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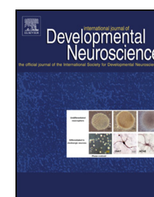

Transgenerational effects of neonatal hypoxia-ischemia in progeny

Smitha K. Infante, Harriett C. Rea, J.R. Perez-Polo*

Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555, United States

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ABSTRACT

Neonatal hypoxia-ischemia (HI) affects 60% of low birth weight infants and up to 40% of preterm births. Cell death and brain injury after HI have been shown to cause long-lasting behavioral deficits. By using a battery of behavioral tests on second generation 3-week-old rodents, we found that neonatal HI is associated with behavioral outcomes in the progeny of HI-affected parents. Our results suggest an epigenetic transfer mechanism of some of the neurological symptoms associated with neonatal HI. Elucidating the transfer of brain injury to the next generation after HI calls attention to the risks associated with HI injury and the need for proper treatment to reverse these effects. Assessing the devastating extent of HI's reach serves as a cautionary tale to the risks associated with neonatal HI, and provides an incentive to create improved therapeutic measures to treat HI.

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1. Introduction

Perinatal hypoxic-ischemic encephalopathy is an outcome after neonatal hypoxia-ischemia (HI), a major cause of neonatal morbidity and mortality, often resulting in developmental neurological deficits such as cerebral palsy, and delayed cognitive and behavioral deficits(Boichot et al., 2006; Zanelli et al., 2008). In the United States, perinatal HI occurs in 0.2–0.4% of term infants, and up to 60% of preterm (<37 weeks) or very low birth weight (<1500 g) infants (Vannucci et al., 1999; Zanelli et al., 2008). Due to the increasing incidence of preterm and low birth weight infants and the lack of adequate treatment for HI, characterization of brain injury after HI remains an extremely relevant area of interest. We tested the hypothesis that the long-lasting impacts of HI on sensory-motor coordination deficits are transmitted to progeny in an established model of rodent perinatal ischemia.

In the Vannucci and coworkers (Rice et al., 1981; Vannucci et al., 1988) rodent model of HI there is increased cell death in brain regions associated with cognitive processes by increasing inflammatory cytokine levels followed by activation of AP-1 and NF-κB-mediated transcriptional regulation of free radical generating enzymes and the prostaglandin pathway, mimicking aspects of observations in infants at risk (Bockhorst et al., 2010; Fabian et al., 2004, 2007, 2008; Grafe et al., 2008; Hu et al., 2005; Perez-Polo et al., 2011; Qiu et al., 2001, 2004; Smith et al., 2008; Tong et al., 2003; Gill and Perez-Polo, 2008; Hu et al., 2003; Xiaoming et al., 2006). HI also induces edema (Ferrari et al., 2010a,b) and via a BAX protein mechanism involving nuclear translocation, a more inflammatory cell death outcome as compared to apoptosis (Dicou and Perez-Polo, 2009; Gill et al., 2008, 2009). HI stimulation of nuclear processes is consistent with epigenetic processes being triggered by HI in animal models (Englander et al., 1999; Martin et al., 2005).

Epigenetic modifications can be manifested by (1) changes in cell phenotype; (2) regulation of cell lineage progress; and (3) a stably inherited phenotype. Numerous studies have shown the transgenerational effects of exogenous factors (Diamond et al., 1972; Denenberg and Whimbey, 1963). We focused on the third manifestation of epigenetics, that of stably inherited phenotype and assessed the behavioral characteristics of 2nd generation pups of rats that experienced neonatal HI with special attention to gender-specific transgenerational transference. Thus, we compared 2nd generation offspring of naive and sham-treated pups, to offspring of HI-exposed parents. We used similar behavioral assays to those used by Ferrari et al. (2010a,b), based on the sensory-motor coordination impairments reported for HI vs sham-treated pups (Ferrari et al., 2010a,b). We used a battery of three behavioral assessments of sensory-motor coordination: bar holding; wire mesh ascending; and sticky dot tests (Tchekalarova *et al.*, 2005). This study is the first to show that some behavioral deficits observed after HI are transferred to the next generation of animals. More importantly, we showed that male progeny are more susceptible to inheriting HI-induced neurological deficits than female progeny, consistent with an epigenetic mechanism being responsible.

2. Experimental procedures

2.1. Animal care

All animal procedures were performed according to the UTMB Animal Care and Use Committee (IACUC)-approved protocol # 9102020. Pregnant Wistar dams were purchased from Charles River at gestation days between E17-20. Dams gave birth

* Corresponding author at: 301 Univ. Blvd., Galveston, TX 77555, United States. Tel.: +1 409 772 3668; fax: +1 409 772-8028.
E-mail address: Regino.perez-polo@utmb.edu (J.R. Perez-Polo).

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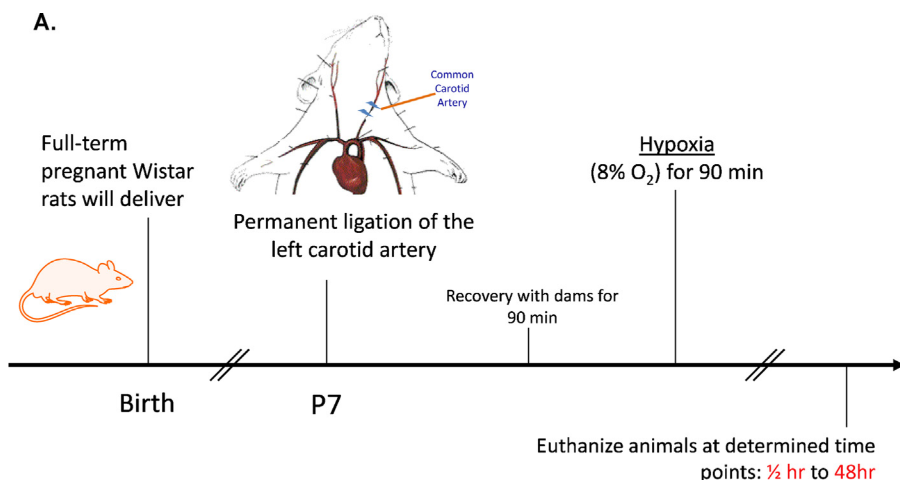

Fig. 1. Representation of HI model.

when ready, generally on E21 or E22. On postnatal day 2 (P2), the litter was culled to 10 pups to ensure that all pups received an equal chance to nurse. On surgery day, P7 pups were separated from dams and placed on a heating pad set at 37 °C, to mimic the mother's body temperature. Each pup was weighed, sexed, numbered, and categorized into the following groups: naive, sham, or HI. Naive animals remained with dams and did not undergo surgery or anesthesia, but were weighed, sexed, and numbered on P7 and before tissue harvest. Post surgery analgesics included daily administration of Buprenorphine (Bupernex) and 2 drops of topical anesthetic on suturing to minimize any localized pain for a maximum of three days post-surgery.

2.2. Surgical ischemia procedure

Littermates were separated into two condition groups: HI and sham. As shown in the procedure timeline in Fig. 1, pups were placed in 2.5% isofluorane anesthesia chamber for a minimum of 5 min. For surgical procedure, pups were moved onto a 37 °C heating pad throughout the surgery and anesthetized using 1.5–2.5% isoflurane by nose cone in an air/O_2 mix. For HI pups, aseptic technique was used to make an anterior off-midline incision in the neck. The left carotid artery was then isolated from surrounding fat and tissue, dually cauterized, and transected between the cauterizations for a permanent ligation. Topical administration of marcaine was provided directly onto the incision, to address any pain or discomfort. The incision was then closed with 6-0 silk suture and cleaned. Topical bitter-flavored skin sealant was applied to the suture to ensure closure of the incision and to deter the dam from removing sutures.

Sham animals were also anesthetized using isofluorane as stated above and placed on a heating pad for surgery. An anterior off-midline incision was made, immediately closed with a 6-0 silk suture, and cleaned. Though an incision was made on sham animals, in order to prevent any minor injuries that could result in ischemia/reperfusion of the left hemisphere, the carotid artery was not isolated.

Anesthesia timings for each pup were monitored, recorded, and never exceeded 45 min. After surgery, pups were placed back onto heating pads and monitored for any signs of bleeding until they recovered from anesthesia. If a pup does not nurse or shows no signs of mobility post surgery, the animal was euthanized. Awake pups were then returned to dams, where they were mobile and nursing within 15 min, which was our indication that they recovered from the surgical procedure.

2.3. Hypoxia procedure

As shown in the procedure timeline in Fig. 1, after 90 min of post-surgery recovery, all pups were separated from their mother. HI pups were placed in a hypoxia (8% O_2) chamber for 90 min, and shams were placed on a heating pad in normal air levels of oxygen (20% O_2) for 90 min. In the hypoxia chamber, animals were monitored for signs of excessive discomfort or immobility. Temperature was also monitored and rigorously maintained at 37 °C to insure reproducibility. After 90 min, all pups were returned to their mothers where they remained until they were sacrificed at specific time points up to 72 h after injury. Litters used for breeding of second generation pups for behavior analyses were returned to the animal resource center facility.

2.4. Animal breeding

For our behavior analyses, we required second generation pups. Fig. 2 depicts our timeline for acquiring second-generation animals. Three pregnant dams were ordered from Charles River, and at P7, each first generation litter was assigned a condition (HI, sham, or naive), and handled as stated above under surgical procedure and hypoxia. After surgery, pups were returned to their mothers and left under the care of UTMB's Animal Resource Center. On P21, first generation pups were

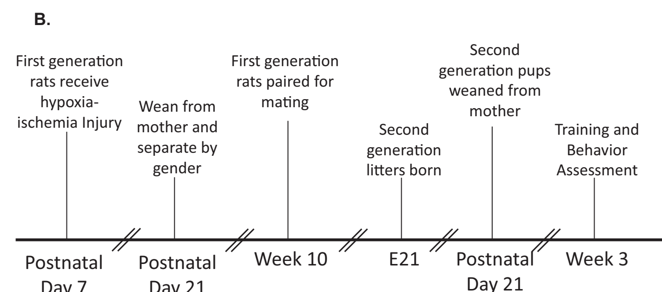

Fig. 2. Experimental plan.

weaned from their mother and separated by gender. On Week 6, pups required further separation as they grew, with a maximum of three females or two males per cage.

Wistar rats are mature and ready to mate at 10 weeks of age. On Week 10, rats were paired into harems containing one male and two females. Rats were paired as in Table 1. Harems were maintained for up to two weeks, or once a female appeared to be pregnant. Pregnant first-generation females were immediately separated into their own cages to prepare for birth. Generally, second generation pups were born within a month after harems were set up. As before, on P2, litters were culled to ten pups. Second generation pups were allowed to nurse with the mother until P21, when pups were weaned and randomly separated into groups of five per cage. At this stage, pups were not separated by gender. Starting on P21, each pup was handled daily to reduce anxiety from fear and acclimate them to being held. Animals were trained for three days beginning on Day 25, with the final behavior testing day being Day 28.

3. Behavior analysis

Three different behavior assays were conducted to assess sensory-motor coordination impairment in second generation pups. The setup for the mesh ascending test and bar holding test, based on Tchekalarova et al. (2005), was identical to that of Ferrari et al. (2010a,b). All behavior analyses were carried out between 8 am and 4 pm, using the same room and setup for each animal. All animals were in room at least 30 min before testing, to ensure

Table 1
First-generation parent crosses for behavior analyses of second-generation pups.

Mother	Father	# Total pups
Naive	Naive	20
Naive	Sham	10
Naive	HI	30
HI	Sham	10
Sham	HI	20
HI	HI	10

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