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Research Report

Auditory processing and learning/memory following erythropoietin administration in neonatally hypoxic–ischemic injured rats

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ABSTRACT

Background: Hypoxia–ischemia (HI) is a common injury arising from prematurity/complications at birth and is associated with later language, auditory, and learning impairments. **Objective:** To investigate the efficacy of two doses (300 or 1000 U/kg) of Erythropoietin (Epo) in protecting against neuropathological and behavioral impairments associated with HI injury in rats. **Methods:** HI injury (right carotid artery cauterization and 120 min of 8% O₂) was induced on postnatal day 7 (P7) and Epo or saline was administered i.p. immediately following the procedure. Auditory processing and learning/memory were assessed throughout development. **Results:** Both doses of Epo provided behavioral protection following HI injury. Rats given 300 or 1000 U/kg of Epo performed significantly better than HI animals on a short duration complex auditory processing procedure, on a spatial Morris water maze assessing spatial learning/reference memory, and a non-spatial water maze assessing associative learning/reference memory. **Conclusions:** Given Epo's extant clinical use (FDA approved for pediatric patients with anemia secondary to prematurity), the current results add to a growing body of literature supporting the use of Epo as a potential protective agent for neurological and behavioral impairments following early HI injury in infants.

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1. Introduction

Brain injury and associated behavioral impairments following neonatal hypoxia–ischemia [HI; associated with prematurity/very low birth weight (VLBW) or term birthing injuries, such as asphyxia] are of significant clinical importance. In addition to increased mortality, seizure, and handicap rates, the incidence of later cognitive and other behavioral deficits in premature infants is approximately 25–50% (Volpe, 2001). Behavioral deficits most strongly associated with early HI insults include: recognition memory and processing speed (Rose and Feldman, 1996; Rose et al., 2005); language-related

abilities (Frisk and Whyte, 1994; Johnston et al., 2001; Kilbride et al., 2004; Largo et al., 1986; Msall and Tremont, 2002; Robertson and Finer, 1985; Volpe, 2001); spatial memory (Curtis et al., 2002); and rapid auditory processing (RAP) ability (Downie et al., 2002; Jansson-Verkasalo et al., 2004). RAP (the ability to discriminate between rapidly changing auditory stimuli, within tens of milliseconds; Benasich and Tallal, 2002) has been shown to be critical to language acquisition, and deficits in this skill have been suggested to play a causal role in emergent language problems (see Tallal, 1976). RAP deficits have also been shown in premature/VLBW children (Downie et al., 2002; Jansson-Verkasalo et al., 2004)

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and may relate to the high incidence of language problems in this population.

To date, no intervention therapy has been found effective in ameliorating behavioral deficits associated with neonatal HI in infants. In fact, most current neuroprotective strategies used in HI animal models are not applicable to humans due to detrimental side-effects. For example, MK-801 (a glutamate receptor antagonist) attenuates brain injury following HI in rats (Hagberg et al., 1994) but has neurotoxic properties that preclude it from human clinical use (Levene, 1992). One agent, an endogenous cytokine (Erythropoietin; Epo), is already routinely given to infants with anemia and has been found to have no adverse side-effects (Reiter et al., 2005). Moreover, Epo has been shown to provide neuropathological protection following HI injury in rodents (Aydin et al., 2003; Kumral et al., 2003, 2004; Spandou et al., 2005; Sun et al., 2005; Wang et al., 2004). When taken together, the protective effects of Epo combined with its clinical safety render it a promising therapeutic intervention for HI injury in infants.

The aim of the current study was to investigate the efficacy of Epo in protecting against neonatal HI-induced behavioral impairments (auditory processing, learning/memory) and to further evaluate the relative effectiveness of two different doses in a rat model. We have previously demonstrated that 300 U/kg of Epo provides significant protection against auditory processing deficits in neonatal HI rat model (McClure et al., 2006). Therefore, we chose to administer Epo at 300 U/kg (a reported dose in use for anemia of prematurity; Reiter et al., 2005), as well as 1000 U/kg (a dose previously reported to provide neurological and memory protection following HI injury in rats; Kumral et al., 2004). Our goal was to compare the effectiveness of Epo at these different doses in protecting against long-term behavioral deficits following neonatal HI.

Spatial and reference memory deficits have previously been identified in a rodent model of HI, as measured by T-maze (Ford et al., 1989) and Morris water maze (MWM; Almlil et al., 2000; Arteni et al., 2003). Since the hippocampal formation plays a major role in reference and spatial learning and memory (Galani et al., 2002; Nanry et al., 1989), and because P7 HI injury typically leads to gross hippocampal damage (Towfighi et al., 1991), we expected to see deficits in animals with induced HI, as well as protection from such deficits following Epo administration. With regard to auditory processing, it has been shown that thalamic and cortical damage typically follow HI injury (Northington and Ferriero, 2001; Vannucci et al., 1999), and since complex and/or rapid auditory processing has been shown to depend on cortical and subcortical contributions (i.e., medial geniculate body of the thalamus; Clerici and Coleman, 1998), we hypothesized that auditory discrimination tasks with RAP requirement (e.g., short duration FM sweeps) would show impairment in HI animals, and similarly show protection with Epo treatment.

2. Results

2.1. Histology

Non-parametric tests based on assessment scores of brain damage rated on a scale from 0 to 3 (0=no damage, 1=minimal

damage, 2=moderate, and 3=severe; assessments performed blind to treatment, see Fig. 1) were performed between the HI group and all other groups. Mann-Whitney U 's revealed significant differences between HI [mean=2] and sham [mean=0] groups ($z=3.70$, $p<0.01$), with HI animals having higher scores on the damage scale. Significant differences were also found between the HI and HI+300 U/kg Epo [mean=1] groups ($z=1.75$, $p<0.05$) and HI and HI+1000 U/kg Epo [mean=0.75] groups ($z=2.27$, $p<0.05$). Subjects who were determined to be outliers based on behavioral data ($n=4$) were also found to have damage inconsistent with their treatment group (i.e., no damage in the HI subject group and severe damage in the HI+Epo subjects), suggesting either incomplete performance of treatments or subject switches (thus the 4 subjects were dropped).

2.2. Auditory

For the single tone procedure, comparison of acoustic startle response (ASR) for cued and uncued trials showed that all groups could discriminate the cue. For all analyses of ATT

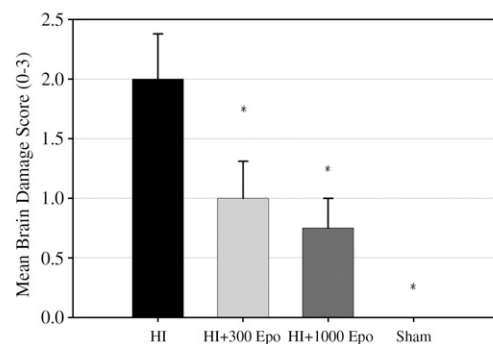
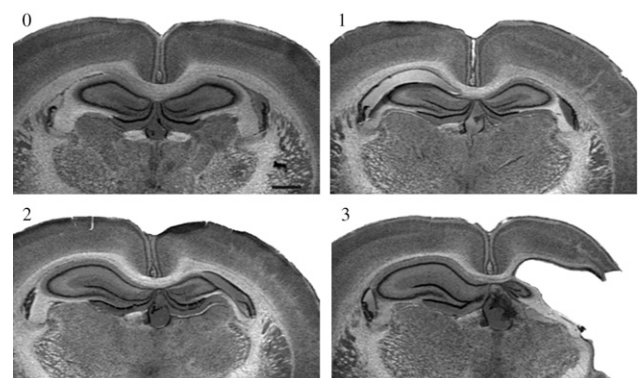


Fig. 1 – Nissl-stained sections representing typical histological quantification of no (0), mild (1; some cortical cell loss and/or slight reduction in the size of the hippocampus), moderate (2; significant cortical cell loss and hippocampal damage), and severe (3; near complete blow out of the right hemisphere) brain damage (scale bar = 1 mm). Means (\pm SEM) for HI, HI-300 Epo, HI+1000 Epo, and shams on the 0–3 damage scale. Comparisons between HI and all other groups showed HI animals given Epo and sham animals had significantly lower scores on the damage scale compared to the HI group supporting Epo's role in neuropathological sparing ($*p<0.05$).

scores, no differences were found between the sham, HI +300 U/kg, HI+1000 U/kg, and the HI group as shown by individual univariate ANOVAs. These results indicate that animals did not differ in their ability to process “easy” auditory stimuli, had no gross hearing deficits, and their startle brain circuitry was intact.

For the FM sweep procedure, analysis of ASR scores showed that all subjects could discriminate the cues. Analyses of ATT scores for sham and HI subjects were then compared using a 2 (FM duration; long vs. short) \times 2 (Treatment) repeated measures ANOVA. This analysis showed a main effect for Treatment [$F(1,14)=4.89$, $p<0.05$], with simple effects analysis showing that HI animals performed significantly worse, specifically on short duration FM sweeps [$t=2.39$, $p<0.05$] as compared to shams (Fig. 2).

Next, both Epo groups were compared to the HI group using separate 2 (FM duration; long vs. short) \times 2 (Treatment) repeated measures ANOVAs. A significant interaction was found for FM duration \times Treatment for the HI + 300 U/kg Epo vs. HI group [$F(1,12)=5.19$, $p<0.05$], and a significant interaction was also found for the HI + 1000 U/kg Epo vs. HI group [$F(1,13)=5.48$, $p<0.05$]. Simple effects analyses revealed that both groups given Epo (300 and 1000 U/kg) performed significantly better, specifically on the short duration FM sweep as compared to the HI group [$t=2.57$, $p<0.05$ and $t=2.79$, respectively, $p<0.01$] (Fig. 2).

2.3. Water escape and MWM

No differences were found between any group on the water escape procedure, indicating comparable performance in swim-

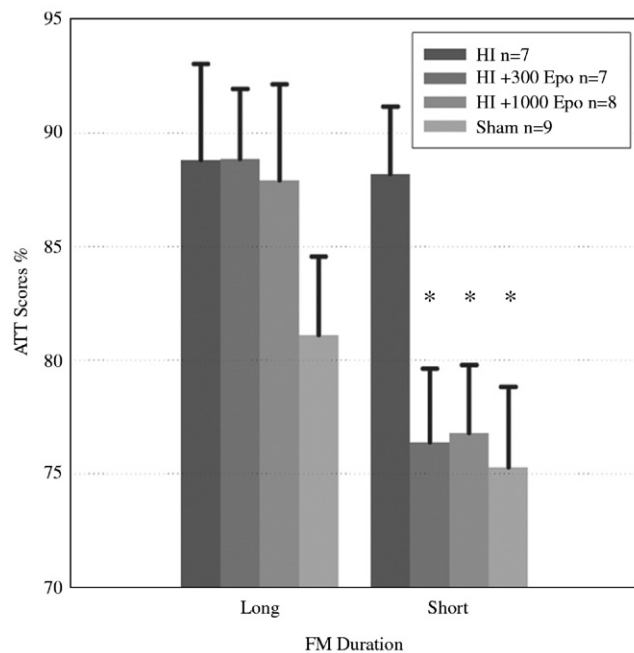


Fig. 2 – Mean (\pm SEM) attenuation response (ATT) scores on the FM Sweep procedure. FM sweeps were grouped into long (≥ 125 ms) and short (≤ 75 ms) durations. HI animals given Epo and sham animals performed significantly better on the short duration FM sweeps as compared to HI animals given saline ($*p<0.05$).

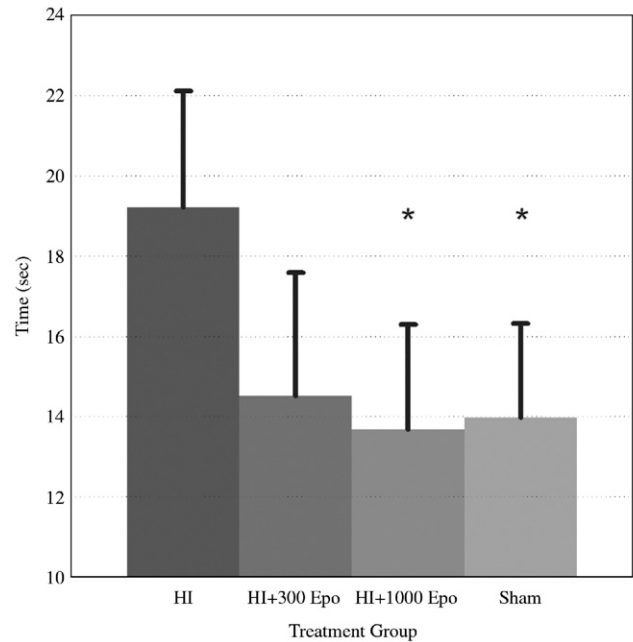


Fig. 3 – Latency to reach platform (\pm SEM) on the spatial version of the Morris water maze (MWM). Time in seconds was averaged over 5 days of testing/4 trials per day for all subjects. Results showed significant differences between the HI group and the HI + 1000 U/kg Epo and sham groups, with HI performing worse ($*p<0.05$).

ming ability and finding a visible platform. For the MWM, three separate 2 (Treatment) \times 5 (Day) repeated measures ANOVAs showed that HI subjects performed worse than all other groups, as indicated by greater latencies to locate the submerged platform (see Fig. 3). Significant differences were found between HI and HI+1000 U/kg Epo [$F(1,13)=3.17$, $p<0.05$], as well as between HI and the sham group [$F(1,14)=3.47$, $p<0.05$]. Independent samples t-tests showed that the HI group took significantly longer to reach the platform on days 4 and 5 as compared to the sham [$t=2.03$ and $t=2.11$, $p<0.05$] and HI+1000 U/kg Epo groups [$t=2.01$ and $t=2.05$, $p<0.05$]. The HI group also performed worse on days 3 and 5 as compared to the HI+300 U/kg group [$t=2.04$ and $t=2.01$, $p<0.05$], although the main effect of Treatment was not significant for this comparison.

2.4. Non-spatial water maze

HI animals once again performed worse than all other groups, as indicated by longer latencies to reach the submerged platform (Fig. 4). A significant main effect for Treatment was found between the HI group and all other groups using 2 (Treatment) \times 5 (Day) repeated measures ANOVAs [Shams, $F(1,14)=3.59$; HI+300 U/kg Epo, $F(1,12)=4.49$; and HI+1000 U/kg Epo, $F(1,13)=5.12$; $p<0.05$ all comparisons].

3. Discussion

In accord with prior results demonstrating sparing of rapid auditory processing (RAP) ability following administration of

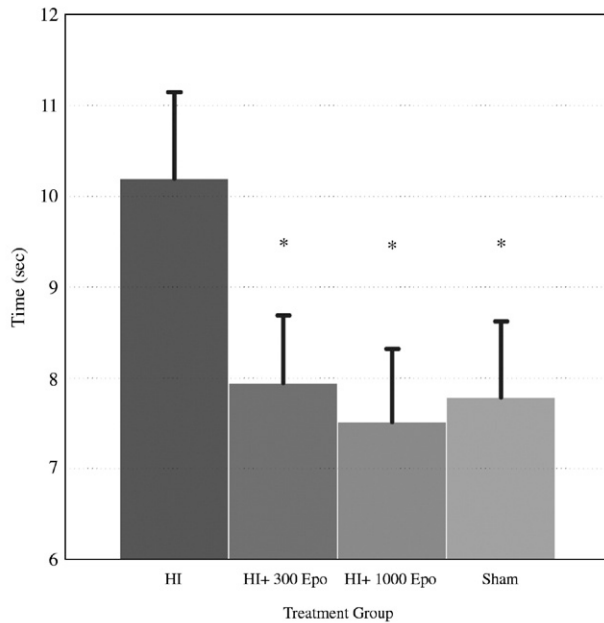


Fig. 4 – Latency to reach platform (\pm SEM) on the non-spatial water maze. Time in seconds was collapsed over 5 days of testing/4 trials per day for all animals. Results showed significant differences between the HI group and all other groups, with HI performing worse ($*p < 0.05$).

Epo in conjunction with P7 HI injury to rats (McClure et al., 2006), the current study replicates a preservation of RAP ability and also provides evidence of protection against other behavioral impairments associated with HI injury (specifically for learning and memory). Two facets of memory were tested using a water maze, spatial and non-spatial. These tasks were chosen because previous research has shown deficits in both aspects of memory following neonatal HI induction (Ford et al., 1989), as well as protection with Epo in spatial memory (Kumral et al., 2004). We have shown that both a small dose (300 U/kg) as well as a larger dose (1000 U/kg) afforded similar protection against RAP ability as well as spatial and non-spatial aspects of memory. Further, the administration of Epo to HI animals resulted in comparative behavioral performance relative to shams and a significant degree of neuropathological sparing compared to HI animals. As a caveat, however, HI animals administered 300 U/kg of Epo did not differ overall from HI animals on the spatial version of the water maze (although there was a near significant effect of treatment, $p < 0.1$) but did perform significantly better on 2 of the 5 days, indicating some degree of preservation of spatial learning and memory ability. Importantly, both HI groups given Epo did not differ from one another on any task supporting the relative efficacy of a small dose of Epo.

The rationale for the administration of a small dose of Epo speaks to the clinical literature in which premature infants are routinely administered Epo for anemia (doses typically vary from 200 U/kg to 700 U/kg, s.c.; Arif and Ferhan, 2005; Polkowska et al., 2004; Reiter et al., 2005). These data contrast the animal literature, wherein higher doses are routinely used

for neuroprotection (e.g., 1000 U/kg, 5000 U/kg, i.p.; Kumral et al., 2004; Sun et al., 2005). While the pharmacokinetics differ between the human and animal studies (i.e., subjectivity to first-pass effect, absorption rate, etc.) resulting in different bioavailability, data comparing different routes of administration (s.c. and i.v.) in premature infants show similar effects on hematocrit levels (Brown et al., 1993). Thus, while 200–700 U/kg of Epo is given s.c. to premature infants, our choice of administering 300 U/kg i.p. to rats likely falls within that range.

In summary, we report that a one-time dose of Epo (300 or 1000 U/kg) provides significant protection from HI-induced RAP deficits and also protection from learning and memory deficits. Moreover, the lower dose (300 U/kg) appears to provide protection nearly indistinguishable from that provided at a higher dose (1000 U/kg). When taken together, our results provide clear clinical significance in suggesting that a currently administered treatment for anemia in premature infants (300 U/kg; Reiter et al., 2005) may afford protection from HI-induced behavioral deficits if given at or near the time of injury. In fact, chronic Epo treatment for premature/VLBW infants at high-risk for intra-cranial bleeds and/or white matter injuries might be considered.

4. Experimental procedures

4.1. Animals/HI induction

Subjects were male Wistar rats, born to time-mated dams (Charles River Laboratories, Wilmington, MA) at the University of Connecticut. Pups were randomly assigned to a treatment group [HI, HI+300 U/kg of Epo, HI+1000 U/kg of Epo, or sham procedure]. All treatments were administered on P7 (see Rice et al., 1981, for surgical reference), and treatments were balanced within litter. On P7, HI pups were anesthetized with isoflurane (2.5%), a midline incision was made longitudinally in the neck, the right common carotid artery was located, separated from surrounding tissue, cauterized, and the incision was sutured. Sham animals received a similar surgical procedure but with no artery cauterization. Pups were then marked with footpad injections, placed under a warming lamp, allowed to recover from anesthesia and returned to their dams for a period of 2 h. HI animals were then placed in a container in which they were exposed to 8% humidified oxygen (balanced with nitrogen) for a period of 120 min while sham animals were placed in a chamber open to room air for an equivalent period of time. Following removal from the hypoxic chamber, one-third of the HI animals and all of the sham animals received an injection of saline (volume equivalent to injections of Epo for HI subjects). The remaining HI animals received either an injection of 300 U/kg of Epo or 1000 U/kg of Epo (Protein Sciences Corporation) intraperitoneally (i.p.; in saline). It is important to note that research shows comparable binding properties for Epo derived from rats and humans as measured in murine cells (Okano et al., 1993), thus supporting the use of recombinant human Epo in the current rodent study. Both sham and experimental pups were returned to their mothers after the procedure, and underwent behavioral testing post-weaning (P21).

4.2. Auditory testing

4.2.1. Behavioral testing: startle reduction

The startle reduction paradigm capitalizes upon the acoustic startle reflex (or ASR), which is a large amplitude motor response to an acoustic startle-eliciting stimulus (SES). The ASR can be measured (in mV) by placing an animal on a calibrated load-cell platform and presenting a SES (in the current study, a 105-dB, 50 ms white noise burst). When the SES is preceded by a benign prestimulus, the ASR is attenuated. The comparison between ASR amplitude when no prestimulus is presented (an uncued trial) and when the prestimulus precedes the SES (a cued trial) yields an objective measure of sensory detection (Marsh et al., 1975). Thus, ASR for cued and uncued trials on a given task serves as a dependent variable. A second comparative measure is calculated by dividing the sum of the ASR for cued trials by the sum of the ASR for uncued trials (mV) and multiplying by 100 (within task for each subject). These percentage scores represent attenuation response (ATT) scores and serve as a second dependent variable. ATT scores close to 100% indicate poor detection of a cue (i.e., the ability to attenuate to the prestimulus). The inter-trial interval between each SES is variable (range 16–24 s) but averages 20 s. In the current study, different tasks (comprising alterations in stimulus properties) were used to look at different aspects of auditory processing. First, a normal single tone procedure was used to assess baseline startle and hearing. Next, a more complex task, an FM sweep procedure, was used to identify any deficits in discriminating rapidly changing frequencies (i.e., as seen in human speech).

4.2.1.1. Apparatus. During startle testing, each subject was placed on an individual load-cell platform (MED Associates, Georgia, VT). The output from the platform was amplified (linear amp PHM-250-60 MED Associates) and acquired by a Biopac MP100WS Acquisition system connected to a Macintosh computer. The amplitude of each subject's ASR was recorded (in mV) following the onset of the SES by extracting the maximum peak value from the 200-ms signal epoch following the presentation of the SES. These values were coded for cued and uncued trials and represented the subject's absolute response amplitude for each trial. Scores were subjected to further analysis by deriving ATT scores as a function of relative performance on cued and uncued trials for each condition, for each subject, as described above (cued/uncued \times 100). 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 Marantz integrated amplifier PM700, and delivered via Cambridge speakers.

4.2.1.2. Single tone procedure (P23). The single tone test session consisted of 104 trials (cued or uncued), presented in a pseudo-random order on a single day (with no more than three of the same type of trial in a row). Uncued trials consisted of a silent background followed by the 105-dB, 50-ms SES. On cued trials, a 75-dB, 7-ms, 2300-Hz tone was followed 50 ms later by the SES.

4.2.1.3. FM sweep procedure (P40–43). A single FM sweep session consisted of 104 trials and involved the repeated pre-

sentation of a background 75 dB, downward FM sweep (2300–1900 Hz) with an upward FM sweep as the cue (1900–2300 Hz). The duration of sweeps lasted 225, 125, 75, or 50 ms, and this variable remained constant within a test session. Sweeps were separated by a between-stimulus interstimulus interval, which was always 200 ms greater than the sweep duration. FM sweeps were grouped into two durations for analysis (225/125 ms = "long", and 75/50 = "short").

4.3. Spatial Morris water maze (MWM) and water Escape (P44–50)

Subjects were first tested on a water escape procedure to test swimming ability. The water escape task involved the use of a visible platform (4 in. in diameter) placed in an oval galvanized tub (40.5 in. \times 21.5 in.) filled with water at 70 °F see (McClure et al., 2005). Subjects were placed in one end of the tub and timed until they swam and climbed onto the platform on the other side of the tub. Latency to escape was recorded for the single trial.

The Morris water maze (MWM) was then used to test spatial learning and memory (i.e., the ability to use spatial cues to locate a submerged platform relative to constant extramaze cues). MWM testing took place in a 48-in. diameter tub with a hidden submerged 8-in. diameter platform located 2 cm below the surface of the water (70 °F). The room had numerous extramaze cues and no intramaze cues to ensure that subjects had to rely on the location of extramaze cues to locate the platform (thus testing spatial learning and memory). Each testing day consisted of 4 trials per animal, with a quasi-randomly selected release location from each compass point (N, E, S, W). On trial 1 of day 1, the animal was first placed on the platform for 10 s, removed from the maze, and then released from the appropriate starting location. The escape platform was placed in the SW quadrant for 5 days. Animals were allowed to remain on the platform for 10 s following completion of the task and, if the platform was not reached during the 45-s trial time, they were guided to the platform and allowed to remain for 10 s. The latency to reach the platform and the release location were recorded for each subject on each trial.

4.4. Non-spatial water maze (P80–85)

The non-spatial water maze has been used to test reference memory, i.e., the ability to consistently locate a hidden platform, using intramaze visual cues that are orthogonal to extramaze space. Testing took place in the same 48-in. diameter tub as the spatial MWM, with the submerged 8-in. diameter platform located 2 cm below the water's surface, but also included an insert characterized by 4 black/white complex visual stimuli (which provided unique intramaze cues for each quadrant of the outer maze wall). The intramaze patterns consisted of black/white vertical stripes; black/white horizontal stripes; a gray panel; and a white panel. These cues were presented on a black background (see, Stavenezer et al., 2002, for more details). Rats were randomly divided into groups, which were assigned to one of the four intramaze patterns as their "positive" stimulus. The platform location and the "positive" stimulus were always paired for each subject, such that escape required an association between the target intra-

maze stimulus and the platform, irrespective of extramaze cues. While the platform remained in a constant within-maze position relative to the 4 quadrants, the maze itself was randomly rotated across trials with respect to the room. Subjects were released from the same compass point (N) on all trials, and latency to reach the platform was recorded for each trial. All other testing parameters were similar to the spatial version of the MWM (number of trials, testing days, and length of time subject was left on platform).

4.5. Histology

Upon completion of testing, animals were weighed, anesthetized, and transcardially perfused with fixative (10% buffered formalin phosphate). Brains were then coded (blind to treatments) on a scale assessing extent of damage [0 = none, 1 = mild (some cortical cell loss and/or slight reduction in the size of the hippocampus), 2 = moderate (significant cortical cell loss and hippocampal damage), 3 = severe (near complete blowout of the right hemisphere; see Fig. 1)].

4.6. Statistics

Four subjects were determined to be outliers with regard to behavioral scores [i.e., were consistently ± 2 standard deviations from the mean of their surgery group (HI=2, HI+300 Epo=1, and HI+1000 Epo=1)]. These subjects were later confirmed to exhibit pathology inconsistent with their treatment group (see below), and thus the 4 subjects were excluded from all analyses. Thirty-one subjects (HI=7, HI+300 Epo=7, HI+1000 Epo=8, and Sham=9) were used for statistical analyses. Comparisons were performed between HI and all other groups (HI+300 U/kg Epo, HI+1000 U/kg Epo, and shams) using repeated measures ANOVAs. Dependent variables included raw ASR (for auditory tasks), ATT Scores (for auditory tasks), and latency in seconds (for water mazes). Significant differences were subject to simple effects analysis (independent samples *t*-tests). Alpha was set at $p < 0.05$ for all analyses, and one-tailed tests of significance were used based on *a priori* hypotheses of specific auditory processing and learning and memory deficits in the HI group relative to Epo treated subjects and shams (Kumral et al., 2004; McClure et al., 2006).

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