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Early testosterone modulated sex differences in behavioral outcome following neonatal hypoxia ischemia in rats

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Abstract

Hypoxia ischemia (HI; reduced blood oxygenation and/or flow to the brain) represents one of the most common injuries for both term and preterm/very low birth weight (VLBW) infants. These children experience elevated incidence of cognitive and/or sensory processing disabilities, including language based learning disabilities. Clinical data also indicate more substantial long-term deficits for HI injured male babies as compared to HI injured females. Previously, we reported significant deficits in rapid auditory processing and spatial learning in male rats with postnatal day 1 (P1), P7, or P10 HI injury. We also showed sex differences in HI injured animals, with more severe deficits in males as compared to females. Given these findings, combined with extant clinical data, the current study sought to assess a putative role for perinatal testosterone in modulating behavioral outcome following early hypoxic-ischemic injury in rats. Male, female, and testosterone-propionate (TP) treated females were subjected to P7 HI or sham surgery, and subsequently (P30+) underwent a battery of auditory testing and water maze assessment. Results confirm previous reports of sex differences following HI, and add new findings of significantly worse performance in TP-treated HI females compared to vehicle treated HI females. *Post mortem* anatomic analyses showed consistent effects, with significant brain weight decreases seen in HI male and TP-treated HI females but not female HI or sham groups. Further neuromorphometric analysis of brain structures showed that HI male animals exhibited increased pathology relative to HI females as reflected in ventricular enlargement. Findings suggest that neonatal testosterone may act to enhance the deleterious consequences of early HI brain injury, as measured by both neuropathology and behavior.

Keywords

HI; language impairment; prematurity; auditory processing

Introduction

Perinatal hypoxic-ischemic injury is a major cause of brain damage in the premature/very low birth weight (VLBW) infant (<1500g), as well as in infants suffering from birth trauma (see [1] for review). In the premature brain, HI is often due to the vulnerability of the

underdeveloped and extremely fragile nature of the neural vascular system [2], which can lead to hemorrhagic injury (termed intraventricular (IVH) or periventricular hemorrhage (PVH) [3]). As a result of intracranial bleeding, brain tissue is compressed, further limiting its blood and oxygen supply (see [4] for review). Non-hemorrhagic HI injuries may also be seen, including reperfusion failure and ischemic injury resulting in periventricular leukomalacia (PVL). Also prevalent in severely premature infants, PVL is associated with loss of white matter surrounding the cerebral ventricles [see 5 for review]. Risk of HI injury and neurological impairment increase with decreasing gestational age at birth [6–7; see 4 for review].

In term infants, hypoxic-ischemic incidents related to birth complications and trauma result in a different pattern of neuropathology as compared to that seen in preterm children, due to the greater vulnerability of gray matter to excitotoxicity at this age [8]. Thus term HI more typically manifests as damage to gray matter structures such as the thalamus and basal ganglia [9, see 1 and 10 for review].

Hypoxic-ischemic insult to the developing brain also results in long-lasting behavioral consequences. Specifically, 25–50% of VLBW infants later show cognitive and behavioral deficits [3, see 11 for review], including delayed language acquisition [12] and deficits in verbal and language domains [13]. Term infants suffering from HI also show decreased language skills [14], and display lower verbal and performance IQs [15]. Auditory processing deficits (suggested to be both predictive of and possibly causal to later language related impairments) have also been reported in premature babies [16–20]. Specifically, rapid auditory processing (RAP; or the ability to detect brief changes in sound stimuli, speech, or non-speech stimuli changing within tens of milliseconds) is critical to accurate speech processing, and early impairments in RAP have been associated with cascading deleterious effects on subsequent language development [9, 17, 21, see 22 for review]. Previous research has reliably measured this ability in animals, and tests of RAP in male rats have revealed deficits in subjects with induced neonatal brain injuries including perinatal hypoxia ischemia [23–27], microgyria (small induced cortical malformations [28–32]), and prenatal teratogenic exposure [33]. These findings suggest that RAP indices may serve as a marker of neural disruptions associated with language-based impairments.

Clinical data also reveal sex differences in the incidences of several brain-based developmental disorders, with most having higher prevalence for males than females [34–38]. For example, males show a higher incidence of prematurity, anoxia, intraventricular hemorrhage, and infection, as well as increased mortality from prematurity or stillbirth [34, 36–39], and evidence suggests female premature/VLBW babies may be at an advantage as compared to their male counterparts. Specifically, IQ measures taken at 4–5 years of age revealed that premature males with ICH fell significantly below matched female counterparts with comparable ICH injury on full scale, verbal, and performance IQ (although no differences in IQ measures were found between matched male and female premature control groups without ICH [40]). Clinically, boys suffer more long-term cognitive deficits, and are more likely than girls to have speech and language disorders (including stutter and dyslexia), as well as learning disabilities [36–37, 39].

The causal factor(s) underlying clinical evidence of heightened vulnerability to cognitive impairments associated with early neurodevelopmental disruption in males remains unknown, but could relate to the substantially higher levels of plasma testosterone in neonatal males. In humans, fetal males show evidence of increased testosterone production relative to females as early as 8 weeks gestation, and plasma levels remain markedly elevated through the first year of life, after which levels drop until the start of puberty [41]. Substantial evidence has shown that this early “surge” in testosterone production is tied to the masculinization of subsequent neural morphology and behavior (see [41] for review). To examine the question of whether poorer outcomes following early brain injury in infant males may relate to this early testosterone exposure, the current study examined well-studied and replicated deficits in behavioral performance caused by early HI injury in male rats, and assessed whether similar HI effects would be seen in females, as well as females treated with early testosterone propionate. This paradigm followed evidence that plasma testosterone in fetal male rats is significantly higher than female littermates beginning at embryonic day 18 (E18; until approximately postnatal day 5; [42]), coupled with substantial evidence that a postnatal regimen of testosterone exposure in female rats leads to masculinization of numerous measures of brain and behavior [43–45]. We hypothesized that treating female rat pups with testosterone propionate (P1–5), prior to the induction of HI, would lead to poorer performance on behavioral tasks, as well as increased brain damage (comparable to that typically seen in male HI rats).

Methods

Subjects

Time-mated female Wistar rats were ordered from Charles River Laboratories and shipped on embryonic day 5 (E5) to minimize prenatal stress. Dams were housed in the University of Connecticut animal facility in a 12-h light/dark cycle where they gave birth. Pups were culled to litters of five males and five females on P1. All pups received subcutaneous injections of either 0.05ml sesame oil, or 0.1mg TP in 0.05ml sesame oil, on the mornings of postnatal days 1–5 and underwent HI or sham surgery on P7 (for N's see Table 1). This regimen of TP administration is well-established as leading to masculinization of female morphology and behavior in rodents [44–45, see 41 for review]. Due to concerns for maternal exposure and contamination of milk, treatment with TP or vehicle (oil) was assigned between litters.

Induction of hypoxia-ischemia

On P7, pups were randomly selected for sham or HI procedure (balanced within litter). At surgery, HI selected pups were anesthetized with isoflurane (2.5%), and a longitudinal midline incision was made in the neck. The right common carotid artery was located, separated from surrounding tissue, and completely cauterized. The incision was sutured, footpad marking injections were made, and pups were returned to dams after recovering from anesthesia under a warming lamp. Approximately two hours after recovery (allowing time to feed), pups were placed under a warming lamp in an air-tight chamber containing 8% humidified oxygen (balanced with nitrogen) for 120 minutes. Sham animals underwent the same procedure, excluding artery cauterization and hypoxia (shams were exposed to

room air in an equivalent chamber for 120 minutes). All pups were returned to their mothers, where they remained housed until weaning on P21.

Behavioral testing, startle reduction

The startle reduction paradigm utilizes the subject's acoustic startle reflex (ASR), a large motor reflex response to a startle eliciting stimulus (SES; 105dB white noise burst), coupled with a benign acoustic stimulus just prior to the SES on cued trials. Termed prepulse inhibition or startle reduction, this procedure provides an indirect measure of cue detectability based on the magnitude of startle attenuation elicited by the prepulse cue (for review see [46]). This procedure allows for analysis of the magnitude of the startle response on cued versus uncued trials as a function of cue properties (e.g., gap duration), thus providing a measure of detectability of the pre-SES cue.

Apparatus, auditory testing

During auditory testing, each subject was placed on a Med Associates PHM-252B load cell platform in an opaque polypropylene cage, in a quiet testing room. Output voltages from each platform were sent from a PHM-250-60 linear load cell amplifier to a Biopac MP100A-CE Acquisition system connected to a Power Macintosh G3. This apparatus recorded the amplitude of each subject's startle reflex (150 ms) from the onset of the SES. The extracted peak value from this interval served as the subject's response amplitude for that trial. Auditory stimuli were generated on a Pentium III Dell PC with custom programmed software and a Tucker Davis Technologies (RP2) real time processor, amplified by a Niles SI-1260 Systems Integration Amplifier and delivered through 10 Cambridge Soundworks MC100 loudspeakers placed 53 cm above the platforms. The SES was always a 105dB, 50 ms burst of white noise.

Normal Single Tone (NST, P25)

On cued trials, subjects were presented with a single 75dB, 7 ms, 2300Hz tone followed 50 ms later by a 105dB, 50 ms SES. On uncued trials, only the 105dB SES was presented. The attenuated response (ATT; cued score/uncued score $\times$ 100) served as a measure of detection of the cue, with higher scores indicating poorer detection (100% = chance). NST measures thus provide a baseline measure of startle attenuation, which can be used to confirm intact hearing and startle circuitry.

Silent Gap (SG, P27–30)

The gap detection task involved 300 trials of randomly presented silent gaps embedded in a continuous broadband white noise 75dB background. The Silent Gap 0–100 task featured gaps of 2, 5, 10, 20, 20, 40, 50, 75, and 100 ms. The gap, serving as the cue, was presented 50 ms prior to the SES on cued trials, while there were no gaps (gap duration = 0 ms) on uncued trials. ITIs of 16, 18, 22, or 24 seconds randomly separated each trial.

Behavioral Testing, water maze, water escape

Water maze testing began with a water escape task on Day 1 (P86). Each rat was released at the end of an oval water filled tub and had to swim to the opposite end where an escape

platform was visible. If the rat did not swim to the platform after 45 seconds, it was guided to the platform, where it remained for 10 seconds. This procedure was followed for each animal in order to acclimate to water tests, as well as to assess any group differences in baseline motor (swimming) behavior.

For all remaining water maze tasks, a Sony Handycam DCR-TRV280 camera mounted above the pool recorded and translated the path taken, as well as the time to the platform (latency), using a Smart Video Tracking System Version 2.5 on a Dell Dimension E521 computer.

Morris water maze (MWM, P89–93)

The MWM is designed to assess spatial reference memory. This task was performed in a round tub filled with water at room temperature, with a submerged platform that remained in a fixed location. Extra-maze cues around the room included large black shapes painted on the surrounding walls, and the doorframe. To begin, each rat was placed into the maze at one of four start positions (N, S, E, or W quadrant wall), where it was required to swim until it found the hidden platform (or until 45 seconds elapsed). It was then returned to its cage under a warming lamp for 2–3 minutes before the next trial. On the 3 remaining trials, the rat was placed into the maze from one of the remaining starting points. This procedure was repeated over 5 days, and employs spatial navigation and memory.

Non-spatial water maze (NSM, P96–100)

Non-spatial maze testing was similar to MWM, however, the location where the rat was placed into the pool remained constant whereas the escape platform was paired with one of four intra-maze visual cues (vertical lines, horizontal lines, white dots on a black background, or black dots on a white background) painted on a circular insert placed just inside the outer wall of the maze tub. The location of the platform, along with the position of the associated cue, were randomly varied (NW, NE, SW, SE quadrants). The escape platform was consistently paired with the same intra-maze cue (vertical lines), and both the platform and the cue changed locations together throughout the 4 trials per day, for 5 days. The center of the platform was approximately 10 inches from the perimeter of the maze wall where the associated cue was located, and animals had 45 seconds to find the platform before being guided to its location. This task was repeated over 5 days, and employs associative learning ability and memory (i.e., associating the platform with a specific cue regardless of spatial cues).

Statistical Analysis

Based on specific *a priori* hypotheses, planned comparisons were performed between specific groups. This includes separate analyses for sham and HI males, sham and HI females, and sham and TP-treated HI females. Planned comparisons between TP-treated HI females vs. male HI animals, and TP-treated HI females vs. oil-treated female HI animals were also conducted to specifically examine the effects of TP on response to HI injury.

In statistical tests examining behavioral outcome for HI and sham males only, one-tail tests were used. This is based on the fact that multiple prior studies have established significant

behavioral deficits in HI male rats on these same tasks [23–27]. Accordingly, P7 HI injury should not lead to improvement in male rats, and we were not testing for effects in this direction. Two-tail tests were used in all other comparisons, because no prior data has established the effects of HI injury on behavioral performance in females or TP-treated females — leaving open the possibility that effects in either direction could be observed.

The pre-pulse inhibition paradigm was used in this context to assess complex acoustic processing of cues in intact and injured animals. As such, it was important to ascertain that baseline differences in hearing, startle response, or simple pre-pulse inhibition did not contribute to reported differences between groups. Such effects would confound our interpretation regarding the effects of HI injury on more complex sound processing, and associated extension of the results to human clinical data from infants with early injuries and language problems. To specifically address the issue of complex acoustic processing — without the confounds of hearing, startle, or PPI differences — baseline single-tone ATT scores were used as a covariate in analyses of gap detection data. This means that any differences in gap processing as measured that were attributable to underlying differences in hearing, startle, or baseline PPI were removed from effects as reported. Although use of the covariate leads to a more conservative statistical test of group differences (by eliminating some of the between-group variance), we feel this procedure is critical to the interpretability of results — particularly since a Sex $\times$ Treatment effect on NST was found (possibly reflecting sex differences in hearing, startle, body weight/strength, or PPI). Although such effects may be of interest in another context, they are not the measure of interest in the current study. Thus, baseline differences in simple PPI were removed from further analyses through use of the NST as a covariate.

Multivariate analyses of variance (ANOVA) were used to analyze both auditory ATT scores and latency to the platform for water maze tasks. Variables presented in the Results section include: Sex (2 levels; male, female); Treatment (2 levels; HI, sham); Hormone (2 levels; TP, oil); Day (4 levels for auditory testing and 5 levels for maze testing); Gap (9 levels); and Task (2 levels; MWM, NSM). All analyses were conducted using SPSS 15.0 with an alpha criterion of .05. Data presented in auditory task graphs is presented as mean attenuation score (cued response/uncued response $\times$ 100) $\pm$ SEM, with higher scores indicating poorer performance (100% = chance levels). Data presented in water maze task graphs is presented as mean latency to the platform $\pm$ SEM in seconds.

Histological Analysis

Upon the completion of behavioral testing, all animals were weighed and deeply anesthetized with an IP injection of a mix of ketamine and xylazine (100mg/Kg and 15mg/Kg), then transcardially perfused with .9% saline followed by 10% buffered formalin. Brains were removed from the skull, and post-fixed in 10% buffered formalin before being sent to Beth Israel Deaconess Medical Center, where they were weighed, embedded in celloidin, cut on a sliding microtome at 40 microns, stained with cresyl violet, and mounted on glass slides (every 10th section). The slides were returned to the University of Connecticut, where each slice was photographed under 1.3x magnification on a Fisher Scientific Micromaster digital microscope using Micron software, and analyzed (blind to treatment or sex) for damage and

structural 3D volume indices for the cerebral ventricles. Measures were derived using a grid overlay and ImageJ software. Cavalieri's point counting estimator of volume was used to estimate total volume of the left and right ventricles separately (see [47]).

Results

Normal Single Tone

Results of a univariate ANOVA performed for male and female oil-treated animals revealed no significant differences of Sex or Treatment. However, a significant Sex $\times$ Treatment interaction was found, [$F(1,59) = 6.45, p < .05$]. In addition, a second univariate ANOVA for TP and oil-treated females revealed a nearly significant Treatment effect, [$F(1,66) = 3.99, p = .05$]. These scores were used as a covariate in all further acoustic analyses.

Silent Gap 0–100

Sex Effects—A repeated measures ANOVA performed on ATT scores across four days of testing for oil-treated male and female animals revealed no significant Sex or Treatment main effects. However, a trend for a Sex $\times$ Treatment interaction was found, [$F(1,55) = 3.59, p = .063$]. Separate analysis of male and female groups revealed a near significant Treatment effect for males [$F(1,27) = 2.60, p = .059$] (one-tailed, HI worse than sham), but no effect of HI on females (see Figure 1, panels A and B respectively).

Next, a repeated measures ANOVA was performed on ATT scores across four days of testing for oil-treated male and female HI animals only. This analysis revealed a significant Gap $\times$ Sex interaction, [$F(8,216) = 2.27, p < .05$], indicating that early HI injury had a less detrimental effect on female animals (Figure 2A).

Hormone Effects—A repeated measures ANOVA performed on ATT scores across four days of testing for TP and oil-treated females revealed no main effect of Treatment, Hormone, or a Treatment $\times$ Hormone interaction. However, a repeated measures ANOVA performed on ATT scores across four days of testing for TP and oil-treated HI females did reveal a main effect of Hormone, [$F(1,27) = 4.92, p < .05$] (TP-treated HI females worse than oil-treated HI females), and a significant Gap $\times$ Hormone interaction [$F(8,216) = 2.70, p < .01$] (Figure 2B). Results indicate that oil-treated HI female animals were better than TP-treated HI females at detecting gaps over 10 ms. Thus, early treatment with TP increased deleterious HI effects on females.

Finally, a repeated measures ANOVA performed on ATT scores across four days of testing for oil-treated HI males and TP-treated HI females revealed no effect of Treatment and no Treatment $\times$ Gap interaction. Results thus indicate that TP-treated female HI animals performed comparably to oil-treated male HI animals.

Water Maze Results

A pseudo-random subset of animals (balanced so that mean Silent Gap ATT scores reflected means from full groups) was taken from the total N for water maze testing. This use of a sub-sample was required due to time limitations.

Water Escape—A univariate ANOVA performed on latency scores for male and female oil-treated animals revealed no significant effects of Sex or Treatment. Similarly, results of a one-way ANOVA performed on latency scores for TP-treated females revealed no significant effect of Treatment. These results indicate that all animals had intact and equivalent visual abilities and swimming capabilities.

Morris Water Maze—A repeated measures ANOVA performed on latency scores across five days of testing for male and female oil-treated animals revealed a significant main effect of Sex, [$F(1,28) = 5.98, p < .05$, (consistent with literature reports; see [48] for review)], Treatment, [$F(1,28) = 5.17, p < .05$], and Day, [$F(4,112) = 13.34, p < .001$]. Therefore, analyses were performed for male, female, and TP-treated female animals separately.

A repeated measures ANOVA of latency scores across five days of testing for male animals revealed a main effect of Treatment [$F(1,14) = 3.81, p < .05$] (one-tailed) and Day [$F(4,56) = 14.30, p < .001$], indicating that male subjects performed better on each consecutive day of testing, with HI males significantly impaired as compared to sham males (see Figure 3). This same analysis performed for oil-treated female animals revealed a significant effect of Day [$F(4,56) = 3.02, p < .05$], but no effect of Treatment, indicating that both sham and HI female animals performed better on each consecutive day, but neither group performed better than the other.

A repeated measures ANOVA performed on latency scores across five days of testing for female TP and oil-treated animals revealed a significant effect of Day, [$F(4,112) = 3.84, p < .01$] and a trend for an effect of Treatment, [$F(1,28) = 3.34, p = .078$]. Analyses were thus performed for oil and TP-treated female animals separately.

A repeated measures ANOVA performed on latency scores across five days of testing for TP-treated female animals revealed a trend towards poorer performance by TP HI females, but no significant effect of Treatment was found, nor were Treatment effects seen for oil-treated females.

Non-Spatial Water Maze—A repeated measures ANOVA performed on latency scores across five days of testing for male and female oil-treated animals revealed a significant main effect of Sex, [$F(1,28) = 15.42, p = .001$, (consistent with literature reports; see [48] for review) and Day, [$F(4,112) = 4.65, p < .05$]. Therefore, analyses were performed for male, female, and TP-treated female animals separately.

A repeated measures ANOVA performed on latency scores across five days of testing for male animals revealed a significant effect of Treatment, [$F(1,14) = 4.39, p < .03$] (one-tailed) and a trend for an effect of Day, [$F(4,56) = 2.39, p = .062$], indicating all male subjects performed better on each consecutive day of testing, while HI males were significantly impaired as compared to sham males (see Figure 4). This same analysis performed for oil-treated females revealed a significant Day effect, [$F(4,56) = 2.62, p < .05$], but no effect of Treatment, indicating that both sham and HI female animals performed better on each consecutive day, but neither group performed better than the other.

A repeated measures ANOVA performed on latency scores across five days of testing for TP and oil-treated female animals revealed no significant effects. Likewise, no effect of Treatment was found for TP-treated female animals, though a significant Day effect was revealed, [$F(4,56) = 2.60, p < .05$], indicating TP females performed better on each consecutive day of testing, but HI and sham animals performed similarly.

Anatomy Results

Brain Weight

To determine Treatment and Hormone effects on brain weight, one-way ANOVA's were performed for total brain weight within specific groups (male, females, and TP females). Results showed male HI brain weight ($2.21\text{g (mean)} \pm .04\text{ (SEM)}$) to be significantly lower than that of male shams ($2.38\text{g} \pm .05$), [$F(1,29) = 7.39, p < .05$; Table 1], while no differences were found between brain weights of oil-treated female HI ($2.09\text{g} \pm .05$) and female sham animals ($2.19\text{g} \pm .02$; see Table 1). Oil-treated female sham brain weight was significantly less than male sham brain weight; however, treatment with TP increased female sham brain weight to nearly equal to that of male shams. Similarly, brain weights were significantly different between TP-treated female HI ($2.18\text{g} \pm .05$) and sham animals ($2.30\text{g} \pm .02$), [$F(1,37) = 5.04, p < .05$], with smaller brains in TP-treated HI animals (Table 1).

Further anatomical analyses of ventricular size (an indirect measure of pathology) were computed within in each group using the difference score (the amount of difference between the left and right ventricle), as measured from serial sections (ImageJ). A univariate ANOVA was computed using the difference score of the ventricular volume for oil-treated male and female animals, and revealed a significant Treatment effect, [$F(1,58) = 2.85, p = <.05$], with HI animals having larger difference scores. One-way ANOVA's performed for oil-treated male and females groups independently revealed a significant effect of Treatment for male HI versus sham only, [$F(1,29) = 4.39, p = <.05$], indicating HI treatment caused an increase in the left-right ventricular volume difference in male animals only. Further simple effects analysis performed for male and female animals independently revealed significant differences between the left and right absolute ventricular volume (right larger than left) for male HI animals ($p <.05$, one-tailed), indicating induced HI injury lead to a significant expansion of the right ventricle in male animals only (Table 1).

A univariate ANOVA computed using the difference score of ventricular volumes for TP and oil-treated female animals did not reveal a significant Treatment or Hormone effect.

Discussion

Perinatal hypoxic-ischemic injury, and term-birth trauma resulting in HI, represent leading causes of brain damage in the neonatal population [2–3, for review see [1] and [4]]. Further, clinical data reveal that early events leading to brain damage [36–39] and multiple brain-based disorders (e.g., learning and language disabilities [34]) occur at higher incidence for males as compared to females. Sex differences in androgen levels represent one principal difference characterizing the male/female neonatal brain, with neonatal ovaries largely quiescent in the perinatal human female, but testicular androgens at near-pubertal levels in

males [41, 49]. This difference may account, at least in part, for sex differences seen in behavioral and neuropathological outcome following early HI injury.

In humans, the testes develop around gestational week (GW) 6, and detectable plasma levels of testosterone (from the testes and adrenals) are circulating in males by GW 8 [41, 50]. Fetal testosterone production is highest from GW 10–20, with a subsequent postnatal surge during the first week of life [41]. Levels of plasma androgens in males remain high for the first year, and peak during the 3rd–4th month at plasma levels similar to the second stage of puberty (200–300ng/dL; [41]). Research has shown that testosterone exerts intra-neuronal masculinizing effects on brain development via the P-450 enzyme, aromatase [see 49 for review], with the intra-neuronal conversion of testosterone to 17- β estradiol allowing the estradiol synthesized within individual neurons to bind to estrogen receptors within the brain of both humans and rodents [see 43 for review, 51–52].

In rats, elevated levels of testosterone are seen in males starting at E18 and lasting through P5 [42]. These differential levels of plasma testosterone may modulate sex differences in early response to neuronal disruption as measured by behavioral outcome. Evidence of these effects includes data showing that male rats and mice with neuronal disruptions including microgyria and ectopia, were found to be impaired on fast, but not slow, auditory discrimination tasks, even though females with identical injuries were able to perform both tasks successfully [31–32, 53]. Anatomical analysis also revealed more small and fewer large neurons in the medial geniculate nuclei (MGN) of male rats with microgyria as compared to shams, while in female animals MGN neuronal size distribution did not differ [54]. Interestingly, androgenizing female rat pups via P1–P5 injections of TP, followed by induction of microgyria, led to a shift in MGN neuronal size distribution similar to that seen in male microgyric rats. Conversely, the brain histology of vehicle-treated females undergoing microgyric surgery was found to be identical to sham females [44]. On tasks of rapid auditory processing, male BXSB/MpJ mice [31] and rats [32] with neonatal cortical malformations (ectopia and microgyria) were found to be significantly impaired as compared to female littermates with equivalent injury. Evidence of sex differences in response to brain injury has also been obtained in non-rodent species. In a classic study by Clark and Goldman-Rakic [45], female rhesus monkeys with prefrontal cortical lesions were unimpaired on reversal learning, while male monkeys and females receiving testosterone (at differing ages) were significantly impaired following lesion when compared to shams. These collective findings suggest that the actions of perinatal gonadal steroids may mediate some of the sex differences seen following early injury to the cortex — a suggestion consistent with evidence that testosterone may enhance neuronal excitotoxicity *in vitro* [55].

Implications of Current Results

Based on clinical data, coupled with prior data gathered from our lab indicating poorer performance by HI male rats as compared to HI female rats on tasks of auditory processing [56], the current study sought to assess a potentially modulating role for perinatal testosterone relative to long-term behavioral response to hypoxic ischemic injury. We found: 1) evidence of more deleterious behavioral effects of early HI injury for male as compared to female subjects, replicating earlier reports [56]; 2) evidence of increased pathology as a

result of HI injury in males relative to similarly treated female animals; and 3) new evidence that early exposure to high levels of testosterone propionate prior to HI injury causes females to exhibit both an increase in neuropathological damage, and also larger behavioral deficits (characteristic of males).

Results from tests of RAP ability in the current study show that male HI animals were significantly worse at detecting silent gaps in broadband white noise as compared to sham counterparts, while both female HI and sham animals were able to perform this task equivalently. Interestingly, treatment with testosterone propionate prior to induced injury led HI females to show significantly impaired performance on the Silent Gap task compared to oil-treated counterparts. Indeed, this impaired performance by TP-treated HI females was equivalent to that of oil-treated HI males. Maze testing results also show a deficit in performance by HI males as compared to sham males for both MWM and the NSM, while female HI and sham animals performed the tasks equally well. Treatment with TP produced a trend for female HI deficits on water maze tasks, but this effect was not significant. Anatomical analysis revealed decreased brain weight in both male HI and TP-treated female HI animals as compared to their sham counterparts, but no brain weight differences between oil-treated female HI and sham animals. Finally, only male HI animals showed increases in ventricular volume relative to sham counterparts. This indicates that though TP-treated HI females had deficits in behavioral performance, the neuropathological indices of brain weight and ventricular volume indicated effects mid-way between intact males and females. Because the developing brain responds to brain injury (or genetic disruption) through wide-spread, but subtle, alterations to the neurodevelopmental cascade, these disruptions are often more difficult to quantify as compared to more overt damage such as that caused by adult stroke. However, studies are underway to examine potential neural correlates to the behavioral deficits described here, such as changes in MGN cell size [54].

The effects of administration of testosterone propionate to female rats in the current study — combined with comparable data such as Rosen [44] and Clark and Goldman-Rakic [45] — provide evidence for a detrimental effect of this predominantly male steroid hormone following early brain injury. Though mechanisms underlying these effects require further assessment, with a goal that the basis for “protection” in females might be better understood (and potentially adapted to males via neuroprotective treatment), it is evident that testosterone acts in some manner to exacerbate response to early hypoxic-ischemic injury in rats as measured by behavioral and neuropathological outcome. This work holds important implications for future research on gender differences in response to early brain hemorrhages/HI injury, including the possible implementation of gender-specific neuroprotectants.

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Figure 1.

Panel A. A near significant Treatment effect in males ($p = .059$ one-tailed; HI worse than sham) was found; Panel B. No effect of Treatment was found for females.

Figure 2.

Panel A. Female HI animals performed significantly better than male HI animals ($p < .05$).

Panel B. TP-treated HI females performed worse than oil-treated HI females ($p < .05$).

Figure 3.

Male subjects performed better on each consecutive day of testing ($p < .001$), but HI males were significantly impaired as compared to sham males ($p < .05$, one-tailed). No effects of HI were seen for female oil-treated animals, although a trend (n.s.) toward worse performance in TP-treated HI females was observed. Average latency (mean seconds $\pm$ SEM) across 5 days of testing follows: male HI 16.47 ± 1.50 , male sham 12.95 ± 1.00 , female HI 19.69 ± 1.83 , female sham 16.72 ± 1.25 , TP female HI 21.76 ± 3.01 , TP female sham 16.42 ± 2.60 .

Figure 4.

Male subjects performed better on each consecutive day of testing ($p < .05$), but HI males were significantly impaired as compared to sham males ($p < .05$, one-tailed). No effects of HI were seen for oil or TP-treated female animals. Average latency (mean seconds $\pm$ SEM) across 5 days of testing: male HI 9.23 ± 0.86 , male sham 6.92 ± 0.69 , female HI 12.29 ± 1.59 , female sham 12.47 ± 1.04 , TP female HI 17.34 ± 3.45 , TP female sham 14.66 ± 2.17 .

Table 1

Mean body weight (g) at time of perfusion (~P110) is presented in column 2. Brain weight (g) was taken just after perfusion and is presented in column 3. Left and right ventricle size as measured using Cavalieri's Estimator of volume is presented in column 4 and 5 respectively.

	Body Weight (g) ± SEM	Brain Weight (g) ± SEM	Left Ventricle (mm ³ ± SEM)	Right Ventricle (mm ³ ± SEM)
Male HI N=15	408.7 ± 11.6	2.21 ± .04	3.60 ± 0.55	9.22 ± 3.09
Male Sham N=15	518.5 ± 15.5	2.38 ± .05	6.69 ± 1.18	5.78 ± 0.82
Female HI N=15	284.0 ± 4.2	2.09 ± .05	1.82 ± 0.28	3.71 ± 1.36
Female Sham N=15	281.3 ± 6.2	2.19 ± .02	2.90 ± 0.46	2.84 ± 0.27
TP Female HI N=18	341.2 ± 11.1	2.18 ± .05	6.68 ± 2.67	10.05 ± 4.26
TP Female Sham N=20	359.5 ± 8.6	2.30 ± .02	4.61 ± 0.43	4.98 ± 0.58