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Regular Article

Mild intermittent hypoxemia in neonatal mice causes permanent neurofunctional deficit and white matter hypomyelination

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ABSTRACT

Very Low Birth Weight (VLBW) premature infants experience numerous, often self-limited non-bradycardic episodes of intermittent hypoxemia (IH). We hypothesized that these episodes of IH affect postnatal white matter (WM) development causing hypomyelination and neurological handicap in the absence of cellular degeneration. Based on clinical data from ten VLBW neonates; a severity, daily duration and frequency of non-bradycardic IH episodes were reproduced in neonatal mice. Changes in heart rate and cerebral blood flow during IH were recorded. A short-term and long-term neurofunctional performance, cerebral content of myelin basic protein (MBP), 2′3′ cyclic-nucleotide 3-phosphodiesterase (CNPase), electron microscopy of axonal myelination and the extent of cellular degeneration were examined.

Neonatal mice exposed to IH exhibited no signs of cellular degeneration, yet demonstrated significantly poorer olfactory discrimination, wire holding, beam and bridge crossing, and walking-initiation tests performance compared to controls. In adulthood, IH-mice demonstrated no alteration in navigational memory. However, sensorimotor performance on rota-rod, wire-holding and beam tests was significantly worse compared to naive littermates. Both short- and long-term neurofunctional deficits were coupled with decreased MBP, CNPase content and poorer axonal myelination compared to controls.

In neonatal mice mild, non-ischemic IH stress, mimicking that in VLBW preterm infants, replicates a key phenotype of non-cystic WM injury: permanent hypomyelination and sensorimotor deficits. Because this phenotype has developed in the absence of cellular degeneration, our data suggest that cellular mechanisms of WM injury induced by mild IH differ from that of cystic periventricular leukomalacia where the loss of myelin-producing cells and axons is the major mechanism of injury.

Introduction

White matter injury (WMI) remains the leading cause of neurologic morbidity in premature infants. It has been reported that 5–10% of children born prematurely exhibit severe sensorimotor impairment and 25–50% develop cognitive and behavioral deficits. Cystic periventricular leukomalacia (PVL) has been recognized as the major form of WMI. However, recently the incidence of PVL has declined and occurs in only ~5% of VLBW infants. In contrast, non-cystic, diffuse WMI has become a predominant lesion, affecting approximately 50% of VLBW infants (Back, 2006; Volpe et al., 2011). Since incomplete myelination remains the pathological hallmark of WMI, the recent evolution of WMI pathology from cystic PVL toward focal and diffuse hypomyelinating disease suggests a mechanistic shift away from necrotic/apoptotic death of myelin-producing cells and their precursors toward dysfunction of myelinating cells. Mechanistic insight into this emerging pattern of WMI requires an animal model in which signs of cellular demise and degeneration are minimal, but a failure of postnatal brain myelination is prominent. In this model the primary insult must be non-lethal for brain cells, yet significant enough to alter axonal myelination. For this reason, well-characterized models of acute neurodegeneration, birth asphyxia, cerebral hypoxia–ischemia and focal stroke do not fully replicate the mechanisms and phenotype of non-cystic WMI. In contrast, the model of WMI induced by a non-lethal, postnatal chronic (8–10 days) hypoxic stress largely reproduces the neuropathologic and neurofunctional phenotype of diffuse WM hypomyelination (Back et al., 2006; Scafidi et al., 2009, 2014). However, it is extremely rare when preterm infants experience such a prolonged hypoxemia, because the maintenance of normoxemia is the most critical therapeutic requirement in neonatology. Clinical data demonstrate, that rather than chronic hypoxemia, VLBW infants daily experience numerous brief episodes of intermittent hypoxemia (desaturation) during several initial weeks of life. Although the majority of these desaturations are brief, the neuropathological

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significance of this IH stress remains unclear (Martin et al., 2011). We hypothesized that non-lethal, brief and repetitive hypoxic stress that occurs during the most active period of myelination, adversely affects postnatal WM myelination—the key component of WMI. To test this hypothesis we developed a mouse model where WMI was induced by non-lethal IH stress and the phenotype was characterized by cerebral hypomyelination associated with permanent neurofunctional deficit, without cell degeneration in the brain. In contrast to reported models of WMI induced by IH mimicking severe hypoxemic event associated with apnea of prematurity (Cai et al., 2012; Oorschot et al., 2013), our paradigm of IH simulates only mild, non-bradycardia-associated, repetitive hypoxemic episodes, the IH-pattern of unknown neurological significance, yet the most commonly seen in human premature infants.

Materials and methods

Clinical guidance for selection of IH paradigm in mice

To devise a clinically relevant experimental paradigm of IH we analyzed physiologic data collected over the initial six weeks of life from ten premature infants born between 24 and 27 weeks of gestational age (GA). All data-collection and analysis were approved by the IRB, Columbia University Medical Center. Data were obtained using BedMaster EX, a software and computer hardware system that interfaces with the patient bedside monitors. The program archived patients' vital signs at 0.5 samples/s and waveforms such as EKG, pulseoximeter, plethysmogram, respiration, and blood pressure at 240 samples/s. Hypoxemia was defined as the value of SaO_2 below 85% recorded continuously for a greater than 10 s, but no longer than 15 min. To minimize potential artifacts, heart rate (HR) readings derived from the electrocardiography signal were compared with the pulse rate measurement obtained from the pulseoximeter. SaO_2 measurement was considered for further analysis only if the difference in HR between the electrocardography and pulse-oximetry signals was less than the standard deviation of the hourly variability in the HR (~8 beats/min) in preterm infants (Sahni et al., 1999). As we were focused on the modeling of non-ischemic, non-cystic WMI, severe IH events associated with bradycardia which compromises cerebral circulation were excluded from analysis. Bradycardia was defined as a decrease in the HR below 100 beats/min. The following parameters of intermittent hypoxemia (IH) were considered for replication in the mouse model: daily incidence IH events associated with no bradycardia recorded over 24 h, cumulative daily time (%) spent by the patient with SaO_2 value <85% recorded over 24 h, and daily minimal SaO_2 during hypoxemic events. These parameters were collected over the initial six weeks of life and expressed as a mean daily value for each patient (Fig. 1A). Because neuropathological significance of IH events is unknown (Martin et al., 2011), the inclusion criteria were restricted only to the gestational age, 24–27 weeks. All patients required different levels of respiratory support during these six weeks of data-recording; intubation and mechanical ventilation (3 infants), mechanical ventilation via nasal cannulae (1 infant), and continuous positive airway pressure with FiO_2 requirements from 0.21 to 0.40 (6 infants).

Intermittent hypoxia reoxygenation paradigm in mice

All animal experiments were done according to the protocol approved by the Columbia University Animal Care and Use Committee (IACUC) and in accordance with AAALAC guidelines.

In premature infants the initial several weeks of life are the most critical age for maturation of myelin-producing cells and brain myelination (Back, 2014). The peak in the frequency of daily desaturation events in human VLBW infants also occurs during the initial several weeks of life (Martin et al., 2011). Therefore, the initial two weeks of mouse life were selected for replication of human neonatal IH stress, as myelination in mice is mostly accomplished by p28 (Vincze et al., 2008). C57Bl/6J neonatal mice of both genders were randomly assigned to the IH group or naïve-control group (Fig. 1B). The IH paradigm consisted of exposure to humidified 8% O_2 balanced with 92% N_2 for 3 min followed by reoxygenation in room air for 5 min. A mean duration of hypoxemia ($SaO_2 < 85\%$) was 2.26 ± 0.11 ($n = 12$) minutes and mean minimal SaO_2 during hypoxemic episode was $66.74 \pm 4.1\%$ ($n = 12$). The duration of a single hypoxemic episode and daily number of episodes was selected to approximate the daily cumulative length of hypoxemia in human neonates during the initial six weeks of life (Fig. 1A). The reoxygenation time of 5 min was chosen to ensure stable normoxia ($SaO_2 > 90\%$) in between hypoxemic episodes. During hypoxic exposure pups were separated from their dams and placed into air-tight chamber with an ambient temperature of 35 °C. Mice were subjected to 30 episodes of IH daily divided into two 15-episode sessions. Between sessions mice were returned to their dams for 2 h for recovery and nursing. The paradigm was initiated at day of life 1 (p1) and continued for 14 consecutive days. Litter-matched pups served as controls. Control mice were also separated from their dams for the same period of time as the experimental animals, but kept in room air in a separate incubator. A separate cohort of mice was used to record changes in cerebral blood flow (CBF), HR and SaO_2 in response to hypoxia and reoxygenation and we compared these data with that of human neonates (Figs. 1 C and D). Upon completion of IH all mice were tested for a short- and long-term neurofunctional performance as described below. One cohort of mice was raised until adulthood and another cohort was used for intermediate assessment of cerebral myelination, neurodegeneration and short-term neurobehavioral testing (Fig. 1B).

Measurements of heart rate, oxygen saturation and cerebral blood flow

Changes in HR, SaO_2 and CBF during hypoxic event and reoxygenation were recorded in p5–7 mice exposed to ten IH episodes prior to a single IH episode used for data-collection. HR and SaO_2 were recorded using a mouse vital sign monitor (Starr Life Sciences, PA), as we described (Niatsetskaya et al., 2012a,b). The SaO_2 probe was wrapped around the neck. Changes in CBF during hypoxemia and reoxygenation were recorded using laser Doppler flowmeter (Peliflux 5000, Sweden), as we described (Matsiukevich et al., 2010). In brief, the Doppler probe via 15–20 cm fiberoptic extention was attached with tissue glue to the skull in the projection of the right cortex (2 mm posterior and 3 mm lateral to bregma). The right side was chosen randomly, because the model produces a global hypoxemia with no experimentally intended regional changes in cerebral circulation. The Doppler probe was placed under brief isoflurane anesthesia. However, the CBF measurements during hypoxic exposure were performed under local anesthesia.

Short-term and long-term neurofunctional evaluation

Acquisition of sensorimotor reflexes

Starting at p3 all pups from both groups were evaluated for the acquisition of three developmental sensorimotor reflexes as described (Ten et al., 2003). Reflexes were tested daily prior to the initiation of the afternoon session of IH exposure. Acquisition of a reflex was considered established after it was executed correctly on two consecutive days and results were recorded as the day of life when an individual reflex was acquired. *Cliff aversion reflex*: pups were placed with overhanging forepaws at the edge of a plank. The time required to turn 90 degrees away from the edge was recorded. A time <2 s was considered a positive result. *Righting reflex*: Mouse pups were placed gently in the supine position. The time (s) required for the mouse to flip to the prone position was recorded. Successful righting in <2 s was considered a positive result. *Negative geotaxis reflex*: Mouse pups were placed head down on board covered with a claw-friendly fabric and inclined at 45 degrees. The reflex was considered positive if the mouse turned head-up (>150°) within 20 s (Hill et al., 2008).