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Immediate and delayed decrease of long term potentiation and memory deficits after neonatal intermittent hypoxia.

Ivan Goussakov, PhD¹, Sylvia Synowiec¹, Vasily Yarnykh, PhD², and Alexander Drobyshevsky, PhD^{1,*}

¹Department of Pediatrics, NorthShore University HealthSystem Research Institute, 2650 Ridge Ave 60201, Evanston, IL, USA

²Department of Radiology, University of Washington, 850 Republican St., Room 255 Seattle, WA, USA

Abstract

Apnea of prematurity is a common clinical condition that occurs in premature infants and results in intermittent hypoxia (IH) to brain and other organs. While short episodes of apnea are considered of no clinical significance, prolonged apnea with bradycardia and large oxygen desaturation is associated with adverse neurological and cognitive outcome. The mechanisms of cognitive deficits in IH are poorly understood. We hypothesized that brief but multiple episodes of severe oxygen desaturation accompanied by bradycardia may affect early and late synaptic plasticity and produce long-term cognitive deficits.

C57BL/6 mouse pups were exposed to IH paradigm consisting of alternating cycles of 5% oxygen for 2.5 min and room air for 5 to 10 min, 2 hours a day from P3 to P7. Long term potentiation (LTP) of synaptic strength in response to high frequency stimulation in hippocampal slices were examined 3 days and 6 weeks after IH. LTP was decreased in IH group relative to controls at both time points. That decrease was associated with deficits in spatial memory on Morris water maze and context fear conditioning test. Hypomyelination was observed in multiple gray and white matter areas on in vivo MRI using micromolecule proton fraction and ex vivo diffusion tensor imaging. No difference in caspase labeling was found between control and IH groups.

We conclude that early changes in synaptic plasticity occurring during severe episodes of neonatal IH and persisting to adulthood may represent functional and structural substrate for long term cognitive deficits.

*Corresponding author A. Drobyshevsky, Department of Pediatrics, NorthShore University HealthSystem Research Institute, 2650 Ridge Ave. Evanston, IL 60201, Tel.:847-570-4074, Fax: 847-570-0231, adrobyshevsky@northshore.org.

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Competing interests

The authors declare that they have no competing interests.

Keywords

intermittent hypoxemia; hypomyelination; memory deficits; synaptic plasticity

1. Introduction

Apnea and bradycardia of prematurity [1] is a common clinical condition that occurs in premature infants, related to instability of respiration control and results in intermittent hypoxia (IH) affecting the brain and other organs. IH events typically stop or decrease by term equivalent or hospital discharge.

Short episodes of apnea (less than 10s, without hypoxemia and bradycardia) cause mild arterial blood desaturation without bradycardia and are considered inconsequential to behavioral outcomes. More prolonged apnea that lasts for more than 15 or 20s and / or apnea associated with bradycardia or oxygen desaturation, may result in short-term disturbances of cerebral hemodynamics and oxygenation, and is associated with adverse neurological and cognitive outcomes, assessed by Bayley scales [2, 3]. Bradycardia is a prominent feature in preterm infants with apnea [4]. A causal relationship remains to be established between such events and short or long-term cognitive outcomes. The severity and effects of apneic episodes are hard to document and isolate from co-morbidities related to prematurity. Childhood sleep disorders that affect breathing, and cause episodes of IH, have been associated with poorer executive function, memory, and general intellectual ability [5]. The magnitude of the problem may be higher than currently recognized due to limitations in detecting and reporting apnea with clinical impedance monitoring of respiration in neonates [3, 6]. Caffeine citrate is the drug of choice to reduce the frequency and severity of apneic spells in preterm infants, but randomized multicenter clinical trials showed that neonatal caffeine therapy did not improve cognitive function at 5 years of age or academic function at 11 years of age [7].

Several animal models were developed that mimic clinical situations with relatively mild IH, a small magnitude of desaturation and no bradycardia events. Mild motor deficits and white matter injury, but very few long-term cognitive abnormalities were found in these models [8–10]. Moreover, several studies utilizing mild neonatal IH report neuroprotection from excitotoxic injury and improvement of long-term cognitive abilities in adulthood [11–13]. It appears that the IH insult must be relatively severe to result in cognitive deficits in rodent models. Mice and rats have evolved to tolerate IH well. Long-term cognitive deficits and hyperactivity are observed after severe prolonged hypoxia mimicking perinatal asphyxia [14–16], but timing, type of insult and a substantial cell loss in the brain [16] in those models is different from the IH models, trying to replicate clinical apnea of prematurity situation [9].

In the current study we examined the long-term cognitive outcome in a mouse model of relatively severe neonatal IH with episodes of large desaturations and bradycardia. In parallel, we examined the hippocampal synaptic plasticity by measuring long term potentiation (LTP), that might underlie observed memory deficits, as well as structural changes and myelination in white matter in adult mice exposed to severe episodes of IH as

neonates. White matter injury is a consistent finding in perinatal brain hypoxia and ischemia injury of different types [8–10, 14] and may also be a determinant of later cognitive abnormalities. Impairments in LTP have been examined in models of single acute hypoxic event during prenatal period [17], but have not been studied in neonatal IH, especially in relation to cognitive outcome. We hypothesize that brief but multiple episodes of severe desaturation accompanied by bradycardia will affect synaptic plasticity and be sufficient to produce long-term cognitive deficits.

2. Materials and Methods

2.1 Experimental Animals and Neonatal Intermittent Hypoxia Model.

The study has received approval by the Institutional Animal Care and Use Committees of NorthShore University HealthSystem.

C57BL/6J mice, originally obtained from the Jackson Laboratory (Bar Harbor, Maine), were bred at the NorthShore University HealthSystem animal facility. Neonatal mice of both sexes from each litter were randomly assigned to the IH or control group. The IH paradigm in this study was modified from a model of IH in neonatal mice by Cai et al. [18] to produce more severe episodes of hypoxia with consistent onset of bradycardia. The IH paradigm consisted of periodic exposure to 5% O₂ balanced with 95% N₂ for 2.5 min followed by reoxygenation in room air for 5 to 10 min. During hypoxic exposure pups were separated from their dams and placed into 250 mL volume air-tight chamber in a temperature controlled neonatal incubator with ambient temperature of 35 °C. The paradigm was initiated at postnatal day 3 (P3, day of birth considered as P0) and continued for 5 consecutive days. Air flow was 4L/min and was switched between hypoxic gas mixture and room air by solenoid valves controlled by a programmable timer. Oxygen concentration and air temperature in hypoxic chamber were continuously monitored with a calibrated oxygen sensor and a thermometer placed in the chamber. Oxygen concentration reached nadir 5% in chamber within 40-sec from the onset of hypoxic period. The number of total daily hypoxic episodes were 20, 20, 16, 14 and 12 for 5 consecutive days, correspondingly, divided into morning and afternoon sessions. Room air periods were 5,5,8,10,10 min durations for days 1 to 5, respectively. Between the sessions mice were returned to their dams for 4 hours for recovery and nursing. Control mice were also separated from their dams for the same period of time as the IH animals, but kept in room air in the same incubator and at the same temperature.

Upon completion of IH course a subset of mice was taken for electrophysiological studies at P11. Another subset was sacrificed at the end of IH at P7 and used for histopathology. The remaining mice were raised until 6 weeks old and used for behavioral assessment, MRI, and electrophysiology and histopathology. Upon completion of behavioral and *in vivo* MRI studies mice were transcardially perfused with saline followed by 4% paraformaldehyde, brains removed and postfixed in the same fixative overnight and stored in 0.01M PBS until used for *ex vivo* MRI and processed for histopathology. The number of samples (animals and slices) used in the study is presented in Table 1.

2.2 Measurements of heart rate, respiration, and oxygen saturation

Changes in heart rate, SaO₂ and respiration during hypoxic event and reoxygenation were recorded in a subset of P3 (n=4) and P6 mice (n=5) during morning session of IH exposure using a MouseOx pulse oximeter (Starr Life Sciences, PA), with the probe wrapped around pups head.

2.3 Electrophysiology

2.3.1 Hippocampal slices preparation and maintenance.—Mice were deeply anesthetized with isoflurane (3%) and decapitated. Brains were rapidly removed and the hippocampus with adjoined cortex dissected out in ice cold artificial spinal fluid (aCSF) of the following composition (mM): NaCl 124, KCl 2.5, NaCO₃ 25, CaCl₂ 2, MgSO₄ 2, NaH₂PO₄ 1.25, D-glucose 10, bubbled with carbogen (95% O₂/5% CO₂) to maintain pH of 7.4. Hippocampal slices were cut 300 µm thick using VF-300-OZ vibrotome (Precisionary Instruments, NC). Slices were maintained on nylon mesh in an incubation chamber filled with room temperature aCSF, bubbled with carbogen for at least 1 hour before recording.

2.3.2 Extracellular Field potential Recording.—For extracellular recordings, slices were transferred to recording chamber (model RC-24, Warner Instruments Hamden, CT, US) and continuously perfused with room temperature oxygenated aCSF bubbled with carbogen (pH7.4) at the rate of 3 ml/min. Extracellular (field) excitatory postsynaptic potential (fEPSP) was recorded in stratum radiatum of CA1 region of hippocampus in response to electrical stimulation of Schaffer collateral/commissural axons with bipolar platinum-iridium electrode. Recording electrodes were pulled from capillary tubes 1B150F-4 (World Precision Instruments Inc., Sarasota FL, US) using P97 puller (Sutter Instrument Co., Novato, CA, USA) and filled with aCSF (resistance 2–3 MΩ). Unipolar stimulus pulses with 0.1 ms width were delivered from stimulus isolator Iso-Flex (I.M.P.I, Israel). Stimuli to evoke fEPSP were applied once per minute in all recordings. Input-output curves were generated by increasing stimulus intensity in 20µA increments from 0 to 300 µA and recording fEPSP amplitudes. Stimulus intensity required to evoke fEPSP of 40% of maximum (before appearance of pop spike) amplitude was determined and used for evoking all subsequent fEPSP. Electrical signals were amplified using Multiclamp-700B amplifier and digitized with Digidata 1440 (Molecular Devices, Sun Jose, CA, USA) and recorded in current clamp mode using pCLAMP-10 software (Molecular Devices). All drugs were purchased from (Sigma-Aldrich Inc., MO, USA).

2.3.3 Physiological long term potentiation (LTP) was elicited with two theta burst stimuli (TBS) separated by 1 min. Each TBS stimulation consisted of 10 trains of 4 pulses each, delivered at 100 Hz with 200 ms between the trains. Time course of fEPSP amplitude was recorded during 20 min for baseline before TBS and 60 min after to measure LTP. LTP time course was calculated as percentage of baseline for each recording and averaged between the recordings. The LTP value was measured as an average of last 10 min of recording and was used for statistical comparison by t-test.

2.3.4 Paired pulse facilitation (PPF) was elicited by applying two consequent stimuli 30 ms apart. PPF was calculated as ratio of the second stimulus fEPSP amplitude over the first

stimulus fEPSP amplitude. PPF characterizes property of presynaptic terminal to increase release of neurotransmitter when an impulse closely follows a prior impulse, and represents a form of short-term synaptic plasticity.

2.4 Behavioral studies.

Testing of mice between 41 and 45 day old took place in a dedicated quiet room. Each testing apparatus was cleaned with 70% ethanol and aired for 3 min after each animal. Animal movements were registered and processed with ANY-maze software ver. 6.5 (Stoelting Co., Wood Dale, IL, USA).

2.4.1 Open Field Test.—Animals were placed individually in the center of an open clear field box (61×61 cm), and their spontaneous motor activity was recorded for 20 minutes. The computer program automatically recorded the following parameters: mobile time, mean speed, traveled distance and time spent traveled in the center (40×40 cm) and peripheral zones.

2.4.2 Elevated Plus Maze Test.—The elevated plus maze, made of white acrylic plastic, consisted of four arms (each 28×5 cm) and a central area (5×5 cm) elevated 50 cm above the floor. Two arms were open and two were closed with 15-cm-high walls made of the same material. Mice were individually placed in the center facing an open arm and allowed to explore for 5 min. Time spent in the closed and open arms was recorded.

2.4.3 Morris water maze test.—The apparatus consisted of a circular 120 cm diameter tank with room temperature (22°C) water, which had been made opaque with the addition of non-toxic, white tempera paint. High contrast black and white images were placed on the walls of the testing room around the tank to serve as visual cues. At the first stage of the test, mice were trained to locate a visible 10 cm diameter platform exposed 1 cm above the water. If they did not find the platform within the 60-second trial limit, they were guided to it and allowed to stay for 20 seconds. The mice were wiped with towels after removal from the pool and placed under a heating lamp to dry. Five trials were performed and the platform location was changed for each trial. At the second stage, mice were trained to find a hidden platform that was submerged 1 cm below the surface in the southeast quadrant. Mice were trained once a day for four consecutive days in the afternoon, five trials at a training session, at least 10 mins between the trials. Starting quadrants varied between the trials each day in the same order for all mice. The latency required to locate the platform and their path lengths was recorded. The probe trial was performed on the 5th day, where mice swam for 60 seconds with no platform in the tank. Time spent in the quadrant where the submerged platform had been in previous stages was recorded.

2.4.4 Fear conditioning test.—The fear conditioning tests assesses the ability of mice to learn and remember an association between environmental cues and aversive experiences. In this test, mice were placed into a conditioning chamber and were given pairings of a conditioned stimulus (CS, an auditory cue) and an aversive unconditioned stimulus (US, an electric foot shock). The conditioning chamber consisted of an opaque acrylic plastic $30 \times 30 \times 21$ cm box with a clear lid and a shocking grid on the floor made of 2 mm diameter

metal rods, 6 mm between runs. During the conditioning stage at day 1, mice were allowed to freely explore the chamber for 120 seconds. Thereafter, a white 55 dB noise auditory cue was presented as a CS for 30 seconds, followed by a 0.3 mA foot shock given as a US during the last 2 seconds of the sound using an animal shocker (San Diego Instruments, CA). The presentation of CS-US was repeated three times per session (120, 240, and 360 seconds after the beginning of the conditioning). Following the final foot shock, the mice were left undisturbed in the chambers for 90 seconds. After the conditioning session had been completed, mice were returned to the same conditioning chamber 24 h later to measure contextually conditioned fear (context test). The mice were placed in the conditioning chamber and were allowed to freely explore the chamber for 300 seconds without CS and US presentations. A cued test was conducted on the same day two hours after the context test. In this test, the shocking grid was removed and the walls of the chamber were covered with checkerboard pattern wallpaper, providing a novel context that was unrelated to the conditioning chamber. Mice were placed into the testing chamber for 3 min. At the end of the first 3 min, the CS auditory cue that had been presented at the time of conditioning was given to mice for 3 min. Fear memory was assessed based on freezing behavior to the conditioned cued or the contexts to which mice were previously exposed. The outcome variables were freezing time in the context test and during the first and last 30 seconds of the cued test.

2.4.5 Social interaction test.—Two social behaviors (social interaction and social memory/novelty recognition) were quantified using a rectangular 3-chamber apparatus that consisted of a 20×45×30 cm middle chamber made of acrylic plastic, with two 10×10 cm openings leading to two separate (left and right) chambers of the same size, each containing a steel cage enclosure. After 5 min acclimation to empty apparatus, each mouse (experimental subject) was placed in the middle chamber and allowed to explore and interact for 10 minutes, with the one side chamber being empty and the another side chamber containing an unfamiliar mouse (Stranger I, non-littermate control mouse of the same sex, housed in a separate cage) held in the steel cage enclosure. Assignment of chambers was randomized between mice. Sociability index was determined as the time spent by the experimental subject in the chamber holding the unfamiliar congener divided by the time spent in the chamber containing an empty enclosure. To measure social memory (or novelty recognition), a new novel stimulus mouse (Stranger II) was subsequently placed in the chamber with the previously empty enclosure. The tested mouse was allowed to explore and interact for 10 min. The stages of the test followed each other without delay. A Social Novelty index was defined as the ratio of time spent by the experimental subject in Stranger II chamber over the time spent in Stranger I chamber.

2.5 Magnetic resonance imaging (MRI).

Imaging was performed on a 14.1 T Bruker Avance imaging spectrometer (Bruker, Billerica, MA) using a 20-mm bird cage resonator.

2.5.1 In vivo macromolecule proton fraction (MPF) imaging.—Mice were sedated with isoflurane (Abbot, IL) inhalation, diluted in air to 5% for induction and 1.5% for maintenance. Each animal's respiration rate and rectal temperature were monitored with

a small animal physiological monitor (SAII's Small Animal Instruments, NY, USA). Body temperature was kept at 35° C by maintaining ambient temperature in bore at 32 ° C using the spectrometer gradient temperature controller.

A novel *in vivo* quantitative MRI method, macromolecular proton fraction (MPF) mapping, was employed to reconstruct parametric maps of a relative amount of macromolecular protons causing the magnetization transfer effect. In the brain the method estimates proportion of water bound predominantly to myelin relative to the rest of brain water and therefore provides a quantitative biomarker of myelination in neural tissues [19, 20]. The method has been validated with histological markers of myelin in the normal rat brain [21] and in a murine model of demyelination [22]. 3D MPF maps were obtained from three source images (Magnetization transfer (MT)-, Proton density (PD) -, and T1-weighted) using the single-point method with the synthetic reference image. PD- and T1-weighted GRE images were acquired with TR/TE = 16/2.6 ms and $\alpha = 3^\circ$ and 16° , respectively. MT-weighted images were acquired with TR/TE = 25/2.6 ms and $\alpha = 9^\circ$. Off-resonance saturation pulse was applied at the offset frequency 6 kHz with the effective saturation flip angle 500° . All images were acquired in the axial plane with whole-brain coverage and resolution $0.125 \times 0.125 \times 0.35 \text{ mm}^3$. All images were obtained with six signal averages and a total scan time of 33 min. In all 3D imaging experiments, linear phase-encoding order with 100 dummy scans, slab-selective excitation, and fractional (75%) k-space acquisition in the slab selection direction were used. To correct for field heterogeneities, 3D B0 and B1 maps were acquired using the dual-TE (TR/TE1/TE2 = 20/2.9/5.8 ms, $\alpha = 8^\circ$) and actual flip-angle imaging (TR1/TR2/TE = 13/65/4 ms, $\alpha = 60^\circ$) methods, respectively[23].

2.5.2 Ex vivo diffusion tensor imaging (DTI).—*Ex vivo* MR imaging occurred from seven to 21 days post-fixation. Brains were removed from skulls, soaked in PBS for 2 days and immersed in non-aqueous media (fomblin Y, Sigma Aldrich, MO). Diffusion experiments consisted of 64 non-collinear directions diffusion weighted spin echo images with $b=0, 0.8, 1.5$ and $3.0 \text{ ms}/\mu\text{m}^2$, $\delta/ = 3.5/7.2 \text{ ms}$. Imaging parameters were TR/TE/NEX 1200/16.3/3, 8 axial slices, 55×100 micron resolution in plane, 0.25 mm thick, 0.2 mm gap. Imaging time was 12 hours 36 minutes.

2.5.3 MRI data processing.—Macromolecule proton fraction maps were reconstructed by solving pulsed magnetization transfer matrix equation by the Gauss–Newton method with standardized constraints for nonadjustable two-pool model parameters and corrections for B1 and B0 field inhomogeneities [19]. All reconstruction procedures were performed using custom-written C-language software.

Magnetization transfer images of mouse brains obtained as a component of MPF experiment and possessing excellent white/gray matter contrast were used for automatic structural parcellation using multi-atlas label fusion method, as detailed in [24]. Briefly, individual mouse heads imaged were processed for brain extraction, intensity non-uniformity correction, affine registration to atlas images and label fusion. The publicly available MRMNAt atlas database was used, containing 10 individually labeled C57BL/6J *in vivo* mouse brains. Twenty-one white and gray matter structures from the atlas were mapped for

each mouse brain [25]. Volumes of individual anatomical areas and values of MPF were compared between control and IH groups.

First order diffusion tensor imaging (DTI) maps were calculated using multivariate linear fitting of signal attenuation from the acquired diffusion weighted images [26]. Fractional anisotropy (FA) maps were calculated [27] using in-house software written on Matlab (MathWorks, Natick, MA, USA). For *ex vivo* data analysis, regions of interest were drawn on white matter tracts as described previously [28] using directionally encoded FA maps. FA and directional diffusivities provide quantitative indexes that are informative in assessment of white matter development and injury, including axonal integrity and myelination.

2.6 Histological analysis

Fixed brains were cryoprotected with 20% sucrose and frozen. Serial 20 micron cryosections were obtained throughout the cerebrum from anterior commissure to the end of the hippocampus every 400 microns. To assess neuronal degeneration, frozen sections were mounted on gelatin coated slides, air dried, immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min, rinsed for 2 min in 70% ethanol and water, incubated in 0.06% potassium permanganate solution for 10 min, and stained in 0.0001% solution of Fluoro-Jade C (Histo-Chem, AK) in 0.1% acetic acid for 10 min, dehydrated in alcohols, cleared in xylene and mounted with DPX and examined on a Leica fluorescent microscope.

For immunostaining of cells undergoing apoptosis, primary antibodies were cleaved caspase-3 rabbit polyclonal (Cell Signaling, cat. 9661). The secondary antibodies were anti-rabbit Alexa-Fluor 488 (ab150077, Abcam). The sections were hydrated in 0.01 M PBS, blocked in 5% normal goat serum, incubated with the primary antibodies at 4 °C overnight, and then with the secondary antibody diluted at room temperature for 60 min and mounted with ProLong Gold Antifade Mountant with DAPI (ThermoFisher Scientific).

2.7 Statistical analysis.

Data are presented as means $\pm$ standard error of means. Comparison across treatment and sex groups for each age was made using two-way ANOVA with interaction term. Post-*hoc* group comparisons were performed using Tukey-Kramer method. Latency time to platform in Morris water maze test was analyzed using repeated measures ANOVA with training day as the repeated factor and treatment as the fixed factor. For comparison of outcome measures in behavioral tests, a predefined limited number of outcome measures (<4) was used and no correction for multiple comparisons were made [29]. The differences were considered significant at $\alpha=0.05$.

3. Results

3.1 Growth rate is not affected by severe IH in neonatal mice

Body weight was slightly less in IH mice during IH course (Figure 1), but the difference did not reach significance even at the last day of IH at P7 ($t_{39}=1.79$, $p=0.083$). There was also no difference between the group at 6 weeks of age for all mice ($t_{25}=0.91$, $p=0.36$), as well as for

females ($t_{32}=1.59$, $p=0.135$) and males ($t_{12}=1.21$, $p=0.083$) separately, with the trend of larger weights in IH group.

3.2 Severe neonatal IH is accompanied with bradycardia

During IH course arterial blood oxygen saturation fell rapidly to approximately 30% within 40 seconds from the onset of a hypoxia period. After 40–90sec of the hypoxia period onset there was a brief period of agitation in pups, followed by a still period. Pattern of heart rate changes was variable, but bradycardia was observed in all studied ages from P3 to P7. In some pups of younger ages P3–P5 heart rate accelerated during hypoxia periods and slowed down during room air periods, as shown for P4 pup on Figure 2A, first 65 min of IH. Starting around P4–P5, heart rate pattern changed to tachycardia with low variability during room air periods, with episodes of bradycardia appearing at the end of hypoxia periods (Figure 2A, starting from 70 min). Starting from P6, bradycardia episodes started at first or second hypoxia cycle and were reliably induced in all later ages during IH course (Figure 2B). At these ages, deceleration of heart rate progressively increased with each hypoxia cycle. Mortality rate in IH group was 27% at the end of IH course and no mortality thereafter.

3.3 Early and late reduction of physiological LTP after neonatal intermittent hypoxia.

Potentiation of fEPSP after TBS was smaller in hippocampal slices obtained from pups at P11–P15, three days after 5-day course of IH, than in controls (Figure 3A). This reduction in LTP in hippocampal slices persisted and increased in mice at 6 weeks after neonatal IH (Figure 3B). Amplitude of fEPSP was not significantly different between adult female and male mice after neonatal IH (123.5 ± 7.2 and 128.3 ± 6.5 % baseline, $p=0.82$). Therefore LTP data were pooled for both sexes to boost statistical power to compare controls and IH groups. The reduction of LTP was statistically significant at both time points (Figure 3C). Paired pulse facilitation in IH group was not different between the groups at P11–P15 ($p=0.21$), but was larger in IH group at 6 weeks ($p=0.0011$), as shown on Figure 3D, suggesting that neurotransmitter release probability was unchanged and decreased, respectively.

Synaptic excitability was increased in neonatal slices at P11–P15 after IH, as evident in increased response to stimulation on input-output curves shown on Figure 3E,F, $p=0.037$ as compared at intensity 300 μ A, but was lower than in controls at 6 week ($p<0.041$).

3.4 Long term behavioral outcome.

Morris water maze.—Control and IH mice were not different in total travelled distance ($F_{1,39}=0.17$, $p=0.68$) or mean speed ($F_{1,39}=0.196$, $p=0.66$), indicating that motor performance did not affect the memory test. Average latency to reach platform during training stage significantly decreased during training for females ($F_{3,72}=8.13$, $p<0.001$), as well as for males ($F_{3,54}=3.08$, $p=0.035$), indicating that both groups successfully learned the paradigm. There was no difference in treatment effect between the groups during the training period (Figure 4A, B). Mice after IH spent significantly less time in platform quadrant in probe trial (Figure 4C, treatment effect $F_{1,39}=6.62$, $p=0.014$, sex effect and treatment-by-sex interaction were not significant). Post hoc comparison revealed that the

difference between controls and IH groups was significant only for males ($p=0.038$). Similarly, control mice made more platform crossings in probe trial (Figure 4D, treatment effect $F_{1,39}=5.88$, $p=0.02$, sex effect and treatment-by-sex interaction were not significant). Post hoc comparison revealed that the difference between controls and IH groups in number of platform crossings was significant only for males ($p=0.015$).

Fear conditioning test.—Treatment effect ($F_{1,39}=4.77$, $p=0.035$) was significant in context fear conditioning test indicating that IH mice had freezing time less than control when placed in the same chamber where conditioning had been performed but no sound cues and shocks were presented. Post-hoc comparison revealed that freezing time in the context fear conditioning test was significantly decreased only in males ($p=0.025$) (Figure 4E). Sex effect was also significant ($F_{1,39}=4.98$, $p=0.032$) with females having larger freezing time than males in both groups. No significant differences between the groups were found in cued fear conditioning test (Figure 4F).

Open field.—No significant difference between control and IH groups in treatment effect was found in total travel distance ($F_{1,39}=0.22$, $p=0.63$) as well as in time spent in center zone ($F_{1,39}=0.29$, $p=0.59$) (Figure 4G,H). Sex effect and treatment-by-sex interactions were also not significant.

Elevated plus maze.—No significant difference between control and IH groups in treatment effect was found in total travel distance ($F_{1,39}=0.22$, $p=0.63$) as well as in time spent in center zone ($F_{1,39}=0.29$, $p=0.59$) (Figure 4). Sex effect and treatment-by-sex interactions were also not significant. Number of entries to the closed arm was larger in males (sex effect $F_{1,41}=6.25$, $p=0.016$, Figure 4I). Treatment effect was not significant for the number of entries to the closed arm (Figure 4J).

Social interaction test.—No significant difference between control and IH groups in treatment effect was found for Sociability index, ($F_{1,39}=0.85$, $p=0.36$) as well for Social Novelty index ($F_{1,39}=1.13$, $p=0.29$) (Figure 4K,L). Sex effect and treatment-by-sex interaction were also not significant.

3.5 No tissue loss after severe neonatal IH on in vivo MRI

No significant differences between control and IH groups were found in regional volumes in white and gray matter structures measured on atlas based segmentation (Figure 6A, B). No difference was found between the groups in total brain volume. Sex factor was significant with males mice having larger brains than females ($F_{1,35}=7.95$, $p=0.0078$)

3.6 Hypomyelination in gray and white matter after severe neonatal IH on in vivo MRI

IH group had lower MPF than in controls in corpus callosum ($F_{1,35}=4.72$, $p=0.036$), anterior commissure ($F_{1,35}=4.65$, $p=0.035$) and fimbria ($F_{1,35}=5.13$, $p=0.029$) (Figure 6C), as well as in hippocampus ($F_{1,35}=4.71$, $p=0.038$), thalamus ($F_{1,35}=4.71$, $p=0.042$) and cortex ($F_{1,35}=6.56$, $p=0.014$), indicating hypomyelination in white and gray matter in adult mice after neonatal IH. Sex factor and treatment-by-sex interaction were not significant in myelination response to IH.

3.7 Microstructural abnormalities in white matter tracts in IH mice on DTI

Diffusion tensor imaging was used to examine differences in white matter tracts in adult mice after neonatal IH (Figure 6E). Significantly lower fractional anisotropy (FA) was found in corpus callosum ($p=0.013$) and fimbria ($p=0.023$). While no significant difference in radial and axial diffusivities was found between the groups (Figure 6F), decrease in FA in IH group was likely due to larger values of axial diffusivities and smaller numbers in axial diffusivities (data not shown).

3.8 No immediate cell death after severe neonatal IH

A few caspase positive cells were observed in cortex and hippocampus both in control and IH groups (Figure 5A,B). No difference in caspase-positive cells count was found between the groups (Figure 5C). FluoroJade C staining was negative for control and IH group (data not shown). To confirm that the FluoroJade assay works, it was confirmed to produce positive staining on slices obtained from a neonatal stroke mouse model.

4. Discussion

The main result of our study was the decreased synaptic plasticity in hippocampal neurons in the form of early LTP in mice, exposed to multiple episodes of severe intermittent hypoxia with bradycardia during the neonatal period that persisted to adulthood. LTP decrease was associated with memory deficits in spatial navigation task on Morris water maze and context fear conditioning tests. The significant memory deficits were found only in males. We confirmed hypomyelination and microstructural injury in white matter tracts, observed in previous studies on rodent models of IH, but also found hypomyelination in cortex, hippocampus and thalamus. Sex factor was not significant in myelination response to IH.

The very preterm infants experience numerous episodes of oxygen desaturation during initial weeks of life [6]. It is still unknown whether these episodes of IH have an adverse effect on brain development. What has been shown is that only prolonged apnea and bradycardia were associated with long-term neurodevelopmental delay in prematurely born infants [6]. Several rodent models have been developed to study effects of IH on brain development. In animal models that mimic clinical situation of relatively mild IH, characterized by small magnitude of desaturations and no bradycardia, very few long term cognitive abnormalities are reported [8–10]. Moreover, mild IH is also reported to improve cognitive performance [13]. Long-term cognitive deficits are reported in models with severe IH [14, 15], modeling perinatal asphyxia and effects of preterm birth. Long-term deficits were also found in the current study where IH was accompanied with episodes of large desaturation and bradycardia, modeling apnea of prematurity.

Bradycardia was observed in rodent models with severe neonatal IH with fraction of inspired oxygen (FiO_2) 3–5.7% and duration of hypoxia period 2 min [18, 30] and 2.5 min in the current study. In the mouse model of Cai et al. nadir FiO_2 was maintained at 5.7% or 8% for 20 seconds [18], while in our model oxygen concentration reached nadir in 40 seconds maintained at 5% for 110 seconds. Reliable occurrence of bradycardia places our model

toward the end with larger severity in the spectrum of IH models. Apparently, not only severity of hypoxia but also duration and number of hypoxia episodes are important to produce brain injury resulting in neurobehavioral deficits. Long-term neurobehavioral deficits, such as hyperactivity and spatial memory impairment, were observed in models with IH regimen similar to chronic hypoxia [31], with moderately low oxygen (around 10%) but long exposure for several weeks.

Injury to oligodendrocytes but no neuronal loss is typically found in IH models [9, 14, 18]. Gozal et al. [32] reported low apoptosis on TUNEL staining seen in hippocampus CA1 at P2-P5 rats, exposed 10% hypoxia/room air alternation for 90 sec for 48 hours, but dramatically increased apoptosis by P15. We found little apoptosis in our model, as well as no tissue loss on *in vivo* volumetric imaging. It is possible that IH may induce a delayed cell death that needs to be assessed at later time points. It is also possible that the pups experiencing most severe desaturations and pronounced bradycardia may develop a larger cell death; however it is unlikely to be dramatic. Absence or minimal cell loss after neonatal IH suggests that the observed cognitive abnormalities are caused by changes in functional properties of preserved networks.

It is widely recognized that long-term synaptic plasticity and LTP are associated with learning and memory formation [33]. A decrease of LTP has been shown in hippocampal slices obtained 3 and 7 days after IH in adult rats [34] and LTP was proportionally lower in adult mice IH after 3 and 14 days of 10% oxygen 90 sec cycle IH [35] that could be rescued by exogenous BDNF. There is a paucity of data in LTP changes after neonatal IH and neonatal hypoxiaischemia, but a decrease of LTP had been found postnatally in spiny mice 5 days after near term acute fetal asphyxia [17] in the model associated with postnatal behavioral abnormalities [36]. Of interest, there was no structural injury observed in this model, but neuronal density and synaptophysin expression and BDNF were increased in hippocampus in male P5 pups, suggesting that the behavioral changes were likely due to changes in synaptic plasticity. Similarly, we found a decrease in LTP immediately after IH that persisted till adulthood and was associated with long-term memory deficits.

In our study we found an early increase in excitability based on steeper input-output curve 3 days after IH course. Increased postsynaptic response may indicate that for a given stimulus intensity there is a greater presynaptic release of mediator or a larger postsynaptic response, for instance, due to changes in expression/trafficking of AMPA receptors. The increase of release probability is unlikely because PPF ratio was not significantly different. The reason for the increase in synaptic efficacy after neonatal IH is currently unclear and requires further studies. A possible mechanism may be an increase in AMPA receptors mediated current due to AMPA receptors phosphorylation and incorporation in to postsynaptic membrane. This pathway was observed after global ischemia [37] and is analogous to physiological LTP [38]. This phenomenon, described as “anoxic LTP”, may also occur during IH episodes with large arterial desaturation and bradycardia, resulting in brain hypoperfusion and hypoxia. In these conditions anoxic neuronal depolarization and large release of glutamate may occur and trigger hypoxic LTP. LTP decrease right after hypoxic insult may be explained in this case by LTP occlusion through saturation of plasticity mechanisms as shown in models of oxygen and glucose deprivation *in vitro* [39, 40] and in

models of global ischemia in vivo [41]. Increased synaptic excitability was also found in neonatal hippocampal slices after oxygen glucose deprivation in vitro [42]. Alteration of synaptic plasticity, indicated by decreased LTP and occurring at the critical developmental period of active synapse formation and maturation [43], may explain observed memory deficits in adulthood in neonatal IH model.

In the current study neuronal excitability was initially increased in hippocampus of neonatal mice after IH and became lower in adults, relative to age matched controls. However, LTP remained reduced at both time points. This observation suggests that additional mechanisms, apart from possible occlusion of LTP shortly after neonatal IH, may play a role in decreased synaptic plasticity and memory deficits in adults after neonatal IH. Among possible reasons for the decrease of initially elevated neuronal excitability can be a reduction in density of axons in Schaffer collaterals, decreased number of synapses and dendrites, decreased dendritic arborization, fewer functioning synapses, structural injury to axons and glia, including hypomyelination.

In the current study we pooled male and female electrophysiology data together for statistical analysis since we did not have sufficient statistical power to analyze LTP data with regard to animal sex. Since significant differences in memory tests were found only in males, sex differences become critical for understanding the significance of the LTP impairment to problems with learning and memory. Sexual dimorphism in behavioral outcome and extension of brain damage is now widely recognized in neonatal hypoxia ischemia[44]. This is a limitation of the study that needs be addressed in the future.

White matter injury and hypomyelination is a consistent finding in neonatal IH models [8–10], including the current study. We found hypomyelination in cortex, thalamus, and hippocampus, as well as in major white matter tracts using a novel *in vivo* quantitative MRI method, macromolecular proton fraction mapping. MPF mapping is a validated biomarker of myelination in neural tissues. The method has high resolution, fast and independent of directional tissue organization, and therefore is uniquely suited to assess myelination in gray matter structures [22, 25, 45]. MPF provided complementary information to diffusion tensor imaging in white and gray matter in this study. The mechanisms of white matter injury have been extensively studied in neonatal hypoxia-ischemia [46] and IH models [8] and point to selective injury to immature oligodendrocytes and failure to progress into mature myelinating stage. The relationship between deficits in myelination after neonatal IH injury and long-term cognitive impairment is not clear, especially in gray matter. In the current study we did not distinguish whether hypomyelination was due to thinner myelin sheets or fewer myelinated axons, which could be resolved by electron microscopy in the future. Hypomyelination likely affects maturation of neuronal network, since myelin facilitates axonal signal transmission [47, 48]. Hypomyelination and the decrease in synaptic plasticity after neonatal IH injury may act synergistically in the origin of long-term cognitive impairment. The impact of myelination deficits in hippocampus after neonatal hypoxia injury on long-term impairment of learning and memory should be further elucidated.

5. Conclusion

The current study presents a modified murine model of neonatal IH that recapitulates features of severe apnea of prematurity with large desaturations and bradycardia, resulting in early and persistent changes of synaptic plasticity and hypomyelination in gray and white matter associated with long term memory deficits.

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Abbreviations:

IH	intermittent hypoxia
LTP	long term potentiation
fEPSP	field excitatory postsynaptic potential
TBS	theta burst stimulation
PPF	paired pulse facilitation
MPF	macromolecule proton fraction
DTI	diffusion tensor imaging

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Highlights

- Severe neonatal intermittent hypoxia (IH) with bradycardia results early decrease in long term potentiation in hippocampus that persist till adulthood.
- Severe neonatal IH with bradycardia results in long term spatial memory impairment
- Memory deficits were gender dependent, larger affecting males
- Hypomyelination after neonatal IH is found in white but also in gray matter

Figure 1.
Body weight in mice during and after neonatal IH. Adult mice were measured at 6 weeks.

Figure 2.

Tracings of heart rate, respiration and arterial oxygen saturation (SaO₂) during IH. Measurements were obtained using MouseOx pulse oximeter using sensors placed around pups' heads. Heart rate pattern changed with age from tachycardia-bradycardia transition on a trace from a P3 pup (A) to episodes of bradycardia (B, P6 pup) during hypoxia periods.

Figure 3.

Synaptic activity measured in CA1 area in hippocampus. Potentiation of fEPSP after high frequency stimulation (TBS) in hippocampal is shown on trace recordings in slices obtained from mice three days (A) and after 6 weeks (B) after IH and from controls. C. LTP, expressed as percentage over baseline during last 10 min after TBS. D. Pared pulse facilitation after IH and in controls. Input –output curves after three days (E) and after 6 weeks (B) after IH and in controls.

Figure 4.

Results of behavioral testing in 6 week mice after neonatal IH. A,B – Average latency to reach platform in session during training in Morris water maze. C. Probe trial in in Morris water maze, time in platform quadrant. Significant decrease was found in IH males. D. Probe trial in in Morris water maze, number of platform crossings. Significant decrease was found in IH males. E, F – fear conditioning test. Significant decrease in freezing time was found in male IH mice in context fear conditioning test (E), but not in cued fear conditioning test (F). I,J. Elevated plus maze. No difference between the groups in time spent in closed arm

(I) or in number of entries in closed arm (J). K,L – 3-chamber social interaction test. No difference between the groups in sociability (K) or social novelty (L) indexes.

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Figure 5. Cleaved caspase immunostaining in CA1 hippocampal. Selective caspase 3 – positive cells were observed in hippocampus both in control (A) and IH (B) mice. Insert shows capase-3 positive cells at higher magnification. No difference between the groups was found in number of caspase 3 positive cells (C). Scale bar 200 μ m.

Figure 6.

Magnetic resonance imaging on 6 weeks mice controls and after neonatal IH.

A-B. Regional volumes on in vivo MRI. Brain images with magnetization transfer contrast were segmented using atlas base segmentation and tissue volumes are compared between control and IH groups in white (A) and gray matter (B). No difference was found in the treatment factor.

C-D. Micromolecule proton fraction (MPF), as an *in vivo* measure of myelin, was averaged for selected white (C) and gray matter (D) regions obtained from atlas based segmentation. Differences on ANOVA between control and IH groups in MPF are indicated by braces.

E-F. *Ex vivo* diffusion tensor imaging revealed lower fractional anisotropy in IH pups in corpus callosum (CC) and Fimbria (E), but not in anterior commissure (AC) or internal capsule (IC). No significant difference was found in radial diffusivity (F) in all white matter structures.

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Table 1.

Number of samples used in experiments.

Experiment	Control group	IH group
Electrophysiology at P11	7 pups/11 slices	8 pups/ 14 slices
Electrophysiology at 6 weeks	10 pups/15 slices	11 pups./25 slices
Behavioral	10 females, 8 males	13 females, 12 males
In vivo MRI	8 females, 8 males	11 females, 11 males
Ex vivo MRI	8 of both sexes	10 of both sexes
Histopathology	6 of both sexes	6 of both sexes