# Syn3 and D-Syn3 attenuate brain infarct volume loss after hypoxic-ischemic brain injury in neonatal rats

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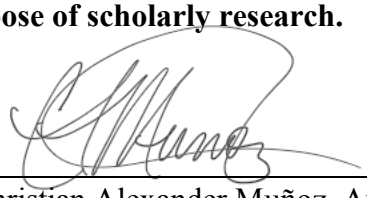
Date 04/24/2024 

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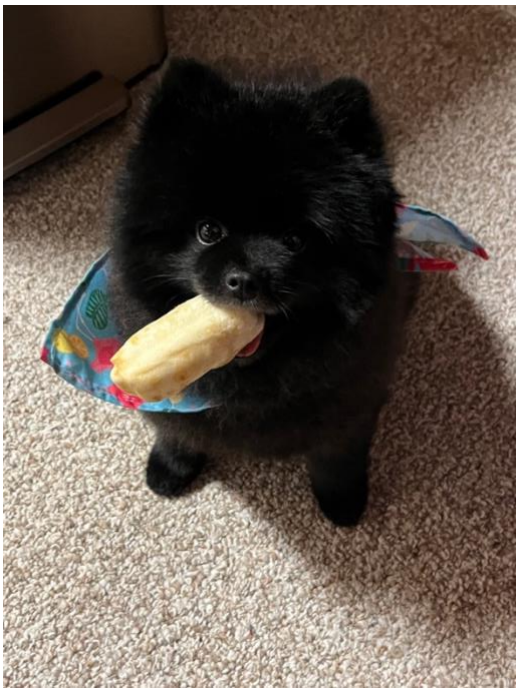
To build a strong structure that will withstand the ages, a strong foundation must be laid. To build a strong foundation there is a necessity to gain wisdom with a steadfast consistency in open-mindedness. This allowance will procure the true growth of the collective into one being. The blossoms that erupt from these converging stories and experiences will create a brilliance that will push humanity forward. From the bottom of my heart, I am appreciative of all the teachings, experiences, observations, and obstacles placed within my path from the individuals that have touched my life in one way or another. I dedicate this project and thesis to my Siti, mother, and my family for always believing in me. Furthermore, I would like to give much thanks to my professors: Dr. Xiaodi Chen, Dr. Diana Horrigan, Dr. John Marshall, and Dr. Jacquelyn Schell, for being patient with me and supporting me when I needed it most. Thank you all and I wish you all nothing but everlasting happiness and success! Last, but certainly not least, I would also like to acknowledge my partner in crime, a secondary shareholder in this degree with me, my pup, Pluto.



While a secondary shareholder, Pluto, also specialized in cuddles, and found himself (potentially against his will) minoring in psychological therapy as well! I could not have done it without everyone on this page and I am beyond grateful for everyone who has contributed to my completion of this project. It was not easy, and I probably made some of these folks pull out their hair, but in the end, I learned many important lessons and I will take them forward with me forever.

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*Abstract of Syn3 and D-Syn3 attenuate brain infarct volume loss after hypoxic-ischemic brain injury in neonatal rats, by Christian Muñoz, ScM, Brown University, May 2024*

Perinatal brain injury (PBI) results from the severe lack of oxygen in the uterus. This can result in brain dysfunction, including hypoxic-ischemic encephalopathy (HIE). PBI affects more than 10,000 infants each year in the United States. Moreover, neonatal HIE brain injury is the leading cause of infant mortality and long-term neurologic disability (e.g., cerebral palsy). Hypothermia, the only approved treatment for HIE, reduces long- and short-term complications from brain trauma or low oxygen. However, this therapy is limited to only treating full-term infants. In this thesis, I investigate how the developing brain responds to hypoxic ischemia (HI) and examine the efficacy of two newly defined neuroprotective and immunomodulatory peptidomimetic drugs, Syn3 and D-Syn3, to attenuate HIE-related brain damage in the neonate. Postnatal day 7 rats were assigned to 4 groups: Placebo (HI-PL n=11), Sham (n=8), HI-Syn3 (n=16), and HI-DSyn3 (n=16). Animals underwent unilateral ligation of the carotid artery followed by administration of 8% oxygen and balanced nitrogen for 90 minutes. 1 mg/kg of Syn3 or D-Syn3 was injected intraperitonially (I.P.) into the rats in group HI-Syn3 or HI-DSyn3 immediately (0-hr abruption), 24-, and 48-hr after hypoxic-ischemic brain injury. Brains were removed, cryo-sectioned, and stained with cresyl violet and analyzed in ImageJ (NIH) to determine infarct volume. Significantly, the infarct volume after HI-brain injury was attenuated (Kruskal-Wallis and Dunn's test, female+males: Syn3, $p$=0.0224; D-Syn3, $p$=0.0067) following treatment with 1 mg/kg b.w. Syn3 or D-Syn3 in male and female neonatal rats.

## Chapter 1: Introduction

### *1.1 Hypoxic Ischemia Encephalopathy*

One of the major causes of neurological disabilities in neonates is hypoxic-ischemia encephalopathy (HIE)(Allen & Brandon, 2011). The HIE rate of incidence is 1 in 6 to 1-8 per 1000 live births in developed nations (Douglas-Escobar & Weiss, 2015; Ristovska et al., 2022). HIE pathophysiology occurs when the brain encounters a decrease in oxygen or blood flow which leads to brain injury, or brain dysfunction (Allen & Brandon, 2011). The magnitude of damage to the brain is dependent on the length of time there was an impaired blood and oxygen flow to the brain. HIE may cause "developmental delay, cognitive impairment, cerebral palsy, or epilepsy, and sometimes these intellectual disabilities are not identifiable until school-age" (Douglas-Escobar & Weiss, 2015).

The number of infants born prematurely before the gestational age of 37 weeks is on the rise, and currently accounts for around 11% of live births (Gopagondanahalli et al., 2016). Pre-term infants are more at risk for complications and serious health issues due to respiratory complications, feeding difficulties, infections, and much more (Gopagondanahalli et al., 2016). Additionally, pre-term infants also have a higher incidence rate for neurological complications including brain injury and HIE (Gopagondanahalli et al., 2016; Ristovska et al., 2022), thus leading to suboptimal neurological development outcomes that impact cognitive, physical, social/ emotional, and behavioral development.

HIE can potentially occur during fetal development in pregnancy, during labor and delivery, or in the postnatal period. Unfortunately, the exact etiology for HIE has yet to be identified. Risk factors that could lead to HIE during pregnancy include placental complications, preeclampsia, gestational diabetes with cardiovascular disease, congenital microbial infections,

drug and/ or alcohol usage, lung and heart malformation, and most likely other causes that have yet to be discovered (Martinello et al., 2017; Rossi & Prefumo, 2019). Potential risk factors for developing HIE during labor and delivery include complications with the umbilical cord, placental abruption or rupture of the uterus, excessive bleeding from the placenta, a prolonged labor, extremely low blood pressure in the mother, and abnormal fetal position in the womb (Martinello et al., 2017; Rossi & Prefumo, 2019). Complications after delivery that may increase the risk of HIE include severe prematurity, severe lung or heart malformations, serious infections, traumatic brain injury, extremely low blood pressure in the neonate, and respiratory failure or cardiac arrest (Boskabadi et al., 2015; Martinello et al., 2017; Rossi & Prefumo, 2019).

The pathophysiology of HIE and the timing of the events make it difficult to identify an optimal window for efficacious treatment of this condition (Iwata et al., 2007; Laptook, 2009). Researchers have identified three phases that are associated with HIE: the primary failure phase, the secondary failure phase, and lastly, the latent phase (Gunn & Thoresen, 2019). Treatment must be delivered during the latent phase when many brain cells show recovery from the insult, which typically lasts 6 hours (Gunn & Thoresen, 2019).

### *1.2 Neuronal Energy Failure*

HIE is an evolving process and is mainly caused by a series of events that begin with an acute perinatal event. During the primary energy failure phase, the hypoxic-ischemic insult promotes anaerobic energy production which leads to prolonged cellular oxygen deprivation (Efstathiou et al., 2017). This phase is also characterized by the depletion of high energy metabolites, such as adenosine triphosphate (ATP) (Efstathiou et al., 2017), and an increased lactate acid production (Allen & Brandon, 2011). With the depletion of these specific metabolites,

mitochondrial membranes begin to depolarize, leading to a chain of reactions, such as intracellular calcium accumulation within neurons, cytotoxic edema, extracellular accumulation of excitatory amino acids, and death of neuronal cells via necrosis (Efstathiou et al., 2017). The magnitude of the primary energy phase is dependent on the severity of the hypoxic ischemic insult. Moreover, the latent period, a quick period of recovery, is also thought to have a time length dependence due to the extent of severity of the hypoxic ischemic insult (Allen & Brandon, 2011). The more serious the insult, the shorter the latent period. The latent period begins with the restoration of blood flow and normal cerebral metabolism (Shalak & Perlman, 2004), and has been deemed the most favorable period for optimal therapeutic interventions (Cotten & Shankaran, 2010). However, the issue that complicates the progression of healing in this type of encephalopathy is that the exact timing of when the phases begin and end and are unknown with regards to severity and other confounding factors.

The exact mechanisms of the secondary failure phase remain unclear, but appear to be associated with oxidative stress, excitotoxicity, and inflammation (Allen & Brandon, 2011). Oxidative stress contributes a large sum of harm to the neonatal brain as the production of free radicals run rampant, causing damage to neuronal cell membranes to produce outcomes of automatic cell death or necrosis. The progression of the fetal brain to a neonatal brain consumes a substantial sum of antioxidants and a high concentration of oxygen, which leads to the decreased ability of the now neonatal brain to combat and eliminate the free radicals (Buonocore & Groenendaal, 2007). Thus, the increased susceptibility leads to an outcome of neuronal tissue damage. Reduced cerebral flow and low ATP levels lead to the release of glutamate and excitotoxicity causing overstimulation of excitatory receptors (Allen & Brandon, 2011). Glutamate plays diverse roles in development and overstimulation of excitatory receptors will subsequently

cause HIE related disruptive effects on vision, somatosensory functioning, learning, memory, and hearing.

*1.3 Hypothermia Therapy to Manage Hypoxic Ischemic Encephalopathy*

There is major opportunity to identify a neuroprotective agent because of the pharmacologic targets that can be found in each phase within the evolving progression of HIE. Hypothermia has become a well-established treatment for asphyxia-related neonatal injury in the past two decades (Tagin et al., 2012). Hypothermia theoretically should be an efficacious treatment as it lowers glutamate levels and free radical production, slows oxygen consumption, and slows necrosis and reduces apoptosis (Allen & Brandon, 2011). Additionally, meta-analysis of clinical trials show that therapeutic hypothermia reduces cerebral injury and improves neurological outcome (Tagin et al., 2012). However, this treatment has not been shown to be translational in preterm infants thus far, and other studies provide mixed results on effectiveness for term neonates (Herrera et al., 2018; Lemyre & Chau, 2018). With garnered real-world evidence, a meta-analysis of 767 infants from multiple studies, including the National Institute of Child Health and Human Development (NICHD) study, and the Total Body Hypothermia (TOBY) study, was used to determine if hypothermia compared to control improved health outcomes for infants with HIE at 18 months (Edwards et al., 2010). The results of the analysis found that there was a reduction in the risk of mortality, severe disability, neurodevelopmental delay, cerebral palsy, and blindness at 18 months for infants impacted by moderate HIE (Edwards et al., 2010). In contrast, individuals with severe HIE damage did not have a significant reduction in death or disability (Edwards et al., 2010). Therefore, it is necessary to continue developing additional treatments for infants who were

not observed in this analysis (e.g., preterm infants), but also to improve infant survival in neonates experiencing severe HIE or infants with baseline developmental outcomes.

*1.4 Syn3 and D-Syn3 as Treatment for Hypoxic Ischemic Encephalopathy*

The Marshall lab has developed Syn3, a neuroprotective and neurorestorative macrocycle peptidomimetic I.P. injectable agent. The purpose of this therapeutic is to provide an intervention on the upregulation of the maladaptive pathways triggered in response to a HI incident. Syn3 was found to target postsynaptic density-95 (PSD-95), a synaptic scaffolding protein, which amplifies brain-derived neurotrophic factor (BDNF)-mediated neuroprotection (Marshall et al., 2017; Marshall et al., 2015). BDNF plays an important role in neuronal growth and apoptosis mitigation, neuronal plasticity, modulates neurotransmitter release, which can play a critical role in learning and memory (Bathina & Das, 2015). BDNF is an endogenous agonist for the tropomyosin-receptor kinase B (TrkB). Activity-dependent release of BDNF and stimulation of TrkB promote the binding of TrkB to the PSD-95 scaffolding protein, forming the PSD-95-TrkB complex, to enhance BDNF signaling (Cao et al., 2013). Some evidence shows that treatment with systemic BDNF is insubstantial due to its brief half-life in serum, poor blood brain barrier penetration, and the activation of the truncated TrkB.T1 isoform, which downregulates TrkB signaling (Cao et al., 2020). However, with Syn3, the cyclic peptide binds to the PDZ3 domain on PSD-95, which enhances the PSD-95-TrkB formation and amplifies BDNF signaling to mitigate neuronal death (Marshall et al., 2017; Marshall et al., 2015). There is evidence that suggests Syn3 enters the central nervous system (CNS) and efficaciously reduces injury in the Vannucci model of HI (Rice et al., 1981).

Advancing forward, the Marshall lab was then able to discover that there was further potential for binding affinity of the Syn3 compound series by studying linear peptide versions than the original cyclic compound because it was more cost effective (Naik et al., 2023). The lab found that substituting Syn3 with D-amino acids could improve binding affinity by 2-fold, and make the new analogue, D-Syn3, less susceptible to proteolysis, which ultimately makes this new compound a more desirable peptidomimetic drug (Naik et al., 2023). D-Syn3 is a novel analogue of Syn3 that substitutes Pro5 for d-Proline residue as well as making the entire arginine tag and cysteines D-amino acids (Naik et al., 2023). Studies of D-Syn3 in animal models by the Marshall lab will be released in separate publications, but the major findings demonstrate that D-Syn3 has comparable binding affinity to the linear SYNGAP peptide (Syn3 without the cyclic component) for the PDZ3 domain on PSD-95 (Naik et al., 2023). This continued research being conducted on Syn3, and D-Syn3 in the Marshall lab is promising and may provide an alternative treatment plan for term infants with HIE and create a new clinical course and treatment plan for preterm infants.

## Chapter 2: Methods

All experimental procedures were first reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Brown University and of Women & Infants Hospital of Rhode Island before the study began.

### *2.1. Preparation of the animal, surgical procedures, experimental design, and autopsy*

Pregnant Wistar rats were purchased from Charles River Laboratories (Wilmington, MA, USA) and were in their 15$^{th}$ or 16$^{th}$ day of gestation. These dames were housed and monitored in a temperature-controlled, 12-hr light/ dark-cycled space with *ad libitum* access to food and water in the Animal Care Facility at Brown University. The delivery date of the rats was designated as the postnatal day (P0). To mitigate intra-litter variability, litter was downsized to a maximum of 10 pups and genders equalized as much as possible on P1.

HI was induced on the pups using the Rice-Vannucci method. Utilizing the labs prior methodology for arranging the groups of neonatal rats, the pups were randomly assigned into one of three groups: Sham-operated controls (Sham), HI placebo-treated (HI-PL), HI Syn-3 or D-Syn-3 treated (HI-Sy3 or D-Sy3). Rat pups were anesthetized with 4% isoflurane and maintained with 2% isoflurane using a Vapomatic Anesthetic Vaporizer (Bickford Anesthesia Equipment, Wales Center, NY, USA) (Barrios-Anderson et al., 2019; Chen et al., 2019; Schuffels et al., 2020). While surgery was being conducted, the pups were placed in a supine position on a heated surgical platform to the body temperatures at 36°C. Continuing to follow the lab's methodology, a 0.5-1 cm vertical incision was made above the suprasternal notch on the right common carotid artery (Barrios-Anderson et al., 2019; Chen et al., 2019; Schuffels et al., 2020). When making the incision, isolation was of top priority as not to damage the trachea, surrounding veins and nerves,

especially the vagus nerve. After the procedure, sterilized 5-0 silk sutures were looped and tied with a double know around the right common carotid artery in the HI-PL and the HI-Syn3 and HI-D-Syn3 groups (Barrios-Anderson et al., 2019; Chen et al., 2019; Schuffels et al., 2020). The same incision was made in the sham group, but the right common carotid artery was not binded (Barrios-Anderson et al., 2019; Chen et al., 2019; Schuffels et al., 2020). Incision in each rat pup was closed and cleaned with betadine and 70% ethyl alcohol (Barrios-Anderson et al., 2019; Chen et al., 2019; Schuffels et al., 2020). Pups were then marked with ink injections on their tails for identification (Neo-9, Animal Identification & Marking Systems, Inc., Hornell, NY, USA). After the ink injections, pups were then returned to the dam for 1.5-2 hrs before exposure to hypoxia.

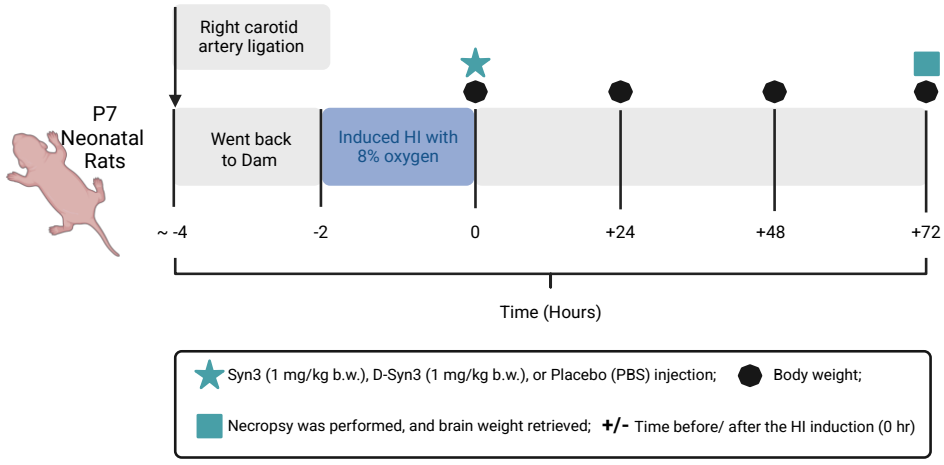


**Fig. 1. Schematic for treatment with Syn3 or D-Syn3**. After the pups had their right carotid artery ligated, they were returned to their dams for around 4 hrs before being exposed to hypoxia for 90 mins at 8% oxygen with balanced nitrogen at a constant temperature of 36°C. Immediately after the hypoxia exposure, the pups were given an I.P. injection of 1mg/kg b.w. of either Syn3, D-Syn3, or placebo (PBS) at 0 hr. The body weight of each subject was measured before the ligation surgery, at 0, 24, 48, and 72 hrs after hypoxia. 72 hr after the hypoxia treatment, the necropsies were performed, and brain weights were retrieved. The +/- signs are indications of time in hours for after and before the termination of hypoxia, respectively. PBS is phosphate-buffered saline.

The Chen/ Stonestreet and Marshall labs have previously shown that exposing the pups to 90 mins of hypoxia (8% oxygen, 92% nitrogen gas) is sufficient in inducing moderate to severe HI-related brain injury in neonatal rats within the HI-PL grouping (Barrios-Anderson et al., 2019;

Chen et al., 2019; Schuffels et al., 2020). The pups were placed in a temperature-controlled airtight chamber (Biospherix, Parish, NY, USA) that would induce the hypoxia ratio labeled above for 90 mins to produce moderate to severe brain damage in the rat pups (Barrios-Anderson et al., 2019; Chen et al., 2019; Schuffels et al., 2020). To monitor body temperature before and during the hypoxia treatment of the litter, a rectal temperature probe (accuracy of 0.1°C; RET-4, Physitemp, Clifton, NJ, USA) was inserted into a non-ligated sentinel neonatal rat. It has been shown that alterations occur to the HI related brain injury in the sentinel rat due to the stress of having the thermometer probed inserted into the rectum, and therefore, the rat pup is no longer included in the experiment (Schuffels et al., 2020). Rectal temperature was recorded every 10 mins during the process of inducing hypoxia, and the chamber was held at a constant temperature of 36 °C (Schuffels et al., 2020). The pups that were within the Sham group were placed in the chamber and given room air for 90 mins.

After exposure to the carotid-ligation surgery and hypoxia for 90 mins, the Syn3 and D-Syn3 experimental groups received one I.P. injection of 1 mg/kg of drug, dependent on rat's group assignment.

Each rat pup was weighed before the ligation surgery and at 72 hr after inducing hypoxia. The subjects were then sedated with a mix of 74 mg/kg of ketamine and 4 mg/kg of xylazine via I.P. injection. The brains were perfused at a flow rate of 3 mL/ min with PBS and a 4% paraformaldehyde (PFA) via cardiac puncture (Schuffels et al., 2020). Brains were extracted, weighed, and placed in PFA for 24 h. Afterwards, the brains were stored in 30% sucrose in phosphate buffer (0.1 M) at 4 °C before cryo-sectioning for infarct volume analysis (Schuffels et al., 2020).

*2.2. Brain sectioning*

The HI-exposed rat pup brains were cut into five sections 2 mm coronal sections using a brain slicer matrix (Zivic instruments, Pittsburgh, PA, USA) to begin the process of measuring infarct volume. The brains were submerged in optimal cutting temperature (OCT) embedding medium (Tissue-Tek, Sakura, CA, USA), then froze the block in a metal container with 2-methylbutane or isopentane (MilliporeSigma) (Chen et al., 2019). The container was put on crushed dry ice to chill the 2-methylbutane. In multiple replicates, the cryosections from a brain were mounted onto gelatin-coated microscope slides (SuperFrost™ Plus; Fisherbrand™, Fisher Scientific International, Inc., Hampton, NH, USA) and stored at -80°C before staining with cresyl violet.

*2.3. Cresyl Violet Staining*

The five 2 mm coronal brain section blocks were sliced down further into histological sections for analysis. Every fourth to fifth cryosection from each block was procured for infarct volume analysis for the different study groups were placed on one slide, five replicate slides were made. These sections were then selected to be stained with cresyl violet for identifying and evaluating HI injury and were then stored at -80°C until ready for staining. When the cryosectioned slides were removed from the freezer, they were left in a fume hood to be air-dried overnight.

To initiate staining protocol, each slide with the cryosections were exposed to 1mL of 1:1 solution of chloroform and 100% ethyl alcohol for 20 mins, the sections were then stained with cresyl violet (0.1%, *w/v*, MilliporeSigma) for 4 mins, and continuously rinsed in Milli-Q water for 1 min. Next, the slides went through a process of differentiation and were exposed to 95% ethyl alcohol for 3-4 mins, dehydrated with100% ethyl alcohol for 3-4 mins, exposed once more with

95% ethyl alcohol for 3-4 mins, and air-dried overnight in a fume hood. The next day, the stained cryosectioned slides would then be mounted with Cytoseal™ XYL (Richard-Allan Scientific™, San Diego, CA, USA).

*2.4. Infarct volume measurement*

To obtain consistent imagery of the stained cryosections, the lab utilized a Micropublishing 6 CCD Camera, (Qimaging, Surrey, British Columbia, Canada). Without having prior knowledge of group assignment, ImageJ (NIH, Bethesda, MD, USA), a software analytical tool, was used to measure infarct volumes of the brains in all the study groups (e.g., sham, HI-PL, D-Syn3, and Syn3). The volumes of all the brains were calculated by multiplying the distance between the five sections of each respective brain. Given the formula, infarct (%) = [1 - (total ipsilateral hemisphere – ipsilateral hemisphere damage)/total contralateral hemisphere)] x 100%, the infarct volume data was determined as a percent ratio of the damaged area within the ipsilateral hemisphere in ratio to the contralateral hemisphere with correction for potential edema in the hemispheres (Barrios-Anderson et al., 2019; Chen et al., 2019; Schuffels et al., 2020).

*2.6. Statistical analyses*

The results found within this thesis are all expressed with mean ± standard deviation (SD). The percent body weight gain over time between the study groups were measured using multiple comparison ANOVA for repeated measures, and the Tukey's Honestly Significant Difference (HSD) test was used as a post hoc test. Brain weight results were normally distributed based on the Shapiro Wilk W normality test. Therefore, the results were analyzed with Kruskal-Wallis and Dunn's test for male+female, and male, and the female group with one-way ANOVA and Tukey's

HSD test. Kruskal-Wallis and Dunn's test were used to analyze the male + female and male rats' infarct volume, and one-way ANOVA and Fisher LSD for female rats' infarct volume. The ROUT method was used to detect outliers. Two outliers were identified in the sham group and one outlier was also discovered in the male D-Syn3 group, and they were removed from the analysis. Statistical analyses for infarct volume and brain weight were completed using GraphPad (GraphPad Software, Boston, MA, USA), and for the change in body weight gain percentages, Statistica (TIBCO, Santa Clara, CA, USA) was utilized. A $p$ value $< 0.05$ was indicative of statistical significance.

## Chapter 3: Results

The purpose of this study was to identify the effectiveness of Syn-3 and D-Syn3 drugs on a HI neonate rat model through measuring infarct volume analysis. The analysis on infarct volume allowed the lab to gather information on HI damage on the ipsilateral side of the brain. The data was obtained by examining a few measurements; body weight at four consecutive 24 hr intervals (i.e., 0 hr, 24 hr, 48 hr, 72 hr); brain weight at extraction at 72 hr after HI, and infarct volume analysis. This chapter presents the results of the data analysis for this thesis' objectives.

### *3.1. Brain and Body Weight Gain after Immediate Treatment with Placebo, Syn3, or D-Syn3 in neonatal rats exposed to severe HI*

In Fig. 2, body weight gain and brain weight gain are plotted separately as a percent and simplified for the whole sample population (i.e., female+male), and then separately, male and female, to distinguish potential gender-based differences in the study cohort. Each group within the study is represented in the figure as Sham, HI-PL, D-Syn3, and Syn3, and the experimental groups were treated immediately after HI exposure.

In brain weight for the total cohort, HI-PL and Syn3 (Kruskall-Wallis and Dunn's test; Male + Female; $p$=1.0000) were more equal in weight and less than the sham group. However, D-Syn3 brain weights were significantly heavier than in the HI-PL (Kruskall-Wallis and Dunn's test; Male + Female; $p$=0.0185) and Syn3 groups (Kruskall-Wallis and Dunn's test; Male + Female; $p$=0.0144), and D-Syn3 was not significantly heavier than Syn3 in the male group but was observed in the female group (one-way ANOVA and Tukey HSD test; Female; $p$=0.0397).

The body weight gain over time was plotted as percentages (%) for all the sub-figures: total sample population, females, and males. The Sham group had the largest increase in body weight

over the time of the study in the total cohort, female, and in the male groups. Moreover, body weight gain was larger in Syn3 and D-Syn3 compared to the HI-PL group across the board.

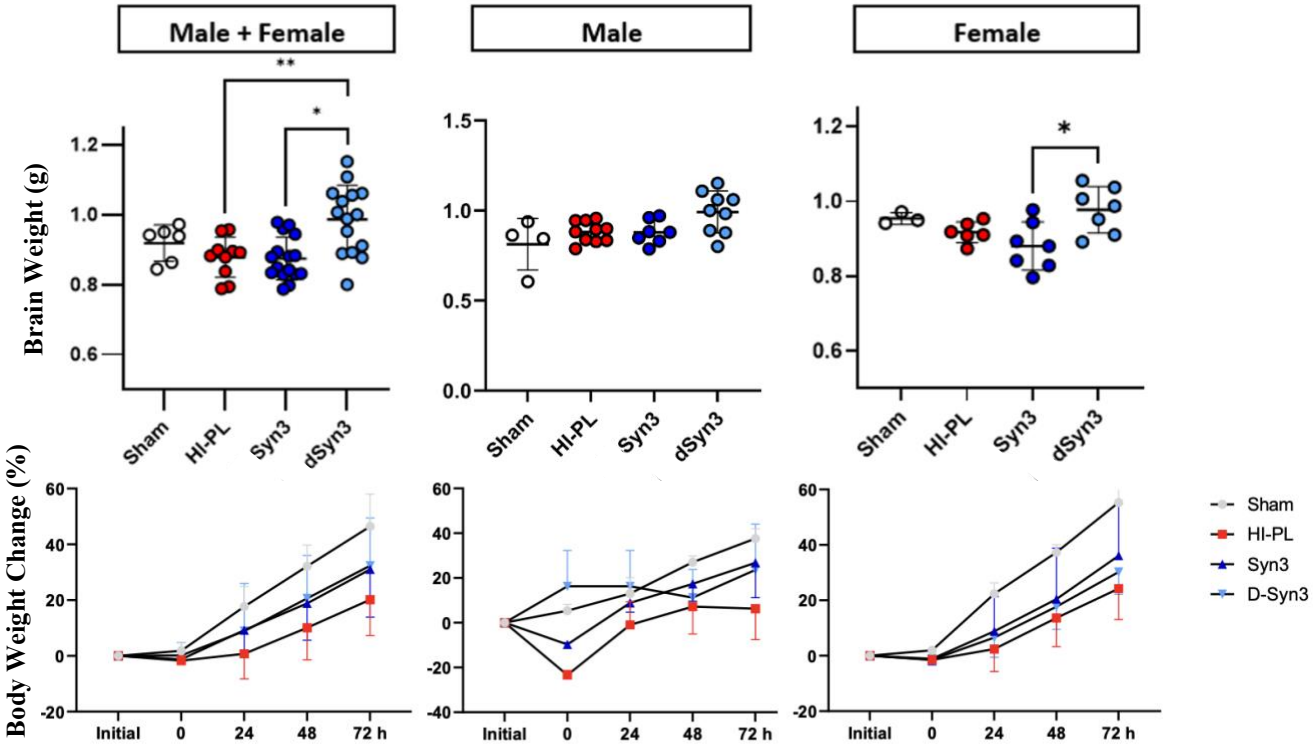


**Fig. 2. Brain weights (g) and change in body weight over time (%)**. These measurements are representative of the Sham, HI-PL, Syn3, and D-Syn3 groups after treatment in neonatal rats exposed to HI related brain injury. The brain weights in the total sample population of male+female, males, and females portrayed as dot plots showing values as mean ± SD. In the male+female group, the HI-PL and Syn3 brains were significantly lighter than the D-Syn3 group. For the female neonates the Syn3 brain weights were significantly smaller than the D-Syn3 group. The percent body weight gain on the y-axis plotted against study time intervals in hours on the x-axis for all the figures (e.g., males + females, males, and females). The body weight gain was lower in the HI-PL, Syn3, and D-Syn3 in the total sample population compared to the Sham group during the study interval. Body weight gain (%) was greater in the Syn3 and D-Syn3 total cohort compared with the HI-PL group during the study. Sham: male $n = 3$, female n = 3; HI-PL: male $n = 11$, female n = 11; Syn3: male $n = 7$, female $n = 6$; D-Syn3: male $n = 9$, female n = 9. Values are mean ± SD. *$p < 0.05$.

Syn3 and D-Syn3 body weights gained at a similar rate, except for the beginning of the study when D-Syn3 males gained more at the 0 hr and 24 hr intervals, than in the Syn3 subjects. The body weight increase over time was greater in the Sham than in the HI-PL (multiple comparison ANOVA, post hoc Tukey HSD test, females+males: 0 hr $p$=1.0000; 24 hr $p$=0.5381; 48 hr $p$= 0.1898; 72 hr $p$=0.2402), Syn3 (multiple comparison ANOVA, post hoc Tukey HSD test,

females+males: 0 hr $p$=1.0000; 24 hr $p$=0.9790; 48 hr $p$= 0.7540; 72 hr $p$=0.5253) and D-Syn3 (multiple comparison ANOVA, post hoc Tukey HSD test, females+males: 0 hr $p$=1.0000; 24 hr $p$=0.9997; 48 hr $p$= 0.9837; 72 hr $p$=0.7604) groups. However, the body weight gain over time differed between the HI-PL and Syn3 (multiple comparison ANOVA, post hoc Tukey HSD test, females+males: 0 hr $p$=1.0000; 24 hr $p$=; 48 hr $p$= 0.9744; 72 hr $p$=0.9989) and D-Syn3 groups (multiple comparison ANOVA, post hoc Tukey HSD test, females+males: 0 hr $p$=1.0000; 24 hr $p$=0.8599; 48 hr $p$= 0.5589; 72 hr $p$=0.5875). Syn3 and D-Syn3 remained similar throughout in the total sample population (multiple comparison ANOVA, post hoc Tukey HSD test, females+males: 0 hr $p$=1.0000; 24 hr $p$=1.0000; 48 hr $p$= 1.0000; 72 hr $p$=1.0000). The male body weight gain in over time was greater in the Sham than in the HI-PL (multiple comparison ANOVA, post hoc Tukey HSD test, males: 0 hr $p$=1.0000; 24 hr $p$=0.8962; 48 hr $p$= 0.3488; 72 hr $p$=0.4263) and in the Syn3 group (multiple comparison ANOVA, post hoc Tukey HSD test, males: 0 hr $p$=1.0000; 24 hr $p$=1.0000; 48 hr $p$= 0.9992; 72 hr $p$=0.9951). The difference between the males in the Syn3 and HI-PL (multiple comparison ANOVA, post hoc Tukey HSD test, males: 0 hr $p$=1.0000; 24 hr $p$=0.9752; 48 hr $p$= 0.8608; 72 hr $p$=0.9751), and HI-PL and D-Syn3 (multiple comparison ANOVA, post hoc Tukey HSD test, males: 0 hr $p$=0.9999; 24 hr $p$=0.6211; 48 hr $p$= 0.1675; 72 hr $p$=0.2254) groups were not significantly different from the Sham group. There was also no significance between the Syn3 and D-Syn3 male groups (multiple comparison ANOVA, post hoc Tukey HSD test, males: 0 hr $p$=1.0000; 24 hr $p$=0.9999; 48 hr $p$= 0.9996; 72 hr $p$=0.9955). The female body weight gain over time was greater in the Sham than in the HI-PL (multiple comparison ANOVA, post hoc Tukey HSD test, females: 0 hr $p$=0.9999; 24 hr $p$=0.0650; 48 hr $p$= 0.0077; 72 hr $p$=0.0004), Syn3 (multiple comparison ANOVA, post hoc Tukey HSD test, females: 0 hr $p$=1.0000; 24 hr $p$=0.7354; 48 hr $p$= 0.3213; 72 hr $p$=0.2669), and D-Syn3 (multiple comparison

ANOVA, post hoc Tukey HSD test, females: 0 hr $p$=1.0000; 24 hr $p$=0.3655; 48 hr $p$= 0.0740; 72 hr $p$=0.0131) female groups. However, differences were not observed between the HI-PL and Syn3 (multiple comparison ANOVA, post hoc Tukey HSD test, females: 0 hr $p$=1.0000; 24 hr $p$=0.9909; 48 hr $p$= 0.9831; 72 hr $p$=0.4868), and most of the HI-PL and D-Syn3 female groups, except for the 72 hr weight change differences (multiple comparison ANOVA, post hoc Tukey HSD test, females: 0 hr $p$=0.9999; 24 hr $p$=0.9362; 48 hr $p$= 0.9644; 72 hr $p$=0.0002). As well as no significance in the female Syn-3 and D-Syn3 groups (multiple comparison ANOVA, post hoc Tukey HSD test, females: 0 hr $p$=1.0000; 24 hr $p$=1.0000; 48 hr $p$= 1.0000; 72 hr $p$=0.9975).

*3.2. Outcomes in ipsilateral hemispheric infarct volume losses after immediate treatment with Syn3 and D-Syn3 when neonatal rats were exposed to severe HI*

To determine infarct volume, cresyl violet was utilized to stain neurons by binding to Nissl bodies, which provides varying shades of purple stain. After application via the Nissl staining methodology, the Cresyl violet coloring attaches to the perikaryon and dendrites (Schuffels et al., 2020). The lighter the shading appears, the greater the damage or neuronal loss in the specific region of the sample (Schuffels et al., 2020). Fig. 2A portrays representative cresyl violet images of coronal brain sections for the Sham, HI-PL, Syn3, and D-Syn3 groups. After reviewing the coronal images, the HI-PL group had increased ipsilateral hemispheric loss compared to the Sham group in both male and female neonatal rats. The D-Syn3 and Syn3 treated groups had decreased lightening compared to the HI-PL group in male, female, and both male and female neonatal rats exposed to moderate-to-severe HI. Percent infarct volume loss confirmed there was less neuronal loss in the D-Syn3 and Syn3 treated groups compared to HI-PL. The ipsilateral hemispheric infarct volumes were significantly larger (47.80 ± 11.31%; SD) in the HI-PL than the other HI-exposed

rat groups in the study (i.e., Sham ($p$=<0.0001), D-Syn3 ($p$=0.0067), and Syn3 ($p$=0.0224); Kruskal-Wallis and Dunn's Test, female+males). Overall, the infarct volume of the Sham cohort was 1.74 ± 1.84% (SD). D-Syn3 treatment of neonatal subjects after exposure to severe HI reduced the infarct volume for the total sample population to 26.07 ± 18.95% (SD), compared to the HI-PL cohort of 47.80 ± 11.31% (SD) (Kruskal-Wallis and Dunn's test, females+males: $p$=<0.0067). Similar to D-Syn3, treatment of the HI-exposed cohort with Syn-3 had a significantly reduced infarct volume of 26.47 ± 16.59% (SD) compared to the HI-PL cohort (47.80 ± 11.31% (SD); Kruskal-Wallis and Dunn's test, female+males: $p$=0.0224). Treatment of females with D-Syn3 in comparison to HI-PL significantly reduced the infarct volumes from 49.12 ± 10.80% (SD) to 33.98 ± 21.39% (SD; one-way ANOVA and Fisher's LSD, female: $p$=0.0485), but was even more effective in males by reducing the infarct volume to 17.18 ± 11.28% for D-Syn3 (SD; Kruskall-Wallis and Dunn's test, males: $p$=<0.0073) compared to HI-PL (46.47 ± 12.16%; SD).

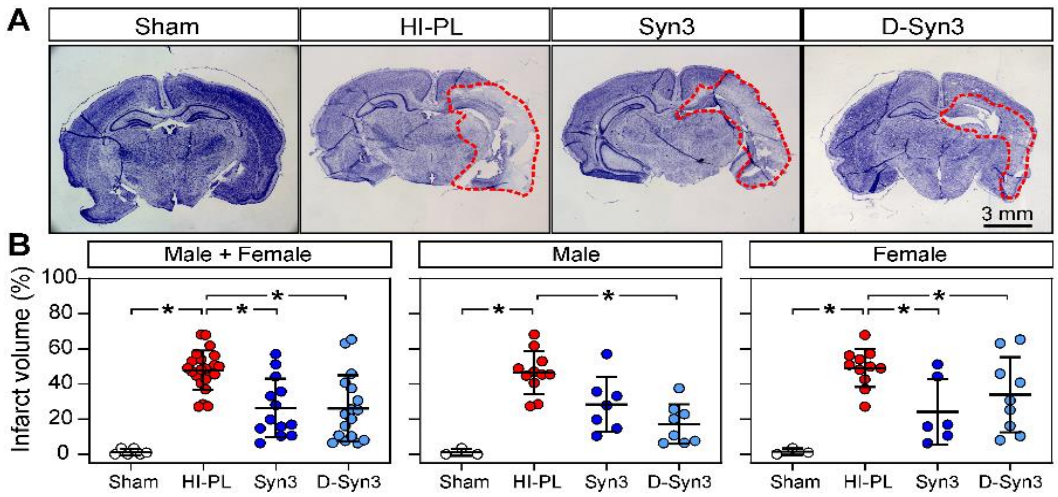


**Fig.3. Syn3 and D-Syn3 reduce injury size. (A)** Representative cresyl violet staining 72 h after HI at P10. Scale bar = 3 mm. **(B)** Percent infarct volume plotted on the y-axis for Sham (male: n=3; female: n=3), HI-PL (male: n=11; female: n=11), HI-Syn3 (Syn3, male: n=7; female: n=6), and HI-D-Syn3 (D-Syn3, male: n=8; female: n=9) groups on the x-axis. Statistics: Kruskal-Wallis and Dunn's test for male+female and male rats, and one-Way ANOVA and Fisher LSD for female rats.

In males, treatment with Syn3 reduced the infarct volume to 28.42 ± 15.72% (SD) compared to HI-PL's 46.47 ± 12.16% (SD), but the comparison between the two groups were not significant (Kruskal-Wallis and Dunn's test, males: $p$=0.3427). However, treatment with Syn3 did significantly reduce infarct volume in females to the mean value of 24.20 ± 18.77% (SD; one-way ANOVA and Fisher's LSD, females: $p$=0.0057). Treatment with Syn3 also showed that even after exposure to HI, the cohort's neonate subjects had a substantial reduced infarct volume of 26.47 ± 16.59% (SD) for the total sample population, compared to the HI-PL's 47.80 ± 11.31% (Kruskal-Wallis and Dunn's test, female+males: $p$=0.0224).

Comparing the effectiveness of Syn3 versus D-Syn3 as a treatment for lowering infarct volume damage was also explored. In the cohort and in the male groups there were no significant differences between the two independent variables (Kruskal-Wallis and Dunn's test, female+males: $p$=0.9999; males: $p$=0.9999). The comparison on solely females treated with Syn3 and D-Syn3 were slightly more differentiated, but still not substantial enough to be considered significant (one-way ANOVA and Fisher's LSD, females: $p$=0.2641).

The findings in this study demonstrate that, for immediate exposure to HI, D-Syn3 and Syn3 attenuated brain volume loss in the ipsilateral hemisphere of the neonatal rats. Interestingly, the drugs appear to be more efficacious in male rats, suggesting there is a potential sex dimorphism with the therapeutics.

## Chapter 4: Discussion and Conclusions

This chapter will summarize the study conducted and discuss the findings, implications for practice, recommendations for further research, research limitations, and the conclusion. The fourth chapter entails a more in-depth narrative of the study's findings. Future research suggestions are provided to portray potential trajectories of this research topic to determine effective studies to identify all sensory and positive/adverse health effects of Syn3 and D-Syn3 either by themselves or compared to other potential nuanced treatment plans. Lastly, a concluding statement is presented to synthesize the essence of what this study has achieved.

### *4.1 Summary of Findings*

The purpose of the study was to quantify and compare brain injury size utilizing infarct volumes of the Sham, HI-PL, Syn3, and D-Syn3 treated neonatal rat groupings. The study hypothesized that the Syn3 and D-Syn3 treated groups would reduce brain injury compared to the placebo-treated HI group when providing treatment immediately after HI-exposure. Most importantly, the study also hypothesized that the Syn3-treated and D-Syn3-treated groups would have comparable protective properties against HIE out of all the experimental groups.

Moreover, the primary objective of the study was to examine the differences between Syn3 and D-Syn3 and identify any measurable changes within the infarct volume analyses or physiological growth in the rat pups. Previous studies found that treatment with Syn3 and D-Syn3 promoted BDNF signaling to restore learning in Angelman syndrome and mitigate depression-like phenotypes, but the neuroprotective and potential neurorestorative properties had not been explored (Naik et al., 2023). This study demonstrated for the first time that treatment with Syn3 or

D-Syn3 immediately after HI-exposure significantly provided neuroprotective effects against HI brain injury in neonatal rats within the perimeters of this small-scale study.

*4.2 Discussion of the Findings*

The cohort's brain weights showed significant differences between HI-PL and D-Syn3, and Syn3 and D-Syn3, with D-Syn3 having the larger brain weights within both comparisons. In females, the brains of animals treated with D-Syn3 were significantly heavier than with Syn3. These data provide compelling evidence that utilizing D-Syn3 and Syn3 as a treatment for immediate exposure to HI attenuates brain volume loss in the ipsilateral hemisphere in neonatal rats. Significantly, after analyzing the infarct volume, the male and female subjects had noticeably different responses to their given treatment of either Syn3 or D-Syn3, suggesting there is potential sex dimorphism with these therapeutics. The infarct volume analysis for Syn3 yielded less damage toward the female neonatal rat brains compared to D-Syn3, while D-Syn3 lessened the damage more for male neonatal rats in contrast to Syn3. Similarly, recent studies by Chen et al., found that treatment with inter-alpha inhibitor proteins (IAIPs) immediately after HI improved brain weights and reduced brain injury only in male rats, indicating sex-related differential effects (Chen et al., 2019).

Substantial evidence suggests that sex dimorphism creates differences in the pharmacokinetics and the pharmacodynamics of drugs and the respective sex (Soldin & Mattison, 2009). In rats specifically, research has indicated sex-specific differences in disease patterns and drug responses (Gerges & El-Kadi, 2023; Shapiro et al., 1995). One factor that could be causing many of these differences within the sexes is the different Cytochrome P450 (P450) enzymes (e.g., CYP1A2, CYP3A, and CYP4A1), which are expressed in the hepatic tissue, but also

extrahepatically in other organs, such as the heart, lungs, kidney, brain, and small intestine (Gerges & El-Kadi, 2023; Shapiro et al., 1995). All these confounding factors could lead to major differences that impact the expression levels of P450 in different organs of male and female rats (Gerges & El-Kadi, 2022), and thus lead to different drug interactions with both genders and their respective metabolism levels. This research is relatively new and continued investigations will be required to shed light on how to better understand differences in pathogenesis, the pharmacodynamics, and pharmacokinetics, between females and males to one day further improve treatment strategies for everyone.

When examining body weight gain as a percentage across the cohort and the isolated gender groups, there were no significant differences in weight gain of note that would further promote sex dimorphism or the interactions between body weight changes and the pharmacodynamic responses of Syn3 and D-Syn3. Interestingly, the neurotrophic factor that Syn3 and D-Syn3 potentiate, BDNF, has been deemed essential for body weight management, and mutations in BDNF processing and impairment in the activation of the TrkB receptor can result in obesity, less energy expenditure, and insatiable hunger (Sonoyama et al., 2020). Evidence shows that drugs (e.g., risperidone) that impact the brain, can potentially interact with BDNF signaling, which can cause weight gain, but this may also depend on the subjects and whether they have the *BDNF* Val66Met polymorphism (i.e., a methionine (Met) substitution for Valine (Val) at codon 66) (Chen et al., 2008). Furthermore, there are many other confounding factors (e.g., complex interplay of hormones, brain circuits and peripheral tissues, and another neurotrophic factor known as ciliary neurotrophic factor (CNTF)) that could produce changes in energy balance, the intersection between energy intake and expenditure, weight gain, and satiety and fullness (Perugini et al., 2022). Moreover, following the release of BDNF at synapses, Syn3 and D-Syn3 increase the

recruitment of PSD-95 to the TrkB receptor to facilitate pro-survival signaling and dendritic spine formation (Cao et al., 2013; Lau et al., 2023; Marshall et al., 2017). Further studies will need to be completed to identify if these specific mechanisms between the drugs of interest, BDNF, and the TrkB receptor, mitigate risk on obesity, satiety and fullness, and energy intake and expenditure.

### *4.2 Implications for Practice*

Continuing to expand on pre-term and term infant treatment alternatives away from, or combined with, the standard of care (i.e., hypothermia), provides potential for further identifying a novel, efficacious, and effective treatment toward vulnerable populations that appears to only be increasing (Gopagondanahalli et al., 2016). This study increases the body of knowledge on Syn3 and D-Syn3 as potential alternate treatments for HIE and will guide the next set of studies. Thus, even minor adjustments, such as comparing different therapeutic time windows that reflect potential real-world scenarios, could make large strides in understanding the mechanisms of these novel therapies that prevent brain injury in neonates and potentially produce outcomes that result in less developmental disabilities in infants. Thus, continuing to explore Syn3 and D-Syn3 as a treatment will continue to potentially show positive outcomes in pre-clinical trials.

### *4.3 Recommendations for Further Research*

Follow-up studies that consider using P 2-3 or P 10 rats to explore the objectives and experimental design of this study and to provide a clearer simulation of premature and mature brain development in portrayal of real-world preterm and term infants. Furthermore, a study focusing on simulated real-world application with providing delayed treatment may also be insightful to further developing effective drugs. Distinguishing differences between Syn3 and D-

Syn3 may be supportive to the cause of more individualized care dependent on meeting or missing the therapeutic window and the effects of sex dimorphism. Lastly, with hypothermia being the current and only approved therapy to treat HI-related injury in human neonates, and if Syn3 and/ or D-Syn3 prove to be an effective and efficacious neuroprotective agent, in future studies it will be of great interest to determine if a combination of Syn3 and/ or D-Syn3 with hypothermia can provide enhanced protection.

### *4.4 Research Limitations*

It is important to discuss the limitations of this study. First, it is pertinent to discuss the limited parameters of this small-scale study, and how there could have been more experiments to study the mechanism of drug action, for example, examining microglial activation by microglial cell counting or other cell counting methodologies to further quantify and provide information on Syn3 and D-Syn3 actions after HI-exposure, that may provide more information regarding sex-specific pharmacology.

Another limitation was the smaller sample size, especially in the Sham group. Therefore, with a limited sample size, this study was underpowered. The data variation between groups at a time or over a short time will most likely be unremarkable.

### *4.5 Conclusion*

The study design focused on two potential treatments to attenuate brain injury after HI-exposure. The data collected within the study suggest that Syn3 and D-Syn3 successfully attenuated brain volume loss. However, potentially due to sex dimorphism, D-Syn3 was more effective in males, while Syn3 was more effective in females. When looking at the entire cohort,

both Syn3 and D-Syn3 provide neuroprotective effects on brain volume. The results from this study expand the growing body of information on these novel therapeutics for mitigating brain injury in neonates.

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