Behavioral and Neuroanatomical Outcomes Following Altered Serotonin Expression With or Without a Hypoxic-Ischemic Injury in a Neonate Rodent Model

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Abstract

Background: Children born prematurely (<37 gestational weeks) are at risk for a variety of adverse medical events. They may experience ischemic and/or hemorrhagic events leading to negative neural sequelae. They are also exposed to repeated stressful experiences as part of life-saving care within the neonatal intensive care unit (NICU). These experiences have been associated with methylation of *SLC6A4,* a gene which codes for serotonin transport proteins, and is associated with anxiety, depression, and increased incidence of autism spectrum disorders.

Purpose: To examine the effects of altered serotonin levels on behavioral and neuroanatomical outcomes in a neonate rodent model with or without exposure to hypoxic-ischemic (HI) injury.

Methods: Wistar rat pups were randomly assigned to either the HI injury or sham group. Pups were treated with a chronic SSRI (Citalopram HBr) or saline, mimicking the effects of *SLC6A4* methylation. Subjects were assessed on behavioral tasks and neuropathologic indices.

Results: HI injured subjects performed poorly on behavioral tasks. SSRI subjects displayed greater anxiety. HI + SSRI subjects learned faster than HI + NS. Histologically, SSRI subjects had predominantly larger brain volumes than NS. This may be related to serotonin-induced neurogenesis.

Conclusion: HI subjects showed cognitive and motor deficits relative to controls. SSRI treated subjects without injury showed patterns of increased anxiety, consistent with theories of *SLC6A4* methylation. HI + SSRI subjects showed a paradoxical trend to improved cognition relative to HI alone, which may reflect an unexpected SSRI neuroprotective effect in the presence of injury.

Keywords:

Introduction

An estimated 15 million infants are born prematurely (<37 GW) each year according to a recent report by the World Health Organization and this number is rising ("World Health Organization," 2018). In North America, preterm infants account for ~8% of all live births (Hamilton, Martin, Osterman, Curtin, & Matthews, 2015). These infants are at increased risk for hypoxic-ischemic (HI) brain injury, a perinatal complication associated with 25% of neonatal deaths globally (Liu et al., 2015). In developed countries, 30% of all neonatal encephalopathy is related to HI and that figure rises to nearly 60% in developing countries (Kurinczuk, White-Konig, & Badawi, 2010). HI occurs when blood and/or oxygen flow to the brain is impaired, leading to energy failure and cellular death (Low, 2004; Vannucci & Hagberg, 2004; Volpe, 2001). In premature infants, the fragile, immature neurovascular system increases the risk of HI due to intraventricular/periventricular hemorrhages as well as inflammation and resulting oxidative stress that occurs during reperfusion (Barrett et al., 2007; Hagberg, Gressens, & Mallard, 2012). Most HI injuries among preterm infants result in white matter damage, thought volume reductions of gray matter in cortical and subcortical regions are also seen, particularly in late preterms (Barret et al., 2007; Inder et al., 1999). HI injuries in late preterm infants (34-37 weeks' GA) are less common, with resulting pathology similar to the deep nuclei grey matter damage seen in term infants with HI (Barrett et al., 2007; Billiards, Pierson, Haynes, Folkerth, & Kinney, 2006; De Vries & Cowan, 2009; Fatemi, Wilson, & Johnston, 2009; Huang & Castillo, 2008).

Additionally, premature infants are exposed to numerous stressors including excessive light and noise levels, parental separation and high levels of pain-related stress that are simply

part of routine life-saving care within the neonatal intensive care unit (NICU) (Casavant, Bernier, Andrews, & Bourgoin, 2017; Casavant et al., 2019). Chronic stress can induce long-lasting effects on neuroendocrine and behavioral responses (Gruneau, 2013; Valeri, Holsti, & Linhares, 2015). One pathway for these effects involve stress effects on epigenetic mechanisms that essentially turn genes "on" or "off" (Weinhold, 2006). Recent research suggest that stress-induced epigenetic modifications may have a role in neurodevelopmental outcomes (D'Agata et al., 2017). One particular candidate involves DNA methylation of *SLC6A4*, a gene which encodes for serotonin transport proteins. Since the serotonergic system is critical in socio-emotional stress response (T. Canli & Lesch, 2007; Hood et al., 2006) and serotonin receptors are found throughout the central nervous system -- appearing early during gestation and then rapidly developing after birth (Gaspar & Cases, 2003), it is expected that epigenetic mechanisms regulating *SLC6A4* transcription have been associated with early adversities and degraded behavioral and neurodevelopmental outcomes (Provenzi, Giorda, Beri, & Montirosso, 2016). Serotonin fibers are found throughout the central nervous system and develop early and rapidly (T. Canli, Lesch, K.P., 2007). This system is managed by feedback mechanisms through the serotonin transport protein coded by *SLC6A4* (Canli & Lesch, 2007). Structural variations affect the amount of serotonin reuptake and is associated with lower socio-emotional outcomes and negative emotionality during early life (Pauli-Pott, Friedl, Hinney, & Hebebrand, 2009). Methylation of *SLC6A4* has been associated with early repeated painful or stressful procedures or treatments (Provenzi et al., 2016). For this study, the administration of selective serotonin reuptake inhibitor (SSRI) injections, was used to mimic epigenetic alterations associated with chronic stress. The purpose of this study was to ascertain the effects of chronic stress and decreased serotonergic tone on behavioral and neuroanatomical outcomes in a preterm infant

rodent model with or without exposure to HI brain injury. HI injuries were induced following established experimental methods. *SLC6A4* methylation following stress was modeled through chronic SSRI administration with or without HI injury (saline treatment control)

Methods

Subjects

Eighteen time-mated female Wistar rats were delivered to the Bousfield vivarium at University of Connecticut from Charles River Laboratories (Wilmington, MA, USA). Six dams arrived on embryonic (E) day 4, six on E 5 and six on E 6. Dams were housed in single cages on a 12-hour light/dark cycle with food and water provided *ad libitum*. Pups were born on approximately E22. On postnatal day (P) 6, pups were culled to litters of 4 males (n=40 males) and 4 females (n=40 females). Pups were weaned on P21 and housed in pairs of same-sex, same-treatment. At P49 (adulthood) they were single-housed for behavioral testing.

Experimental Design

On P6, an age that mirrors a 32-35 week gestational age premature infant (Workman, Charvet, Clancy, Darlington, & Finlay, 2013), pups were randomly selected to receive HI or sham procedure, and were also assigned to the chronic SSRI or saline condition. The four conditions thus were: HI chronic SSRI (n=10 per sex), HI saline (n=10 per sex), sham (SH) SSRI (n=10 per sex) and SH saline (n=10 per sex).

Induction of Hypoxic-Ischemic Injury

Pups were anesthetized with isoflurane 2.5% and local bupivacaine subcutaneousy (SC). A vertical incision was made on the neck at midline. The right common carotid artery was identified and cauterized to restrict blood flow to the right cerebral hemisphere. The incision was sutured, and footpads tattooed for individual identification purposes. Sham animals underwent a

similar surgical procedure without cauterization, and with comparable duration of isoflurane (maximum 9 minute exposure in all groups to avoid confounding neuroprotective or neurotoxic effects) (Wei & Inan, 2013). Every effort was made to avoid potential animal suffering with oversight and approval from the University of Connecticut's Institutional Animal Care and Use Committee (IACUC). Pups in the chronic SSRI condition were injected SC with 10mg/kg of citalopram HBR diluted in 9% normal saline (NS) to a volume of 1/100th their weight (15 grams = .15 mL diluted citalopram HBR) immediately after surgery and then daily until P21. Shams were injected subcutaneously with a volume of 1/100th of their weight of 9% NS daily until P21.

Post-surgery, pups were returned to the dams to nurse for 60 minutes. After feeding, hypoxic conditions were induced among pups in the HI group by placing them in an airtight container on a warming tray (maintained at nest temperature) and subjected to 8% oxygen with a nitrogen balance for 90 minutes. Sham pups were placed in an open container on a warming tray for 90 minutes. Subjects were then returned to the dams. Every morning from P6-P21 pups in the chronic SSRI condition received SC injections of 10mg/kg citalopram HBR diluted with 9% normal saline while shams received SC injections of NS.

Behavioral Evaluation

Seventy-two animals (40 female and 32 male) were tested on a battery of behavioral paradigms. No significant sex differences were found, therefore group comparisons on outcome scores are collapsed across sex. Results are provided for HI vs. sham within saline and SSRI groups separately, as well as between SSRI and saline groups with sham injury.

Sensorimotor Task: Rotarod (P35-39)

The rotarod task was used to measure balance, motor coordination and motor learning on an accelerating rotarod (Buitrago, Schulz, Dichgans, & Luft, 2004). Animals were placed on a

rotating rod gradually accelerating from 4 rotations per minute (rpm) to 40 rpm, during a 5-minute period. Animals were given four trials per day for three days. Latency to falling off the rod as well as rpm at time of fall was recorded per trial (in seconds) with the average latency per day being used for analysis.

Anxiety: Elevated Plus Maze (EPM) (P54-P58)

The Elevated Plus Maze was used to assess general anxiety behavior. Rats were transported to the testing room inside their home-cages to minimize distress and remained in their cages until testing. The plus maze was constructed of black plastic and elevated 50 cm above the floor. The apparatus consisted of four arms, 50 cm long and 10 cm wide aligned perpendicularly. Two arms were enclosed by 30 cm high walls and the other two arms were exposed. The exposed arms each had a 0.9 cm lip to prevent subjects from falling off. The maze was placed in the center of a quiet room with only ambient lighting.

Each experimentally naïve subject was placed in the center of the platform, facing an open arm and were allowed to freely explore for 5 min. After each observation, the EPM was cleaned with Virkon disinfectant cleaner to remove debris and scent cues left from the preceding subject. Each subject was recorded through a video camera and video files stored in a secure computer for later analysis.

Anxiety: Open Field (P61-65)

The Open Field task is a simple sensorimotor test used to determine general activity levels, gross locomotor activity, and exploration habits in rodent models of CNS disorders (Gould, Dao, & Kovacsics, 2009). Assessment takes place in a square Plexiglas box. The field is marked with a grid and squares with a center square marked by colored tape, the middle square surrounding the center is marked by a different colored tape and the outside square reaches the

walls of the box. The animal is placed in the center arena and allowed to freely move about for 10 minutes while being recorded by an overhead camera. The footage is then analyzed by an automated tracking system for the following parameters: distance moved, velocity, and time spent in pre-defined zones.

Anxiety: Marble-Burying (P66-69)

The marble-burying behavioral task is another measure of anxiety or obsessive-compulsive traits. Rodents are placed for thirty minutes in a standard cage filled with wood chip bedding 5cm deep. There are 10 marbles evenly spaced in the cage. After 30 minutes the number of marbles buried is measured.

Learning and Memory Assessments (P70-74)

Screening for general visual or motor deficiencies was conducted prior to Morris Water Maze (MWM) testing in a one-day water escape task. The task was conducted in an oval tub (40.5 x 21.5 in.) that was filled with room temperature water and a visible escape platform at the end opposite from where the animal was introduced to the water. Once placed in water, animals were timed until they reached the platform or 45 seconds had elapsed. Those who were not able to locate the platform during the 45-second swim were led to it and allowed to detect cues for 2 seconds. Maze testing took place in adulthood.

Morris Water Maze (Two batches: P75-79/P82-86)

The Morris water maze task assesses spatial learning and memory.(Morris, 1984; Vorhees & Williams, 2006). Animals were divided into two equal batches of males and females given the large sample size. Batch 1 completed the task during P75-79 while Batch 2 completed the task during P82-86. This task requires animals to use spatial cues outside the maze (large black geometric shapes painted on the surrounding walls, lighting and the experimenter) to locate

a platform. Testing was conducted in a 48-in. diameter inflexible black tub that contained a 6-in. diameter escape platform submerged 1 in. below the water line, making it invisible to the animals. The escape platform was located in the same quadrant of the tub for each trial. There were no intra-maze cues, requiring subjects to use external room-based spatial strategies. Animals participated in four trials per day where the start position varied (North, South, East or West). Start position was not repeated in the same day, and the order of start positions was not repeated on subsequent days. Each animal's attempt was recorded with a Sony Digital 8 video camera connected to a Dell Dimension E21 computer with SMART Version 2.5 tracking software recording animal latency (measured in s) to reach the platform, as well as average swim speed (measured in cm/s). During each trial the animal was given 45s to locate the submerged platform. If the animal failed to reach the platform it was guided to the platform and allowed to sit and survey the room for 5 s.

Histological Examination

After behavioral testing was complete, rats were anesthetized with ketamine (100mg/kg) and xylazine (15mg/kg), and transcardially perfused with 0.9% saline solution followed by 10% buffered formalin. Brains were extracted from the skulls and placed in 10% formalin. Due to the large n, only shams were quantified, specifically to determine the effect of the SSRI versus normal saline to brain volume. Brains were sliced coronally using a Leica VT1000 vibratome at a thickness of 60μm. Every other slice was mounted on a chrom-alum subbed slide then received Nissl staining. To measure gross structural volumes of the structures associated with emotionality (caudate putamen, globus pallidus, amygdala, hippocampus)(Paxinos & Watson, 1982), Stereo Investigator Microbrightfield software and an Axio 2 Zeiss Microscope were utilized. Volumes were quantified using 2.5x magnification with Cavalieri's Estimator software

and a grid overlay. Fewest number of sections were counted to achieve a coefficient error of less than 0.05 which was considered stereologically valid. Every third mounted slice was examined and all measurements were performed blind to treatment group.

Statistical Analysis

Statistical analyses were conducted in SPSS 24.0 software, alpha criterion 0.05 (IBM, Armonk, NY, USA) and R (Team, 2019) and figures were produced using the package ggplot2 (Wickham, 2016). Data were collapsed across sexes as appropriate for some tasks when there was not enough evidence showing the sex effect.

Two quantities were analyzed separately by linear regression for the Elevated Plus Maze task. The ratio of entries into open arm versus closed arm shows the tendency to enter the open arm, while the difference of time spent on open arm versus on closed arm measures the inclination to stay on the open arm. Open Field task was analyzed using one-way ANOVA. Marble Burying task was analyzed by linear model with certain box-cox transformation applied, and nonparametric testing procedure. Rota-rod task was analyzed by repeated measure ANOVA. Four trials in day 1 were treated as the warm-up period for rats so that data for day 1 was eliminated in the statistical analysis. Morris Water Maze was analyzed by mixed-effect model. The models of surgery/treatment interacting with days were considered since we are interested in different performance in learning rate (slope).

Histology measures were analyzed using independent t-tests since only sham SSRI and sham NS were examined.

Results

Significant associations between serotonergic anomalies were discovered that have also been associated with chronic stress, and an HI injury typical of preterm populations.

In our study, females showed less anxious behavior than male animals on the Elevated Plus Maze by displaying a higher value on the difference of time spent in open versus closed arms (Figure)($p < 0.001$), and the ratio of entries.

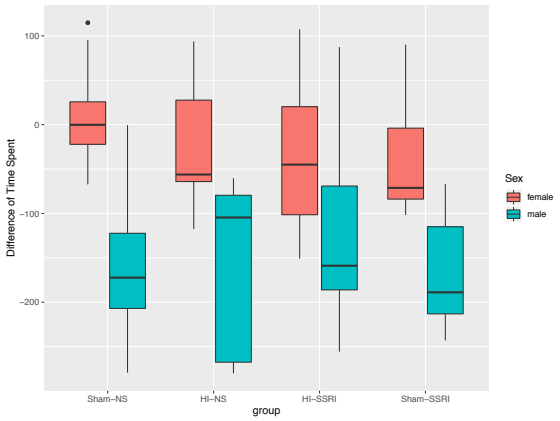

Figure 1 EPM, Boxplot for difference of time spent on open arm and closed arm (TimeOpen - TimeClosed)

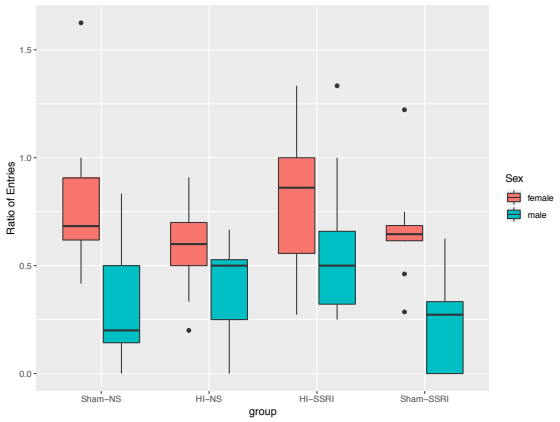

Figure 2, EPM, Boxplot for ratio of entries into open arm against closed arm (#OpenEntry/#ClosedEntry)

In the Open Field (OF) test, neither sex effect nor group effect was statistically significant in any zone. In the Marble Burying task, male animals buried fewer marbles than females (p = 0.015), but surgery/treatment effects did not reach significance.

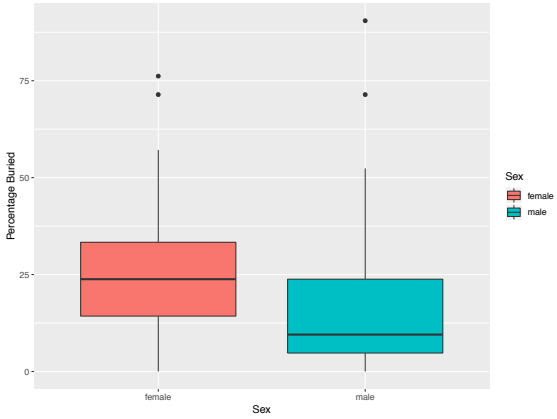

Figure 3 Marble Burying, Boxplot of percentage buried across sexes

Gross motor function, balance and coordination

After eliminating Rota-rod data from day 1, a 2 (Day) x 4 (Treatment) repeated measures ANOVA on the Rota-rod task showed an overall treatment effect, with almost statistically significantly greater latency to fall in SSRI animals compared to the Saline group (p = 0.059). As expected, those in the Sham-NS group showed greater latency to fall than those in the Sham-SSRI group, although it was not statistically significant (p=0.14).

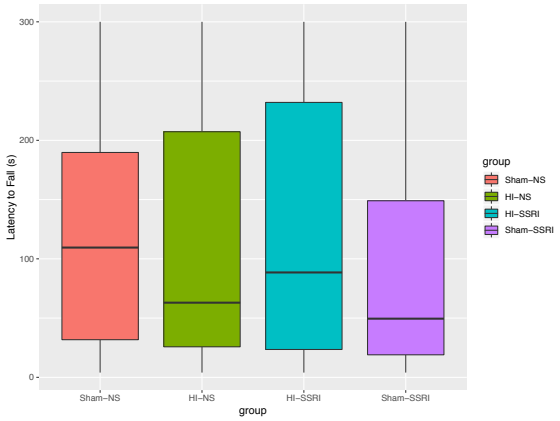

Figure 4 Rota-rod, Boxplot of latency to fall after dropping day 1 and treating day 2-3 as repeated measures.

Spatial learning and memory

Consistent with other reports of learning impairments in rats with induced P6 or P7 HI injury (Alexander, Garbus, Smith, Rosenkrantz, & Fitch, 2014; Ikeda et al., 2001) subjects that sustained an HI injury had more difficulty learning tasks such as the Morris Water Maze (MWM), than Shams ($p=0.015$) (see Figure 5).

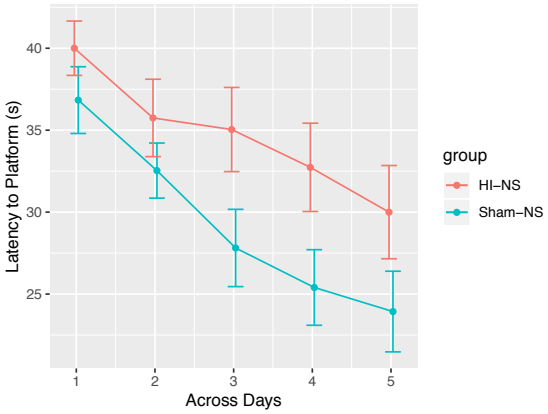

Figure 5 Morris Water Maze, comparison between HI-NS and Sham-NS (mean level for each group across days with error bar representing standard error)

As clearly shown in Figure 6, HI groups perform worse than Sham groups at day 1. At day 5, HI-NS group still performed much worse than Sham groups, but the gap between HI-SSRI group and Sham groups was no longer as considerable. Subjects with both HI and SSRI trended toward learning more quickly over time points than those with only the HI injury, differences between HI-NS and HI-SSRI were statistically significant (one-sided p-value = 0.04).

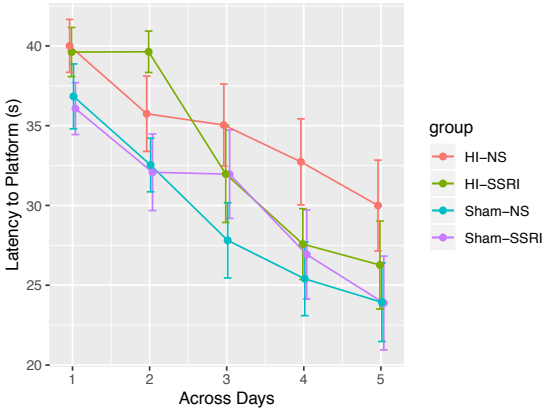

Figure 6 Morris Water Maze (mean level for each group across days with error bar representing standard error)

Histology

Since the treatment effect of interest related to the effects of the SSRI v. NS, only surgical sham rats were examined histologically. An independent samples t-test revealed no significant treatment effect of the Citalopram for either left or right side in any of the structures examined (caudate putamen and anterior cingulate gyrus, globus pallidum, dorsal hippocampus or the amygdala). It is important to note that structural integrity of tissue varied, and as such, not all structures related to emotionality were able to be examined. In addition, the number of samples varied per structure (Figures 7-10).

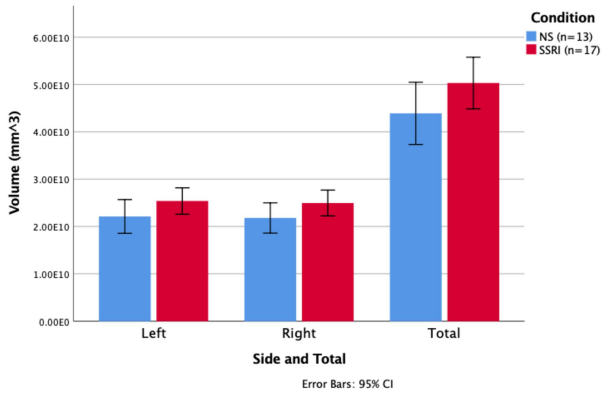

Figure 7 Volume of Caudate Putamin and Anterior Cingulate Gyrus

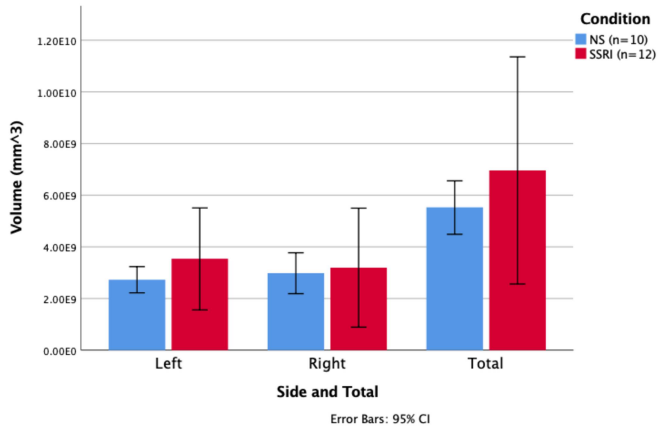

Figure 8 Volume of Globus Pallidum

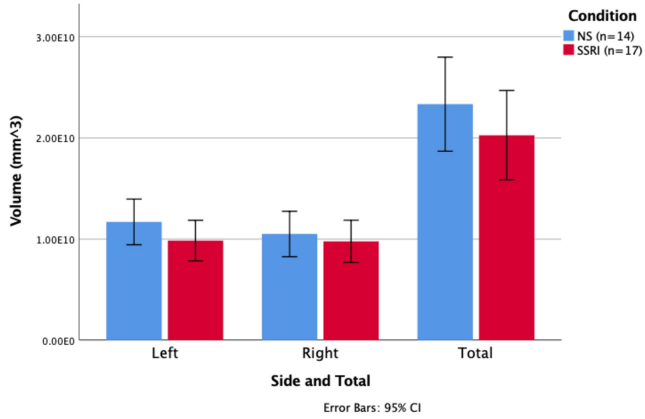

Figure 9 Volume of Dorsal Hippocampus

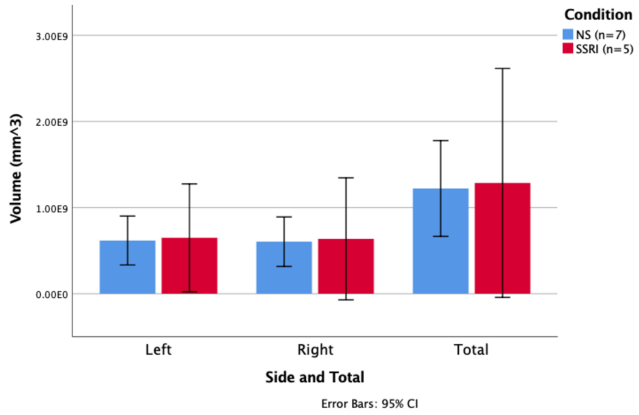

Figure 10 Volume of Amygdala

Of interest is that although these volumes did not reach statistical significance, those who received the Citalopram trended toward larger brain volumes in striatal and limbic structures assessed except the hippocampus.

Discussion

Recent research suggests that infants who undergo numerous stressful events per day including skin-breaking procedures while in the NICU demonstrate reduced serotonin expression later in life (Provenzi et al., 2016). The current study sought to examine the effect of altered serotonergic tone in rodents who underwent a hypoxic-ischemic injury at P6, an age that mimics a moderately preterm infant. Our findings demonstrate that exposure to SSRIs during vulnerable stages of neurodevelopment may evoke detrimental effects on brain development and behavior. Previous studies have found that neonatal exposure to SSRIs results in alterations in behaviors associated with emotion, such as increased depressive-like behavior (Hansen, Sancehz, & E., 1997), decreased exploration and increased anxiety (Ansorge, Lira, Hen, & Gingrich, 2004). Perinatal exposure to citalopram results in behavioral alterations that persist into adulthood

(Sprowles et al., 2016). While the behavioral effects of the hypoxic-ischemic injury were consistent with previously reported studies, the results following chronic administration of citalopram in combination with the injury in this study were novel and suggest a trend toward possible neuroprotective effect of SSRI in the presence of HI injury. This could reflect beneficial evolutionary effect of early chronic stress in the presence of injury, even though the effects of chronic SSRI (mimicking chronic stress) were deleterious in healthy (sham) subjects. Specifically, rodents injected with citalopram from P6-P21, an age in which 5-HT ontogeny and synaptogenesis occurs (Homberg, Schubert, & Gaspar, 2010) displayed learning impairment compared to sham saline rodents. In contrast, subjects who sustained an HI injury and received the citalopram trended toward learned more quickly on the MWM than subjects with an HI injury who received saline. Thus, it is possible that the SSRI may have acted as a neuroprotectant with a p-value 0.04. Additional research may prove useful in determining neuroprotective capacity of SSRIs in learning and memory tasks for those with brain injuries.

Histologically, there was no statistical significance in brain volume differences between Sham-NSal subjects and Sham-SSRI subjects. However, it is interesting to note that with the exception of the dorsal hippocampus, SSRI subjects trended toward larger brain volumes than Sal subjects in striatal and limbic structures associated with anxiety. Our findings mirror others which indicate that exposure to serotonin reuptake inhibitors spurs neurogenesis, creating larger structural volumes related to emotionality (Powell et al., 2017). Limitations of this study include, that we were not able to conduct polymerase chain reactions to assess true methylation status; small sample size for histology and that structural integrity of tissue varied, as such, not all structures related to emotionality were able to be examined; in addition, the number of samples varied per structure.

Conclusion

In conclusion, the current study provides a direct comparison of anatomic and behavioral outcomes following HI injury or sham surgery in a near term (P6) rodent model. Further, rodents were exposed to chronic stressor of injection with SSRI or saline until P21, the equivalent of early childhood (Sengupta, 2013). The SSRI was used to mimic altered DNA methylation and thus, altered serotonin expression. As seen in previous literature, HI subjects displayed a significant treatment effect of diminished learning and memory as evidenced in the Morris Water Maze task ($p=0.015$). In addition, there was a trend towards increased anxiety in HI versus Sham subjects. Nonetheless, HI subjects who received chronic SSRI treatment displayed a trend to learn faster than those who received saline on the MWM task ($p=0.04$). Further research might elucidate whether the SSRI acted as a neuroprotectant. From a neuroanatomical perspective, SSRI rodents trended toward larger brain volumes in striatal and limbic structures which may be a result of serotonin-related neurogenesis. Further studies that simply examine the effects of chronic stress via peritoneal injections of SSRI versus saline from late preterm to early childhood and then examine behavioral and anatomical results might provide greater insight into the long-term effects of chronic stress.

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Field Code Changed

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