Calbindin-1 Expression in the Hippocampus following Neonatal Hypoxia-Ischemia and Therapeutic Hypothermia and Deficits in Spatial Memory

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Keywords

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Abstract

Hippocampal injury following neonatal hypoxia-ischemia (HI) leads to memory impairments despite therapeutic hypothermia (TH). In the hippocampus, the expression of calbindin-1 (Calb1), a Ca^{2+} -buffering protein, increases during postnatal development and decreases with aging and neurodegenerative disorders. Since persistent Ca^{2+} dysregulation after HI may lead to ongoing injury, persistent changes in hippocampal expression of Calb1 may contribute to memory impairments after neonatal HI. We hypothesized that, despite TH, neonatal HI persistently decreases Calb1 expression in the hippocampus, a change associated with memory deficits in the mouse. We induced cerebral HI in C57BL/6 mice at postnatal day 10 (P10) with right carotid ligation and 45 min of hypoxia ($\text{FiO}_2 = 0.08$), followed by normothermia (36°C, NT) or TH (31°C) for 4 h with anesthesia-shams as controls. Nissl staining and glial fibrillary acidic protein (GFAP) immunohistochemistry (IHC) were used to grade brain injury and astrogliosis at P11, P18, and P40 prior to the assessment of Calb1 expression by IHC. The subset of mice followed to P40 also performed a memory behavior task

(Y-maze) at P22–P26. Nonparametric statistics stratified by sex were applied. In both anterior and posterior coronal brain sections, hippocampal Calb1 expression doubled between P11 and P40 due to an increase in the cornu ammonis (CA) field (Kruskal-Wallis [KW] $p < 0.001$) and not the dentate gyrus (DG). Neonatal HI produced delayed (P18) and late (P40) deficits in the expression of Calb1 exclusively in the CA field (KW $p = 0.02$) in posterior brain sections. TH did not attenuate Calb1 deficits after HI. Thirty days after HI injury (at P40), GFAP scores in the hippocampus ($p < 0.001$, $r = -0.47$) and CA field ($p < 0.001$, $r = -0.39$) of posterior brain sections inversely correlated with their respective Calb1 expression. Both sexes demonstrated deficits in Y-maze testing, including approximately 40% lower spontaneous alterations performance and twice as much total impairment compared to sham mice (KW $p < 0.001$), but it was only in females that these deficits correlated with the Calb1 expression in the hippocampal CA field ($p < 0.05$) of the posterior sections. Hippocampal atrophy after neonatal HI also correlated with worse deficits in Y-maze testing, but it did not predict Calb1 deficits. Neonatal HI produces a long-lasting Calb1 deficit in the hippocampal CA field during development, which is not mitigated by TH. Late Calb1 deficit after HI may be the result of persistent astrogliosis and can lead to memory impairment, particularly in female mice. © 2019 S. Karger AG, Basel

Introduction

Hypoxia-ischemia (HI) is one of the most common causes of perinatal brain injury. Complete or partial disruption of blood flow to the brain impairs normal brain function by reducing the delivery of oxygen and glucose necessary for aerobic metabolism [1–3]. Along with the basal ganglia and the cortex, the hippocampus is one of the regions with the highest metabolic demands and vulnerability to HI [3, 4]. Harmful increases in intracellular Ca^{2+} concentrations lead to mitochondrial failure and neuronal death [2, 4, 5]. Ca^{2+} dysregulation may persist long after the initial HI insult and leads to neuroinflammation and ongoing injury [6].

Neurons use EF-hand type Ca^{2+} -binding proteins (CBPs) to buffer out excess calcium and achieve homeostasis after an insult [2, 7, 8]. These CBPs, including calbindin-1 (Calb1), are also involved in many neuronal functions, such as synaptic transmission, cell signaling, the release of neuropeptides, gene expression and activation, and cell death [7, 9–12]. Inhibition of Ca^{2+} influx attenuates neuronal cell damage from ischemic injury [8]. Thus, the relative expression of CBPs, or the lack thereof, plays an important role in neuronal injury in response to an insult such as HI. Although these proteins often work in conjunction with each other, there are distinct differences in their basic functions. Specifically, Calb1, mainly expressed by GABAergic interneurons of the cornu ammonis (CA) subfields of the hippocampus and the granular cells of the dentate gyrus (DG) [7, 11], has the highest affinity to buffer calcium [11], and has been linked to the preservation of hippocampal-dependent memory in the mouse [11, 12].

Neonatal HI injures the basal ganglia-thalamus and cortical tracks and results in motor impairments, which are attenuated by therapeutic hypothermia (TH) [13–16]. However, a significant proportion of survivors of neonatal HI brain injury suffer from cognitive and memory impairments, suggesting that TH does not fully protect the hippocampus, an essential brain region for the consolidation of memory [7, 12, 13, 16]. Persistent disturbance in Calb1 expression within the hippocampus may play a critical role in memory outcomes following neonatal HI injury. There is a segregation of the rodent hippocampus along the dorsoventral axis, with the dorsal hippocampus (anterior in the mouse) functionally linked to spatial learning and memory [17], so the study of the relationship between Calb1 and memory outcomes requires stratification according to hippocampal section in the anteroposterior axis. Here, we aimed to study the expression

of hippocampal Calb1 following HI and TH, and the link with the degree of astrogliosis and memory outcomes in a mouse model. We hypothesize that, despite TH, neonatal HI persistently impairs Calb1 expression in the hippocampus, a change linked to memory deficits in the mouse.

Materials and Methods

Mice

Male and female C57BL6 (Charles River Laboratories, Wilmington, MA, USA) pups were used for these experiments, totaling 215 mice. All efforts were made to minimize the number of mice used and their suffering.

Neonatal HI Injury and TH

Neonatal HI brain injury of postnatal day 10 (P10) mice was induced using the Rice-Vannucci model adapted for neonatal mice as previously published. In short, the mice were anesthetized with an inhaled isoflurane (3% for induction and 1% for maintenance) mixture with nitrous oxide prior to the induction of brain HI by unilateral right carotid artery ligation. They were returned to their cages for a 1-h resting period followed by 45 min of hypoxia at $\text{FiO}_2 = 0.08$ in an acrylic glass chamber. After HI, mice were randomized to normothermia (NT) at 36 °C or TH at 31 °C for 4 h, and then returned to their cages. Surgery lasted 3–5 min. Mice randomly assigned to the sham group received an inhaled isoflurane/nitrous oxide mixture for 5 min at similar concentrations as described above for the NT and TH groups and then returned to their cages. This mouse model of neonatal HI and TH has been validated by T2 MRI techniques, demonstrating sustained neuroprotection against cerebral and cortical atrophy at 8 and 20 days after HI injury at P10 and a lack of sustained protection of the hippocampus [18]. This differential regional protection afforded by TH in this model resulted in protection of the motor domains and a lack of protection of the memory domains of neurodevelopment [19]. After exclusions, we studied the brains in 3 different cohorts of animals at P11 (sham $n = 9$; NT $n = 10$; TH $n = 11$), P18 (sham $n = 9$; NT $n = 7$; TH $n = 14$), and P40 (sham $n = 37$; NT $n = 31$; TH $n = 35$) on immunohistochemistry (IHC). Mice were killed via exposure to 20% (v/v) mixture of isoflurane in propylene glycol by the 1-drop exposure method [20] prior to being exsanguinated with 0.1 M cold PBS (pH 7.4) via cardiac perfusion in the left ventricle. Brains were perfused with 4% paraformaldehyde (PFA) at 4 mL/h and postfixed overnight on 4% PFA prior to being cryoprotected in 30% sucrose in PBS until they sank. All brains were then frozen and stored at –80 °C prior to being cut on a freezing microtome at 50 μm .

Terminology

The use of the term “anterior” or “posterior” reflects the position of the coronal brain section in the anteroposterior axis. For all experiments, we evaluated the hippocampal sections located within the mouse brain. Sections were classified as anterior if the fasciola cinereum was adjacent to the dorsal third ventricle and before the emergence of the dorsal subiculum. They were classified as posterior if the CA3 subfield crossed the level fasciculus retroflex-

us but was positioned prior to the emergence of the DG granular cell layer in the ventral portion of the coronal section [9]. Based on the description above, the hippocampus located in our anterior coronal brain sections corresponds to the dorsal/septal hippocampus, while that located in our posterior coronal brain sections corresponds to the transition between the dorsal/septal and ventral/temporal hippocampus. Thus, the hippocampus in the posterior coronal sections of the brain does not correspond exclusively to the ventral hippocampus, but shows an arrangement of the dorsal and ventral hippocampus in the upper and lower areas, respectively.

Inclusion and Exclusion Criteria

The hippocampus within the anterior and posterior coronal brain sections was assessed for severity of injury using Nissl staining and glial fibrillary acidic protein (GFAP) scoring [9]. Evidence of early (P11) cell death with nuclear changes characterized by pyknosis, karyorrhexis, and karyolysis, apoptotic cell bodies, and delayed (at P18 and P40) columnar injury of the CA1 and CA3 subfields without obliteration on Nissl staining were included in the analysis. GFAP scores of 3–7 per subfield or 9–21 in the whole-hippocampus analysis on IHC suggested injury/astrogliosis and were included in the analysis [9]. Pups demonstrating either minimal or no injury (2 at P11, 4 at P18, and 5 at P40) or an obliterated hippocampus (2 at P11, 4 at P18, and 9 at P40) were not included in the analysis.

IHC for GFAP and Calb1

For IHC, tissue was fixed with 4% PFA and cryoprotected with a 15/30% sucrose gradient as previously described prior to storage at -80°C , and then cut at $50\text{ }\mu\text{m}$ with a freezing microtome [21]. Floating IHC was performed as previously described [21] with whole-rabbit antisera anti-Calb1 (Cell Signaling Technology, Inc, Beverly, MA; 1:250), or anti-GFAP (DAKO/Agilent Technologies, Santa Clara, CA, USA; 1:1,000) followed by goat anti-rabbit antibody (1:200) used as the secondary antibody and DAB. Cresyl-violet (CV) staining was performed to assess injury at P11, P18, and P40 and for volumetric analysis.

Antibodies

Calb1 (CS13176; RRID:AB_2687400): rabbit monoclonal antibody raised against a recombinant protein specific to the N-terminus of whole Calb1 protein of human origin detecting a product at 28 kDa ($2\text{ }\mu\text{g/mL}$). GFAP (DAKO Z0334; RRID:AB_10013382): rabbit polyclonal antibody raised against GFAP isolated from cow spinal cord with no reported cross reactivity ($1\text{ }\mu\text{g/mL}$).

Analysis of Calb1 Immunoreactivity and Hippocampal Volume

Calb1 IHC and CV-stained slides were imaged using a light microscope (Nikon Eclipse E400, Nikon), to produce high-resolution photomicrographs, and then analyzed using ImageJ v1.8.0 software (NIH, Bethesda, MD, USA) (Rasband 1997–2018).

Calb1 Immunoreactivity

Using the free draw tool, the whole hippocampus and the DG field were outlined separately. All images were converted to 8-bit images and globally calibrated to optical density (OD). The background was removed from the images to measure the Calb1 staining. The background was obtained by drawing 10 circles free of Calb1 staining, 5 in the lacunosum molecular layer and 5 in the

oriens layer. The average OD score from these 10 background circles was subtracted from the whole-hippocampus average OD score to create the adjusted mean OD for the hippocampus. The mean OD of the CA field was determined by the following formula: $\text{CA mean OD} = [(\text{mean hippocampal OD} \cdot \text{hip area}) - (\text{mean DG OD} \cdot \text{DG area})] / (\text{hippocampal area} - \text{DG area})$. Thus, the Calb1 OD used for the analysis and correlations was the average of the OD measured in every pixel contained within the region of interest (whole hippocampus, CA field, or DG).

Hippocampal Volume

We calculated residual volumes using the hippocampal areas (mm^2) obtained from 5 representative $50\text{-}\mu\text{m}$, CV-stained coronal brain sections positioned $600\text{ }\mu\text{m}$ apart in the anteroposterior axis of the brain at P40. Total residual volume was extrapolated using the following formula:

$$\text{Hippocampal volume (mm}^3\text{)} = \sum_{i=1}^n (n=5) [S_i \times 0.05] + \sum_{i=1}^n (n=4) [(S_i + S_{i+1}) \times 0.6/2]$$

where S = area of hippocampal section in mm^2 , i = section position in the anteroposterior axis, and n = number of sum repeats.

Behavioral Testing

The P40 cohort underwent behavioral testing (Y-maze) between P22 and P26 before harvesting the brain at P40. Testing was conducted in a noise-free room between 1 and 6 p.m. The order of testing the mice was randomized for each test within the litters. Each litter had a maximum of 6 pups, with up to 2 litters during each behavioral session. Pups were habituated in the experimental room for 30 min prior to the start of testing. All scored tasks were recorded using videos which were reviewed and scored manually by 2 team members blinded to the treatment groups (JGH and DS), and electronically using AnyMaze v5.1 (Stoelting Co. Wood Dale, IL, USA). The mice were returned to their cages following testing.

Y-Maze

The Y-maze test was used to assess spatial working memory in rodents. Uninjured mice will choose to investigate a new arm before returning to one recently visited. The apparatus was made of acrylic glass forming a “Y” with 3 identical arms positioned at 120° angles. There are 2 phases each lasting 5 min: phase 1 to assess working memory and phase 2 to assess spatial and recognition memory. During both phases, the mice started the test in the center of the maze. During phase 1, the sequence of arm entries was recorded. Spontaneous alterations performance (SAP) assesses working memory as the mice visit 3 consecutive different arms of the maze. Impaired working memory is determined by higher alternate-arm returns (AAR), i.e., 3 consecutive arm visits in which the first and third arm visit is the same, and same-arm returns (SAR), i.e., 2 successive visits to the same arm. Phase 2 was performed 3 days after phase 1. During the prerecorded phase, mice explore the maze with 1 arm blocked (the novel arm) for 5 min. After a 30-min resting period, all arms were open for the mice to explore the maze once more for 5 min. The percentage of time spent and the number of entries into the novel arm were analyzed.

GFAP Scoring to Assess Astrogliosis

Along with CV staining, GFAP IHC was used to assess the degree of astrogliosis as a surrogate hippocampal injury in order to

exclude those mice demonstrating minimal or no injury. We used a semiquantitative GFAP-derived scoring system developed in our laboratory [9] for this purpose and also to establish the correlation between persistent astrogliosis and changes in Calb1 expression. This scoring system takes into account the dispersion of hippocampal immunoreactivity and the morphological characteristics of astrocytes [22, 23] in the CA1 and CA3 subfields and in the DG in the hippocampus positioned in the anterior and posterior coronal brain sections. Scoring was based on the abundance of GFAP staining and glial scars, astrocyte body size, the number and thickness of the branches, and the overlap of astrocytic domains at a higher magnification [9]. Representative high-magnification photomicrographs showing GFAP-stained astrocytes within the hippocampal CA1 subfield at P40 can be seen in online suppl. Fig. 1 (see www.karger.com/doi/10.1159/000497056 for all online suppl. material). The presence of astrocytes with small somas and few or many thin branches without overlapping domains was granted 1 or 2 points for the subfield, respectively (online suppl. Fig. 1A₁, A₂). The identification of astrocytes with large somas and many thick branches with overlapping domains was granted 3 points (online Suppl. Fig. 1A₃), while the presence of astrocytes with even larger soma, forming 1, 2, or ≥ 3 glial scars, was granted 4, 5, or 6 points, respectively (online suppl. Fig. 1A₄). Lastly, diffuse astrocytic activation through the subfield was granted 7 points. The cumulative score was the sum of the scores for each of the 3 subfields (CA1, CA3, and DG), which varied from 1 to 7. This resulted in a cumulative score of 3–21 for the whole hippocampus and 2–14 for CA field.

Statistics

Multiple groups were analyzed using nonparametric Kruskal-Wallis (KW) *H* one-way ANOVA stratified by sex, with post hoc pair analysis using the Dunn-Bonferroni test. All results were presented as box and whisker plots, where the box was limited by the 25th and 75th percentiles and the solid line represented the median. Significance was assigned by $p \leq 0.05$ adjusted for multiple comparisons in all cases. Nonparametric Spearman Rho correlations were applied between Calb1 expression, hippocampal residual volume, GFAP score, and memory outcome. Analysis was performed using SPSS v24v (IBM Corp., Armonk, NY, USA).

Results

Developmental Changes in Calb1 Expression in the Hippocampus

In the hippocampal sections located within both the anterior and posterior coronal sections, Calb1 expression increased between P11 and P40. In the hippocampus within the anterior coronal sections, Calb1 expression increased from 0.167 arbitrary units (a.u.) (IQR 0.157–0.177) at P11 to 0.204 a.u. (0.161–0.263) at P18, and 0.334 a.u. (0.277–0.339) at P40 (Fig. 1A₁–A₄). The expression in the CA field within the anterior coronal sections was, on average, 0.105 a.u. (0.103–0.110) at P11, 0.117 a.u. (0.093–0.147) at P18, and 0.284 a.u. (0.196–0.367) at P40 (H 22.7; df 2; KW p <

0.001; P11 vs. P40 and P18 vs. P40 adjusted p = 0.001; Fig. 1A₅). Thus, in the anterior brain sections, the 2-fold increase in hippocampal Calb1 expression between P11 and P40 (H 19.9; df 2; KW p < 0.001; P11 vs. P40 adjusted p = 0.001) was specifically linked to the increase within the CA field (Fig. 1A₅), but not the DG (Fig. 1A₆). Similarly, in the posterior brain sections, hippocampal Calb1 expression increased from 0.239 a.u. (0.194–0.352) at P11 to 0.415 a.u. (0.362–0.445) at P40 (H 20.4; df 2; KW p < 0.001; P11 vs. P40 adjusted p = 0.002; Fig. 1B₁–B₄). The 75% increase in the hippocampal Calb1 expression between P11 and P40 was dependent on the increase within the CA field of the posterior brain sections (H 12.4; df 2; KW p = 0.002; P11 vs. P40 adjusted p = 0.003; Fig. 1B₅) but not the DG (Fig. 1B₆). Collectively, the expression of hippocampal Calb1 increased by 54 and 96% between P11 and P18 (H 29.2; df 2; KW p < 0.001; adjusted p < 0.001) and P18 and P40 (adjusted p < 0.001), respectively. This change was linked to the increase in the CA field (H 27.2; df 2; KW p < 0.001; P11 vs. P18 and P18 vs. P40 adjusted p < 0.001; data not shown).

Impaired Expression of Calb1 following HI and Lack of Protection by TH

Neonatal HI did not alter the developmental increase in hippocampal Calb1 expression between P11 and P18 in the anterior coronal brain sections (Fig. 2A₁–A₃). However, deficits in expression of Calb1 were documented in the hippocampus as early as 8 days after injury (P18, Fig. 2B) in the posterior brain sections. Specifically, the expression of Calb1 within the CA field was 18% lower 8 days after HI injury (H 7.1; df 2; KW p = 0.02; adjusted p = 0.05 vs. sham) and TH did not provide protection (adjusted p = 0.02 vs. sham; Fig. 2B₂). Impairment in the expression of Calb1 in the hippocampus progressed by P40 (30 days after injury) in the posterior brain sections. At P40, hippocampal Calb1 expression in the posterior brain sections of injured mice was 20% lower than in shams (H 22.4; df 2; KW p < 0.001; adjusted p $\leq$ 0.001 vs. sham) and TH did not prevent this deficit (–24%; adjusted p = 0.002 vs. sham; Fig. 3A₁). The developmental increase in Calb1 expression in the CA field between P11 and P40 (Fig. 1B₅) was impaired by neonatal HI injury in the posterior brain sections (–25%, H 10.8; df 2; KW p = 0.005; adjusted p = 0.03 vs. sham; Fig. 3A₂). Even the mice treated with TH after HI injury had 32% lower Calb1 expression in the CA field compared to shams (p = 0.008; Fig. 3A₂). Again, no differences in Calb1 expression were seen in the DG at P40 (Fig. 3A₃). Calb1 deficits in the hippocampus and CA

Fig. 1. Developmental changes in Calb1 expression in the hippocampus. Representative Calb1 IHC of hippocampal sections in anterior (**A1–A3**) and posterior (**B1–B3**) coronal sections at P11 (**A1, B1**), P18 (**A2, B2**), and P40 (**A3, B3**). Scale bar, 1,000 μ m. Two photomicrographs ($\times 4$) of the hippocampus in the posterior coronal section (i, ii), were used to compose the final Figure (**B1–B3**). Box and whiskers plots represent mean optical densities (OD) adjusted (Adj) for background for Calb1 immunoreactivity in ante-

rior and posterior coronal brain sections for the whole hippocampus (**A4** and **B4**, respectively), the CA field (**A5** and **B5**, respectively), and DG (**A6** and **B6**, respectively). Boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Analysis by Kruskal-Wallis ANOVA with Dunn-Bonferroni correction; * adjusted $p < 0.05$. CA, cornus ammonis; DG, dentate gyrus.

Fig. 2. Impaired hippocampal expression of Calb1 in posterior coronal sections following HI and lack of protection by TH at P18. Box and whiskers plots represent mean OD adjusted (Adj) for background for Calb1 immunoreactivity in whole hippocampus (**A1, B1**), CA field (**A2, B2**), and DG (**A3, B3**) in anterior and posterior sections, respectively. Boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside

the box). Analysis by Kruskal-Wallis ANOVA with Dunn-Bonferroni correction; * adjusted $p < 0.05$. Representative Calb1 IHC section hippocampus within posterior coronal brain sections of sham (**B4**), NT (**B4'**), and TH (**B4''**). Scale bar, 1,000 μ m. Two photomicrographs ($\times 4$) of the hippocampus (I, ii) within the posterior coronal section were used to compose the final figure (**B4**). CA, cornus ammonis; DG, dentate gyrus; NT, normothermia; TH, therapeutic hypothermia.

Fig. 3. Persistent Calb1 deficits in the hippocampus in posterior coronal sections following HI linked to persistent astrogliosis at P40. Box and whiskers plots represent mean OD adjusted (Adj) for background for Calb1 immunoreactivity in whole hippocampus (**A1**), CA field (**A2**), and DG (**A3**) in anterior and posterior coronal sections. Boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Analysis by Kruskal-Wallis ANOVA with Dunn-Bonferroni correction; * adjusted $p < 0.05$. Representative Calb1 IHC hippocampal section in posterior brain sections of sham (**A4**), NT (**A4'**), and TH (**A4''**).

Scale bar, 1,000 μm . Two photomicrographs ($\times 4$) of the hippocampus (I, ii) within posterior coronal sections were used to compose the final figure (**A4**). Calb1 expression in the whole hippocampus (**B1**), CA field (**B2**), and DG (**B3**) of posterior coronal sections were correlated with their respective GFAP score. Spearman correlations were applied. The continuous line represents the predicted linear regression and discontinuous lines represent the projection to the 95% confidence interval. $p < 0.05$ was considered significant. CA, cornus ammonis; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; HIP, hippocampus; NT, normothermia; TH, therapeutic hypothermia.

field were similar in male and female mice in the posterior brain sections (online suppl. Fig. 2).

Correlation between Persistent Astrogliosis and Decreased Calb1 Expression at P40

Our previously published GFAP-derived scoring system [9] was applied to grade astrogliosis and correlate with Calb1 expression at P40. Representative photomicrographs show GFAP staining of the hippocampus within the anterior (online suppl. Fig. 1B) and posterior (Fig. 4) coronal brain sections at P11, P18, and P40. The median (IQR) GFAP scores of sham, HI/NT, and HI/TH mice in the hippocampus in the anterior brain sections were 3 (3–4), 16 (11–20), and 12 (10–16) (H 68.7; df 2; KW $p < 0.001$), respectively; in the posterior brain sections they were 3 (3–4), 14 (10–21), and 12 (10–15) (H 71.5; df 2; KW $p < 0.001$), respectively. Median GFAP scores of sham, HI/NT, and HI/TH mice in the CA field in the anterior sections were 2 (2–3), 12 (8–14), and 9 (7–12) (H 71.6; df 2; KW $p < 0.001$), respectively; in the posterior sections they were 2 (2–3), 11 (7–14), and 9 (8–11) (H 71.4; df 2; KW $p < 0.001$), respectively. The GFAP scores in the hippocampus (Spearman Rho $p < 0.001$, $r = -0.47$; Fig. 3B₁) and the CA subfield (Spearman Rho $p < 0.001$, $r = -0.39$; Fig. 3B₂) from the posterior brain sections inversely correlated with their respective Calb1 expressions at P40. There was no correlation between GFAP score and Calb1 expression in the DG of the posterior sections (Fig. 3B₂).

Lower Expression of Hippocampal Calb1 following Neonatal HI and Impaired Memory

The percentage SAP in Y-maze phase 1 was lower in HI-injured males by 40% (H 28.9, df 2, KW $p < 0.001$, adjusted $p < 0.001$) and females by 38% (H 19.3, df 2, KW $p < 0.001$, adjusted $p < 0.001$) when compared to shams (Fig. 5A). Accordingly, the percentage AAR was 2.6-fold higher in HI-injured males (H 22.6; df 2; KW $p < 0.001$; adjusted $p < 0.001$) and 1.9-fold higher in females (H 23.5; df 2; KW $p < 0.001$; adjusted $p = 0.001$) when compared to shams (data not shown). This resulted in 2.2- and 2-fold higher total impairment after HI injury in males and female HI-injured mice, respectively (KW $p < 0.001$ for both; Fig. 5B) versus shams. The time spent exploring the new arm during phase 2 of the Y-maze was approximately 50% less in male (KW $p = 0.002$; adjusted $p = 0.03$) and female (KW $p = 0.02$; adjusted $p = 0.05$) injured mice versus shams (Fig. 5C). TH did not attenuate any of the deficits documented during the Y-maze testing. In general, Calb1 expression in the whole hippocampus

(Fig. 5A₁–C₁), and specifically in the CA field (Fig. 5A₂–C₂) within the posterior coronal brain sections, directly correlated with percentage SAP (Fig. 5A₁, A₂) and time spent exploring the new arm (Fig. 5C₁, C₂) and inversely correlated with the percentage of total impairment (Fig. 5B₁, B₂). However, the link between Calb1 expression and the performance in the Y-maze was more robust in females than in males. In female mice only, Calb1 expression in the CA field of the posterior brain sections directly correlated with the percentage SAP and time spent exploring the new arm (Fig. 5A₂, C₂). There were no significant correlations between hippocampal Calb1 expression in the anterior brain sections and Y-maze performance.

Residual Hippocampal Volumes, Calb1 Expression, and Memory Impairment

Neonatal HI produced a 32% (H 17.5; df 2; KW $p < 0.001$; adjusted $p < 0.001$ vs. sham) and 25% (H 16.7; df 2; KW $p < 0.001$; adjusted $p = 0.006$ vs. sham) decrease in the hippocampal volume at P40 in male and female mice, respectively. TH did not mitigate hippocampal atrophy (adjusted $p < 0.001$ [males] and 0.001 [females] vs. sham; Fig. 6A). Residual hippocampal volumes did not correlate with hippocampal Calb1 mean OD at P40 (Fig. 6B). Nevertheless, residual hippocampal volumes predicted memory outcomes using Y-maze testing. Hippocampal volume was directly correlated with percent SAP in Y-maze phase 1 ($r = 0.45$, $p = 0.02$ [males]; $r = 0.47$, $p = 0.01$ [females]), percentage of time spent exploring the new arm during Y-maze phase 2 ($r = 0.39$, $p = 0.03$ [females]) and was inversely correlated with the percentage of total impairment ($r = -0.42$, $p = 0.03$ [males]; $r = -0.44$, $p = 0.02$ [females]).

Discussion

We show that hippocampal Calb1 expression developmentally increases between P11 and P40, specifically within the CA subfields, but not in the DG in the antero-posterior axis of the brain. Neonatal HI produces delayed and late deficits in Calb1 expression exclusively within the CA field of the hippocampus in the posterior sections, but not in the anterior sections, of the mouse brain. These persistent deficits in hippocampal Calb1 expression after neonatal HI in the posterior sections of the brain are: (i) similar in both sexes, (ii) inversely correlated with the degree of astrogliosis, (iii) not correlated with hippocampal atrophy, and (iv) not attenuated by TH. Although neonatal

Fig. 4. Temporal evolution of GFAP immunoreactivity from P11 to P40 in sham and HI-injured mice treated with normothermia (NT) or therapeutic hypothermia (TH). Representative GFAP IHC of posterior brain sections containing hippocampi of sham, NT, and TH mice. Scale bar, 1,000 μ m. Two photomicrographs

($\times 4$) of the hippocampus (i, ii) within the posterior coronal section, were used to compose the final Figure. White arrows, areas of columnar injury or cellular loss within the CA pyramidal cell layer (Py); white arrowheads, glial scars with the CA Py; black arrowheads, area of diffuse astroglia within the CA Py.

tal HI produced deficits in spatial working memory in the Y-maze in both male and female mice, a direct correlation of deficits in the expression of Calb1 in the CA subfields is only documented in the posterior brain sections and more robustly in the female mice at P40. To our knowledge, this is the first report documenting the persistent deficits in expression of the Ca^{2+} -buffering protein Calb1 after neonatal HI, the lack of response to TH, and a correlation with memory deficits.

The developmental increase in hippocampal expression of Calb1 throughout the anteroposterior axis of the brain was specifically linked to the increase within the CA subfields. Our findings are in agreement with the developmental increase of Calb1 described between P6 and P35 in the rat hippocampus [24], and between P7 and P22 in other regions of the brain [25]. The increase in Calb1, and other CBPs, is mostly linked to the postnatal maturation of interneurons within the CA subfields and of the mossy fiber system between the DG and CA3 [9, 26–31]. Calb1 plays an important role in protecting the brain against stroke, traumatic brain injury [32], neurodegenerative disorders [33–37], and aging [38]. In the developing brain, massive shifts in intracellular Ca^{2+} produced by perinatal brain injury or kainic acid administration induce significant hippocampal injury that is attenuated by the upregulation of Calb1 expression [39]. Similarly, study of the aging human brain suggests that subtle changes in Ca^{2+} homeostasis produced by a decrease in CBPs lead to drastic deficits in cognitive function and increases vulnerability to neurodegeneration [40].

Behavioral, functional, anatomical, and connectivity analysis support the segregation of the hippocampus into anterior, middle, and posterior sections in primates, and dorsal, intermediate, and ventral in rodents [17, 41–45]. While the dorsal hippocampus (positioned anteriorly in rodents and corresponding to the posterior hippocampus in primates) is primarily involved in cognitive function and the retrieval of memories [41, 46, 47], the ventral hippocampus (mostly positioned posteriorly in rodents and corresponding to the anterior hippocampus in primates) is involved in anxiety and emotions [41]. The function of the intermediate hippocampus is not well defined, but it appears to overlap the functions of the dorsal and ventral hippocampus and also plays a role in spatial learning and memory [41, 48].

Identification of the boundaries of the dorsal, intermediate, and ventral hippocampus in coronal sections of the mouse brain, as in our experiments, is challenging. Based on the anatomical limits used to classify the anterior and posterior coronal brain sections that we used (Methods

section: “Terminology”), we consider that our anterior brain sections indisputably contained sections of the dorsal hippocampus, while our posterior brain sections contained a combination of the dorsal and intermediate hippocampus, and ventral hippocampus to a lesser extent, transitioning from the top to bottom of the section, as described by Fanselow and Dong [41]. Here, we show that neonatal HI produces deficits in Calb1 exclusively in the CA subfields of the hippocampus contained in the posterior coronal sections but not in the anterior sections of the brain.

In our model, the CA subfields were more selectively vulnerable to HI injury than the DG [49], but there is no obvious explanation for the differential expression of Calb1 between hippocampal sections closer to the septal pole (anterior brain sections) and those positioned immediately between the septal and temporal poles (posterior brain sections). Our analysis of astrogliosis as a surrogate of injury did not suggest differential injury between the hippocampal CA subfields in the anterior and posterior coronal sections (a GFAP score of 12 and 11, respectively). However, given that the developmental increase in Calb1 in the CA subfields in the anterior brain sections occurred after P18 (Fig. 1) and that in the posterior brain sections occurred before P18, HI injury at P10 most likely altered the maturational mechanisms leading to Calb1 expression occurring closer to the insult, and so predominantly affected the hippocampus in the posterior coronal brain sections.

Thirty days after HI, Calb1 deficit within the CA subfields contained in the posterior brain sections directly correlated with worse spatial memory outcomes. This link appears to be more robust in female mice than in male mice and is not dependent on the degree of hippocampal atrophy resulting from HI injury. Although functionally spatial working memory has been linked to the ventral hippocampus as well [48, 50], the strongest evidence [41] suggests that the dorsal, and likely the intermediate, hippocampus (contained within the posterior coronal brain sections) would have the strongest influence on the Y-maze outcomes. However, the lack of correlation between Calb1 expression in the dorsal hippocampal sections (contained within the anterior brain sections) and the memory outcomes in our experiments was puzzling. We speculate that the limited variability in Calb1 OD due to the lack of effect of the HI insult compared to in sham mice in the most dorsal portion of the hippocampus did not allow for a directional correlation with memory outcomes. Alternatively, the intermediate hippocampus may have a more important role in memory outcomes than previously thought.

Decrease in Calb1- and parvalbumin-expressing interneurons in the CA subfields of the mouse hippocampus 8 days after neonatal HI was reported recently by our group [9]. Calb1-positive interneurons are essential for cognition and memory preservation [51–54]. The GABAergic control of pyramidal cells by hippocampal interneurons allows the preservation of mechanisms of long-term plasticity, a molecular proxy of memory, and so a GABAergic deficit leads to memory impairments [55]. Evidence between changes in Calb1 and memory outcomes has been extensively described in traumatic brain injury [56], hippocampal sclerosis [57], and Alzheimer's disease [58]. Any reduction in Calb1 expression appears to be critical enough to impair the ability of the hippocampus to function normally [59]. Our results led us to speculate that the loss of Calb1

immunoreactivity is, at least in part, linked to the decrease of Calb1-positive interneurons in the postnatal CA subfields contained in the posterior murine brain. To our knowledge, this is the first report providing evidence that, similar to many other neuropsychiatric and neurodegenerative disorders, neonatal HI may impair Calb1 expression, in this case by preventing normal development.

We evaluated astrogliosis using the semiquantitative GFAP-derived scoring system recently developed in our laboratory [9]. The severity of astrogliosis as determined by this scoring system, inversely correlated with Calb1 expression in the CA subfields of the posterior brain sections. We were unable to determine whether persistent astrogliosis led directly to impaired Calb1 expression in the CA subfields by 30 days after HI, but it appears that the severity of HI injury,

Fig. 6. Hippocampal atrophy at P40 resulting from neonatal HI at P10 does not correlate with Calb1 deficits. **A** Box and whiskers plots represent the residual hippocampal volumes in mm³ for sham, normothermia (NT), and therapeutic hypothermia (TH). Boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Analysis

by Kruskal-Wallis ANOVA with Dunn-Bonferroni correction; * adjusted (Adj) $p < 0.05$. **B** Calb1 expression in the whole hippocampus correlated with residual hippocampal volumes in males and female mice. Spearman correlations were applied. Continuous line represents the predicted linear regression and discontinuous lines represent the projection to the 95% confidence interval.

Fig. 5. Memory deficits in Y-maze task correlated with Calb1 deficits in the hippocampus of posterior coronal sections following HI at P40. Box and whiskers plots represent percentage of spontaneous alternation performance (SAP; **A**), impairment (sum of alternate arm returns and same arm returns; **B**), and time spend in new arm during Y-maze phase 2 (**C**) stratified by sex. Boxes are limited by the 75th and 25th percentiles (IQR) and whiskers are limited by the last data point within 1.5 times the IQR from the median (continuous line inside the box). Analysis by Kruskal-Wallis ANOVA

with Dunn-Bonferroni correction; * adjusted (Adj) $p < 0.05$. Calb1 expression in the whole hippocampus (**A1–C1**) and CA field (**A2–C2**) in posterior coronal sections correlated with % SAP (**A1, A2**), % impairment (**B1, B2**), and % time in new arm (**C1, C2**) stratified by sex (i, males; ii, females). Spearman correlations were applied. The continuous line represents the predicted linear regression and discontinuous lines represent the projection to the 95% confidence interval. CA, cornus ammonis; ns, nonsignificant; NT, normothermia; TH, therapeutic hypothermia.

as suggested by the degree of hippocampal atrophy, does not fully explain the Calb1 deficits. Although associations between Calb1 loss and astrogliosis in the hippocampus have been previously described following kainic acid injury [60], ethanol exposure [61], viral infections [62], and even exposure to radiofrequency [63], the causal mechanism for these 2 events is still missing and further study is required.

We acknowledge several limitations to our study. We evaluated the hippocampal sections contained within the anterior and posterior coronal sections of the mouse brain. This enabled us to study the dorsal hippocampus closest to the septal pole, but not the intermediate or ventral hippocampus individually, as the hippocampal sections contained in the posterior coronal section are a combination of all of these (as previously published [41]). Thus, the role of the isolated intermediate hippocampus in memory outcomes requires further investigation in association with Calb1 deficits. Additionally, our neonatal HI model produced unilateral injury, and thus compensation from the contralateral (hypoxia-exposed) hemisphere may have attenuated the behavioral outcomes. Despite these limitations, our laboratory has extensive experience in behavioral testing using this mouse model and has obtained consistent results demonstrating impaired memory outcomes (using Y-maze testing) and a lack of response to TH [18, 19]. We did not analyze the electrophysiological properties of Calb1-positive interneurons within the CA subfields to assess function and alterations in the mechanisms of long-term synaptic plasticity to link Calb1 deficit to memory deficits. Lastly, we acknowledge that we only included mice without an obliterated hippocampus due to technical limitations when processing the samples and because HI injury almost fully eliminated the CA subfields from the analysis.

In conclusion, neonatal HI produces a long-lasting impairment in the increase of Calb1 in the CA subfields of the hippocampus during a critical stage of development, and TH does not attenuate the deficit. Late Calb1 deficit correlates with prolonged astrogliosis. Further studies are needed to understand the mechanistic link between deficits in spatial memory and Calb1 expression and the functional role of the intermediate rodent hippocampus.

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Statement of Ethics

The experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine and carried out with standards of care and housing in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services 85–23, 2011.

Disclosure Statement

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Author Contributions

J.G.-H. and R.C.-V.: experimental design, imaging and analysis, and initial draft preparation; J.G.-H., D.S., and J.D.: behavioral testing; J.G.-H. and D.F.: immunohistochemistry; R.C.-V.: statistical analysis; and all authors: critical reviews of the manuscript and approval of the final version.

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