Rats that underwent the surgical occlusion of the left carotid artery followed by the 2-hour hypoxia were considered the hypoxic-ischemic (HI) group. The control group consisted of rats that followed the identical procedure as the HI group but received a sham surgery with no hypoxia treatment.

Sildenafil Preparation and Administration

Sildenafil was prepared in a similar way to how it is prepared when treating human newborns for persistent pulmonary hypertension. A 100 mg tablet of Viagra® (Pfizer) was crushed using a mortar and pestle until ground to a very fine powder. The fine powder was then suspended in a 50/50 blend of Ora-Blend® suspension media (Perrigo) and sterile water, which results in a 50 mg/ml solution. This is typically done by mixing the fine powder in 1 ml of sterile water and 1 ml of the Ora-blend® suspension media. For vehicle, the 50/50 blend is made but without the inclusion of the sildenafil powder.

Rat pups from both the HI and sham surgery groups were weighed every morning and then administered sildenafil or vehicle twice daily via oral gavage. The first dose was started 12 hours after HI and then given every 12 hours for 7 consecutive days from P11 to P17. Rats in the P12 group only received three doses until they were sacrificed on P12. Rats in the P17 and P30 groups received the full treatment (i.e., 14 doses) before being sacrificed.

Western Blot Analysis

Western blotting was used to quantify expression levels of target proteins in the brain. The left hippocampus and left infarcted cortical tissue were used for protein analysis at the three time-points (P12, P17, & P30). Proteins in the left hippocampus and cortex were analyzed separately to see if sildenafil affects these areas differently. Proteins from each sample were first extracted by combining the tissue with RIPA buffer and protease inhibitor solution. Each sample was then sonicated with a set AMP at 40-50% for a duration of two minutes. Samples were then centrifuged at 12,000 RPM for 20 minutes under 4°C. The supernatant of each sample was collected and used to determine the protein concentration using a BCA assay. 25 uL of standards and of each tissue sample were pipetted into a microplate. The microplate was then read using BioTek Gen5 software to quantify the protein extracts for each sample. The absorbance value of each sample was then used to determine the volume of sample needed to add to the western blot gel in order to have 30 ug of protein for each sample. The gel was then loaded and placed in the gel electrophoresis chamber for approximately 1.5 hours to allow the

proteins to separate according to molecular size. After the run was completed, the gel was then placed in a transfer chamber for a duration of 16 hours at 30 V to transfer the proteins to a PVDF membrane. The membrane was then blocked with milk for one hour, followed by incubation with a primary antibody of interest overnight. The membrane was then incubated with the appropriate horseradish peroxidase (HRP) secondary antibody (goat anti-mouse IgG-HRP (H + L)-HRP conjugate, 1706516 or goat anti-rabbit IgG (H + L)-HRP conjugate, 1706515; dilution 1:3000, BioRad, Hercules, California, USA) for one hour. The membrane was then washed with tri-phosphate buffered saline (TBS) for 30 minutes before detection using enhanced chemiluminescence.

Aim 1 – Neuronal Protein Markers

Various markers were used to investigate the effects of HI +/- sildenafil in the cortex and hippocampus (**Appendix A**). For Aim 1, neuronal nuclear protein (NeuN) (mouse anti-NeuN, MAB377; Millipore, Burlington, Massachusetts, USA; dilution 1:500) was used as a marker for mature neurons (Gusel'nikova & Korzhevskiy, 2015). For cell pathway regulation and cell division, we looked at phosphatase and tensin homolog (PTEN) (mouse anti-PTEN, sc-7974; Alexa Fluor, Oregon, USA; dilution 1:1000) (Lachyankar et al., 2000). To determine whether neuronal changes translated to the axonal level, we measured the levels of the growth-associated protein 43 (GAP-43) (Sheep anti-GAP-43, NBP1-41123; Novus Biologicals, Centennial, Colorado, USA; dilution 1:1000), a protein highly expressed in neuronal growth cones as well as during axonal regeneration

(Jacobson, Virag & Skene, 1986; Chung, Shum & Caraveo, 2020). For the synapse, Glutamate Receptor 1 (GluR1) (mouse anti-GluR1, ab31232; Abcam, Cambridge, UK; dilution 1:1000) was used, which plays a role in synaptic strength via its ligand-gated ion channel (Kopec et al., 2007; Andrásfalvy et al., 2003). Levels of early growth response 1 (EGR1) (rabbit anti-EGR1, 55117-1-AP; Proteintech; Rosemont, Illinois, USA; dilution 1:1000) were tested, which plays various roles in neuron development and integrating complex interactions between genes (Duclot & Kabbaj, 2017).

Aim 2 – Necroptosis and Autophagy

For Aim 2, two important markers of necroptosis were looked at: receptor-interacting protein kinase 3 (RIPK3/RIP3) (rabbit anti-RIPK3/RIP3, NBP1-77299; Novus Biologicals, Centennial, Colorado, USA; dilution 1:1000), and mixed lineage kinase domain-like protein (MLKL) (rabbit anti-MLKL, NBP1-65729; Novus Biologicals, Centennial, Colorado, USA; dilution 1:1000). RIP3 is a receptor protein kinase that, when phosphorylated, binds to MLKL and disrupts the plasma membrane and leads to cell lysis (Yu et al., 2021). Autophagy is a regulated process where cells under stress sequester damaged proteins and organelles into autophagosomes (Menon & Dhamija, 2018). The main autophagic protein studied was Beclin-1 (rabbit anti-Beclin-1, ab207612; Abcam, Cambridge, UK; dilution 1:1000), a protein kinase activated in the first steps of autophagosome formation (Kang et al., 2011).

Aim 3 – Cell Signalling Pathways

For Aim 3, to evaluate the activity of the Pl3k/AKT/mTOR pathway, we quantified levels of phosphorylated protein kinase B (pAKT) (rabbit anti-phospho-Akt Ser473 D9E, 4060; Cell Signaling Technology, Danvers, Massachusetts, USA; dilution 1:1000). We also looked at levels of phospho-mTOR serine 2448 (rabbit anti-p-mTOR Ser2448, 2971; Cell Signaling Technology, Danvers, Massachusetts, USA; dilution 1:1000), which reflect the mammalian target of rapamycin complex 1 (mTORC1) activity; phospho-mTOR Ser2481 (rabbit anti-p-mTOR Ser2481, 2974; Cell Signaling Technology, Danvers, Massachusetts, USA; dilution 1:1000), which is for mammalian target of rapamycin complex 2 (mTORC2) activity, and the levels of mTOR antibody (rabbit anti-mTOR, 2972; Cell Signalling Technology, Danvers, Massachusetts, USA; dilution 1:1000). For the Wnt pathway, we measured levels of wingless-related integration site 3a (Wnt3a) (mouse anti-Wnt-3a, sc-136163; Alexa Fluor, Oregon, USA; dilution 1:1000) to understand its role in neurogenesis after HI (Chang et al., 2020).

Quantification and Data Analyses

ImageJ software (Image Processing and Analysis in Java) (Rasband WS, ImageJ) was used to measure the intensity of the bands on the membrane. The background intensity was first subtracted from the intensity of the target protein in order to remove any variability from the signal generated by nonspecific bands or smears. In order to normalize the western blot data, the target protein signal for each sample was divided by the signal value for the internal loading control (i.e.

Actin; mouse anti-actin, A5441; Millipore, Burlington, Massachusetts, USA; dilution 1:5000). This was done to reduce the variability by technical limitations such as pipetting, transfer conditions, and buffer concentrations. All procedures and techniques were validated by reviewing recent literature on the optimal approaches to western blot methodology and analysis (Pillai-Kastoori et al., 2020; Kurien, 2021). Data was then analyzed in Graphpad Prism using a non-parametric Kruskal-Wallis test to assess statistical significance for any main effect differences. This was followed by Dunn's pairwise comparisons test to analyze differences between the groups. A p value < .05 was considered significant.

Results

The following findings demonstrate the effects of HI +/- sildenafil on neuronal markers, markers of necroptosis and autophagy, and markers for the PI3K/Akt/mTOR and Wnt pathways in the cortex and hippocampus. Graphs with individual data points can be found in **Appendix B**.

Sildenafil alters expression of neuronal markers NeuN, Egr1, and PTEN in the cortex and hippocampus

NeuN expression was significantly decreased to almost undetectable levels after HI compared to the control group at P30 in the cortex (p = .02, **Figure 1**). The HI-sildenafil group showed some rescue of NeuN expression to levels not significantly different than the control group. However, the HI-sildenafil group was also not significantly different from the HI-vehicle group, indicating a trend where sildenafil may rescue some expression of NeuN. No significant differences between groups were found for P12 and P17.

Egr1 expression was tested in the cortex. Egr1 at P12 was significantly decreased after HI compared to the control group (p = .04, **Figure 2**). Sildenafil restored levels to those not anymore significantly different from the control group. At P17, sildenafil treatment significantly increased Egr1 expression compared to both the HI-vehicle group (p = .04) and the control group (p = .03). No significant differences between groups were found at P30.

PTEN expression was tested in the hippocampus. PTEN was significantly decreased after HI compared to the control group at P12 (p = .006) and P30 (p = .01), while the sildenafil-treated group was not anymore significantly different compared to the control group (**Figure 3**). No significant differences between groups were found at P17.

Sildenafil alters marker of axonal growth in the hippocampus

We looked at GAP-43 in the hippocampus. The HI-vehicle group showed a significant decrease in GAP-43 expression after HI at P17 (p = .008) and P30 (p = .01) compared to the control group; no difference was seen at P12 between these two groups (**Figure 4**). After sildenafil treatment, GAP-43 was not anymore significantly different compared to the control group at the different time-points, and was significantly increased compared to the HI group at P12 (p = .005).

Sildenafil alters marker of synaptic strength in the hippocampus

GluR1 was tested in the hippocampus. There was no difference in glutamate receptor 1 (GLuR1) expression between the HI group and the control group at the different time-points. GLuR1 was significantly reduced after HI and sildenafil treatment compared to the control group at P17 (p = .01) and P30 (p = .03); no significant differences were found at P12 (**Figure 5**).

Sildenafil alters markers of necroptosis and autophagy

Markers for necroptosis and autophagy were tested in the hippocampus and cortex. Necroptotic marker MLKL was decreased at P12 in the cortex after HI-sildenafil treatment compared to the HI-vehicle group (p = 0.03); no significant differences were found at P17 and P30 (**Figure 6)**. No changes were found in the hippocampus.

RIP3 was significantly increased at P12 in the hippocampus after HI compared to the control group (p = 0.01, **Figure 7**). After sildenafil treatment, RIP3 was not anymore significantly different from the control group. No other significant differences were found at P17 and P30. No changes in RIP3 expression were found in the cortex.

Beclin-1 was significantly decreased in the hippocampus at P17 after HI compared to the control group (p = 0.01); Beclin-1 was not anymore significantly different from the control group after HI-sildenafil treatment (**Figure 8**). No other significant differences were found at P12 and P30. No changes were found in the cortex.

Sildenafil alters the expression of markers of the PI3K/Akt/mTOR and Wnt pathways in the hippocampus

Markers for the PI3K/Akt/mTOR and Wnt pathways were tested in the hippocampus. pAKT was significantly decreased at P12 after HI compared to the control group (p = .005, **Figure 9**). pAKT was significantly increased at P17 (p =

.003) and P30 (p = .02) after HI-sildenafil treatment compared to the HI-vehicle group.

mTORC1 was significantly decreased at P12 (p = .02) and P17 (p = .008) after HI compared to the control group; mTORC1 was not anymore significantly different after HI-sildenafil treatment compared to the control group (**Figure 10**). No significant differences were found at P30.

mTORC2 was significantly decreased at P12 after HI compared to the control group (p = .001); mTORC2 was not anymore significantly different after HI-sildenafil treatment compared to the control group (**Figure 11**). No significant differences were found at P17 and P30.

Wnt3 expression was significantly decreased at P17 after HI compared to the control group (p = .04); Wnt3 expression was not anymore significantly different after HI-sildenafil treatment compared to the control group. No other significant differences were found at P12 or P30 (**Figure 12**).

Discussion

Overall, the present study demonstrated that injury from HI in a rat model at term-equivalent age leads to significant impairment in the neurons and in the signalling pathways found in the hippocampus and cortex. Sildenafil administration was found to reverse these impairments by improving expression levels to those comparable to the control group. This is consistent with previously reported findings demonstrating the impact of HI in these areas of the brain and the effects of sildenafil treatment to treat HI (Yazdani et al., 2021; Zinni et al., 2021; Yazdani et al., 2016; Charriaut Marlangue et al., 2014).

There was a reduction in the expression of mature neurons in the cortex after HI. Similar changes in mature neuron expression have been previously demonstrated in the hippocampus (Yazdani et al., 2021). In addition, a significant decrease in the number of neurons was found near the infarct boundary zone after HI compared to the control group (Yazdani et al., 2016). Our results thus confirm that HI is impairing neurons in both hippocampus and cortex tissue.

Egr1 was found to be reduced in the cortex after HI. Egr1 is a transcription factor known as an immediate early gene, which is a crucial component that provides the framework for gene x environment interactions (Duclot & Kabbaj, 2017). Egr1 is a major regulator of neuronal activity in the central nervous system (CNS). In adult neural stem cells obtained from mice, increased Egr1 expression led to increases in cell proliferation, and is suggested to be one of the regulatory genes involved in neural stem cell proliferation (Cera et al., 2018). In the context

of HI, neurospheres from P4-P6 rats exposed to oxygen-glucose deprivation showed that greater Egr-1 expression led to an increased number of neural precursors improving recovery after HI (Alagappan et al., 2013). At P12, sildenafil treatment restored Egr1 expression to levels not different than control. At P17, sildenafil increased Egr1 expression to levels higher than the control group, which suggests that sildenafil may thus improve cell proliferation after neonatal HI.

We found that PTEN was reduced in the hippocampus after HI compared to the control. PTEN has been involved in modulating neuronal apoptosis, axon regeneration and synaptic vesicle recycling in axons, and regulating Akt (Zhao et al., 2013). A study by Li (2014) and colleagues examined the role of PTEN after adult ischemic stroke via cerebral artery occlusion in 3-month-old rats. Levels of PTEN were found to be significantly reduced as early as one hour after ischemia (Li et al., 2014). PTEN knockout mice also had a significantly greater lesion size seen 10 days after ischemia (Li et al., 2014). Sildenafil treatment after HI may restore PTEN levels which may translate into less neuronal apoptosis and improved axon regeneration. These changes thus contribute to the decreased infarct size observed in this context (Yazdani et al., 2016).

GAP-43 has been associated with axonal growth and is widely distributed in the rat CNS (Chung et al., 2020; Jaccobson, Virag, & Skene, 1986). We demonstrated that HI impaired axonal growth by lowering levels of GAP-43 in the hippocampus. This is similar to previous studies that have reported injury to axons in the hippocampus evident 24 hours after HI in the mouse Vannucci model (Stone et al., 2008). We also found that sildenafil treatment after HI restored the

expression of this marker. This has been previously demonstrated in an adult mice model of multiple sclerosis, where sildenafil treatment led to preserved axon ultrastructure (Nunes et al., 2012). Our study suggests that impaired axonal growth may be restored with sildenafil treatment in the neonatal HI model.

GLuR1 has an established role in synaptic plasticity, where its insertion into the synaptic membrane plays part in increasing synaptic strength, helping to promote synaptic plasticity in the hippocampus (Kopec et al., 2007). In the context of neonatal HI, GluR1 was found to play a protective role by activating Akt signalling, as well as reducing cell death via apoptosis attenuation (Huang et al., 2018). Administering sildenafil after neonatal HI led to a significant decrease in the expression of GluR1 in the hippocampus compared to the control group. This is in agreement with previous findings, where a pharmacological agent Glyzargin, which decreases GluR1 levels, was found to improve learning ability and reduce neurotoxicity in rats with mild controlled cortical impact (Danilenko et al., 2012). Sildenafil treatment has also been shown to reduce GluR1 expression in the hippocampus of rats with hepatic encephalopathy, which was associated with lower neuroinflammation and improved spatial learning (Hernandez-Rabaza et al., 2015). These effects were visible at P17 and P30; similarly, increases in pAKT levels in the sildenafil-treated group were found at the same time-points, suggesting a possible association between the two markers. Previous studies have highlighted that GluR1 activity may be related to the activation of the Akt signalling pathway (Huang et al, 2018). It has also been suggested that reduced levels of GluR1 may be a consequence of increased interleukin-1 beta (IL-1β), where IL-1β

exposure reduced expression of GluR1 in hippocampal neurons, and an IL-1β antagonist prevented this reduction (Lai et al., 2006). IL-1β is found to be increased in the first 24 hours of life in HIE infants compared to control, and is correlated with the severity of brain injury seen (Aly et al., 2006). Further studying the relation between IL-1β and GluR1 after HI and sildenafil treatment and its impact on synaptic plasticity and Akt pathway activation may improve our understanding of sildenafil's effects.

We also demonstrated the consequences of HI on cell death and sildenafil's impact on necroptotic and autophagic pathways in the cortex and hippocampus. In the cortex, we found that MLKL was significantly reduced after HI and sildenafil compared to HI without sildenafil treatment. These findings are in agreement with previous results in a rat model of neonatal HI, where after injury was induced at P10, MLKL inhibition improved brain damage (Qu et al., 2016). MLKL is the main executor of necroptosis. Increased cell stress causes the protein RIP1 to activate RIP3 which, in turn, leads to the phosphorylation of the MLKL protein (see **Figure 13**). This causes MLKL to oligomerize, leading to necroptosis by increasing the secretion of chemokines and cytokines. This attracts immune cells to destroy the damaged neurons. Sildenafil's effects on decreasing MLKL after HI may be decreasing the activity of this pathway, leading to less necroptosis in the cortex.

Necroptosis was also affected in the hippocampus, where RIP3, an important regulator of MLKL, was significantly increased after HI. Increases in RIP3 are known to indicate higher levels of necroptosis (Moriwaki & Chan, 2013).

In vitro studies looking at HT-22 (immortalized mouse hippocampal cell line) cells also demonstrated an increase in RIP3 expression levels after energy depletion from oxygen and glucose deprivation (Yang et al., 2017). Sildenafil-treated rats did not show the same increase in RIP3 seen after HI, indicating once again that sildenafil may play a role in reducing necroptosis.

Interestingly, the necroptotic pathway appeared to be affected differently in the hippocampus and cortex. Changes to RIP3 expression were found in the hippocampus, but not in the cortex. A study by Nikseresht and colleagues (2017) looked at patterns of necroptosis in the hippocampus and cortex after a 15 ug intracerebroventricular injection of bacterial lipopolysaccharide (LPS) to induce necroptosis in rats. In the hippocampus, RIP3 levels were highest 24 hours after LPS injection, and returned to baseline levels 96 hours after. While in the cortex, an increase in RIP3 levels was found eight hours after injection, lasting for 24 hours before returning to baseline levels 48 hours after LPS was injected. Furthermore, after LPS exposure, apoptosis and necroptosis were shown to start simultaneously in the hippocampus, while apoptosis started before necroptosis in the cortex (Nikseresht et al., 2017). These results suggest that changes to RIP3 may be location- and time-dependent and may explain why we did not find any effect in the cortex at P12.

We also found that HI led to lower levels of Beclin-1 at P17 in the hippocampus. This is in line with previous findings, where P7 rat pups with HI injury had significantly lower Beclin-1 expression 5 days after injury was induced (Carloni et al., 2008). Beclin-1 plays a crucial role in autophagy by interacting with several

co-factors to regulate the crosstalk between autophagy and apoptosis (Kang et al., 2011). In HI rat pups, blocking autophagy with wortmannin in the hippocampus decreased Beclin-1 expression and increased necroptosis (Carloni et al., 2008). Lower levels of Beclin-1 expression after HI could indicate the brain's response to injury to favour necroptosis over autophagy (Seo et al., 2020). Sildenafil administration restored levels of Beclin-1 to those not significantly different from the control group. Similar effects of sildenafil have been found in a mouse model of multiple sclerosis (Duarte-Silva et al., 2021). Subcutaneous injections of 25 mg/kg sildenafil given to mice three days after experimental autoimmune encephalomyelitis (EAE) led to increased beclin-1 levels compared to control, preventing the progression of motor dysfunction and alleviating EAE (Duarte-Silva et al., 2021). Overall, these results suggest that sildenafil may be reducing cell death by decreasing necroptosis and promoting autophagic pathways after HI.

The activation of the PI3K/Akt/mTOR pathway plays an important role in increasing neurogenesis and reducing neuroinflammation, leading to improved cell survival (Licausi & Hartman, 2018). Blocking the mTOR pathway with rapamycin decreased cell proliferation and increased neuronal apoptosis in P10 rats exposed to HI injury via right carotid artery ligation and 2.5 hours of 8% oxygen compared to rat pups that did not receive rapamycin (Chen et al., 2012). mTOR has two multiprotein complexes composed of different binding proteins to regulate cell growth and metabolism, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Jhanwar-Uniyal et al., 2019). mTORC1 activation promotes

differentiation by producing new neuroblasts from neuronal stem cells in the neonatal subventricular zone (Mahoney et al., 2016). In the HI model, increased mTORC1 activity through GSK-3β inhibition reduced axonal injury from HI and increased synaptogenesis in P10 rat pups, improving recovery (Xiong et al., 2018). Activation of mTORC2 is known to improve cell survival after cellular stress by regulating apoptosis (Zou et al., 2015; Kazyken et al., 2019). In our study, HI decreased levels of pAkt, mTORC1 and mTORC2 in the hippocampus, suggesting that HI dysregulated the PI3K/Akt/mTOR pathway. Treatment with sildenafil after HI improved expression levels to levels no longer significantly different from the control group. Thus, sildenafil may be activating the PI3K/Akt/mTOR pathway leading to the recovery observed after HI (Yazdani et al., 2021). Increased pAkt levels have been found to rescue cells from apoptosis in the hippocampus and cortex (Zhou et al., 2000). In adult rat subventricular progenitor cells, sildenafil administration was found to increase pAkt levels, which was associated with increased neurogenesis (Wang et al., 2005). When a PI3K blocker (LY 294002) was introduced with the sildenafil treatment, levels of pAKT significantly dropped to control levels. LY 294002 also blocked sildenafil increased proliferation in the subventricular zone, further confirming a role for sildenafil in PI3K/Akt/mTOR pathway activation (Wang et al., 2005).

The Wnt signalling pathway is one of the mechanisms highly involved in early development and in CNS maturation (Oliva et al., 2018). By binding to frizzled receptors, Wnt activates the canonical and noncanonical Wnt/β-catenin pathways and regulates neurogenesis at different stages of growth (Arredondo et al., 2020).

Wnt3 is one of the key molecules in Wnt signalling, responsible for regulating neural stem cell differentiation in the hippocampus (Xu et al., 2020). Overexpression of Wnt3 was found to be sufficient to increase neurogenesis in the hippocampus in both *in vivo* and *in vitro* experiments, and blocking Wnt almost completely reversed these effects (Lie et al., 2005). Wnt3 expression was reduced in the hippocampus after HI, and sildenafil restored levels to those comparable to the control group. These results are in agreement with previous research, where Wnt3 expression was associated with an increased proliferation of neural stem cells in the hippocampus after giving hyperbaric oxygen treatment to rat pups with HI, suggesting a role for Wnt3 in promoting neurogenesis in HI neonatal rats (Wang et al., 2007). Neonatal HI may thus reduce Wnt pathway activation, and sildenafil may promote this pathway to induce neurogenesis.

The western blot analyses done in this study were conducted on the entirety of the ipsilateral cortex and hippocampus to the HI injury. However, the level of injury from HI can vary in different affected parts of the brain. Chavez-Valdez and colleagues (2018) demonstrated this by investigating the morphology of different parts of the hippocampus (CA1, CA3 and the dentate gyrus) after implementing the Vannucci model on P10 rats. They found that injury from HI resulted in more cell death in CA1 and CA3 than in the dentate gyrus. HI also led to more extensive necroptotic death in pyramidal neurons than in interneurons (Chavez-Valdez et al., 2018). It has been proposed that areas that are most vulnerable to damage from HI include neurons with higher firing rates, which are associated with higher levels

of metabolism leading to increased levels of reactive oxygen species (Lana et al., 2020). Since pyramidal neurons in CA1 have higher firing rates and hence, increased energy demands than CA3 neurons, CA1 pyramidal neurons seem to be the most vulnerable to damage from HI (Lana et al., 2020). Future research should investigate the different markers analyzed in this study by immunohistochemistry in different areas of the hippocampus to study if there are differences in expression patterns throughout the hippocampus. This will allow us to determine if sildenafil is targeting areas of the hippocampus differently based on differences in severity of damage in the different hippocampal regions.

As with all techniques, western blotting has its limitations. First, western blotting is known to vary in different experimental conditions, where 80% of the variance seen has been attributed to inter-operator variation (Ghosh, Gilda & Gomes, 2014). This can affect the reliability of the results as it may be more difficult to obtain the same results in different lab environments. Second, western blot quantification can be difficult to keep consistent when measuring the levels of the protein of interest along with the loading control on the same blot. This is because housekeeping genes like actin and tubulin are more abundant and hence require a much smaller amount of sample to be loaded than the target proteins (Bell, 2016). This may lead to the oversaturation of the densitometry data that is generated from the housekeeping genes, making it more difficult to identify differences in protein levels in different samples. When possible, this was avoided in the current study by measuring the levels of the loading control (i.e. actin) separately from the target protein to prevent the bands from overexposure.

Nevertheless, it is recommended that future experiments confirm their western blot results by including another experimental method such as enzyme-linked immunosorbent assay (ELISA) or immunofluorescent staining to confirm the reliability of the western blot data.

The results of this study show changes in protein levels in the cortical and hippocampal regions. Western blotting is used to determine protein abundance in a sample. However, how the levels of these proteins changed in different cells within these regions was not measured (Butler et al., 2019). This limitation could be overcome by analyzing levels of protein in specific cell types through immunohistochemistry and immunofluorescent staining. This will allow us to demonstrate how our results are represented in the different cell-types in the cortical and hippocampal regions, expanding further on the impact HI is having in neurons and how sildenafil is repairing these cells after injury.

Conclusion

In conclusion, HI affected neuron growth, cell death pathways and signalling pathways important for cell survival. Sildenafil after HI appears to play different roles in reducing cell death and promoting neurogenesis in the cortex and hippocampus. Sildenafil treatment improved mature neuron growth, neural stem cell differentiation and proliferation, and axon regeneration after they were reduced by HI. Sildenafil also appeared to influence cell survival by inhibiting necroptotic pathways and promoting autophagic regulation, encouraging repair in the brain. Possible cellular pathways responsible for these neuroprotective and neurorestorative effects of sildenafil have also been identified, with sildenafil restoring the activity of the PI3K/Akt/mTOR pathway and the Wnt pathway after they were impaired by HI. Future studies are required to improve our understanding of the mechanisms of repair induced by sildenafil after neonatal HI, before establishing sildenafil as an efficacious treatment for neonatal HIE, with the goal to improve the lives of the many newborns suffering from HIE around the world.

Figures

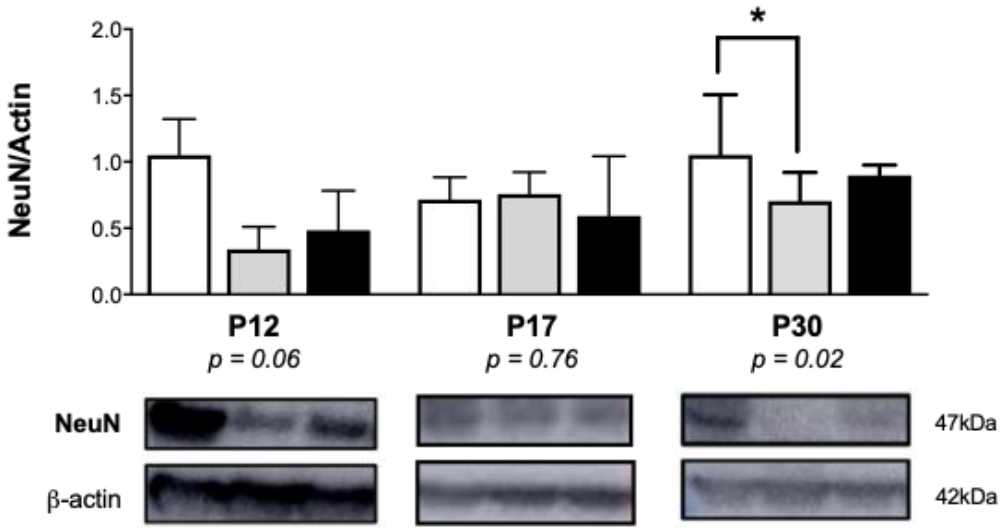

Figure 1. Neuronal nuclear protein (NeuN) expression at P12, P17 and P30 in left cortex tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

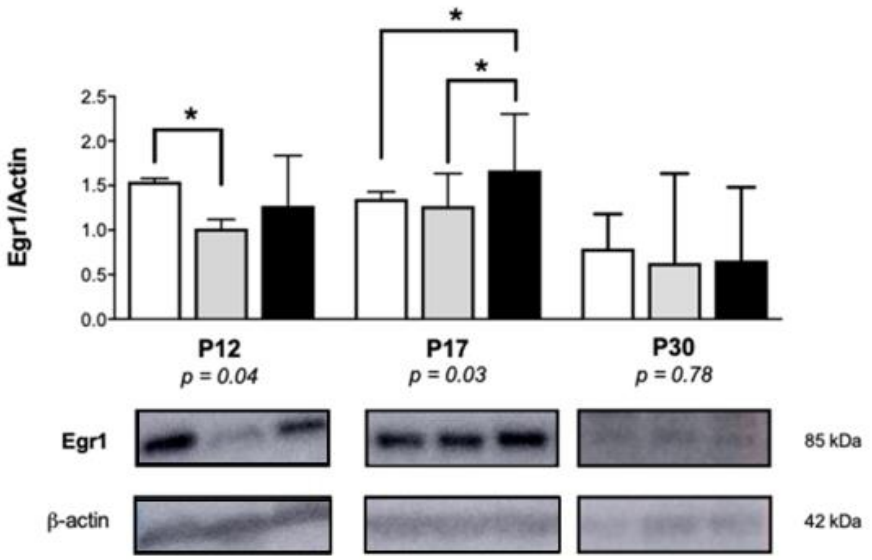

Figure 2. Early growth response protein (Egr1) expression at P12, P17 and P30 in left cortex tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

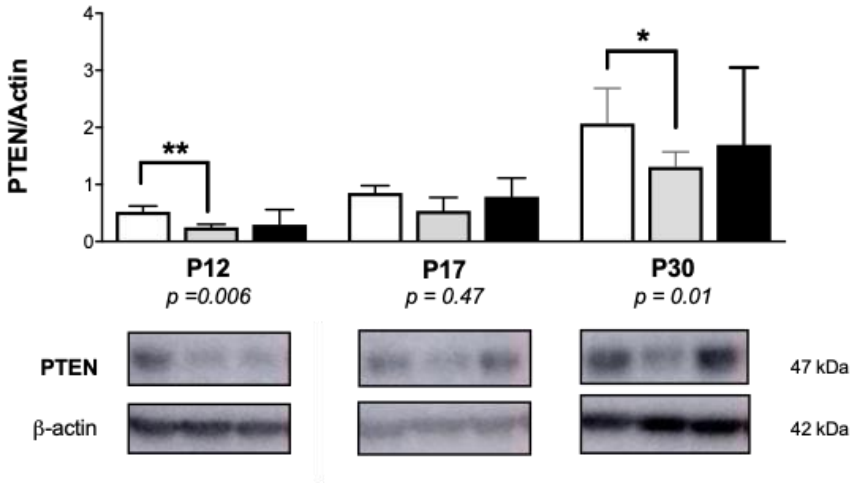

Figure 3. Phosphatase tensin homologue (PTEN) expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

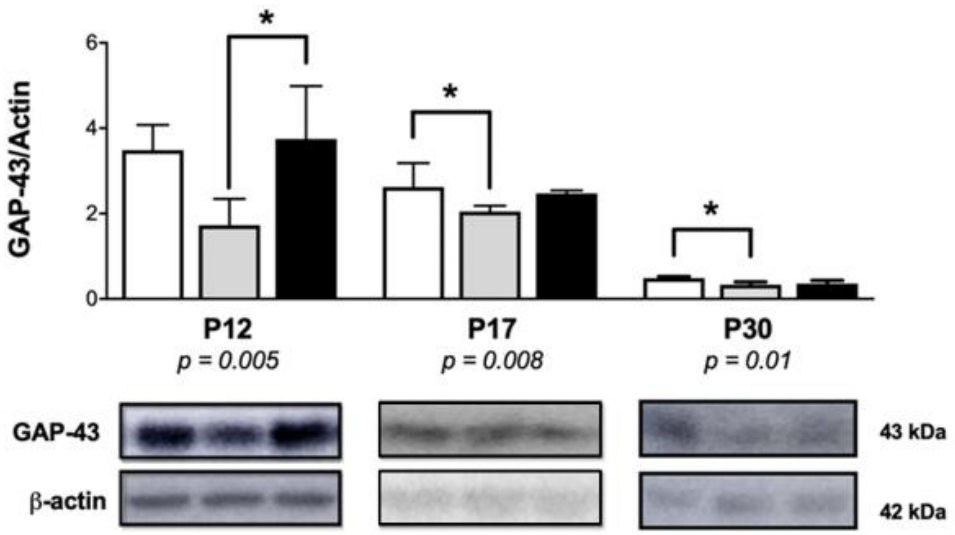

Figure 4. Growth Associated Protein 43 (GAP-43) expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

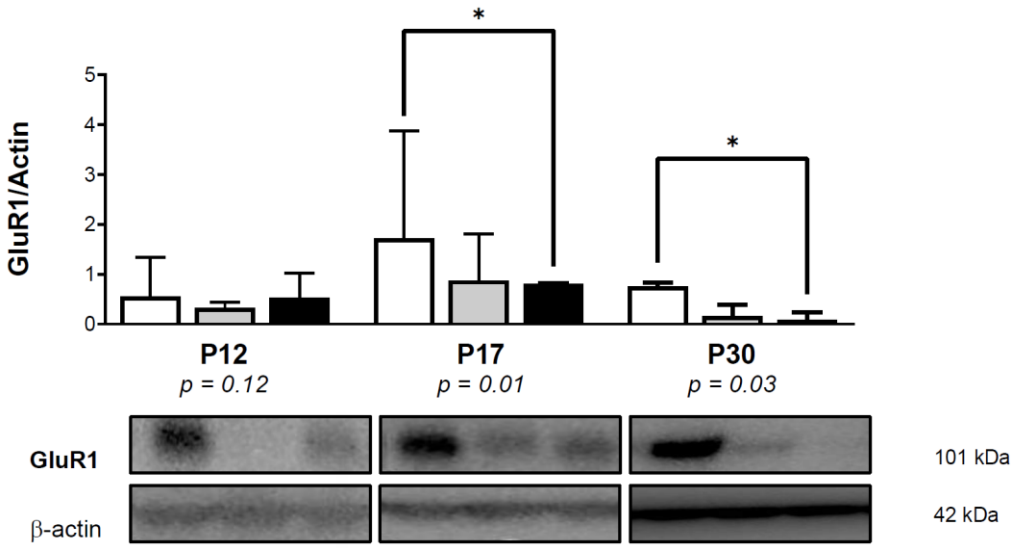

Figure 5. Glutamate receptor 1 (GluR1) expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

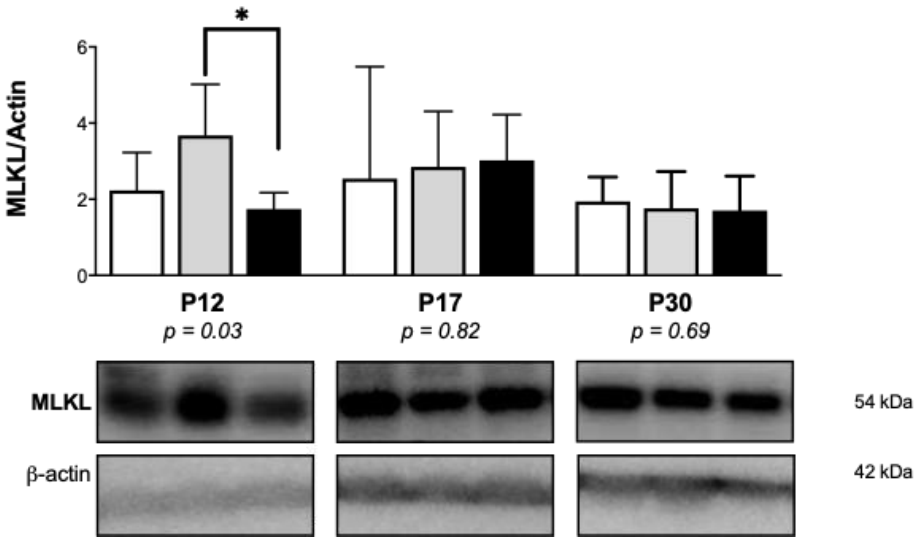

Figure 6. MLKL expression at P12, P17 and P30 in left cortex tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

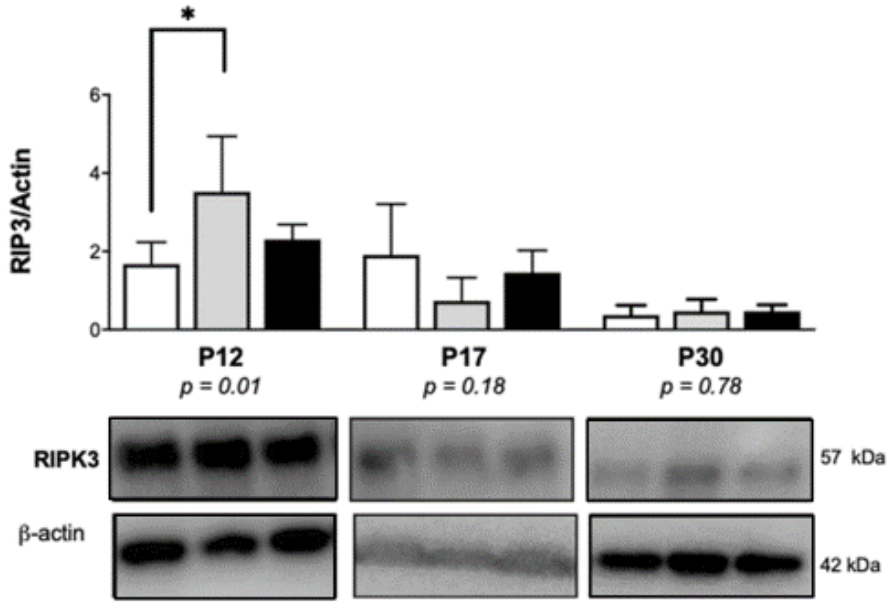

Figure 7. Receptor-interacting protein kinase-3 (RIP3) expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

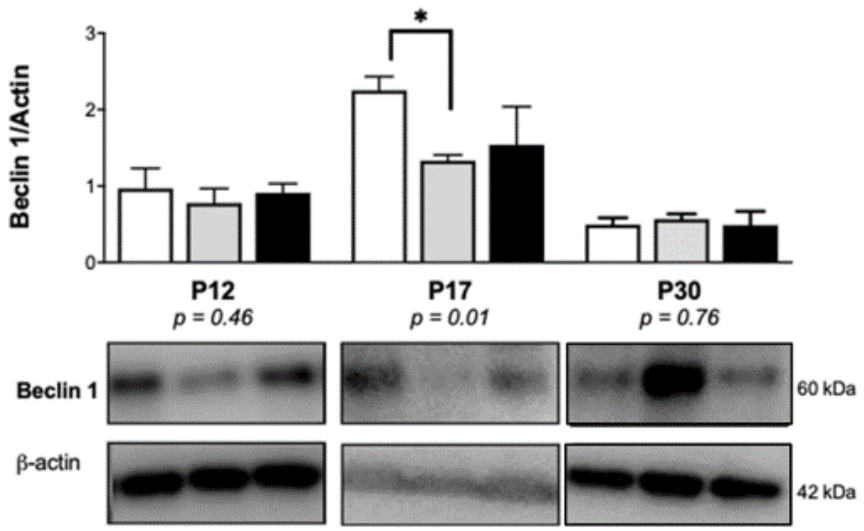

Figure 8. Beclin-1 expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

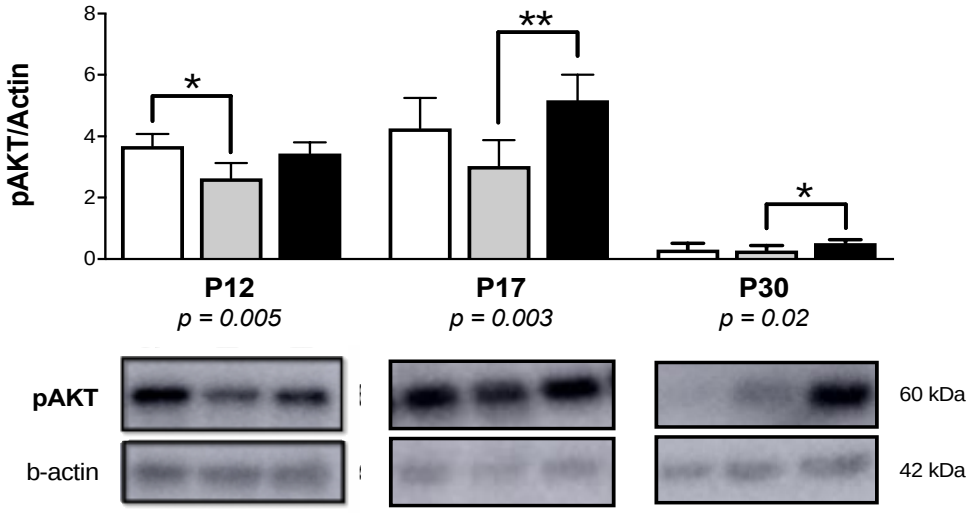

Figure 9. pAKT expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$, ** $p < 0.01$.

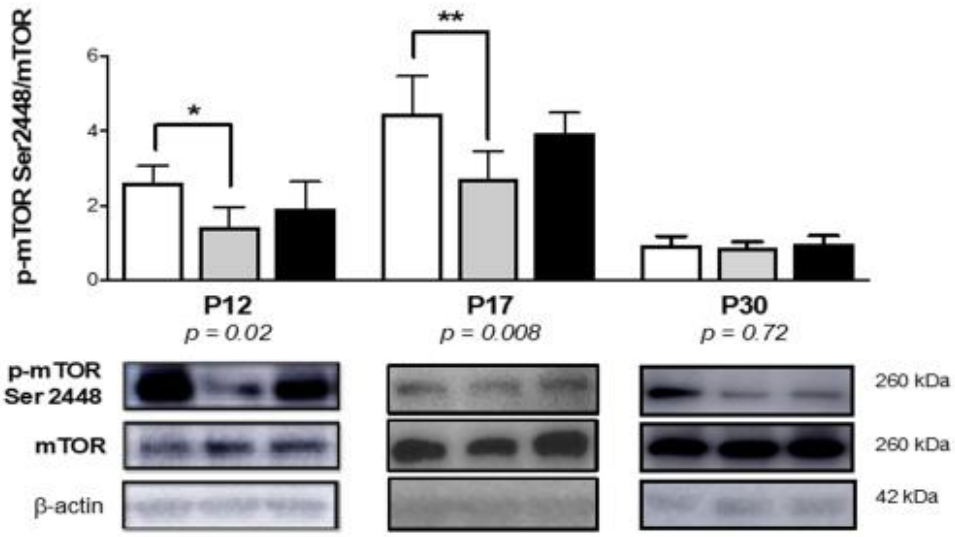

Figure 10. p-mTOR ser2448 (mTORC1) expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$, ** $p < 0.01$.

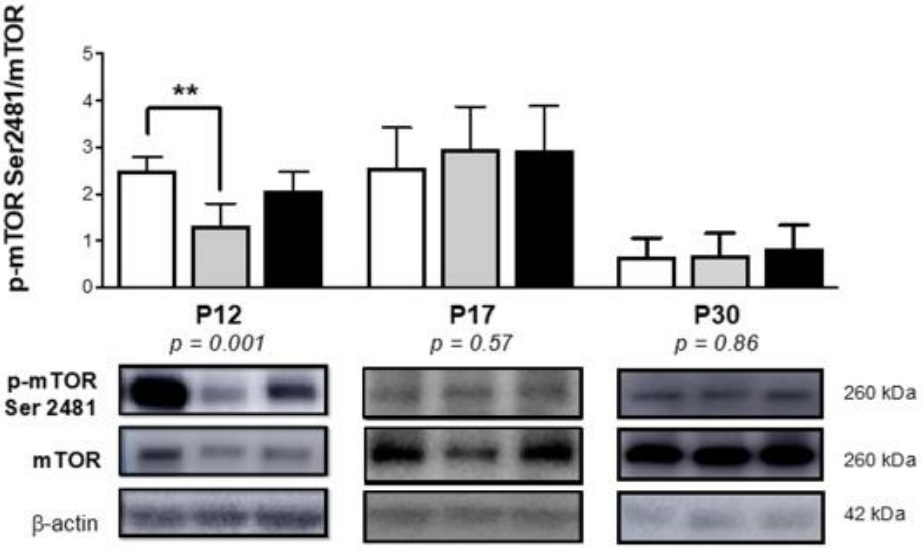

Figure 11. p-mTOR ser2481 (mTORC2) expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: ** $p < 0.01$.

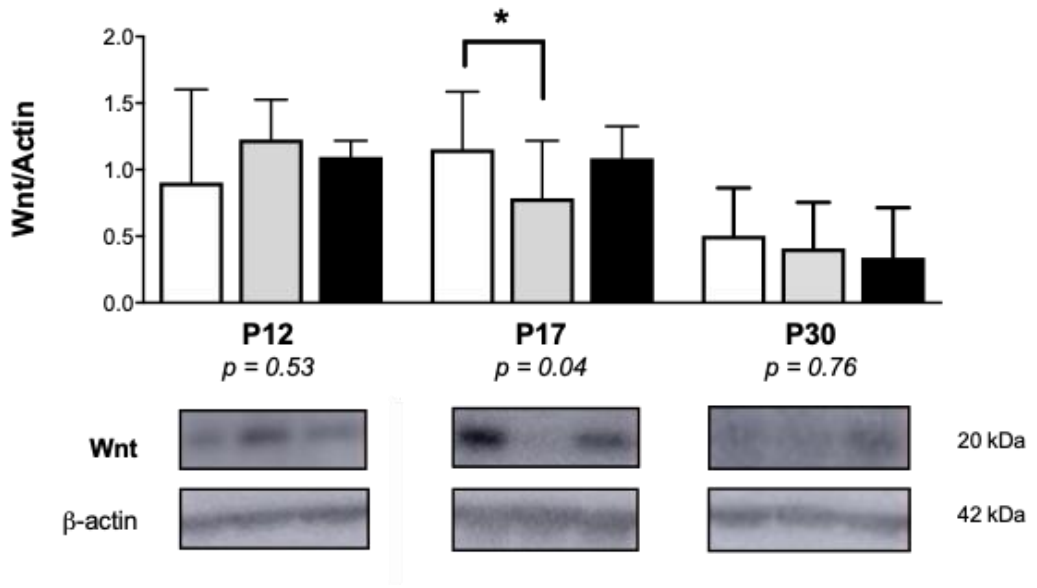

Figure 12. Wingless-related integration site 3 (Wnt3) expression at P12, P17 and P30 in left hippocampus tissue for sham-vehicle (white bars), HI-vehicle (gray bars), and HI-sildenafil groups (black bars). Bars represent the average band intensity of each group ± SD. Bands below provide an example of one sample from each group. Significance: * $p < 0.05$.

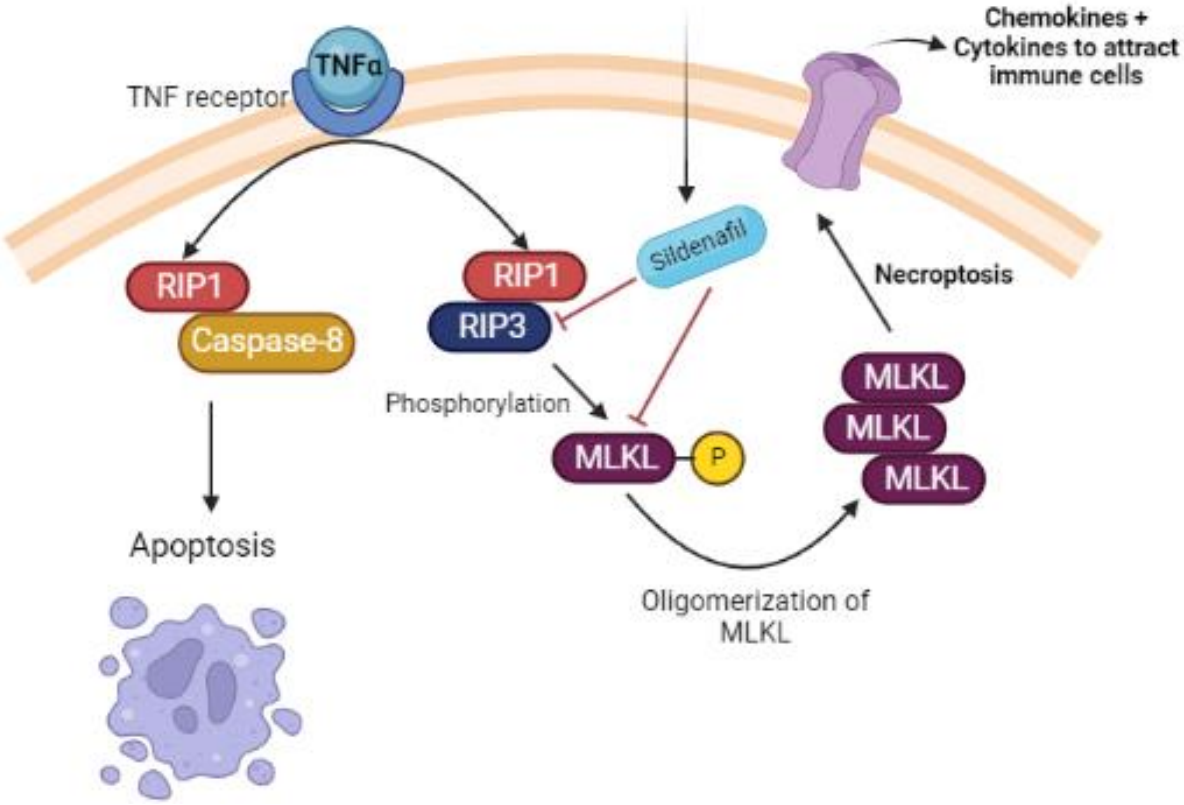

Figure 13. Schematic for sildenafil's proposed effects on the necroptotic signalling pathway in neurons. Abbreviations: *Tumor necrosis factor alpha* (TNFα), *Receptor-interacting protein kinase 1/3* (RIP1/3), *Mixed lineage kinase domain-like protein* (MLKL). Cell stress attracts TNFα which binds to the TNF receptor. This causes the activation of RIP3 by RIP1. Activated RIP3 triggers modifications to the MLKL protein, leading to its phosphorylation. This causes MLKL to undergo oligomerization and integrate into the cell membrane. MLKL triggers necroptosis by secreting chemokines and cytokines which attract immune cells to destroy the neuron. Sildenafil is proposed to inhibit this process by decreasing the expression of RIP3 and MLKL.

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Appendix A

List of Western Blot Markers

Neuronal Markers

Marker	Description	Tissue tested
NeuN	Mature neuron count	Cortex
Egr1	Transcription factor	Cortex
GAP-43	Axonal growth	Hippocampus
PTEN	Neural stem cell differentiation	Hippocampus
GLuR1	Synaptic strength	Hippocampus

Necroptosis and Autophagy

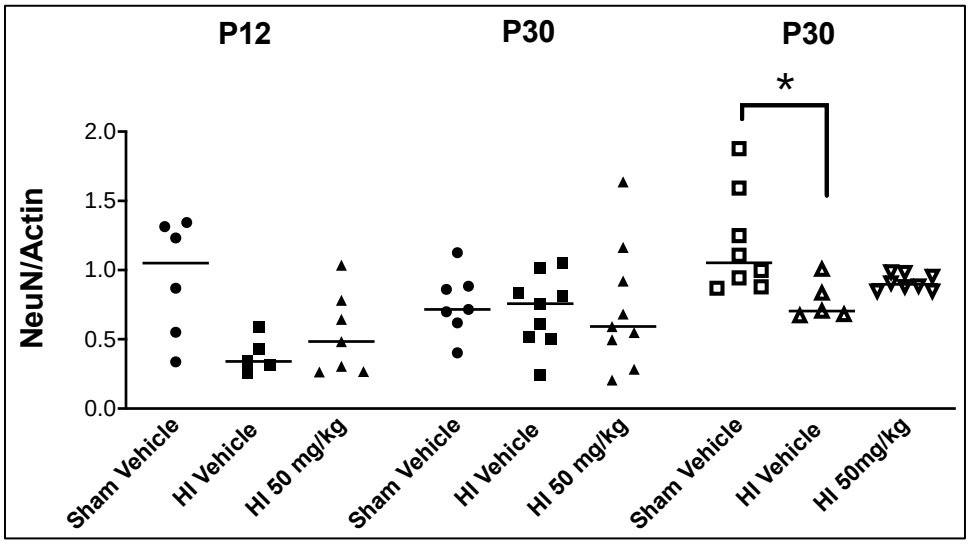
Marker	Description	Tissue tested
MLKL	Main protein in necroptosis	Cortex & Hippocampus
RIP3	Involved in necroptosis regulation	Cortex & Hippocampus
Beclin-1	Autophagy regulation	Cortex & Hippocampus

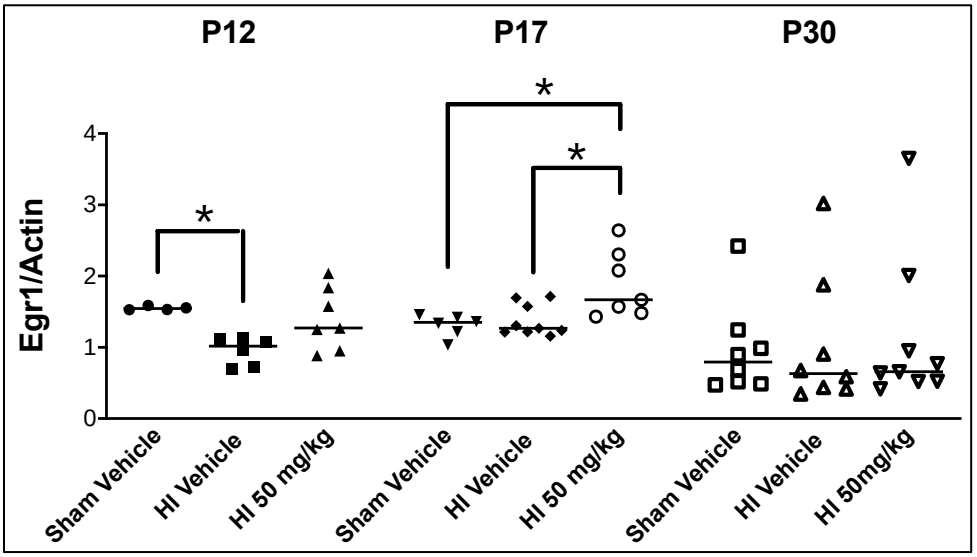
Cellular Mechanisms

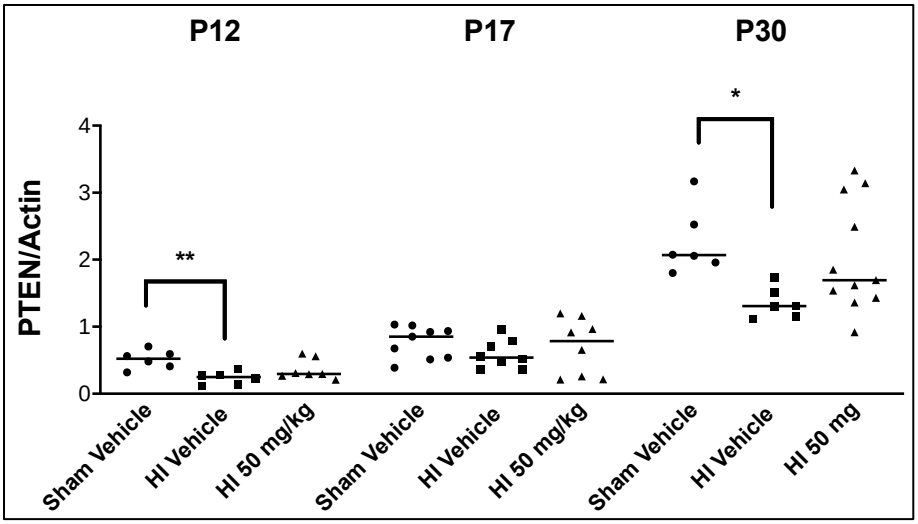
Marker	Description	Tissue tested
Wnt	Neuron differentiation	Hippocampus
pAKT	Early neuronal processes	Hippocampus
mTORC1	Regulates cell metabolism	Hippocampus
mTORC2	Regulates cell metabolism	Hippocampus

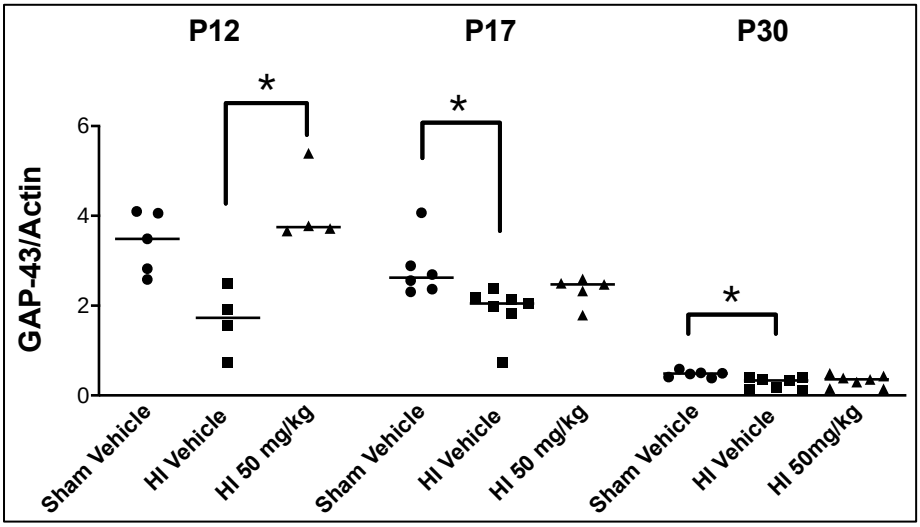
Appendix B

Representation of the results with individual data points. Each data point represents band intensity from one rat. Line indicates the average of each group. * $p < .05$, ** $p < .01$.






P12
P17
P30
PTEN/Actin
0
1
2
3
4
**
*
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50 mg


P12
P17
P30
GAP-43/Actin
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2
4
6
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Sham Vehicle
HI Vehicle
HI 50 mg/kg
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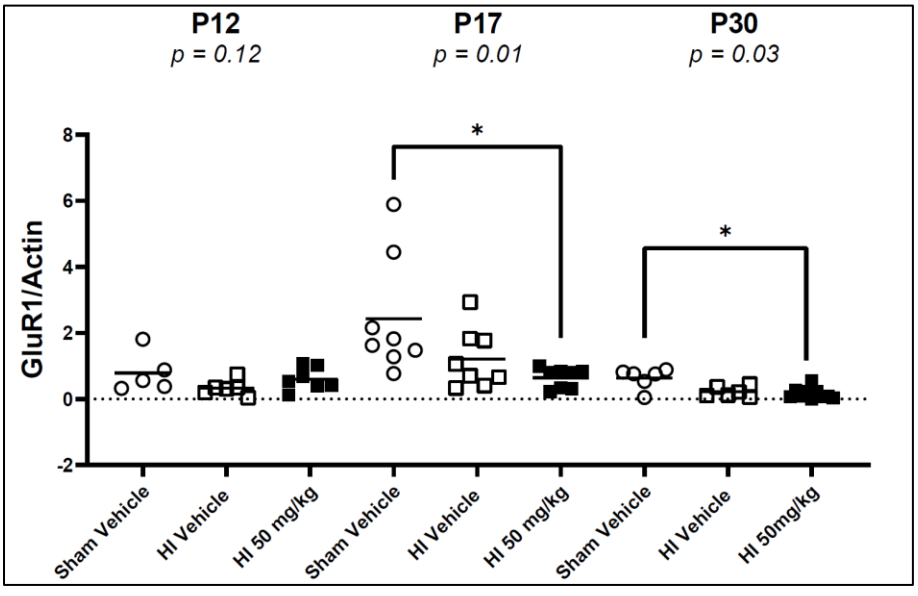
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50mg/kg


P12
p = 0.12
P17
p = 0.01
P30
p = 0.03
GluR1/Actin
-2
0
2
4
6
8
*
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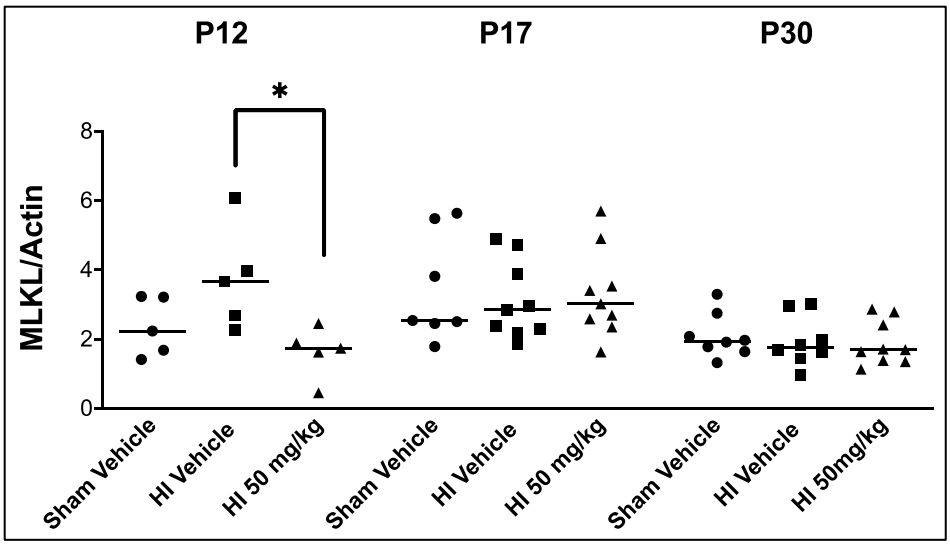
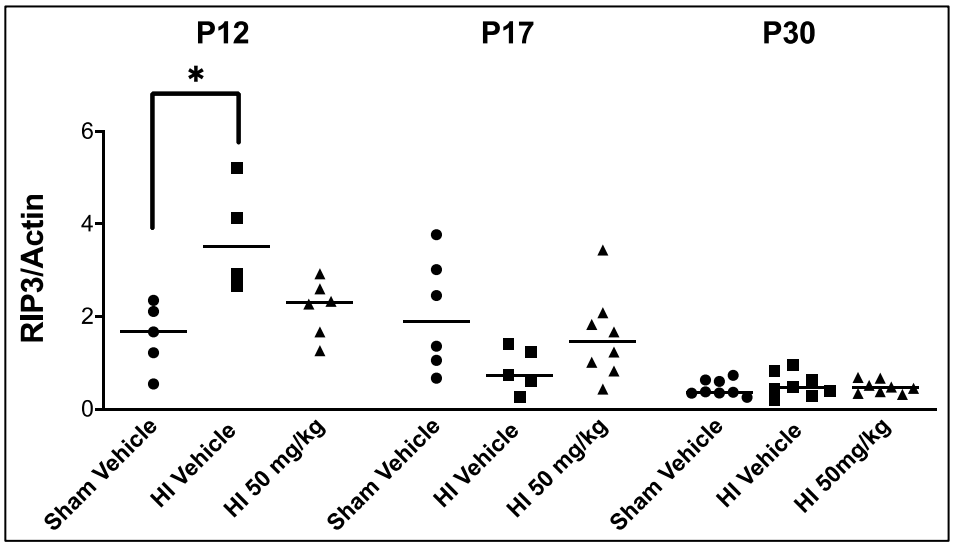
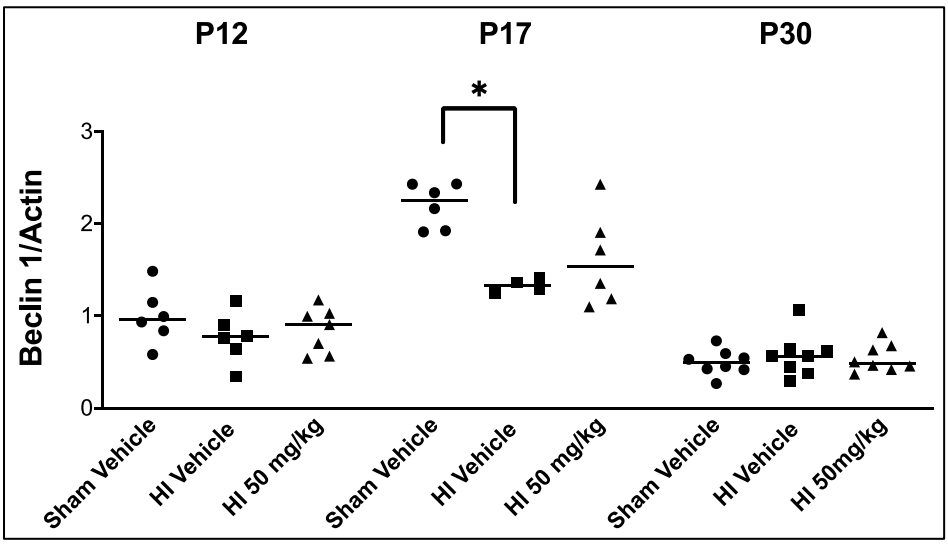
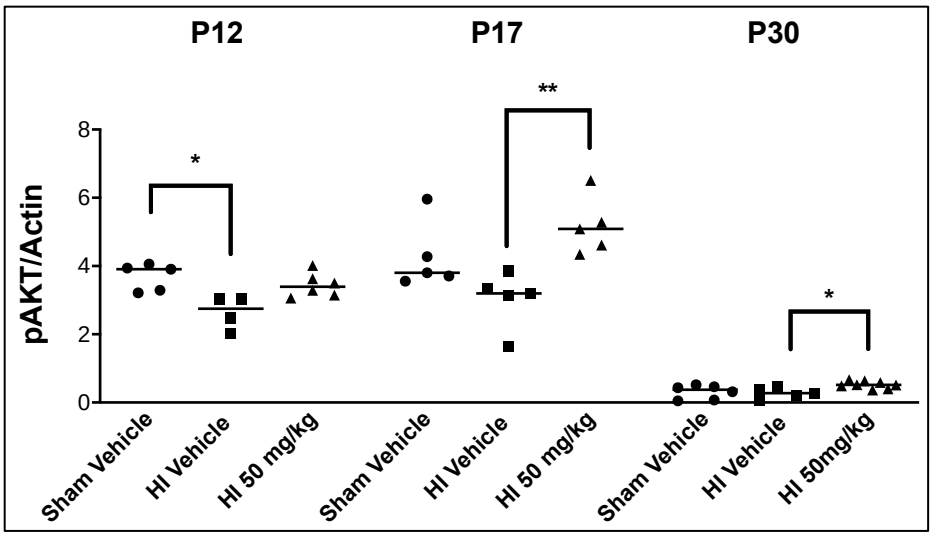
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50mg/kg


P12
P17
P30
*
MLKL/Actin
8
6
4
2
0
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50mg/kg


P12
P17
P30
*
RIP3/Actin
6
4
2
0
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50mg/kg


P12
P17
P30
*
Beclin 1/Actin
3
2
1
0
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50mg/kg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pAKT/Actin
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Sham Vehicle
HI Vehicle
HI 50 mg/kg
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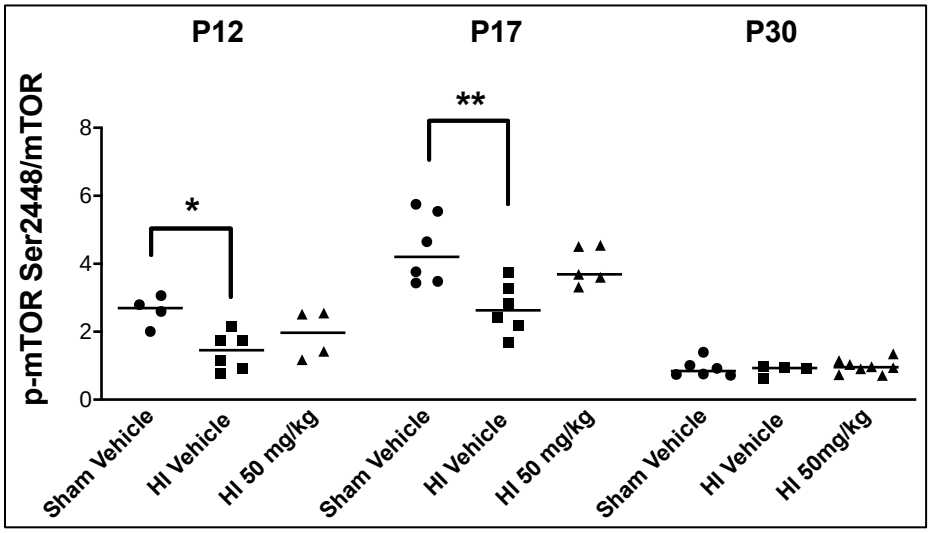
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50mg/kg


P12
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p-mTOR Ser2448/mTOR
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2
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Sham Vehicle
HI Vehicle
HI 50 mg/kg
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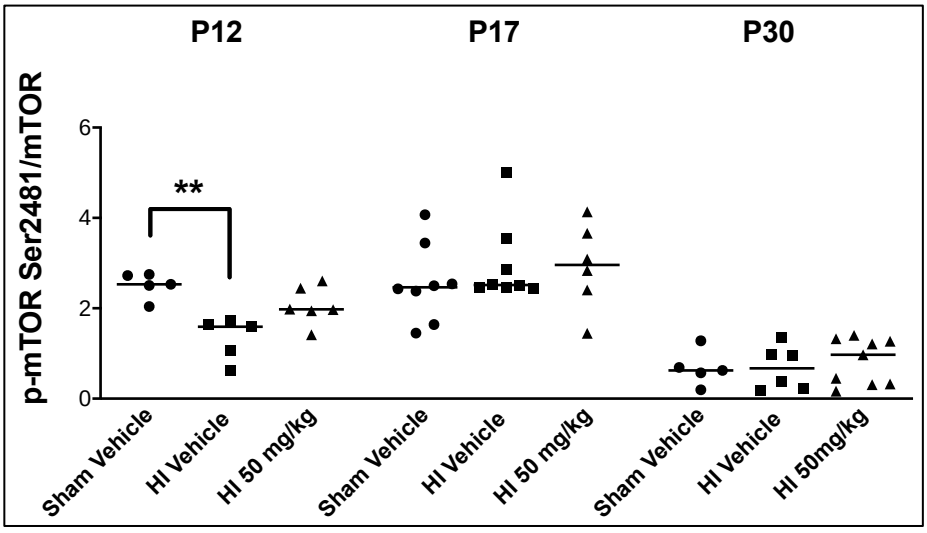
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50mg/kg


P12
P17
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p-mTOR Ser2481/mTOR
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Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
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HI 50 mg/kg
Sham Vehicle
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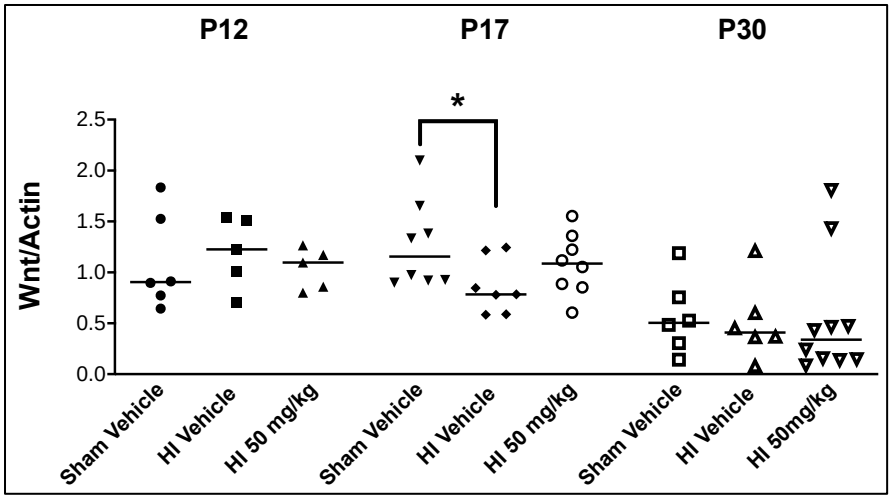
HI 50mg/kg


P12
P17
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Wnt/Actin
2.5
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1.5
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Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
HI Vehicle
HI 50 mg/kg
Sham Vehicle
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