Inhibition of X-Linked Inhibitor of Apoptosis with Embelin Differentially Affects Male versus Female Behavioral Outcome following Neonatal Hypoxia-Ischemia in Rats

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Key Words

Hypoxia-ischemia • Language impairment • Auditory processing • Sex differences • Apoptosis

Abstract

Hypoxia-ischemia (HI; concurrent oxygen/blood deficiency) and associated encephalopathy represent a common cause of neurological injury in premature/low-birth-weight infants and term infants with birth complications. Resulting behavioral impairments include cognitive and/or sensory processing deficits, as well as language disabilities, and clinical evidence shows that male infants with HI exhibit more severe cognitive deficits compared to females with equivalent injury. Evidence also demonstrates activation of sex-dependent apoptotic pathways following HI events, with males preferentially activating a caspase-independent cascade of cell death and females preferentially activating a caspase-dependent cascade following neonatal hypoxic and/or ischemic insults. Based on these combined data, the 'female protection' following HI injury may reflect the endogenous X-linked inhibitor of apoptosis (XIAP), which effectively binds effector caspases and halts downstream cleavage of effector caspases (thus reducing cell death). To test this theory, the current study utilized neonatal injections of vehicle or embelin (a small molecule inhibitor of XIAP) in male and female

rats with or without induced HI injury on postnatal day 7 (P7). Subsequent behavioral testing using a clinically relevant task revealed that the inhibition of XIAP exacerbated HI-induced persistent behavioral deficits in females, with no effect on HI males. These results support sex differences in mechanisms of cell death following early HI injuries, and suggest a potential clinical benefit from the development of sex-specific neuroprotectants for the treatment of HI.

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Introduction

Hypoxic-ischemic insult during the perinatal period is a major cause of mortality and long-term neurologic morbidity in both premature/very-low-birth-weight infants, and in term infants suffering birth complications (e.g. cord asphyxia, prolonged labor, placental distress) [for reviews, see 1, 2], with cell death following neonatal hypoxia-ischemia (HI) resulting from necrosis and/or apoptosis [3, 4]. Causes of HI in premature infants can include intraventricular or periventricular hemorrhage [2, 5–7], or reperfusion failure leading to periventricular leukomalacia (a loss of white matter around the ventricles) [for reviews, see 2, 6, 7]. In term infants, complications of birth compromising placental function or cord

blood flow can lead to HI events in the brain and varied subsequent encephalopathies including periventricular leukomalacia and/or loss of gray matter [1, 2, 5–7].

Up to 50% of infants with HI die in the newborn period, and up to 25% of survivors exhibit permanent neuropsychological dysfunction [for a review, see 8]. Subsequent impairments include cognitive and behavioral deficits [2, 9; for a review, see 10], and many infants with or at risk for HI demonstrate delayed language acquisition [11] as well as deficits in verbal and language domains [12, 13]. Auditory processing deficits have also been reported in infants at risk for HI, and these deficits have been suggested to be both predictive of and possibly causal to later speech and language-related impairments [14–18]. In fact, indices of rapid auditory processing (RAP) are consistently impaired in populations with language disability, possibly reflecting anomalies in underlying neural ‘machinery’ critical to language development and processing [15, 19, 20; for a review, see 21]. Importantly, RAP can be measured in animal models, and is consistently impaired in male rodents with induced neonatal brain injuries including perinatal HI [22–26], microgyria and ectopia (small perinatally occurring cortical malformations) [27–35], and prenatal teratogenic exposure [36]. Interestingly, female rodents fail to show significant behavioral RAP deficits associated with any comparable perinatal brain injury, including HI [30, 31, 37, 38].

Human clinical data also reveal sex differences in behavioral outcome following early neonatal insults, with premature/very-low-birth-weight male infants showing more long-term cognitive deficits as compared to females, even when matched for injury [39–43]. For example, prematurely born males with intracranial bleeds or respiratory complications leading to HI risk show significantly reduced IQ at early school age as compared to matched females [44, 45]. In fact, males overall show a significantly increased risk for developmental speech and language disorders, stutter, dyslexia, autism, and learning disabilities as compared to females [40, 41, 43] and the cause of this sex difference remains unknown.

Interestingly, recent work exploring cell death mechanisms in animal models has revealed important sex differences in apoptotic pathways following early induced HI insult, with evidence indicating neural cell death due largely to caspase-independent activation of apoptosis in males, and caspase-dependent activation in females (though mechanisms are not exclusive to sex) [for reviews, see 46, 47]. Specifically, the caspase-independent pathway mediated by the DNA repair enzyme, poly (ADP-ribose) polymerase 1 (Parp-1) is highly activated in

male but not female mice with early HI injury [48], and significant neural protection from this injury (measured by decreased injury scores) has been shown in Parp-1 knockout male mice but not females [49]. Conversely, the caspase-dependent pathway is mediated by cytochrome c and the activation of caspases [for reviews, see 46, 47], which are higher in female versus male mice after early HI injury [48], and inhibition of caspase cleavage has been shown to be neuroprotective in female rats only following neonatal HI (measured by decreased injury scores [50] and infarct volume [51]). Measures of sex differences in caspase-dependent and -independent apoptotic pathways, as well as gender-specific protection, have also been demonstrated in animal models of adult ischemic injury or stroke [52–55; for a review, see 56].

Within the caspase-dependent pathway, inhibitors of apoptosis proteins serve as endogenous inhibitors of cell death [for reviews, see 57, 58], the most potent being X-linked inhibitor of apoptosis protein (XIAP) [59]. XIAP stops both intrinsic and extrinsic apoptosis by binding to the initiator caspase (caspase-9) and halting further cleavage of downstream caspases (caspases 3 and 7) [59]. XIAP has also been shown to bind and inhibit caspases 3 and 7 directly [for a review, see 57], and its expression has been confirmed in both rodent and human brains following ischemic injury [60]. Because XIAP acts on the caspase-dependent pathway of cell death, it may play a selective role in the protection afforded to females following early HI injury. Though recent studies of XIAP knockout [61] and overexpression [62] have yet to reveal sex differences in the degree of tissue loss following neonatal HI, these results are likely due to compensatory changes in other inhibitor of apoptosis protein family members [63–65] and XIAP still remains a probable source of protection for females.

Based on clinical evidence of sex differences in response to early HI injury, coupled with the above data on differing pathways of cell death between the sexes following early HI injury, the current study sought to assess the caspase-dependent progression of apoptosis in a neonatal HI model. Because XIAP is known to act specifically in the caspase-dependent pathway [for reviews, see 57–59], agents acting as inhibitors of XIAP should increase behavioral deficits and anatomical damage following neonatal HI injury in females. To test this hypothesis, the current study employed embelin – one of the most potent inhibitors to block the binding of XIAP to caspases [66]. Long-term outcome was measured by behavioral assessment of RAP – a clinically relevant measure associated with language outcomes – and via neuromorphometry.

Methods

Subjects

Time-mated female Wistar rats were ordered from Charles River Laboratories and shipped on embryonic day 5 to minimize prenatal stress. Dams were housed in the University of Connecticut animal facility in a 12-hour light/12-hour dark cycle where they gave birth. Pups were culled to litters of 5 males and 5 females on postnatal day 1. All pups received subcutaneous injections of either 20 mg/kg embelin in dimethyl sulfoxide (DMSO), or an equivalent volume (approx. 0.05–0.06 ml) of DMSO or saline, on P5–7 (1 injection in the morning on P5–6, and a third injection approx. 30 min before surgery on P7) and underwent HI or sham surgery on P7. Treatment with embelin or vehicle (DMSO or saline) was assigned between litters, while sham and HI surgery were balanced within litters. Two vehicle groups, saline and DMSO, were utilized to ensure that DMSO did not have its own effect on HI outcome. Food and water were available ad libitum.

Induction of HI

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 was completely cauterized. The incision was sutured, footpad marking injections were made, and pups were returned to their dams after recovering from anesthesia under a warming lamp. Approximately 2 h 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 min [for a review, see 67]. Sham animals underwent a comparable procedure, excluding artery cauterization and hypoxia (shams were exposed to room air in an equivalent chamber for 120 min). All pups were returned to their mothers following sham or HI procedures, where they remained until weaning on P21.

Behavioral Testing, Startle Reduction

The startle reduction paradigm utilizes the subject's acoustic startle reflex – a large motor reflex response to a startle-eliciting stimulus (SES; 105 dB white noise burst) – coupled with a benign acoustic stimulus just prior to the SES on cued trials. Termed prepulse inhibition (PPI) 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 a review, see 68]. 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. sweep reversal), thus providing a measure of detectability of the pre-SES cue [for a review, see 68]. Such assessments measure RAP abilities of rodents through the use of presentation of various prepulse cues, effectively modeling human RAP tasks that tap fundamental speech processing mechanisms [14–16], and thus provide a clinically relevant measure of outcome.

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 through a PHM-250-60 linear load cell amplifier

and into a Biopac MP100A-CE acquisition system connected to a Power Macintosh G3. This apparatus recorded the amplitude of each subject's startle reflex (within a 150-ms epoch), starting with 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 105-dB, 50-ms burst of white noise.

Normal Single Tone (P25)

On cued trials, subjects were presented with a single 75-dB, 7-ms tone (2.3, 5, 8, or 8 kHz) followed 50 ms later by a 105-dB, 50-ms SES. On uncued trials, only the 105-dB SES was presented. Intertrial intervals (ITIs) of 16, 18, 22, or 24 s randomly separated each trial to prevent anticipation of the cue. 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). Normal single tone (NST) measures thus provide a baseline measure of startle attenuation, which can be used to confirm intact hearing and PPI in all subjects.

Silent Gap (P27–30; Juvenile)

The silent gap (SG) detection task involved 300 trials of randomly presented silent gaps embedded in a continuous 75-dB broadband white noise background. The SG 0–100 task featured gaps of 2, 5, 10, 20, 30, 50, 75, and 100 ms embedded in the white noise. 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) in white noise on uncued trials. ITIs of 16, 18, 22, or 24 s randomly separated each trial to prevent anticipation of the cue.

Frequency-Modulated Sweep [P55–58 (Young Adult) and P83–86 (Adult)]

The frequency-modulated (FM) sweep discrimination task involved the repeated presentation (104 trials per session) of a 75-dB, downward FM sweep (2,300–1,900 Hz), with an upward FM sweep serving as the cue (1,900–2,300 Hz) on cued trials. Sweeps lasted 175, 125, 75, or 25 ms (one full session per duration), and ITIs again ranged between 16, 18, 22, and 24 s. Sweep duration remained constant throughout 1 day of testing, while ITIs were varied to prevent anticipation of the cue.

Histological Analysis

Upon the completion of behavioral testing, all animals were weighed and deeply anesthetized with an intraperitoneal injection of a mix of ketamine and xylazine (100 and 15 mg/kg), then transcardially perfused with 0.9% saline followed by 10% buffered formalin. Brains were removed from the skull, and postfixed 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 μ m, 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 $\times 1.3$ magnification on a Fisher Scientific Micromaster digital microscope using Micron software, and analyzed (blinded to treatment or sex) for damage and structural 3-dimensional volume indices for the cerebral ventricles. Mea-

tures 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 [69].

Statistical Analysis

Based on a priori hypotheses that differences would exist between male and female HI animals [37, 38], as well as between vehicle-treated HI animals and embelin-treated HI animals, planned comparisons were performed between specific groups. This included separate analyses for sham and vehicle/embelin-treated HI males, sham and vehicle/embelin-treated HI females, and vehicle-treated HI and embelin-treated HI animals.

The PPI paradigm is used in the context of these experiments to assess complex acoustic processing of cues in intact and HI-injured animals. As such, it is important to ascertain that baseline individual differences in hearing, startle response, or baseline PPI do 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 brain injuries and language problems. To specifically address the issue of complex acoustic processing, without confounds of hearing, startle, or PPI differences, baseline NST ATT scores were used as a covariate in analyses of RAP data. This means that any differences in acoustic processing that were attributable to underlying differences in hearing, startle, or baseline PPI were removed from effects as reported. Although the 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 marginal drug effect ($p = 0.058$) on NST was in fact seen (possibly reflecting differences in hearing, startle, or simple PPI). Although NST 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 the use of NST ATT scores as a covariate.

Multivariate analyses of variance (ANOVAs) were used to analyze auditory ATT scores. Variables are presented in the Results section according to the following: sex (2 levels: male, female), treatment (2 levels: HI, sham), vehicle (2 levels: saline, DMSO), drug (2 levels: vehicle, embelin), day (4 levels), gap (9 levels, for SG only), interstimulus interval (ISI) (4 levels, for FM only), and age (2 levels: young adult, adult for FM only). All analyses were conducted using SPSS 15.0 with an alpha criterion of 0.05. Data presented in auditory task graphs are depicted by ATT, or mean attenuation scores (cued response/uncued response $\times 100$) $\pm$ SEM, with higher scores indicating poorer performance (100% = chance).

Results

To ensure DMSO did not have an independent effect on sham or HI animals, analyses of both auditory tasks (SG and FM) were completed for male and female, saline and DMSO animals. A sex $\times$ treatment $\times$ vehicle $\times$ day $\times$ gap repeated-measures ANOVA performed on ATT scores from SG 0–100 (P27–30) revealed no significant

effect of vehicle. Likewise, a sex $\times$ treatment $\times$ vehicle $\times$ age $\times$ ISI repeated-measures ANOVA performed on ATT scores from FM sweep (P55–58, P83–86) revealed no significant effect of vehicle. These results indicated no differences in scores between animals treated with saline and DMSO and therefore sham saline and sham DMSO groups, as well as HI saline and HI DMSO groups, were combined to form vehicle-treated male sham ($n = 14$), vehicle-treated male HI ($n = 16$), vehicle-treated female sham ($n = 14$), and vehicle-treated female HI ($n = 15$) groups. Embelin-treated groups consisted of male HI ($n = 14$) and female HI ($n = 16$) embelin-treated animals.

Normal Single Tone (P25)

Results of a univariate ANOVA performed for all groups revealed no significant effects, but as discussed above, NST scores were used as a covariate in all further acoustic analyses.

Silent Gap 0–100 (P27–30 Juvenile)

A repeated-measures ANOVA performed on ATT scores across 4 days of testing for male and female animals revealed no significant effects of sex, treatment, or drug. These results indicate that all groups were able to perform this simple silent gap detection task equivalently.

FM Sweep (P55–58 Young Adult, P83–86 Adult)

A repeated-measures ANOVA performed for all groups across both young adult and adult ATT scores revealed a significant sex $\times$ treatment interaction [$F(1, 83) = 7.139, p < 0.01$], a significant sex $\times$ drug interaction [$F(1, 83) = 5.070, p < 0.05$], a significant main effect of age [$F(1, 83) = 37.914, p < 0.001$], and a significant main effect of ISI [$F(3, 249) = 10.335, p < 0.001$]. Separate analyses were then performed for male and female groups to further characterize the nature of effects.

Male FM Sweep

A repeated-measures ANOVA performed for male sham, HI, and embelin-treated HI animals across both young adult and adult ATT scores revealed significant effects of age [$F(1, 40) = 15.066, p < 0.001$], ISI [$F(3, 120) = 4.186, p < 0.01$], and treatment [$F(1, 40) = 6.180, p < 0.05$]. These results indicate that ATT scores improved with increasing age and with decreasing ISI for all animals (note that improvement with decreasing ISI is likely a reflection of test experience from prior days). However, male HI animals (including embelin-treated) were significantly impaired compared to male shams (fig. 1a).

Fig. 1. **a** FM sweep (all male animals). A repeated-measures ANOVA for male animals revealed significant effects of age ($p < 0.001$), ISI ($p < 0.01$), and treatment ($p < 0.05$), with male HI animals (including embelin-treated) significantly impaired compared to male shams. **b** FM sweep (males, sham vs. HI vehicle). Similar analysis for vehicle-treated male HI and male shams revealed significant effects of age ($p < 0.05$), and treatment ($p < 0.05$), with male HI animals significantly impaired compared to shams. **c** FM

sweep (males, sham vs. HI embelin). Similar analysis for male shams and embelin-treated HI animals revealed significant effects of age ($p < 0.001$) and ISI ($p = 0.001$). **d** FM sweep (males, HI embelin vs. HI vehicle). Similar analysis for male HI and male embelin-treated HI animals revealed significant effects of age ($p < 0.01$) and ISI ($p < 0.05$). Importantly, no drug effect was found indicating that vehicle-treated HI and embelin-treated HI males did not differ in performance.

A repeated-measures ANOVA was then performed for vehicle-treated male HI and male sham animals only, across young adult and adult ATT scores. This analysis revealed significant effects of age [$F(1, 27) = 4.429$, $p < 0.05$] and treatment [$F(1, 27) = 4.587$, $p < 0.05$]. Thus ATT scores improved with increasing age, but male HI animals were significantly impaired compared to shams (fig. 1b). This analysis was also performed for male shams and embelin-treated HI animals, revealing significant effects of age [$F(1, 25) = 23.095$, $p < 0.001$] and ISI [$F(3, 75) = 5.767$, $p = 0.001$]. Results indicate that ATT scores improved with increasing age and decreasing ISI for both male shams and embelin-treated male HI animals (fig. 1c).

A repeated-measures ANOVA was then performed for male HI and male embelin-treated HI animals across young adult and adult ATT scores. Results showed significant effects of age [$F(1, 27) = 8.961$, $p < 0.01$] and ISI [$F(3, 81) = 3.427$, $p < 0.05$], indicating that ATT scores improved with increasing age and with decreasing ISI. Importantly, there was no effect of drug, indicating that vehicle-treated HI and embelin-treated HI males did not differ in performance (fig. 1d).

Female FM Sweep

A repeated-measures ANOVA was performed for female sham, HI, and embelin-treated HI animals across young adult and adult ATT scores. Results showed significant effects of age [$F(1, 41) = 22.344$, $p < 0.001$] and

Fig. 2. **a** FM sweep (all female animals). A repeated-measures ANOVA performed for female animals revealed significant effects of age ($p < 0.001$), ISI ($p < 0.001$), and importantly, drug ($p = 0.001$), indicating that embelin-treated HI females were significantly impaired compared to both vehicle-treated sham females and vehicle-treated HI females. **b** FM sweep (females, sham vs. HI embelin). Similar analysis performed for female sham and embelin-treated HI animals revealed significant main effects of age ($p < 0.001$), ISI ($p < 0.001$), and treatment ($p < 0.05$), indicating that embelin-treated HI animals were significantly impaired relative to shams. **c** FM sweep (females, sham vs. HI vehicle). Im-

portantly, similar analysis performed between female sham and vehicle-treated HI animals revealed significant main effects of age ($p < 0.001$) and ISI ($p = 0.001$), but no effect of treatment, indicating that vehicle-treated female HI animals were able to perform this RAP task as well as female shams. **d** FM sweep (females, HI embelin vs. HI vehicle). Similar analysis for female vehicle-treated HI and female embelin-treated HI animals revealed significant main effects of age ($p < 0.01$) and ISI ($p < 0.05$). Importantly, a drug effect was found ($p = 0.001$), indicating that female embelin-treated HI animals were significantly impaired relative to vehicle-treated HI females.

ISI [$F(3, 123) = 8.509$, $p < 0.001$], indicating that ATT scores improved with increasing age and with decreasing ISI for all animals. Importantly, a significant effect of drug was also found [$F(1, 41) = 11.754$, $p = 0.001$], indicating that embelin-treated HI females were significantly impaired compared to both vehicle-treated sham females and vehicle-treated HI females (fig. 2a).

To assess the extent of impairment in embelin-treated HI animals, a repeated-measures ANOVA was performed for female sham versus embelin-treated HI animals across both young adult and adult ATT scores. Results showed a significant main effect of age [$F(1, 27) = 19.350$, $p < 0.001$], ISI [$F(3, 81) = 7.409$, $p < 0.001$], and treatment

[$F(1, 27) = 5.180$, $p < 0.05$], indicating that ATT scores improved with increasing age and decreasing ISI, but that embelin-treated HI females were significantly impaired relative to shams (fig. 2b). Importantly, this same comparison performed between female sham and vehicle-treated HI animals revealed significant main effects of age [$F(1, 26) = 16.321$, $p < 0.001$] and ISI [$F(3, 78) = 6.289$, $p = 0.001$], but no effect of treatment, showing that vehicle-treated female HI animals were able to perform this RAP task as well as female shams (fig. 2c).

To further characterize the extent of embelin-induced deficits, a repeated-measures ANOVA was performed for female vehicle-treated HI and female embelin-treated HI

Fig. 3. FM sweep (male HI vs. female HI animals). A repeated-measures ANOVA was performed for vehicle-treated male HI and vehicle-treated female HI animals across young adult and adult ATT scores revealed a significant effect of sex ($p < 0.03$, one-tail), with male HI animals displaying poorer RAP abilities than female HI animals.

animals across young adult and adult ATT scores. Results showed significant main effects of age [$F(1, 28) = 9.710$, $p < 0.01$] and ISI [$F(3, 84) = 3.446$, $p < 0.05$], indicating that ATT scores improved with increasing age and decreasing ISI. Importantly, a significant effect of drug was also found [$F(1, 28) = 12.808$, $p = 0.001$], showing that female embelin-treated HI animals were significantly impaired relative to vehicle-treated HI females (fig. 2d).

Male versus Female FM Sweep

Given clinical literature indicating poorer long-term outcome in males following neonatal HI, a repeated-measures ANOVA was performed for vehicle-treated male HI and vehicle-treated female HI animals across young adult and adult ATT scores. Given prior findings, a one-tail test was used [37, 38]. This analysis revealed a significant effect of sex [$F(1, 28) = 4.521$, $p < 0.03$, one-tail], with male HI animals displaying poorer RAP abilities than female HI animals (fig. 3).

Anatomical Analysis

Since blood and oxygen flow to the right hemisphere is reduced when the right carotid artery is permanently cauterized, the right hemisphere was expected to show increased pathology relative to the left hemisphere in HI animals. Analysis of anatomical damage was thus computed in each group for ventricles, cortex, and hippocampus via comparison of the left versus right volumes, as well as total volumes (left and right combined) across groups.

Ventricles

Anatomical analysis of ventricular size was computed within each group through comparison of left and right lateral ventricular volumes. A paired-samples t test revealed no significant difference for male shams ($p > 0.05$). However, larger right lateral ventricles were seen in both male HI animals ($p < 0.01$) and embelin-treated HI males ($p < 0.05$; fig. 4). Importantly, volumetric measures of the right ventricle did not differ between vehicle-treated HI and embelin-treated HI males ($p > 0.05$; fig. 4). Left and right ventricles of female shams ($p > 0.05$) and female HI ($p > 0.05$) animals also did not differ (fig. 4). Importantly, however, the right ventricles of embelin-treated HI females were significantly larger than the left ventricles ($p < 0.05$; fig. 4). In addition, a univariate ANOVA performed on all animals for total ventricular volume revealed a significant effect of sex [$F(1, 82) = 4.227$, $p < 0.05$] (male larger than female; likely due to the ventricular enlargement seen specifically in HI males and not HI females). These results show that HI treatment alone increased the right ventricle volumes of males, but not females, relative to the left. However, HI combined with embelin treatment significantly increased the right ventricle volume relative to the left in females. Thus, inhibition of XIAP with embelin following early HI led to a more deleterious effect on brain tissue in female animals. Future studies will confirm this effect given the close mean differences between saline-treated HI and sham females and embelin-treated HI and sham females.

Cortex

Anatomical analysis of cortical volume was computed within each group, and paired-samples t tests revealed no significant differences in left versus right cortical volumes for any group. However, a univariate ANOVA performed on all animals for total cortical volume revealed significant effects of sex [$F(1, 80) = 9.913$, $p < 0.01$] (male larger than female), and drug [$F(1, 80) = 7.944$, $p < 0.01$] (embelin-treated larger than vehicle-treated; data not shown). Further analysis revealed that embelin-treated male HI animals had cortical volumes equal to that of male shams ($p > 0.05$), and significantly larger than vehicle-treated HI males ($p < 0.05$; data not shown). Similar analyses in female animals revealed no effects. These results suggest that treatment with embelin preceding neonatal HI may increase cortical volume in males, although apparently this was not reflected in protection from behavioral deficits. Future research may address this finding by assessing other markers of embelin in neonatal HI males and females (e.g. histological markers of newly

Fig. 4. Left versus right ventricle volume. Paired-samples *t* tests revealed significant increases in right ventricle volume relative to left for vehicle-treated male HI animals (** $p < 0.01$), embelin-treated male HI animals (* $p < 0.05$), and embelin-treated female HI animals (* $p < 0.05$).

born cells, dying cells, microglia, or other indices of transient cell loss and/or preservation).

Hippocampus

Anatomical analysis of hippocampal volume was computed within each group through comparison of the left and right hippocampi and paired-samples *t* tests revealed no significant differences in any group. However, a univariate ANOVA performed on all animals for total hippocampal volume again revealed significant effects of sex [$F(1, 82) = 16.123, p < 0.001$] (male larger than female) and drug [$F(1, 82) = 6.559, p < 0.05$] (embelin-treated larger than vehicle-treated; data not shown). Further analysis revealed that embelin-treated male HI animals again had hippocampal volumes larger than that of both male shams ($p < 0.05$) and vehicle-treated HI males ($p < 0.05$; data not shown), with no significant differences in hippocampal volumes found for females.

Discussion

HI is one of the most common causes of neonatal neurological impairment, and a high proportion of those affected go on to experience cognitive and behavioral deficits [2; for reviews, see 8, 10], including difficulty with language acquisition and verbal ability [11, 12]. Deficits in RAP have been suggested to be predictive of such language impairments in these and other populations [14–18]. Tests of RAP have been successfully developed for use in animal models, and such tasks reveal deficits in ro-

dents with induced brain injuries including HI [22–26, 37, 38], microgyria and/or ectopia [27–35], and prenatal teratogenic exposure [36]. However, these models have never shown comparable deficits in female rodents with early injury [30, 31, 37, 38], consistent with clinical data indicating poorer prognosis for male infants suffering HI as compared to matched females.

Recent laboratory work has begun to explore these intriguing sex differences in outcome following neonatal HI. Results suggest a putative role of sex-specific cell death pathways [for reviews, see 46, 47], with males preferentially utilizing caspase-independent apoptosis (as confirmed by increased Parp-1 activation) [48], and females preferentially utilizing caspase-dependent apoptosis (as confirmed by increased cleaved caspases [48] and neuroprotection following inhibition of caspase cleavage [50, 51]). By binding to caspases 3, 7, and 9, XIAP is the most potent endogenous inhibitor of caspase-dependent cell death [59], and thus may be a crucial factor in the apparent protection afforded to females following hypoxic ischemic insult.

The current study employed embelin – the most potent cell-permeable inhibitor of XIAP – to further characterize mechanisms underlying the behavioral outcome following neonatal HI in rodents. Embelin effectively inhibits XIAP by binding to its BIR3 domain (the binding site of caspase-9), rendering it inactive – and thus leading to a potential increase in cell death via the caspase-dependent pathway [66]. We specifically sought to assess the potentially modulating role of embelin following neonatal HI injury and found: (1) evidence of deleterious behav-

ioral effects and increased pathology as a result of early HI injury in males, with no such effects in vehicle-treated HI female subjects (see earlier reports) [37, 38]; (2) evidence of increased deleterious behavioral effects and increased pathology as a result of early HI injury in vehicle-treated HI males as compared to vehicle-treated HI females, replicating earlier reports [37, 38], and (3) novel and exciting evidence that inhibition of XIAP (via embelin) prior to neonatal HI injury led to both an increase in behavioral deficits in embelin-treated HI females compared to vehicle-treated HI females, and an indication of a similar effect on neuropathology. No comparable effect of XIAP inhibition was seen in males.

Though our anatomical analyses revealed increased right ventricular volume relative to left in both vehicle and embelin-treated male HI animals, we also found embelin treatment to paradoxically increase the total cortical and hippocampal volume of male HI animals relative to vehicle-treated male HI animals. Though there was no effect of embelin on our behavioral tasks for HI males (vehicle-treated equal to embelin-treated), vehicle-treated HI males were significantly impaired relative to shams, while embelin-treated HI males only trended towards poorer performance on the FM task ($p = 0.09$) relative to male shams. Though future studies would need to further assess mechanisms of XIAP inhibition following neonatal HI injury, it seems possible given our anatomical and behavioral data here that suppression of endogenous mechanisms of inhibition of caspase-mediated cell death (i.e., XIAP) may paradoxically protect the male brain (though to a minor extent). Similar paradoxical effects have been seen in an adult stroke model where inhibition of caspase-independent mechanisms of cell death (the typical 'male pathway') have led to increased deleterious effects in females and beneficial effects in males [70]. It is thought that these effects could reflect sex-dependent 'shifts' in apoptotic pathways which benefit males, but are deleterious to females [70].

Conclusions

Results from the current study confirm sex differences in behavioral and anatomical outcome following HI, as well as important and novel sex differences in potential apoptotic mechanisms that may underlie these sex differences. Specifically, vehicle-treated HI and embelin-treated HI males were significantly impaired on FM detection tasks as compared to male shams, and

there were no differences in performance between vehicle-treated male HI and embelin-treated male HI animals. Moreover, vehicle-treated HI males demonstrated increased behavioral deficits and anatomical damage relative to vehicle-treated HI females, while both vehicle-treated female HI and sham animals were able to perform FM tasks equally well (consistent with the prior data). The critical and novel finding of this study was that inhibition of XIAP prior to HI caused a significant increase in both behavioral deficits and anatomical abnormalities (ventricular enlargement) in embelin-treated HI females relative to vehicle-treated HI females. Taken together, these findings suggest that XIAP acts to protect the female brain from the deleterious effects of early HI injury, while elimination of this protection via an XIAP inhibitor exacerbates both damage and behavioral deficits.

Though it is evident that inhibition of XIAP had a detrimental effect on HI outcome for female animals, recent work exploring testosterone-modulated effects of neonatal HI injury also indicates that perinatal hormone levels (specifically testosterone) may contribute to sex differences in response to early brain injury [37]. Future studies will be needed to assess a potential interaction between the early (or concurrent) presence of specific hormones and activation of sex-specific apoptotic pathways, which may further relate to underlying baseline sex differences in cortical development [71]. Extensive work by Arnold and colleagues [72–75] supports the idea that sexual differentiation of the brain occurs due to both hormonal exposure as well as genetic differences in sex chromosome gene expression within brain cells, and thus response to early injury may be influenced by a combination of factors. The results presented here, combined with prior data [37], suggest that the differing effects of HI on the sexes may reflect hormonal factors, an orthogonal genetic factor(s), or a combination of both.

In closing, apoptosis is a major contributor to neuronal cell death and tissue loss following HI injury in the developing brain, and inhibitors of apoptosis represent valuable candidates for therapeutic intervention. However, if cell death proceeds in a sex-dependent manner, then inhibitors of apoptosis will be most effective if they target the specific pathway most utilized by each sex. The results presented here are the first (to our knowledge) to compellingly show behavioral deficits in one sex (female) following manipulation of sex-specific pathways of cell death (caspase-dependent). Moreover, behavioral changes induced via this manipulation persisted through adult-

hood and have relevance to language outcome measures in neonates, thus emphasizing the importance of a better understanding of neonatal mechanism of cell death following brain injury. Our data clearly support the need for further research on sex-dependent apoptotic mechanisms in relation to neonatal HI injury, and have implications for the potential use of sex-specific neuroprotectants in clinical practice.

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