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# Effects of Neonatal Hypoxic-Ischemic Injury and Hypothermic Neuroprotection on Neural Progenitor Cells in the Mouse Hippocampus

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**Key Words**
Hypoxic-ischemic injury · Neurogenesis · Hypothermia · Neural stem cell

**Abstract**
Neonatal hypoxic-ischemic injury (HI) results in widespread cerebral encephalopathy and affects structures that are essential for neurocognitive function, such as the hippocampus. The dentate gyrus contains a reservoir of neural stem and progenitor cells (NSPCs) that are critical for postnatal development and normal adult function of the hippocampus, and may also facilitate the recovery of function after injury. Using a neonatal mouse model of mild-to-moderate HI and immunohistochemical analysis of NSPC development markers, we asked whether these cells are vulnerable to HI and how they respond to both injury and hypothermic therapy. We found that cleaved caspase-3 labeling in the subgranular zone, where NSPCs are located, is increased by more than 30-fold after HI. The population of cells positive for both proliferating cell nuclear antigen and nestin (PCNA+Nes+), which represent primarily actively proliferating NSPCs, are acutely decreased by 68% after HI. The NSPC population expressing NeuroD1, a marker for NSPCs transitioning to become fate-committed neural progenitors, was decreased by 47%. One week after HI, there was a decrease in neuroblasts and immature neurons in the dentate gyrus, as measured by doublecortin (DCX) immunolabeling, and at the same time PCNA+Nes+ cell density was increased by 71%. NSPCs expressing Tbr2, which identifies a highly proliferative intermediate neural progenitor population, increased by 107%. Hypothermia treatment after HI partially rescues both the acute decrease in PCNA+Nes+ cell density at 1 day after injury and the chronic loss of DCX immunoreactivity and reduction in NeuroD1 cell density measured at 1 week after injury. Thus, we conclude that HI causes an acute loss of dentate gyrus NSPCs, and that hypothermia partially protects NSPCs from HI. 

## Introduction

Nearly a million newborns per year are afflicted with neonatal hypoxic-ischemic encephalopathy, a devastating multifactorial disorder that occurs at a frequency of approximately twice in every thousand live births [1, 2], and more than half of surviving infants manifest clini-

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cally apparent brain damage. In moderate encephalopathy a third of children are cognitively impaired, and in severe cases mental retardation is almost universal [3, 4]. These deficits occur because the developing forebrain is very sensitive to hypoxic-ischemic injury (HI) [5]. Treatment options for neonatal HI are extremely limited, and hypothermia is the only widely used and well-validated therapy that is effective in reducing rates of permanent neurocognitive disability [6–8]. Since the fundamental pathophysiology of neonatal HI involves massive neuronal death [9], one promising pathway to substantial recovery is through repopulation of lost cells by enhancement of neural stem cell activity.

Importantly, cell loss in the brain can stimulate proliferation of neural stem and progenitor cells (NSPCs) that contribute to some degree of regenerative repair [10]. This phenomenon has been described in adult animals in the context of stroke and traumatic brain injury [11, 12]. However, neonatal HI differs substantially from adult injury as it occurs in the setting of high levels of NSPC proliferation that are a component of normal early postnatal brain development. It has been shown that neonatal HI stimulates expansion of NSPCs in the subventricular zone (SVZ) [13] that can repopulate both the neocortex [14] and striatum [15]. While hypoxic injury may stimulate neurogenesis, it may also inhibit neural regeneration by eliminating NSPCs or by reducing rates of proliferation. Studies of the SVZ in neonatal rodents show that HI causes loss of NSPCs via mechanisms of apoptotic cell death [16–18], suggesting that neuroprotection for NSPCs could be an important means of promoting regeneration and repair.

It is unclear how hypothermia, the mainstay of clinical therapy for neonatal HI, affects NSPCs in the context of HI. Potentially informative studies of related stem cell populations give mixed results. A study of a preterm sheep model of HI showed that oligodendrocyte precursor cell proliferation in the periventricular white matter is unaffected by hypothermia [19], whereas in a rat model in the corpus callosum hypothermia was shown to enhance oligodendrocyte precursor cell proliferation and maturation. Interestingly, in the fetal sheep HI model, hypothermia did not increase or decrease expression of Ki-67, a nonspecific marker for proliferation, in the SVZ, where cells with potential for both neuronal and glial fates are actively dividing [20]. In the absence of injury hypothermia can actually suppresses NSPC proliferation in the hippocampus [21], suggesting that there is the potential for an adverse effect of this therapeutic approach.

The hippocampal dentate gyrus also has the capacity to generate new neurons that incorporate into functional brain circuits [22, 23]. While neurogenesis in the dentate gyrus persists throughout adulthood, proliferation rates peak in early postnatal life while this structure is still developing [22]. Proliferative cells in the dentate gyrus with the potential to generate newborn neurons reside in the subgranular zone (SGZ), and they appear to have characteristics of embryonic radial glia [24, 25]. Neurogenesis in the SGZ appears to consist of multiple stages. The first stage consists of proliferation of uncommitted NSPCs and the second involves differentiation of NSPCs to neuronal fate-committed intermediate neural progenitors, which in turn give rise to neuroblasts and then immature neurons [26, 27]. At the end of the neuroblast stage, these cells migrate into the granular layer of the hippocampus where they incorporate into networks as they continue to differentiate and grow [28]. The dentate gyrus is critical for learning and memory functions [29], and hippocampal-dependent memory formation is mediated in part by ongoing neurogenesis in this region [30]. Thus, it is of great interest to examine the process of injury and repair in the dentate gyrus SGZ after neonatal HI, as NSPCs residing there contribute both to structural development and normal function during the period of injury and recovery.

Here, using a mouse model and immunohistochemical analysis of neural precursor markers, we ask whether the NSPCs of the dentate gyrus SGZ are acutely vulnerable in neonatal HI and whether neurogenesis is enhanced in response to this injury. We also test whether therapeutic hypothermia in HI mice functions as a neuroprotectant for NSPCs acutely and assess the effects of hypothermia on neurogenesis in the dentate gyrus during recovery.

## Methods

*Animals*

All procedures relating to animal care and treatment conformed to Johns Hopkins Medical Institute Animal Care and Use Committee guidelines and the standards set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Early postnatal C57BL/6 WT male mice used for these experiments were obtained from Charles River Co. or bred in-house. Littermates were divided randomly into study groups on postnatal day 10 (P10), and at least two litters are represented in each experimental condition.

*HI Model*

The HI group was induced in mice using the murine adaptation of the well-validated Vannucci procedure [31]. Briefly, the right common carotid artery was permanently ligated using a 5.0 silk suture under isoflurane anesthesia (3.0% for induction and 1.5%

for maintenance during surgery) to induce ischemic injury. After 1 h of recovery time with the dam, animals were exposed to a hypoxic mixture of humidified 8% oxygen/balance nitrogen for 45 min in an airtight chamber resting in a water bath held at 37°C. In experiments employing hypothermic neuroprotection after HI (HH group) animals were transferred to a room-air chamber in a water bath held at 28.5°C for 4 h, whereas HI animals were moved to a room-air chamber in a 37°C water bath for an equivalent time. In conjunction with HH experiments, one group of animals received hypothermia without artery ligation or hypoxia (HT group). All pups were returned to their dams until sacrifice, which occurred at 24 h or 7 days. Naïve controls (Con group) were littermates that remained with the dam and did not undergo any experimental manipulation.

*Tissue Processing and Immunohistochemistry*

Animals were anesthetized with high-dose isoflurane and sacrificed by intracardiac perfusion with cold 0.9% saline (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Whole brains were dissected out and postfixed with 4% PFA/PBS overnight and transferred to a cryoprotection solution of 30% sucrose for at least 3 days. Brains were embedded in Tissue-Tek OCT matrix (Sakura). Serial coronal sections of 30 μm thickness of the hippocampus ipsilateral to carotid artery occlusion were prepared from the hippocampus using a cryostat at –20°C and collected on slides. Our intent was to study mild-to-moderate HI, and thus we excluded from the study any brains with severe tissue damage and from which high-quality sections could not be prepared. Specifically, in the P11 cohort 1 of 11 brains were excluded from the HI group, and in the P18 cohort 3 of 14 from the HI group and 1 of 9 from the HH group were excluded. The tissue sections were subjected to heat-induced antigen retrieval using Dako antigen retrieval solution (Agilent) for 30 min at 95°C. After TBS/0.05% Triton X-100 washes, sections were incubated with primary antibodies in 3% donkey serum/0.05% Triton X-100 at 4°C overnight. Primary antibodies for this study included: (1) nestin (chicken polyclonal, Aves, 1:500); (2) proliferating cell nuclear antigen (PCNA; goat polyclonal, Santa Cruz, 1:100); (3) cleaved caspase3 (cl-Casp3; rabbit polyclonal, Cell Signaling, 1:300); (4) doublecortin (DCX; goat polyclonal, Santa Cruz, 1:250); (5) neuronal nuclei (NeuN; guinea-pig polyclonal, Millipore, 1:500); (6) neurogenic differentiation 1 (NeuroD1; goat polyclonal, Santa Cruz, 1:25); (7) T-box brain gene 2 (Tbr2; rabbit polyclonal, Abcam, 1:200), and (8) sex-determining region Y box 2 (Sox2; goat polyclonal, Santa Cruz, 1:300). Following TBS/0.05% Triton X-100 washes, sections were incubated with secondary antibodies for 2 h at room temperature. Secondary antibodies included: (1) donkey anti-chicken FITC; (2) donkey anti-goat Cy3; (3) donkey anti-rabbit Cy5; (4) donkey anti-guinea pig Alexa 594, and (5) donkey anti-goat Alexa 488 (all obtained from Jackson ImmunoResearch and diluted 1:250). Sections were again washed in TBS/0.05% Triton X-100 and mounted in 2.5% polyvinyl alcohol mounting media with DABCO (Sigma). Negative controls consisting of secondary antibody only were used for each antibody to confirm appropriate labeling and imaging conditions. Antibody labeling for groups was done simultaneously in all but 2 cases, and in these cases control groups were compared between immunohistochemistry runs and found not to be significantly different (t test showed P11 nestin and PCNA, $p > 0.99$, and P11 PCNA, $p = 0.27$) indicating consistency of labeling between groups.

*Microscopy*

A minimum of 4 and a maximum of 6 animals were used per condition, and exact numbers per group and condition are given in the figure legends. For each condition one slide representing posterior and one representing anterior hippocampus was selected from each animal, with the anatomical level determined by morphology (posterior sections drawn approx. from –2.5 to –3.25 mm from the Bregma show the extension of the hippocampus to the ventral surface of the brain, anterior sections drawn approximately from –1.25 to –2.0 mm from the Bregma do not show extension of the hippocampus to the ventral surface). A minimum of 3 and maximum of 6 sections were selected from each slide depending on the number of sections per slide that exhibited perfectly definable anatomical features of the hippocampus. Consecutive sections were not analyzed, thus, in a slide with 12 sections 6 was the maximum used.

Microscopic images were collected by an investigator blinded to the condition, and each set of experiments was imaged by a single individual blind to the experimental condition to ensure consistency and to minimize bias. Images of one blade of the dentate gyrus per section were taken on a Leica TCS confocal using a 20 × 0.7 numerical aperture objective. The pinhole size was set at 1 Airy unit, and a 1.5-zoom factor was employed. Solid state 488-, 532- and 635-nm lasers were used at a consistent intensity for all images taken. Photo detection windows remained consistent throughout, no averaging functions were used, and gain was generally consistent with only minor changes (<5%) to account for differences in labeling intensity as no measures of fluorescent intensity were used in this study. Stacks of 3 images were taken over a z-distance of 2 μm, which was shown in pilot studies to be effective in capturing both soma and associated neurites in the dentate gyrus. Stacks were collapsed into single-plane and TIF files, and exported for analysis.

*Data Analysis and Presentation*

All offline analysis was performed by an investigator blinded to the condition. The SGZ was defined either as a 50-μm window centered on the intersection of the maximal PCNA and nestin labeling or as a 50-μm window below the granular cell layer as assessed by NeuN labeling or brightfield imaging. Pilot testing showed no difference between these criteria in control or HI samples either in terms of cell counts or in terms of the area defined. Cells positive for PCNA and nestin (PCNA+Nes+) were defined as substantial colocalization of the two markers or the clear emergence of a nestin process from a PCNA+ cell body with minor colocalization. Measurements of area were performed by an investigator blinded to the condition using Metamorph software (Molecular Software). Images were separated into color components and thresholded. cl-Casp3 and NeuN labeling was expressed as a percentage area of immunoreactivity divided by the total area of the dentate gyrus visible in the slide. For analysis of DCX, 3 square sampling areas were placed equidistantly in the molecular layer (ML) and another 3 in the granular layer as defined by NeuN staining. Thresholding was performed and DCX labeling was expressed as a percentage of the total sample area.

Raw data from all image analysis was collected in a spreadsheet, and after unblinding the data were imported into Prism software (GraphPad) for statistical analysis and figure preparation. With the exception of the control dataset for cl-Casp3 analysis, skewness was within ±1 and kurtosis within ±3 for all datasets, indicating no

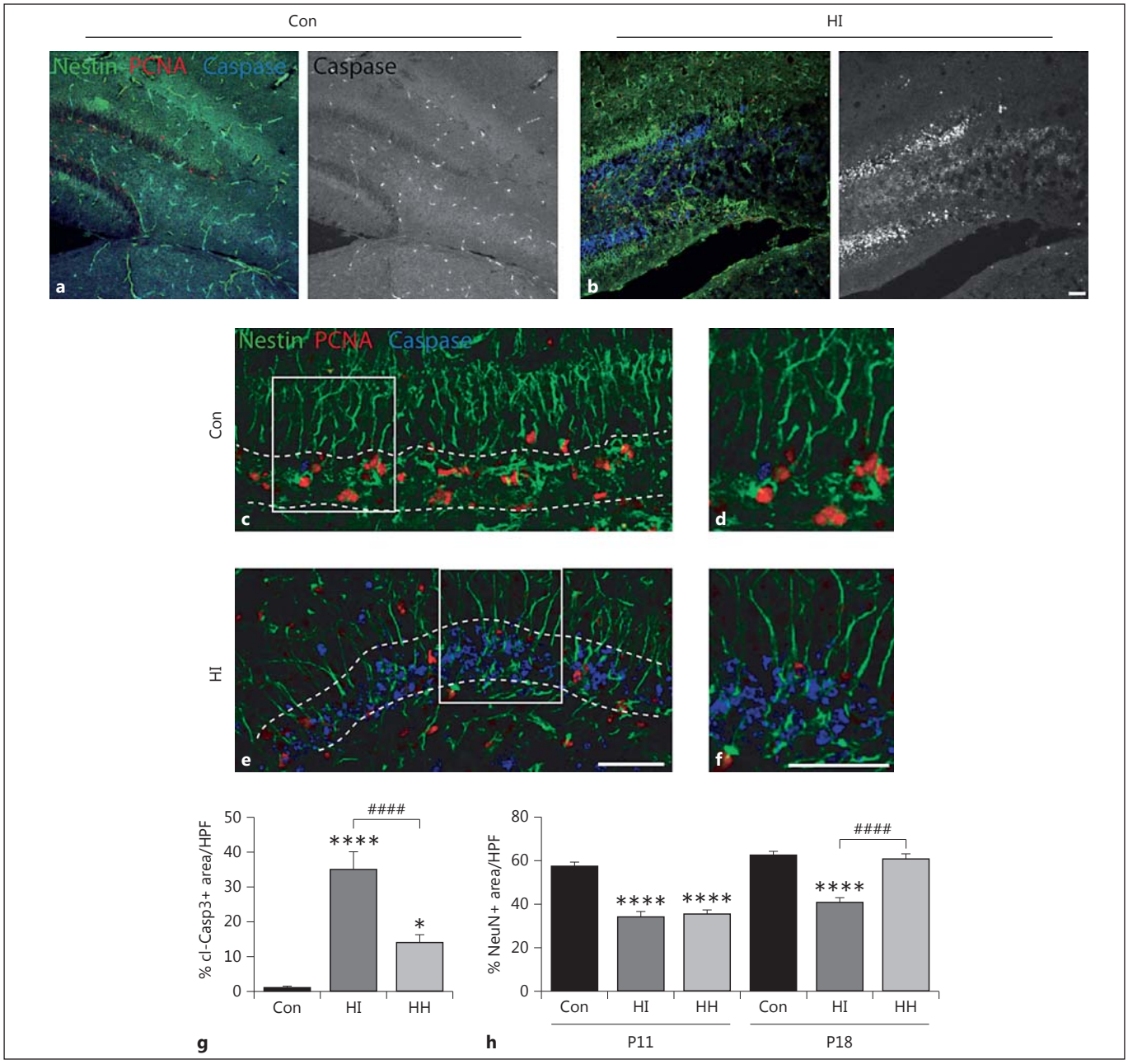


**Fig. 1.** HI causes an acute increase in apoptosis and a sustained loss of neurons in the dentate gyrus. Low-power examples of control (**a**) and the HI hippocampus (**b**) are shown with fluorescence immunolabeling for PCNA (red), nestin (green) and cl-Casp3 (blue) in the left panel, with a monochrome representation of cl-Casp3 separately in the right panel. There is little cl-Casp3 immunolabeling in control conditions, whereas with HI there is an increase in immunolabeling, particularly in the dentate gyrus. The dentate gyrus alone is shown at higher power with a line delineating the SGZ (dotted line) for control (**c**) and HI (**e**) conditions. There is very little labeling for cl-Casp3 (blue) apparent in control conditions (zoom of the solid-line box in **c** shown in **d**), and very substantial labeling with HI (zoom of the solid-line box in **e** shown in **f**). A comparison of the mean percentage of the cl-Casp3-positive area within the SGZ at 24 h after injury (P11) shows a significant increase with HI alone (HI) and with HI in conjunction with hypothermia (HH), and a significant reduction in the cl-Casp3 area in HH compared to HI alone (**g**). There is a significant decrease in the percent area of labeling for the mature neuron marker NeuN in the dentate gyrus at 24 h (P11) after HI, and this decrease can still be observed 1 week (P18) after injury (**h**). Interestingly, there is no significant difference between HH and HI at 24 h, but at 1 week HH is significantly increased compared to HI and not significantly different from the control (**h**). **g** n = 108 sections, 14 animals (5 Con, 4 HI, 5 HH). **h** n = 420 sections, 29 animals (P11: 5 Con, 4 HI, 6 HH; P18: 5 Con, 4 HI, 5 HH). Scale bar = 50 μm (**b**, **e**, **f**). Error bars represent SEM. * $p < 0.05$ and **** $p < 0.0001$ compared to Con; #### $p < 0.0001$ compared to HI. HPF = High-power field.

substantial departure from normality. The minimum criterion for statistical significance was set a priori at $p < 0.05$, and all results discussed as differences meet this criterion. ANOVA was used for comparisons, and post hoc intergroup comparison was accomplished with a Holm-Sidak test. In the case of the cl-Casp3 analysis, significance was confirmed with a Kruskal-Wallis test, which showed $p < 0.001$. Graphical representations of the data were created in Prism. Bars represent group means and error bars denote the standard error of the mean (SEM). Significance is denoted graphically for comparison to the Con group (*) or the HI group ($^{\#}$), according to the following scheme: * or $^{\#}$ $p < 0.05$, ** or $^{\#\#}$ $p < 0.01$, *** or $^{\#\#\#}$ $p < 0.001$, **** or $^{\#\#\#\#}$ $p < 0.0001$. Representative images for display were collected and underwent postprocessing in Photoshop software (Adobe).

## Results

We first asked whether the cells of the dentate gyrus SGZ exhibit substantial cell death due to HI in the neonatal mouse. Because much of the cell death observed in HI occurs via apoptotic pathways [32], we used fluorescence immunohistochemistry to compare cl-Casp3 labeling in the control and HI groups at 24 h after injury. Labeling for cl-Casp3 was broadly increased throughout the hippocampus in the HI group as compared to controls, and in particular there was an increase in immunolabeling in the dentate gyrus (fig. 1a, b), which is concentrated in the SGZ (fig. 1c–f). Quantitative analysis presented in figure 1g showed a significant increase in cl-Casp3 labeling in the HI group compared to controls ($p < 0.0001$) and a partial rescue by hypothermia (significantly decreased compared to HI, $p < 0.0001$, and significantly increased compared to controls, $p < 0.05$). Taken together, these data suggest that HI causes cell death in the SGZ, which can be mitigated by hypothermia.

Next we investigated whether HI causes neuronal cell loss in the more mature cells of the dentate gyrus, in order to determine whether the generation of new neurons would be necessary for repair of this structure. Using quantitative fluorescence immunohistochemistry with the mature neuron marker NeuN, we observed a significant reduction ($p < 0.001$) in the area of labeling at 24 h after injury (fig. 1h), with no rescue by HH. This loss was sustained 1 week after HI ($p < 0.001$), but there was a significant rescue effect with HH at this age (significantly increased compared to HI, $p < 0.001$, no difference from controls; fig. 1h). Thus, hypothermia exerts a protective effect in the granule cell layer (GCL) that is not present immediately but evolves over time.

To test the effects of HI on neurogenesis in the SGZ, we examined the populations of NSPCs. PCNA identifies actively proliferating cells, but might include some dividing cells that are not part of the neurogenesis stem cell pool such as invading microglia, reactive glia or hematologic cells [27]. PCNA+Nes+ cells are more specific for the pool of NSPCs, but may include cells with both neuronal and glial fate [27]. At 1 day after injury, compared to naïve controls (fig. 2a), we found a significant reduction in both PCNA+ and PCNA+Nes+ cells in the HI group ($p < 0.0001$; fig. 2b). Interestingly, hypothermic treatment after HI (the HH group) showed a significant reduction in both PCNA+ and PCNA+Nes+ cells ($p < 0.0001$) relative to the control group, but a significant increase over the HI group ($p < 0.0001$), suggesting a partial rescue (fig. 2c). Hypothermia alone in the absence of HI (the HT group) appeared to have no substantial effects relative to the controls. These results are summarized graphically in figure 2d and e.

To further understand the effects of HI and hypothermia, we examined the expression of three well-characterized markers along the spectrum of early NSPC development at 1 day after injury. These markers included: (1) Sox2, which is highly expressed in the quiescent NSPCs known as type 1; (2) Tbr2, which is strongly expressed in the rapidly proliferating intermediate precursors known

**Fig. 2.** The population of NSPCs in the dentate gyrus SGZ is acutely decreased by HI, but this decrease is attenuated by treatment with hypothermia. Representative examples of immunofluorescence labeling for PCNA (red) and nestin (green) in the SGZ shows that, compared to the control (**a**), there is a significant reduction in PCNA+ and PCNA+Nes+ cell numbers with HI (**b**) at 24 h after injury. The HH group (**c**) shows significant reduction in PCNA+ and PCNA+Nes+ cells compared to the control and a significant increase compared to HI. White arrows indicate PCNA+Nes+ cells, and the white arrowhead denotes the relatively infrequent cells that expressed PCNA but did not have colocalizing or apposing nestin immunoreactivity. Results are presented graphically for PCNA+ (**d**) and PCNA+Nes+ (**e**), and include a bar for a hypothermia-alone control (HT) that was not significantly different from naïve controls. There was no significant difference between groups for either Sox2 (**f–h**, **o**) or Tbr2 (**i–k**, **p**) cell numbers. In contrast, compared to the control (**l**), there was a significant decrease in NeuroD1 cell numbers in HI (**m**). For the HH group (**n**) NeuroD1 cell numbers were not significantly different from the control, but were significantly increased compared to HI. **q** Results for NeuroD1 are summarized graphically. **d**, **e** n = 142 sections, 20 animals (5 each in Con, HT, HI, HH). **o–q** n = 100 sections, 20 animals (5 each in Con, HT, HI, HH). **c**, **n** Scale bar = 20 μm. Error bars represent SEM. **** $p < 0.0001$ compared to Con; $^{\#\#\#\#}$ $p < 0.0001$ compared to HI. HPF = High-power field.

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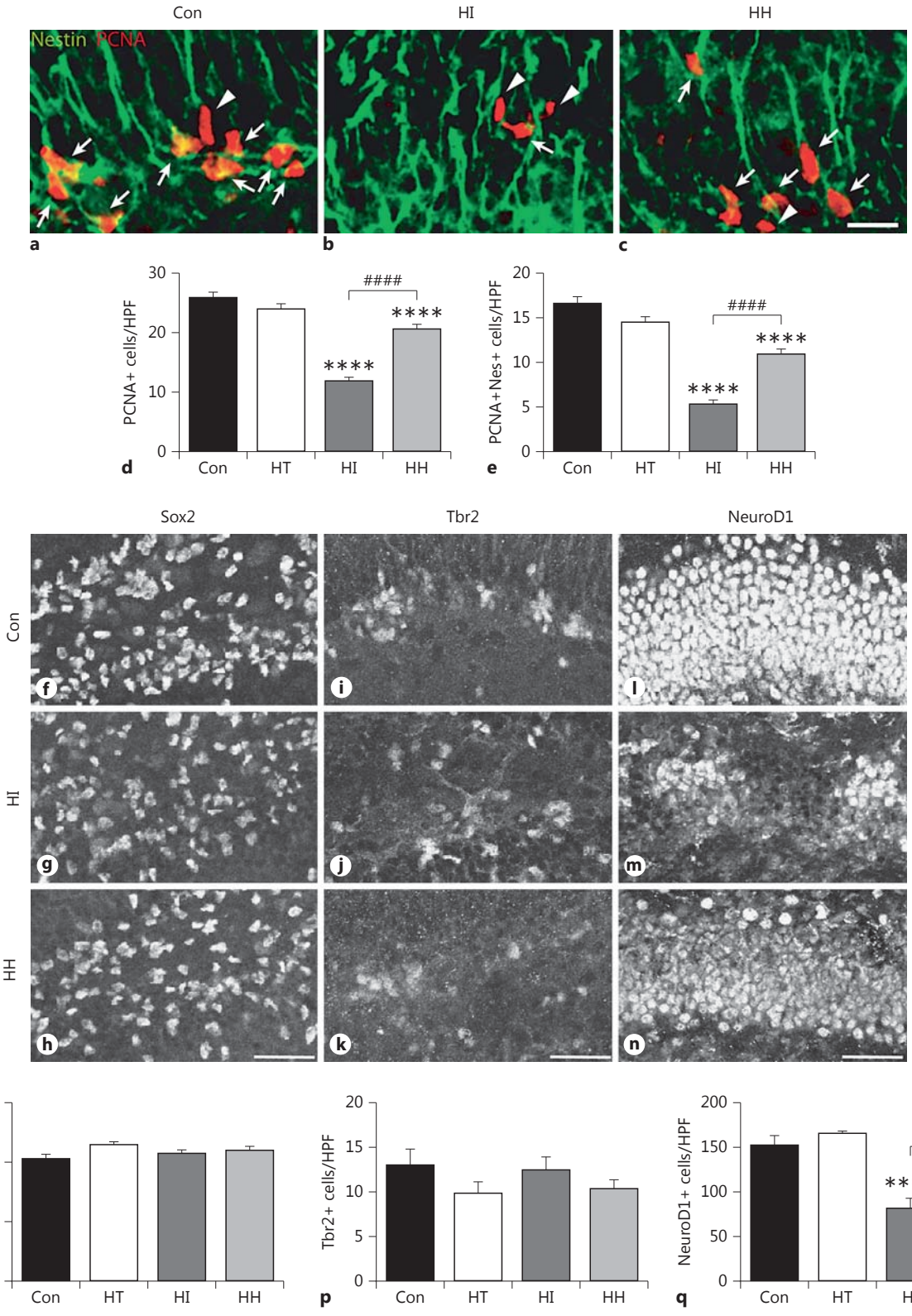


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as type 2, and (3) NeuroD1, which is expressed in cells transitioning to neuroblasts known as type 3 [27]. There is very substantial overlap between all of these markers, and thus they must be considered as a group. There was no significant change in Sox2+ cell numbers (fig. 2f–h, o) and no significant change in Tbr2+ cell numbers (fig. 2i–k, p). Compared to the controls, there was a significant decrease in NeuroD1 cell numbers for HI ($p < 0.0001$), and in HH there was no significant difference from the controls, which represented a significant increase compared to HI ($p < 0.0001$; fig. 2l–n, q). Taken together, these data indicate that the acute damage of HI occurs primarily in stage 3 NSPCs, and that this population of cells can be partially protected with hypothermic therapy.

We then asked whether the acute reduction in the SGZ NSPCs after HI translated into a persistent loss of newborn neurons that could account for the observed loss of postmitotic neurons in the dentate gyrus. To this end, 1

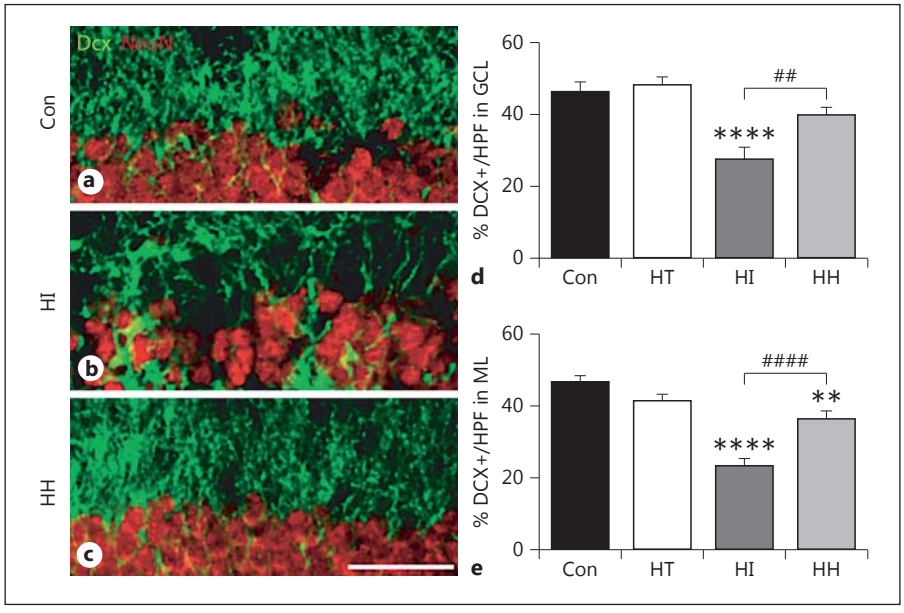


**Fig. 3.** The population of neuronal precursor cells in the dentate gyrus is reduced during recovery from HI, but partially rescued by hypothermic treatment. Immunofluorescence labeling of DCX (green) is shown in the dentate gyrus (NeuN in red defines the GCL and the ML is above). Compared to the control condition (**a**), the density of DCX processes is significantly reduced by HI (**b**), and in the HH group there is a significant reduction in the ML but not the GCL. In the HH group there is a significant increase in the percent area of labeling compared to HI in both GCL and ML. The results are presented graphically for both GCL (**d**) and ML (**e**). n = 285 sections, 19 animals (5 Con, 5 HT, 4 HI, 5 HH). **c** Scale bar = 50 μm. Error bars represent SEM. ** $p < 0.01$, **** $p < 0.0001$ compared to Con; ## $p < 0.01$, #### $p < 0.0001$ compared to HI. HPF = High-power field.

**Fig. 4.** The proliferation of neural stem/progenitor cells is stimulated during recovery from HI and further enhanced by hypothermic therapy. Representative examples of immunofluorescence labeling for PCNA (red) and nestin (green) in the SGZ shows that compared to the control (**a**) there is a significant increase in both PCNA+ and PCNA+Nes+ cell numbers in HI (**b**) at 1 week after injury. In HH (**c**) there is a significant increase compared to the control and also a significant increase compared to the HI group in both PCNA+ and PCNA+Nes+ cells. These results are summarized graphically for PCNA+ alone (**d**) and PCNA+Nes+ (**e**), along with a bar representing a hypothermia-alone condition (HT), which is not significantly different from naïve controls. There is no significant difference between groups for Sox2 (**f–h**, **o**) cell numbers. Compared to the control, there is a significant increase in Tbr2 cell numbers in both the HI and HH conditions (**i–k**, **p**). There is a significant decrease in NeuroD1-positive cells in HI compared to controls, but there is no significant difference in NeuroD1-positive cells between the HH and control condition. A comparison between HI and HH shows a significant increase in NeuroD1-positive cells in the HH group (**l–n**, **q**). **d**, **e** n = 102 sections, 20 animals for (5 each in Con, HT, HI, HH). **o** n = 20 animals, 105 sections (5 each in Con, HT, HI, HH). **p**, **q** n = 22 animals, 100 sections (5 Con, 5 HT, 6 HI, 6 HH). **c**, **h**, **k**, **n** Scale bar = 20 μm. Error bars represent SEM. *** $p < 0.001$, **** $p < 0.0001$ compared to Con; # $p < 0.05$, ### $p < 0.001$ compared to HI. HPF = High-power field.

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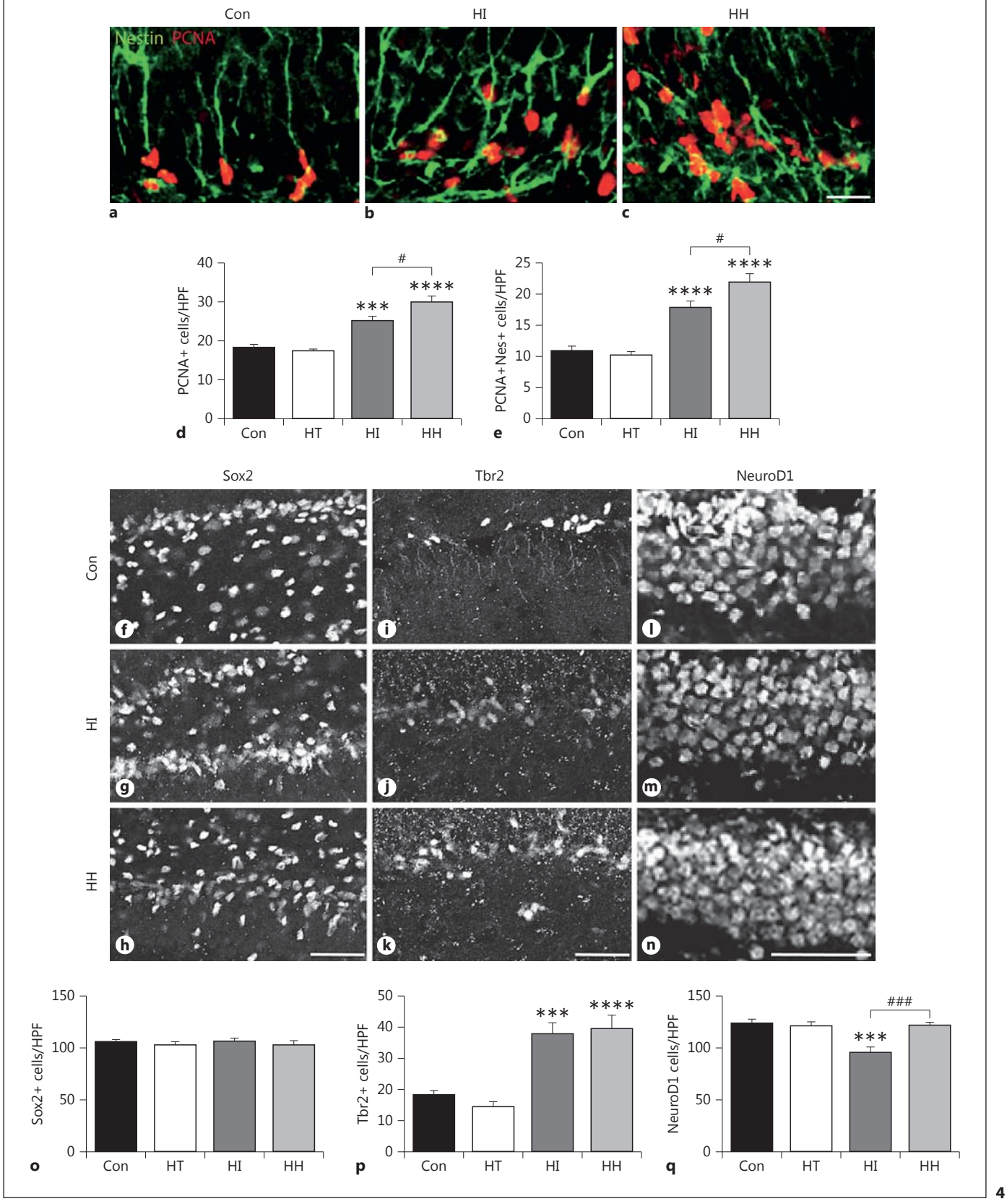


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week after injury we examined labeling for DCX, a neurite marker primarily associated migrating neuroblasts and immature neurons in the dentate gyrus [27]. The density of DCX labeling was quantified both in the GCL, where it should arise more from migrating neuroblasts, and in the ML, where it should arise more from immature neurons that have recently reached their destinations. The results were found to be reminiscent of the pattern seen in early-stage NSPCs at 24 h (fig. 2). Compared to the controls (fig. 3a) the labeling for DCX was significantly decreased in the HI group ($p < 0.0001$; fig. 3b) and rescued in the HH group (fig. 3c). In the HH group there were some anatomical distinctions: in the dentate GCL there was no significant difference from the control and there was a significant increase compared to HI ($p < 0.01$), whereas in the ML there was a significant decrease relative to the controls ($p < 0.01$) and a significant increase compared to HI ($p < 0.0001$). These results, presented graphically in figure 3d and e, indicate that the loss of earlier-stage NSPCs translates into a continued loss in neuroblasts and immature neurons, which can be rescued with hypothermic therapy.

While NSPCs can replenish their numbers through division to a point, injuries that ablate substantial populations can exhibit reduced neurogenesis. To assess whether the reduction in NSPCs seen in HI is likely to result in a persistent reduction in neurogenesis, we assayed for nestin and PCNA in the SGZ 1 week after HI, which falls during the period of recovery and regeneration. The results differed strikingly from what was seen at 1 day postinjury. Relative to naïve controls (fig. 4a) a significant increase in PCNA+ ($p < 0.001$) and PCNA+Nes+ ($p < 0.0001$) cells was seen in the HI group (fig. 4b). Interestingly, PCNA+ and PCNA+Nes+ cell numbers were significantly higher in the HH group (fig. 4c) compared to both the control ($p < 0.0001$) and the HI groups ($p < 0.05$). The results are summarized graphically in figure 4d and e. This finding suggests that HI leads to enhanced neurogenesis that could have the potential to promote repair after injury, and that hypothermia may act by preserving some NSPCs.

To better identify what subtypes of NSPCs are affected 1 week after HI, we assayed for Sox2, Tbr2 and NeuroD1. We found that Sox2 was not significantly altered by HI or HH (fig. 4f–h, o). Interestingly, Tbr2-positive cells were significantly increased in both HI and HH ($p < 0.001$ and $p < 0.0001$, respectively; fig. 4i–k, p), although there was no significant difference between these conditions. By contrast, NeuroD1 cell counts were significantly reduced in HI ($p < 0.001$), whereas in HH NeuroD1 cell numbers were not different from the controls, which represents a significant increase compared to HI ($p < 0.001$; fig. 4l–n, q). Taken together, these findings suggest a marked increase in rapidly proliferating stage 2 NSPCs is caused by HI. Furthermore, it suggests that the increased number of PCNA+Nes+ cells seen in HH is due to a reduced loss of stage 3 transitioning neuroblasts, rather than to a further increase in rapidly proliferating stage 2 cells.

## Discussion

In this study we examined the effects of HI on neurogenesis in the dentate gyrus of the hippocampus of neonatal mice. We found that NSPCs are vulnerable to HI, and that the loss of NSPCs correlated with a later reduction in immature neurons. A subsequent stimulation of neurogenesis occurs in the recovery period 1 week after HI, which is most evident in an increase in the rapidly proliferating population. Hypothermia provides partial protection for NSPCs that are vulnerable to HI and does not impair proliferation of NSPCs during recovery.

In our initial examination of the effects of HI on the dentate gyrus (fig. 1b), we noted widespread immunolabeling with cl-Casp3 throughout the structure, suggesting that numerous cells are vulnerable to apoptotic death as a result of this insult. While we did not conduct an analysis in which cell type-specific markers were colabeled with cell death markers, we can make some inferences from our data as to which cell types are likely to be vulnerable to HI. We see a reduction in NeuN labeling in the dentate gyrus granule layer (fig. 1h), which is consistent with previous reports showing that mature dentate gyrus granular neurons are vulnerable to apoptotic cell death induced by HI [33]. Furthermore, we observed an acute reduction in cells that were positive for PCNA, PCNA and nestin, and NeuroD1 (fig. 2d, e, q). We saw no significant change in Sox2- or Tbr2-positive neurons (fig. 2o, p). Studies of GFP expression under a nestin promoter in transgenic mice suggest that nestin is broadly expressed in type 1 and 2 NSPCs, but is absent from type 3 cells [34]. Sox2 is largely restricted to the types 1 and 2b [35], and Tbr2 is strongly expressed in type 2b NSPCs, but is also substantially expressed in type 2a NSPCs [36]. NeuroD1 has its highest expression in type 2b and type 3 cells (Nes–DCX+), and colocalizes to a modest degree with the cell division marker Ki-67, which is analogous to the PCNA marker used here, and is only rarely coexpressed with Sox2 [37]. Additionally, there is a population of NSPCs committed to astrocytic rather than neuronal fate that

may continue to coexpress nestin and a proliferative marker [38]. Our findings suggest that mature neurons and type 3 NSPCs are the most vulnerable to apoptotic cell death induced by HI (NeuN and NeuroD1). Also, although we did not observe a significant change in Tbr2, we speculate that type 2b neurons might also be vulnerable given that NeuroD1 is expressed in this population and the robust change in nestin- and PCNA-positive cells that was observed suggests an effect on a population of cells at an early stage than the neuroblast transition. Alternately, we speculate that the earliest stage 3 cells may still express nestin and PCNA. Additionally, the reduction in the nestin- and PCNA-positive population may reflect differentiating fate-committed astrocytes that continue to express these markers. Thus, a large spectrum of NSPCs appears to be affected by HI.

Moderate hypothermia has long been appreciated as a potential means of neuroprotection in neonatal HI [39–42]. This effect is operative in multiple cell types and brain regions, including in the hippocampus, where hypothermic treatment in a rat model of HI has been shown to attenuate neuron loss in the CA1 region [43]. Human clinical trials have shown that hypothermia is a relatively safe intervention [44]. In general, clinical trials have shown improvements in outcomes, such as mortality, seizure rate and neurological outcomes [6]. However, some reports have failed to definitively demonstrate improvements [45]. Even when applied optimally, it appears that hypothermia alone is certainly not a sufficient therapy for neonatal HI, and thus hypothermia is likely to function primarily as an adjunct therapy. In particular, if hypothermia can exert neuroprotective effects on NSPCs, it might be a valuable adjunct for therapeutic strategies that seek to harness endogenous sources of NSPCs to effect repair of damaged structures. By limiting tissue damage, hypothermia may act on NSPCs primarily by preserving environmental features that are conducive for NSPC proliferation. However, we did not detect a difference in Tbr2 levels between HI and HH groups during recovery (fig. 4p), suggesting that the environmental support for proliferation was roughly equivalent. Alternately, hypothermia might act primarily by preserving some NSPCs that are at earlier stages of development than the damaged population of cells. These competing hypotheses are not mutually exclusive, and both may be important features of hypothermic neuroprotection. Future studies may examine exactly how hypothermic neuroprotection is operating in this capacity, i.e. by direct effects on NSPCs or indirect effects via environmental factors, in order to better harness and direct its use as a therapeutic modality.

While it has not been studied extensively, there is some evidence that hypothermic therapy can enhance neurogenesis after brain injury due to hypoxia, trauma or radiation in adult models [46]. Our manuscript is one of two reports showing that hypothermia can protect NSPCs in the dentate gyrus SGZ in the setting of neonatal HI. Xiong et al. [47] found that hypothermia reduced infarct size in the SGZ, and acted on differentiation of neural stem cells such that neuronal fate was increased at the expense of reduced glial generation. Our findings also show an important role for hypothermia in preserving neurogenesis in the SGZ through neuroprotection of NSPCs. We found a significant increase in NSPCs at 1 week after HI with hypothermia. Our data appear at first glance to be at odds with a report by Kanagawa et al. [21], which showed that hypothermia in the absence of injury suppresses neurogenesis. However, the exposure to hypothermia in their experiments lasted for 21 h, whereas ours was for only 4 h, which likely represents a substantial biological difference, particularly in terms of reduced stress for the exposed animals.

There is considerable interest in the use of exogenous cell-based therapy for repair after neonatal HI [48, 49]. Transplantation of mesenchymal stem cells in rodents with neonatal HI have been shown to improve neurocognitive outcomes [50], although it appears that the mechanism of repair actually involves an enhancement of endogenous neurogenesis [51]. Transplantation of neural stem cells into rodent models of neonatal HI has been shown to improve functional recovery. However, it is difficult to determine whether mechanisms of recovery may lie more with effects on endogenous regrowth or on the creation of new neuronal circuits by transplanted cells [52]. Our finding that neurogenesis 1 week after HI is substantially enhanced by hypothermia that was induced immediately after injury suggests that hypothermic therapy may be critical to preserving the optimal environment for neuronal growth. Consistent with this hypothesis, a recent study found that neural stem cell transplantation in conjunction with postinjury hypothermia yielded better neurologic outcomes than stem cell transplantation alone, and that graft survival was enhanced in the hypothermia group [53].

Our study has a number of limitations that must be acknowledged. Hypoxic-ischemic encephalopathy in human neonates is an incompletely defined entity that occurs as a result of a diverse range of pathologic insults to oxygenation or circulation [54]. Creating an animal model which accurately represents such a disorder is inherently challenging. While the Vannucci model and its mu-

rine adaptation is very widely used because it has accurately replicated critical features of the human disease, including the distribution of injury, generation of postinjury seizures and the range of neurologic dysfunction [55], it is not a perfect recreation of the range of pathology that leads to HI encephalopathy in humans. Our experiments were designed to study mild-to-moderate injury, both by the length of exposure we used and by the exclusion criteria for severe injury. Thus, our results, including putative benefits of hypothermia, may not apply well to cases of severe injury. Also, we have used a randomly distributed sampling approach rather than a stereologically designed study, which could perhaps have detected more subtle effects. Additionally, the study of early postnatal dentate gyrus neurogenesis has been conducted primarily in rodent models, and the extent to which it translates to the human patient is uncertain. There is convincing evidence that neurogenesis occurs in the mature human dentate gyrus [56], and 25% of dentate gyrus neurons are generated in the first 3 months of life in nonhuman primates [57], which strongly suggests that dentate gyrus neurogenesis is likely to be a target for both injury and potential repair in human patients with neonatal HI. Nevertheless, the time scale of mouse brain development is so compressed relative to the human, it may be that even a relatively brief exposure to HI has considerably exaggerated consequences relative to what would be seen over the course of human brain development. Thus, the findings of this study and other rodent work on neurogenesis in neonatal HI should be replicated in a large animal model. Additionally, it will be of great importance to follow up the findings of this study with an examination of survival and network incorporation of the NSPCs generated in such abundance in the hypothermic rescue condition during recovery.


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