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Short Communication

The effects of erythropoietin on auditory processing following neonatal hypoxic–ischemic injury

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ABSTRACT

Neonatal hypoxia–ischemia (HI) is a common cause of brain damage and subsequent behavioral deficits in premature/term infants. Rapid auditory processing deficits have been suggested to play a role in later language impairments in this population. We have previously shown auditory deficits in rats with neonatal HI injury and now report novel effects of behavioral sparing and neuroprotection following treatment with a low dose of Erythropoietin using this HI injury model.

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Hypoxia–ischemia (HI) refers to the diminished perfusion of blood and decreased delivery of oxygen to the brain. These mechanisms represent a common cause of damage to the perinatal brain and can occur via injuries associated with premature birth as well as during term birth. HI-injured infants go on to demonstrate increased incidence of cognitive and behavioral deficits, including language-related disabilities (Frisk and Whyte, 1994; Johnston et al., 2001; Largo et al., 1986; Msall and Tremont, 2002; Robertson and Finer, 1985; Volpe, 2001). Premature/very low birth weight (VLBW) children also show deficits in rapid auditory and visual processing (Downie et al., 2002, 2003), which have in turn been linked to cascading deleterious effects on language (Tallal, 1976). For example, thresholds for rapidly changing auditory cues in infants with a family history of language impairment are significantly higher than for controls and predict later language performance in both control and at-risk children (Benasich and Tallal, 2002). Interestingly, parallels in both pathology and behavior have been shown between mildly premature infants and a rodent model of HI injury induced on postnatal day 7 (P7; corresponding to a 34- to 36-week-old human fetus in terms of brain development (Vannucci et al., 1999; McClure et al.,

2005a,b). Similarities are seen in both patterns of gray matter damage resulting from HI, as well as deficits in rapid auditory processing (RAP), which may, in humans, relate to later language difficulties.

A major focus of research on the behavioral consequences of HI injury naturally follows to agents that might be used pharmacologically to spare function (i.e., neuroprotectants). Erythropoietin (Epo), a candidate neuroprotectant, is a cytokine which acts on bone marrow to regulate the production of red blood cells, specifically by inhibiting apoptosis of erythroid progenitor cells (Marti, 2004). Epo administration is effective in increasing red blood cell count in premature infants with anemia (Arif and Ferhan, 2005). In addition to its hematopoietic role, Epo has been shown to have nonhematopoietic protective effects. Epo and its receptor are both expressed in the brain [glial cells, endothelial cells, and neurons (Marti, 2004)] and are prevalent during early development (Juil et al., 1999). Epo's protective roles include cytoprotection, by way of promoting the survival of cerebral microvascular endothelial cells and preventing apoptosis (Bombeli et al., 1997; Chong et al., 2002). The genetic expression of Epo is triggered by the transcription factors hypoxia-inducible factors 1 and 2 (HIF-1/

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2) (Bauer, 1995; Ran et al., 2005), and expression is increased in areas of damaged cortex within 24 h following HI insult (Spandou et al., 2004). The location of Epo and its receptor in the central nervous system implicate it as a promising target for neuroprotection, and Epo has shown neuroprotective effects in a rodent model of neonatal HI injury (Weber et al., 2002; Wang et al., 2004; Aydin et al., 2003; Spandou et al., 2005).

Specifically, Epo's neuroprotective effects have been shown in rats following early postnatal HI insult via the improvement of synaptic transmission in hippocampal slice cultures (Weber et al., 2002), prevention of apoptosis in CA1 coupled with a decrease in glial activation in corpus callosum (Wang et al., 2004), and significant reduction of infarct volume (Aydin et al., 2003; Spandou et al., 2005). However, few studies have addressed effects of Epo on behavior [but see Spandou et al., 2005; Wang et al., 2004].

Given prior reports of RAP deficits in HI-injured rats (comparable to those seen in children with language impairment and premature children with periventricular injury (Benasich and Tallal, 2002; Downie et al., 2002; Jansson-Verkasalo et al., 2004)), we sought to assess the potential neuroprotective effects of Epo. Specifically, we sought to determine whether a clinically relevant dose of Epo, administered following HI-induction on P7, could produce anatomical neuroprotection and/or a sparing of RAP ability that we have previously shown to be impaired in a HI model (McClure et al., 2005a,b).

Subjects were 21 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 ($n = 15$) or sham ($n = 6$) on P7; see Rice et al., 1981], both treatments assigned 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 subjects received similar surgical procedure but with no artery cauterization. Pups were 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 subjects were placed in a container in which they were exposed to 8% humidified oxygen (balanced with nitrogen) for a period of 120 min. Sham subjects were placed in a chamber open to room air for an equivalent period of time. Following removal from the hypoxic chamber, a subset of HI subjects ($n = 6$) and sham subjects ($n = 3$) received an injection of 300 U/kg of Epo (Protein Sciences Corporation) intraperitoneally (in phosphate-buffered saline; PBS) or an equivalent injection of PBS ($n = 9$ HI, $n = 3$ sham). The dose of Epo was chosen based on clinical dosage for premature infants (Reiter et al., 2005). Both sham and experimental pups were returned to their mothers after the procedure.

On postnatal day 21, subjects began auditory testing using a startle reduction paradigm (also referred to as prepulse inhibition or PPI). The startle reduction paradigm capitalizes upon the acoustic startle reflex (ASR), a large amplitude motor response to a startle-eliciting ("startling") stimulus (SES). Each individual subject's ASR can be measured in millivolts (mV) by a load-cell platform (which transduces the ballistic motor response) and thus serves as a raw dependent variable. Raw

ASR scores collected for each subject on each task session can be further classified for cued and uncued trials. In a cued trial, the SES is preceded by a benign prestimulus. If this prestimulus is detected by the subject, the ASR is attenuated. In an uncued trial, there is no prestimulus (or cue) presented. In this way, the relative decrement in mean cued vs. uncued ASR represents the detectability or discriminability of a stimulus, for a subject. When variable cues are used (e.g., gaps of variable duration), some cues may be detected, while others are not. A relative comparison of mean cued and uncued ASRs for each subject provides a quantitative index of detectability of the prestimulus (or cue) for each subject, which is measured by dividing the mean ASR (in mV) for cued trials by the mean ASR (in mV) for uncued trials, and multiplying the outcome by 100. This dependent variable is referred to as attenuation response scores (ATT scores) and is expressed as a percentage. ATT scores provide a relative measure for each subject, thus controlling for differences across subjects in baseline startle, as well as for within-subject variance between tasks/days. ATT scores closer to 100% indicate poor detection of a cue (i.e., the ability to attenuate to the prestimulus), since responses on cued and uncued trials are equivalent. Thus, 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), while comparison of ATT scores across groups provides information about group differences in discrimination performance.

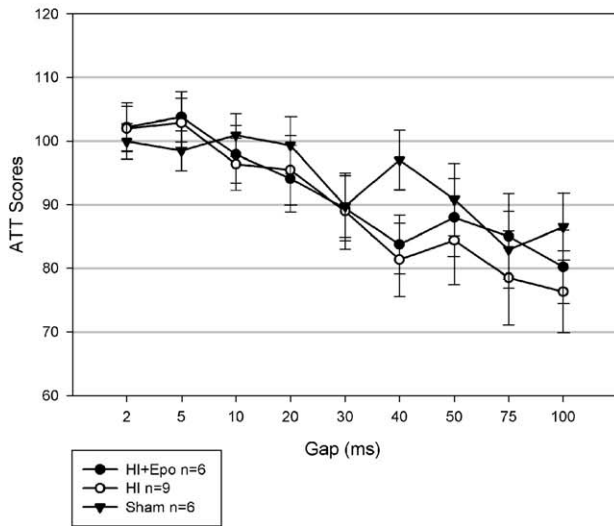
In the studies presented here, the SES was always a 105-dB, 50-ms white noise burst. The simplest task, a normal single tone procedure, employed a 75-dB, 7-ms, 2300-Hz tone prestimulus presented 50 ms before the SES (which is generally easy for rats to detect). In subsequent tasks, the complexity of the cue was increased by changing the nature of the prestimulus. Specifically, more difficult tasks included a 0- to 100-ms silent gap procedure (used to identify deficits in processing of brief acoustic signal changes), and a frequency-modulated (FM) sweep procedure (used to identify deficits in discriminating rapidly changing frequencies, i.e., as seen in human speech). Silent gaps of variable duration (0, 2, 5, 10, 20, 30, 40, 50, 75, and 100 ms) were embedded in continuous 75-dB broad-band white noise and presented 50 ms prior to the SES. Trials with a "gap" of 0 ms represent uncued trials. The FM Sweep procedure involved the repeated presentation of a background 75 dB, downward FM sweep (2300–1900 Hz), with an upward FM sweep as the cue (1900–2300 Hz). Sweeps were separated by an interval 200 ms greater than the sweep duration. The duration of sweeps lasted 225 or 125 ms, and this remained constant within a test session (for further procedural details, see McClure et al., 2005a). More difficult tasks were also presented over numerous days, and ATT scores were generally found to decrease in response to the same task over repeated testing (indicating improved detection/discrimination with experience; see Friedman et al., 2004).

Following completion of auditory testing, subjects were anesthetized and transcardially perfused with fixative (10% buffered formalin phosphate). Brains were removed, placed in formalin, and later serially sectioned in the coronal plane at 150 μm . Every fifth section was mounted on glass slides,

stained with cresyl violet, and coverslipped with Permount. Histological quantification of the hippocampus (volume) and cortex (area) was completed (blind to treatment group) using a Fisher Micromaster II digital microscope. Measures were obtained by overlaying a grid using ImageJ and computed via Cavalieri's estimator of volume and area (Gundersen and Jensen, 1987). All procedures conformed to approved University of Connecticut Institutional Animal Care and Use Committees (IACUC) protocols.

Results from the auditory tests showed that the sham groups (saline, $n = 3$; and Epo, $n = 3$) did not differ on any task.

A ATT Scores for Day 1 of Silent Gap 0-100 ms Procedure HI+Epo, HI, and Sham Subjects



B ATT Scores for Day 2 of Silent Gap 0-100 ms Procedure HI+Epo, HI, and Sham Subjects

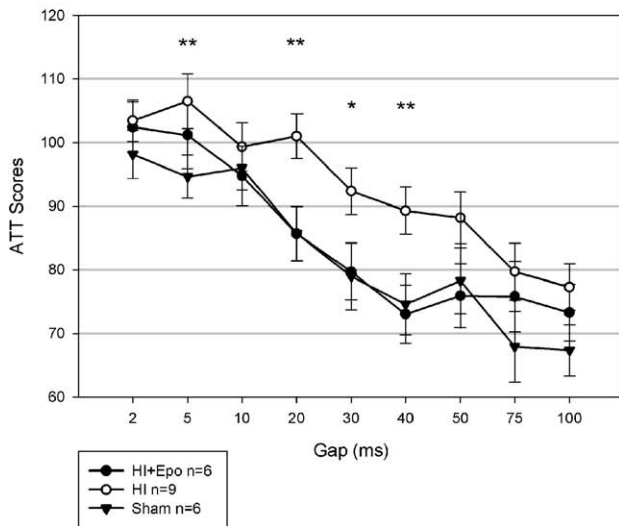


Fig. 1 – Mean (\pm SEM) of attenuation response (ATT) scores for 2 days of 0- to 100-ms silent gap procedure. Note scores closer to 100 indicate no detection of variable duration embedded silent gaps (the cue), i.e., poor performance. * $P < 0.05$, comparison between HI + Epo/HI groups. ** $P < 0.05$, comparison between HI + Epo vs. HI and HI vs. sham groups.

ATT Scores on FM Sweep Procedure
2 Days of 225 ms and 2 Days of 125 ms
HI+Epo, HI, and Sham Subjects

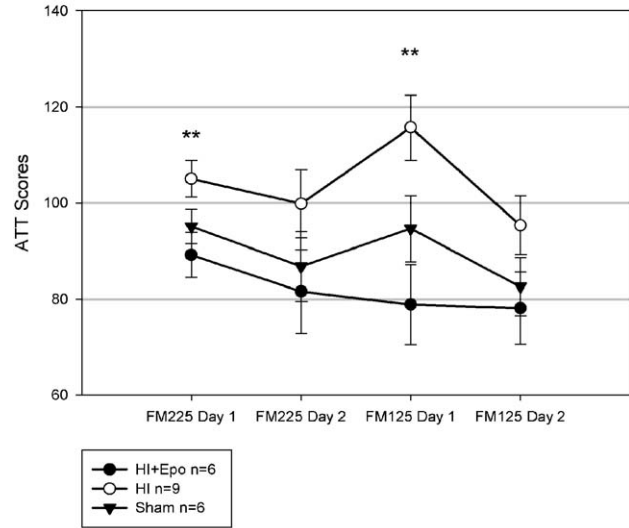


Fig. 2 – Mean (\pm SEM) of attenuation response (ATT) scores for 2 days of the 225 ms FM Sweep procedure and 2 days of the 125 ms FM Sweep procedure. The length of between sequence interstimulus interval (time between FM Sweeps) was always the FM Sweep length (225 or 125) plus 200 ms. Note that scores closer to 100 indicate no detection of the reversal of the FM sweep (the cue), i.e., poor performance. ** $P < 0.05$, comparison between HI + Epo vs. HI and HI vs. sham groups.

Note that although the n values were very small in this comparison, means on virtually all measures were nearly identical for the subgroups, and it was anticipated that we could pool these subjects based on concurrent evidence obtained from our lab (McClure et al., unpublished data). In summary, sham subjects were pooled ($n = 6$) for all subsequent analyses. All groups (HI vs. HI + Epo, and HI vs. sham) failed to differ on an “easy” task, the normal single tone procedure [$F(1,13) = 0.027$ and $F(1,13) = 1.81$, $P > 0.1$] as shown by one-way ANOVAs. Thus, subjects did not differ in their general ability to detect and attenuate to a simple prestimulus. Subsequent ANOVAs using 9 (Gap) \times 2 (Treatment) repeated measures, were performed on ATT scores for the first day of 0- to 100-ms silent gap. These comparisons also showed no Treatment effects [$F(1,13) = 0.181$, HI vs. HI + Epo; $F(1,13) = 0.507$, HI vs. sham, $P > 0.1$]. However, ANOVAs using 9 (Gap) \times 2 (Treatment) repeated measures performed on the second day of 0- to 100-ms silent gap showed that HI subjects performed significantly worse than HI + Epo, as well as significantly worse than sham subjects [$F(1,13) = 4.94$, $P < 0.05$ and $F(1,13) = 12.36$, $P < 0.01$, respectively], indicating that the HI group was unable to improve on this the task with repeated testing (decreases in ATT scores are normally seen with repeated testing; see Fig. 1). On the FM Sweep procedure, two separate 2 (Day) \times 2 (FM Sweep duration) \times 2 (Treatment) repeated measures ANOVAs were again performed for ATT scores and again showed a main effect of Treatment for HI vs.

sham subjects [$F(1,13) = 10.08, P < 0.01$] as well as a main effect of Treatment for HI vs. HI + Epo subjects [$F(1,13) = 8.88, P < 0.05$] (Fig. 2). This result clearly indicates that HI subjects were impaired in the detection and attenuation of more complex auditory stimuli (FM sweeps).

In accordance with these behavioral results, Epo administration to HI subjects was also found to show neuroprotective effects as evident in the hippocampus and cortex (Fig. 3). Paired samples *t* tests were computed within each group between right and left hippocampal volumes, as well as the area of the right and left cortices (at comparable sections between subjects), in order to determine if there was a significant reduction in these areas in the damaged hemisphere for HI and/or HI + Epo subjects. These comparisons served as relative measures of HI-related damage. No significant differences were found between right and left hippocampal volume, or right and left cortical area, for the sham or the HI + Epo groups, indicating that there was not a significant reduction of these structures in the damaged hemisphere in these groups [$t = 1.68$ and $t = 2.14, P > 0.05$]. Likewise, no significant differences were

found between the area of the right and left cortex for sham or HI + Epo groups [$t = 1.2$ and $t = 2.06, P > 0.05$; see Fig. 3]. However, significant differences were found in the HI group for both the hippocampus and cortex [$t = 7.04$ and $t = 4.85, P < 0.01$], with the volume of the right hippocampus and area of the right cortex being smaller than the left (Fig. 3). One-way ANOVAs for difference scores computed between the right and left hippocampus and cortex (left minus right) for the HI vs. the HI + Epo group did not show significant differences [$F(1,13) = 0.46$ (hippocampus) and $F(1,13) = 1.33$ (cortex)]. However, significant differences were found between difference scores for the right and left hippocampus and cortex for the HI vs. the sham group [$F(1,13) = 27.5$ (hippocampus) and $F(1,13) = 17.56$ (cortex), $P < 0.01$]. Results show that the immediate administration of Epo following injury had beneficial effects on behavioral deficits and showed some sparing of neuropathology normally found after HI injury (although not to the level of shams, see Fig. 3).

Many neuroprotective agents directed to ameliorating perinatal injury have been tested in animal models.

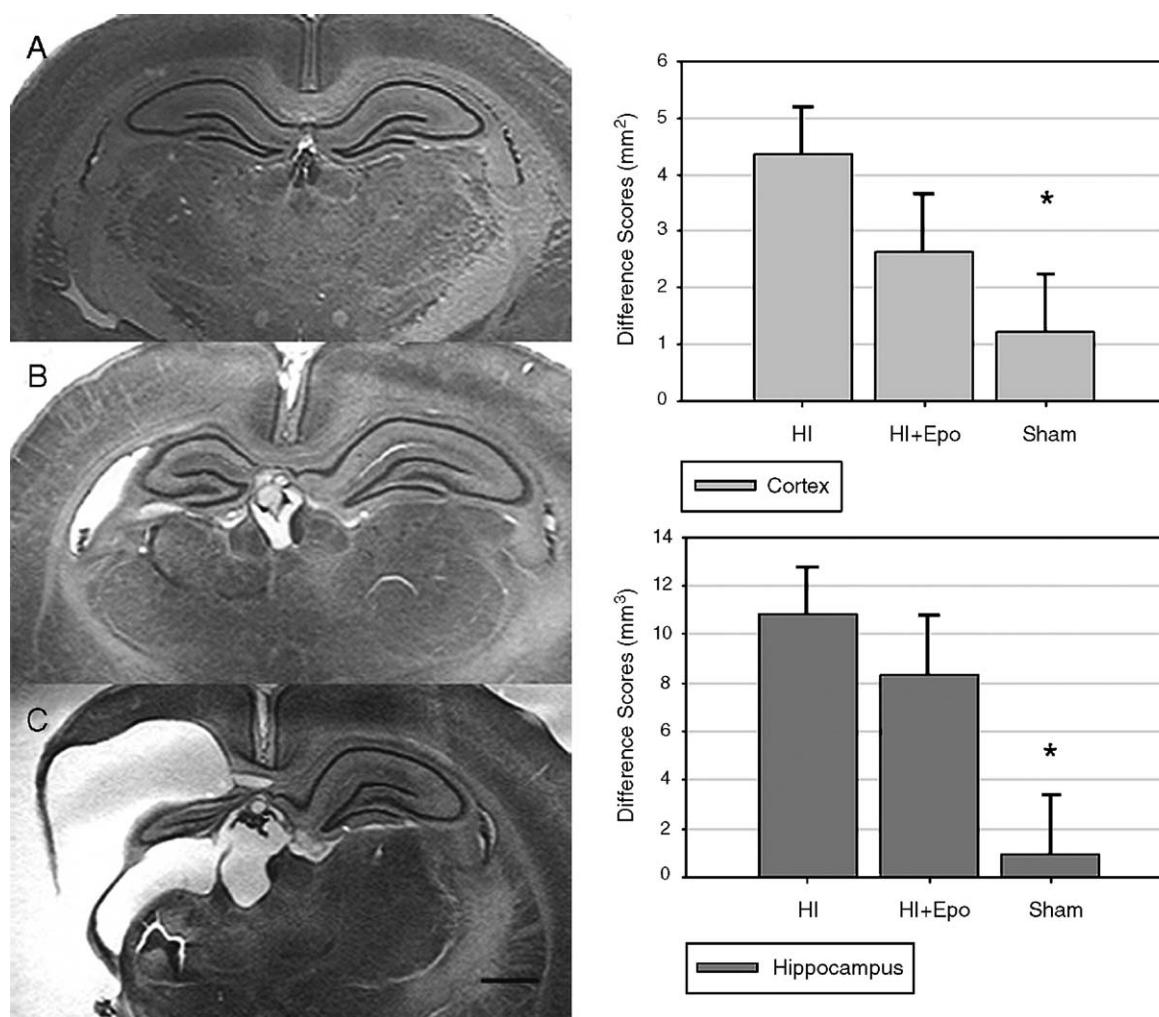


Fig. 3 – Nissl staining of a typical sham (A), HI + Epo (B), and HI (C) brain and graph demonstrating extent of damage between groups illustrated by means (\pm SEM) of absolute difference scores between right and left area of the cortex (at comparable sections) and right and left volume of the hippocampus. Scale bar = 1 mm. * $P < 0.05$, comparison between HI vs. sham groups.

Unfortunately, many of these strategies cannot be applied clinically due to side effects, or are not effective when administered to humans (Peeters and van Bel, 2001). Indeed, few therapies have been shown to reduce HI injury in humans and to date, no neuroprotective agent has been found to reverse HI-related behavioral impairments in humans. However, Epo represents an endogenously produced cytokine and is already given to premature infants to prevent anemia. Importantly, this preventative therapy has not been found to exert adverse side effects (Arif and Ferhan, 2005).

Administration of Epo to rodents pre- or post-HI injury has been shown to decrease the degree/progression of brain injury at various doses including; 5000 U/kg (Matsushita et al., 2003), 2000 U/kg (Spandou et al., 2005), and 1000 U/kg (Kumral et al., 2003, 2004). The current study extends previous research by showing significant protection against auditory processing impairment (associated with untreated HI-injury) in rats administered Epo post-injury at a low dose [comparable to clinical treatments (300 U/kg (Reiter et al., 2005))]. This study adds to the growing body of literature showing anatomical and behavioral protective effects for this drug, as well as presenting the novel finding that a low dose of Epo following injury still affords some neural and behavioral protection. Interestingly, Epo only provided minimal neuropathological sparing but had significant effects on auditory processing, such that HI subjects administered Epo performed comparably to shams. Given Epo's lack of adverse side effects, this study lends critical support to the clinical use of Epo for high-risk premature/VLBW and/or term HI-injured infants.

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