Delayed and Abbreviated Environmental Enrichment after Brain Trauma Promotes Motor and Cognitive Recovery That Is Not Contingent on Increased Neurogenesis

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Abstract

Environmental enrichment (EE) confers motor and cognitive recovery in pre-clinical models of traumatic brain injury (TBI), and neurogenesis has been attributed to mediating the benefits. Whether that ascription is correct has not been fully investigated. Hence, the goal of the current study is to further clarify the possible role of learning-induced hippocampal neurogenesis on functional recovery after cortical impact or sham injury by utilizing two EE paradigms (i.e., early + continuous, initiated immediately after TBI and presented 24 h/day; and delayed + abbreviated, initiated 4 days after TBI for 6 h/day) and comparing them to one another as well as to standard (STD) housed controls. Motor and cognitive performance was assessed on post-operative Days 1–5 and 14–19, respectively, for the STD and early + continuous EE groups and on Days 4–8 and 17–22, for the delayed + abbreviated EE groups. Rats were injected with bromodeoxyuridine (BrdU, 500 mg/kg; intraperitoneally) for 3 days (12 h apart) before cognitive training and sacrificed 1 week later for quantification of BrdU+ and doublecortin (DCX+) labeled cells. Both early + continuous and delayed + abbreviated EE promoted motor and cognitive recovery after TBI, relative to STD ($p < 0.05$), and did not differ from one another ($p > 0.05$). However, only early + continuous EE increased DCX+ cells beyond the level of STD-housed controls ($p < 0.05$). No effect of EE on non-injured controls was observed. Based on these data, two novel conclusions emerged. First, EE does not need to be provided early and continuously after TBI to confer benefits, which lends credence to the delayed + abbreviated EE paradigm as a relevant pre-clinical model of neurorehabilitation. Second, the functional recovery observed after TBI in the delayed + abbreviated EE paradigm is not contingent on increased hippocampal neurogenesis. Future studies will elucidate alternate viable mechanisms mediating the benefits induced by EE.

Keywords: behavioral outcome; controlled cortical impact; environmental enrichment; functional recovery; learning and memory; Morris water maze; traumatic brain injury

Introduction

OVER 10 MILLION PEOPLE WORLDWIDE incur a traumatic brain injury (TBI) each year,¹ and approximately 2 million of those individuals reside in the United States.² In the U.S. alone, more than 120,000 cases involve long-term or permanent neurological dysfunction in motor and cognitive ability. While the affective toll of TBI on a stricken individual's interpersonal relationships with significant others is immeasurable, the economic cost to society accounts for billions of dollars each year.³ TBI is undeniably a significant healthcare issue that is compounded fur-

ther by the paucity of treatment options. In this vein, various pre-clinical pharmacotherapeutic approaches have been and continue to be evaluated,^{4,5} albeit with poor bench-to-bedside translatability.^{6,7} Hence, non-pharmaceutical strategies are gaining momentum as feasible rehabilitative treatments that may facilitate translation from the laboratory to the clinic.^{8,9} One viable alternative approach that confers significant benefits after TBI, as well as in other central nervous system (CNS) injury models, is environmental enrichment (EE).

EE consists of a complex living arrangement that includes a vast living space, novel stimuli, and increased social interaction that

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collectively confers motor and cognitive recovery after TBI versus standard (STD) housing.^{8–11} Because of the benefits instilled by EE after TBI as well as the sensory, motor, and cognitive stimulation that it provides, it is touted as a pre-clinical model of neurorehabilitation.^{8,9,12–17} Like the early-and-continuous exposure strategy (i.e., initiated immediately after TBI and presented 24 h/day), delayed and/or abbreviated EE paradigms, which are more akin to clinical rehabilitation in terms of timing and duration, also enhance neurobehavioral and cognitive recovery.^{16,18,19} Increased neurogenesis has been attributed for the improvement in behavioral outcomes mediated by EE, albeit this premise has yet to be rigorously tested after TBI.

Although developmental neurogenesis and gliogenesis represent the origin of the vast majority of brain cells, neural stem cells (NSCs) continue to produce neurons throughout life.^{20,21} In the hippocampus, adult born cells generate from the neurogenic niche located in the subgranular zone (SGZ) of the dentate gyrus (DG) where they integrate into existing neural circuits and contribute to cognitive recovery.²² These newly generated neurons are mainly incorporated into memory circuits where they enhance memory encoding.²³ Moreover, spatial learning influences the production and the fate of new neurons generated immediately before the training, and these early stages of newly generated neurons have been shown to be necessary for learning and memory.^{24–27} Hence, promoting hippocampal neurogenesis as a therapeutic strategy that could increase functional recovery after TBI has been the focus of a substantial body of research.^{22,28,29}

The effect of EE on hippocampal neurogenesis and behavior has been widely studied. It is well established that EE modulates proliferation and survival of newly generated cells,^{30–32} but still controversial is whether increased neurogenesis is necessary for improved cognitive performance.³³ Moreover, recent evidence has shown that many of the beneficial effects of EE on hippocampal neurogenesis in non-injured animals are mediated by the effect of exercise on a running wheel and that environmental complexity does not directly enhance or modulate neurogenesis.³⁴ On the other hand, EE has consistently been shown to promote recovery after CNS injury. In rodents, EE protects against age-dependent cognitive decline,^{35,36} reduces lesion size and behavioral deficits post-stroke,^{37,38} and decreases inflammation after chronic cerebral hypo-perfusion.³⁹ Numerous studies from our group have demonstrated that EE *sans* a running wheel but that includes physical, sensory/cognitive, and social interaction, consistently promotes robust motor and cognitive recovery after controlled cortical impact (CCI) injury.^{8,9,12–14,16,17,40} EE also has been shown to increase the survival of new cells generated during the early post-injury period,⁴¹ but the effect of EE on learning-induced hippocampal neurogenesis has not been studied (i.e., cells generated immediately before training). Hence, the aim of this study is to test the hypothesis that learning-induced hippocampal neurogenesis mediates cognitive recovery after TBI utilizing the typical EE paradigm where EE is initiated immediately after TBI and provided continuously, which is not an approach typically seen in the clinic, and a novel EE design where enrichment is delayed and abbreviated (i.e., introduced 4 days after injury and provided for only 6 h per day), and has some semblance to timing in the clinic.

Methods

Subjects and pre-surgical procedures

Fifty-seven adult male Sprague-Dawley rats (Harlan, Indianapolis, Indiana) were paired in ventilated polycarbonate rat

cages and maintained in a temperature-controlled ($21 \pm 1^\circ\text{C}$) and light-controlled (on 0700–1900 h) environment with food and water available *ad libitum*. During the week of habituation, the rats were pre-trained on the motor task to ensure that they could balance on the beam for the allotted 60 sec. On the morning of surgery, the rats were pre-assessed for beam-balance to determine baseline performance (Fig. 1A). Following training the rats were randomly assigned to the following conditions, which included both TBI and respective sham controls: standard (STD) housing (TBI, $n = 11$; sham, $n = 8$), early + continuous EE (TBI, $n = 11$; sham, $n = 8$), and delayed + abbreviated EE (TBI, $n = 11$; sham, $n = 8$). All experimental procedures were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee and the National Research Committee of the Mexican Social Security System (R-2014-784-057). All efforts were made to limit the number of rats used as well as to minimize their discomfort.

Surgery

Rats weighing 300–325 g on the day of surgery underwent a CCI injury as previously described.^{15–17,42} Briefly, surgical anesthesia was induced and maintained with inspired concentrations of 4% and 2% isoflurane, respectively, in 2:1 $\text{N}_2\text{O}:\text{O}_2$. After endotracheal intubation, the rats were secured in a stereotaxic frame, ventilated mechanically, and temperature maintained at $37 \pm 0.5^\circ\text{C}$ with a heating blanket. Adhering to aseptic protocol, a midline scalp incision was made, the skin and fascia were reflected to expose the skull, and a craniectomy was made in the right hemisphere (encompassing bregma and lambda and the sagittal and coronal sutures) with a hand-held trephine. The bone flap was removed and the craniectomy was enlarged further to ensure that penetration of the impact tip (6 mm, flat) was unobstructed. Subsequently, the impacting rod was extended and the impact tip was centered and lowered through the craniectomy until it touched the dura mater; then, the rod was retracted and the impact tip was advanced 2.8 mm farther to produce a brain injury of moderate severity (2.8 mm tissue deformation at 4 m/sec). Immediately after the CCI, anesthesia was discontinued and the incision was promptly sutured. The rats were subsequently extubated and assessed for acute neurological outcome. Sham-operated rats underwent similar surgical procedures, but were not subjected to the impact.

Acute neurological evaluation

Hindlimb reflexive ability was assessed immediately following the cessation of anesthesia and removal from the stereotaxic apparatus by gently squeezing the rats' paw every 5 sec and recording the time to elicit a withdrawal response. Return of the righting reflex was determined by the time required to turn from the supine to prone position. These sensitive neurological indices are used to determine the level of injury severity.^{15–18}

Housing conditions (environmental manipulation)

After the effects of surgical anesthesia abated (as evidenced by spontaneous movement in the holding cage), the rats were placed in their assigned home environments. Specifically, one-third were immediately placed in the EE cages (i.e., early + continuous EE group). Briefly, the EE is a specifically designed three-tiered stainless steel-wire cage ($91 \times 76 \times 50$ cm) with ladders to ambulate from one level to another and contains various toys (e.g., balls, blocks, and tubes), nesting materials (e.g., paper towels), and *ad libitum* food and water.^{14–18} To maintain novelty, the objects were rearranged every day and changed each time the cage was cleaned, which was twice per week. Ten to 12 rats, which included TBI and sham controls, were housed in the EE together to minimize variability among the groups. The remaining two-thirds were placed in typical laboratory shoebox cages ($37 \times 25 \times 18$ cm, two rats per

cage; STD environment) with *ad libitum* food and water. Beginning at 9:00 AM on post-injury Day 4, the rats randomized to the delayed + abbreviated EE condition (6 h of EE) were removed from the STD cages and placed in the EE cages until 3:00 PM, at which time they were returned to STD housing. These rehabilitative manipulations occurred each day for 22 days.

Motor performance

The well-established beam-balance test was utilized to assess motor function.^{15–18,43} Briefly, the task consisted of placing the rat on an elevated narrow beam (1.5 cm wide) and recording the time it remained on for a maximum of 60 sec. Baseline performance was assessed 1 h prior to surgery. Testing consisted of three trials (60 sec allotted time with an inter-trial interval of 30 sec) per day and was conducted on post-operative Days 1–5 for the STD and early + continuous EE groups and on Days 4–8 for the delayed + abbreviated EE groups. The average daily scores for each rat were used in the statistical analyses.

Cognitive function

Acquisition of spatial learning. Spatial learning was assessed in a Morris water maze (MWM) task that has been shown to be sensitive to cognitive function/dysfunction after TBI.^{15,19,44–47} Briefly, the maze consisted of a plastic pool (180 cm diameter; 60 cm high) filled with tap water ($26 \pm 1^\circ\text{C}$) to a depth of 28 cm. The maze was in a room with salient visual cues that remained constant throughout the study. The platform was a clear Plexiglas stand (10 cm diameter, 26 cm high) that was positioned 26 cm from the maze wall in the southwest quadrant and held constant for each rat. Training began on post-operative Day 14 for the STD and early + continuous EE groups and on Day 17 for the delayed + abbreviated EE groups. Assessment of spatial learning consisted of providing a block of four daily trials (4-min inter-trial interval) for 5 consecutive days to locate the platform when it was submerged 2 cm below the water surface. On Day 19 for the STD and early + continuous EE groups and on Day 22 for the delayed + abbreviated groups the platform was made visible to the rats by raising it 2 cm above the water surface as a control procedure to determine the contributions of non-spatial factors (e.g., sensory-motor performance, motivation, and visual acuity) on cognitive performance. For each daily block of trials, the rats were placed in the pool facing the wall at each of the four possible start locations (north, east, south, and west) in a randomized manner. Each trial lasted until the rat climbed onto the platform or until 120 sec had elapsed, whichever occurred first. Rats that failed to locate the hidden platform within the allotted time were manually guided to it. All rats remained on the platform for 30 sec before being placed in a heated incubator between trials. The times of the four daily trials for each rat were averaged and used in the statistical analyses.

Probe trial

On Day 19 for the STD and early + continuous EE groups (TBI and sham) and on Day 22 for the delayed + abbreviated EE groups (TBI and sham), the rats were subjected to a probe trial to test their memory retention. Briefly, the platform was removed from the pool and the rat was provided a single 30-sec trial to swim freely. The percentage of time spent in the quadrant where the platform was situated during training (i.e., target quadrant) was recorded using ANY-maze video tracking software. Swim speed also was recorded during this testing period. The percent time spent in the target quadrant was used in the statistical analysis.

BrdU administration

To determine whether EE affects learning-induced neurogenesis in the hippocampus, rats were injected for 3 days (12 h apart) with

bromodeoxyuridine (BrdU, Sigma-Aldrich 500 mg/kg; intraperitoneally) 10 days before sacrifice. Injections were conducted on Days 12–14 for the STD and early + continuous EE groups and on Days 15–17 for the delayed + abbreviated EE groups.

Histology

Immunostaining. After completion of cognitive testing, the rats were anesthetized with Fatal-Plus[®] (0.25 mL, intraperitoneally) and then perfused transcardially with 200 mL 0.1 M phosphate-buffered saline (pH 7.4), followed by 300 mL 4% paraformaldehyde (PFA). The brains were extracted, post-fixed in 4% PFA for 24 h and cryoprotected in serial sucrose-phosphate-buffered saline solutions. To visualize BrdU+ and DCX+ cells, 40 μm coronal sections were cut in a systematic random series and immunostained as previously described.⁴⁸ All immunostaining and immunofluorescence procedures were performed on free-floating sections and all antibody incubations were carried out in a PBT solution (phosphate buffer +4% Triton X-100, Sigma-Aldrich, St Louis, MO) containing 5% horse serum (GIBCO, Paisley, UK). After permeabilization (10 min, PB +10% H_2O_2 + 4% Triton X-100), the sections were rinsed $3 \times$ (PB 3×10 min), incubated for 2 h in formamide (50% in SSC, Sigma-Aldrich) at 74°C , and washed for 5 min in pre-heated SSC buffer. DNA denaturation was performed in HCl (1 N) for 30 min at 37°C . Sections were incubated for 10 min in borate buffer (pH 8.4) and rinsed $3 \times$ in PB (3×10 min). For BrdU peroxidase immunostaining, we used mouse anti-BrdU immunoglobulin G (IgG; 1:5000; Roche Applied Sciences, Indianapolis, IN) and for DCX goat anti-human doublecortin (1:3000; Santa Cruz Biotechnology) was used for 12 h at 4°C . Brains were rinsed in PB (3×10 min), incubated for 2 h in a biotinylated secondary goat anti-mouse IgG or guinea pig anti-goat (1:750; Vector Laboratories, Burlingame, CA), washed (PB 3×10 min) and further incubated with the avidin-biotin peroxidase complex solution (5 $\mu\text{L}/\text{mL}$ in PBT, Vectastain Elite ABC kit; Vector Laboratories) for 90 min. Immunostaining was visualized using a DAB staining kit for 10 min (Vector Laboratories). Sections were mounted on gelatin coated slides, cover-slipped, and counter stained with hematoxylin-eosin (Vector laboratories).

For BrdU/glia fibrillary acidic protein (GFAP)/NeuN triple immunofluorescence, we used a mixture of primary rat anti-BrdU IgG (1:200; ABD; Serotec, Oxford, UK), rabbit anti-GFAP IgG (1:400; ABCAM, Cambridge, MA), and mouse anti-NeuN IgG (1:300; Millipore, Temecula, CA) overnight at room temperature. Sections were washed, incubated for 3 h in a secondary donkey anti-rat IgG (1:300; Alexa 405; Jackson Immuno Research Labs, Pennsylvania, PA), goat anti-mouse IgG (1:300; Alexa 643; Jackson Immuno), and guinea pig anti-rabbit IgG (1:300; Alexa 488; Jackson Immuno), and mounted and cover-slipped with DAKO (Glostrup, Denmark). For BrdU/NeuN/DCX triple immunofluorescence, sections were incubated with a mixture of primary rat anti-BrdU IgG (1:200; ABD Serotec, Oxford, UK) and mouse anti-NeuN IgG (1:300; Millipore) overnight at room temperature. Sections were washed, incubated for 3 h in a secondary donkey anti-rat IgG (1:300; Alexa 405; Jackson Immuno) and goat anti-mouse IgG (1:300; Alexa 643; Jackson Immuno) and rinsed with PB. After visualization of BrdU and NeuN, sections were then incubated overnight in a mixture of two primary antibodies that detect different DCX epitopes, guinea pig anti-DCX (1:400; Millipore) and goat anti-DCX (1:200; Santa Cruz). Sections were incubated for 2 h in a mixture of a secondary antibody biotinylated goat anti-guinea pig IgG and donkey anti-goat IgG (1:300; Vector Laboratories). This two-phase immunostaining procedure was done to ensure that no cross reactivity was observed between Alexa 643 goat anti-mouse fluorescent antibody used to visualize NeuN and donkey anti-goat biotinylated secondary used to visualize DCX. Brains were rinsed in PB (3×10 min) and incubated for 90 min with the fluorescent avidin-FITC substrate kit (Vector laboratories),

washed (3×10 min in PB), mounted and cover-slipped with DAKO mounting media (DAKO, Glostrup, Denmark). Negative controls without primary antibodies were carried out for all immunostaining and immunofluorescence protocols. Negative controls for mouse and rat IgG cross reactivity were also performed to determine working dilutions.

Cell counting

For stereology, sections were evaluated as previously described.⁴⁸ Briefly, the series of brain sections ($240\text{-}\mu\text{m}$ apart from one another) were used for generating stereological counts of BrdU+ nuclei along the extent of the hippocampus. The boundaries of the granular and subgranular zone (SGZ) of the DG were digitally outlined with the aid of image analysis software (axiovision 4.6; Karl Zeiss). Total cell number and density in the areas of interest were obtained from both the dorsal and ventral blades of the DG. DG volume was quantified according to the Cavalieri principle, where the volume is the sum of the areas of each $40\text{-}\mu\text{m}$ section.^{48,49}

Statistical analysis

Statistical analyses were performed on data collected by observers blinded to treatment conditions using Statview 5.0.1 software. The motor and cognitive data were analyzed by repeated-measures analysis of variance (ANOVA). The acute neurological assessments, swim speed, visible platform performance, and immunostaining data, were analyzed by one-factor ANOVAs. When the overall ANOVAs revealed a significant effect, the Newman-Keuls post-hoc test was utilized to determine specific group differences. The data are presented as the mean $\pm$ standard error of the mean (SEM) and are considered significant when corresponding p values are ≤ 0.05 .

Results

There were no surgical or health problems with any of the subjects throughout the experiment and thus the statistical analyses are based on all fifty-seven rats. EE, regardless of the paradigm used, had no effect on sham controls (supplemental data, Fig. S1) and therefore the data were pooled to make up a single Sham group.

Acute neurological assessments

No significant differences were observed among the TBI groups for return of hind limb reflex ability after a brief paw pinch (range for right: 144.5 ± 5.6 – 147.2 ± 6.0 ; range for left: 149.6 ± 5.8 – 151.4 ± 5.9) or righting reflex latency (range = 361.4 ± 15.3 – 389.4 ± 19.5). The lack of significant differences in post-surgical neurological assessments among the TBI groups indicates that all rats received similar levels of anesthesia and injury severity.

Early + continuous EE and delayed + abbreviated EE confer robust motor and cognitive benefits after TBI

No pre-surgical motor differences were observed among groups as all rats were capable of balancing on the beam for the allotted 60 sec (Fig. 1B). However, after the cortical impact, all rats were markedly impaired relative to the Sham group, which maintained balance for the full 60 sec. The ANOVA revealed significant Group ($F_{3,47} = 10.404$; $p < 0.0001$) and Day ($F_{5,235} = 33.862$; $p < 0.0001$) differences, as well as a significant Group $\times$ Day interaction ($F_{15,235} = 6.029$; $p < 0.0001$). *Post hoc* analyses revealed that the Sham group was better than all TBI groups, regardless of housing condition ($p < 0.05$) and that the TBI groups receiving early +

continuous EE or delayed + abbreviated EE performed better relative to the STD controls ($p < 0.05$), but did not differ from one another ($p > 0.05$).

Analysis of the water maze data revealed significant Group ($F_{3,47} = 21.746$; $p < 0.0001$) and Day ($F_{4,188} = 36.967$; $p < 0.0001$) differences. *Post hoc* analyses revealed that relative to the STD-housed controls, both EE conditions significantly decreased the time to locate the escape platform ($p < 0.05$; Fig. 1C) and reduced the distance traveled to the platform ($p < 0.05$; Fig. 1D), which indicates that EE facilitated the ability to learn the association of the spatial cues and the location of the platform. Additionally, both EE conditions afforded the rats more time in the target quadrant during the probe trial, relative to STD-housed controls ($p < 0.05$), which is indicative of memory retention (Fig. 1E, 1F). Specifically, the early + continuous EE and delayed + abbreviated EE groups spent $37.8 \pm 1.4\%$ and $38.4 \pm 1.4\%$ of their time, respectively, in the target quadrant, while the STD control group performed just slightly above chance level with $28.6 \pm 2.9\%$. The Sham group spent $38.5 \pm 3.0\%$ of its time in the target quadrant, which did not differ from the EE groups, regardless of paradigm ($p > 0.05$). No differences were observed in any of the water maze outcome parameters between the early + continuous and delayed + abbreviated EE groups ($p > 0.05$). Additionally, no significant differences in swim speed (range = 27.9 ± 1.3 cm/sec to 32.9 ± 0.7 cm/sec) or visible platform acquisition (Fig. 1C, 1D) were observed among the groups, suggesting that there were no motor impairments or visual disparities that precluded the accurate assessment of place learning.

Early + continuous EE, but not delayed + abbreviated EE, increased training-induced neurogenesis after TBI

Analysis of BrdU+ and DCX+ stereology data revealed significant Group differences in the volume of the neurogenic niche located in the SGZ of the dorsal dentate gyrus ($F_{3,46} = 3.393$; $p < 0.05$), DCX+ cell number ($F_{3,46} = 8.798$; $p < 0.0001$), and DCX+ cell density ($F_{3,46} = 3.543$; $p < 0.05$). *Post hoc* analyses revealed that relative to the STD-housed controls, early + continuous EE, but not delayed + abbreviated EE, selectively increased DCX+ cell numbers ($p < 0.01$), and DCX+ cell density ($p < 0.05$), without increasing BrdU-labeled nuclei ($p > 0.05$, Fig. 2B, 2C). Additionally, we observed that relative to Sham controls, early + continuous EE, but not delayed + abbreviated EE, increased the volume of the neurogenic niche located in the SGZ of the dorsal dentate gyrus ($p < 0.05$), DCX+ cell numbers ($p < 0.01$), and DCX+ cell density ($p < 0.05$).

To further investigate whether EE-induced effects on neurogenesis are correlated to cognitive performance after TBI, the time in the target quadrant during the probe trial (i.e., memory retention) was plotted as a function of BrdU+ (Fig. 2D) or DCX+ cells (Fig. 2E). No significance was observed using Pearson's correlation analysis for behavior and the number of BrdU+ nuclei. A negative correlation between DCX+ cells and time in the target quadrant was observed ($p < 0.05$), which indicates that the EE-induced increase in hippocampal neurogenesis does not correlate with cognitive recovery in the MWM.

We analyzed the density of BrdU+ nuclei and DCX+ cells as a function of the rostro caudal axis (Fig. 3). No effect of housing, TBI, or the rostro caudal axis was observed in the number and density of BrdU+ nuclei ($p > 0.05$). However, we observed that the effect of EE on DCX+ cell density was selective to the more rostral part of the dorsal hippocampus (Fig. 3B). An ANOVA showed a significant effect of Group ($F_{3,419} = 21.217$; $p < 0.0001$) and the

FIG. 1. (A) Rats were subjected to either a controlled cortical impact or sham injury and randomly assigned to standard (STD) housing, early + continuous environmental enrichment (EE), or delayed + abbreviated EE (i.e., EE for 6 h a day commencing 4 days after surgery). EE had no effect on the sham groups and therefore those data were pooled. (B) Time to maintain balance on the beam was evaluated on post-operative Days 1–5 for the STD and early + continuous EE groups, while the delayed + abbreviated EE groups were tested on Days 4–8. Mean $\pm$ SEM (standard error of the mean) * $p < 0.05$ vs. Sham, TBI + abbreviated EE, and TBI + continuous EE. # $p < 0.05$ vs. Sham and TBI + STD. No differences were revealed between the TBI + abbreviated EE and TBI + continuous groups ($p > 0.05$). (C) Time to locate the escape platform and (D) distance traveled to the platform in the Morris water maze were evaluated on Days 14–18 for the STD and early + continuous EE groups and on Days 17–21 for the delayed + abbreviated EE groups. Mean $\pm$ SEM * $p < 0.05$ vs. Sham, TBI + abbreviated EE, and TBI + continuous EE. # $p < 0.05$ vs. Sham and TBI + STD. No differences were revealed between the TBI + abbreviated EE and TBI + continuous EE groups ($p > 0.05$). (E) A probe trial was provided on Day 19 for the STD and early + continuous EE groups and on Day 22 for the delayed + abbreviated EE groups. Dotted line represents chance performance. Mean $\pm$ SEM % time in the target quadrant. * $p < 0.05$ vs. Sham, TBI + abbreviated EE, and TBI + continuous EE. # $p < 0.05$ vs. TBI + STD. No differences were revealed between the TBI + abbreviated EE and TBI + continuous EE group ($p > 0.05$). (F) Representative heat maps demonstrating swim trajectories on Days 1 and 5 of spatial learning as well as the probe trial (i.e., memory retention). Sham, $n = 24$; TBI + STD, $n = 11$; TBI early + continuous, $n = 11$; TBI delayed + abbreviated EE, $n = 11$.

rostral-caudal axis ($F_{9,419} = 2.370$; $p < 0.05$) on DCX+ cell density in the dorsal hippocampus. In the ventral hippocampus (Fig. 3C), no effect of housing or TBI was observed in the SGZ/GCL (i.e., granule cell layer) volume ($p > 0.05$), or number ($p > 0.05$) and density ($p > 0.05$) of BrdU+ and DCX+ cells. However, statistical

analysis revealed significant Group differences ($F_{3,46} = 3.507$; $p < 0.05$) in the volume of the ventral neurogenic niche (SGZ). Multiple comparisons showed that TBI caused an increase in the SGZ volume ($p < 0.05$) compared with the Sham group that was reversed by early + continuous EE ($p < 0.05$).

FIG. 2. (A) Rats were subjected to either a controlled cortical impact or sham injury and randomly assigned to standard (STD) housing, early + continuous environmental enrichment (EE), or delayed + abbreviated EE (i.e., EE for 6 h a day commencing 4 days after surgery). On post-operative Days 12–14 for STD-housed and continuous EE groups, and Days 15–17 for abbreviated EE, the rats were injected twice a day with BrdU (500 mg/kg) and further tested on a Morris water maze task. Rats were sacrificed 48 h after the probe trial and the total number of BrdU+ and doublecortin (DCX+) cells were estimated by stereology. (B) Representative images of BrdU (hematoxylin and eosin counterstain) immunostaining and stereological estimation of granule cell layer (GCL) and subgranular zone (SGZ) volume (mm^3) and total BrdU+ nuclei number and density (nuclei number/ mm^3). Inserts illustrate higher magnifications of cells depicted by arrows. (C) Representative images of DCX immunostaining (scale bar = 500 μm) and stereological estimation of subgranular zone (SGZ) volume (mm^3) and total DCX+ cell number and density (cell number/ mm^3). Inserts illustrate higher magnifications of cells depicted by arrows. Mean $\pm$ SEM (standard error of the mean) ** $p < 0.001$ vs. Sham, # $p < 0.05$ vs. STD, ## $p < 0.01$ vs. STD. Sham, $n = 23$, TBI + STD, $n = 8$, TBI + continuous EE, $n = 8$, TBI + abbreviated EE, $n = 8$. (D) No correlation between BrdU+ nuclei density and behavior was observed, but (E) a negative correlation was observed for DCX+ cell number and behavior (Pearson's r ; * $p < 0.05$).

FIG. 3. (A) Rats were subjected to either a controlled cortical impact or sham injury and randomly assigned to standard housing (STD), early + continuous environmental enrichment (EE), or delayed + abbreviated EE (i.e., EE for 6 h a day commencing 4 days after surgery). On post-operative Days 12–14 for STD and early + continuous EE groups and Days 15–17 for delayed + abbreviated EE groups, the rats were injected twice a day with BrdU (500 mg/kg) and tested in the Morris water maze. (B) Rats were sacrificed 48 h after the probe trial (i.e., measure of memory retention) and the density of BrdU+ and doublecortin (DCX+) cells were plotted as a function of the rostro-caudal axis of the dorsal hippocampal formation. (C) The number of BrdU+ and DCX+ cells in the ventral hippocampus was estimated by stereology. Mean $\pm$ SEM (standard error of the mean) * p < 0.05 vs. Sham, # p < 0.05 vs. TBI + STD. Sham, n = 23; TBI + STD, n = 8; TBI + continuous EE, n = 8; TBI + abbreviated EE, n = 8.

FIG. 4. Rats were subjected to either a controlled cortical impact or sham injury and randomly assigned to standard (STD) housing, early + continuous environmental enrichment (EE), or delayed + abbreviated EE. On post-operative Days 12–14 for STD and continuous EE groups and Days 15–17 for the delayed + abbreviated EE groups, the rats were injected twice a day with BrdU (500 mg/kg) and further tested on a Morris water maze task. Rats were sacrificed 48 h after the probe trial and the phenotype of BrdU+ cells was evaluated by confocal microscopy. **(A)** Representative images of confocal imaging z-stack orthogonal reconstruction of triple immunofluorescent staining for BrdU (red), GFAP (green) and NeuN (blue). Insets show higher magnification of cells depicted by white arrows (scale bar = 200 μm). **(B)** Percentage of BrdU+ nuclei co-localized with the different cell markers. Mean ± SEM (standard error of the mean) ** $p < 0.01$ and * $p < 0.05$ vs. TBI + STD. TBI + STD, $n = 7$; TBI + continuous EE, $n = 7$; TBI + abbreviated EE, $n = 7$. **(C)** DCX+ cells were subdivided into spindle-like cells lacking protrusions (Type 2b), migrating cells with small protrusions (Type 3), and early post-mitotic cells with complete dendritic arborization. Mean ± SEM * $p < 0.01$ vs. Sham, # $p < 0.05$ vs. TBI + STD. Sham, $n = 18$; TBI + STD, $n = 6$; TBI + continuous EE, $n = 6$; TBI + abbreviated EE, $n = 6$.

Early + continuous EE, but not delayed + abbreviated EE, accelerates DCX+ cell maturation

To evaluate whether EE affects the differentiation rate of newly generated neurons we performed triple immunofluorescence confocal analysis for BrdU, DCX and NeuN cells (Fig. 4A) and quantified the percentage of DCX+ cells that could be classified into one of the three main maturation subtypes (Type 2b, Type 3, and early post-mitotic). Ten days after injection, we observed that there was no effect of housing on the percentage of BrdU+ nuclei that co-localized with DCX or the percent of BrdU+/NeuN+ cells (Fig. 4B). However, we observed significant Group differences in the percentage of post-mitotic DCX+ cells ($F_{3,35} = 6.494$; $p < 0.001$). Multiple comparisons showed that compared to TBI + STD, early + continuous EE increased the proportion of post-mitotic DCX+ cells ($p < 0.05$; Fig. 4C). Additionally, we observed that TBI + STD and delayed + abbreviated EE lead to a decrease in the proportion of post-mitotic DCX+ cells compared to Shams ($p < 0.05$).

EE promotes differentiation into GFAP+ phenotype

We further evaluated whether EE affects the differentiation rate of newly generated cells after TBI by performing triple immuno-

fluorescence confocal analysis of BrdU+ cell phenotypes (Fig. 5A). Ten days after BrdU injection, half of the labeled cells presented a mature phenotype (BrdU+/NeuN+) and this proportion was not affected by housing (Fig. 5B). A smaller proportion of BrdU+ cells co-localized with GFAP. Statistical analyses showed a significant effect on the percentage of BrdU+/GFAP+ cells that survive 10 days after injection ($F_{2,20} = 6.753$; $p < 0.01$). Multiple comparisons showed that early + continuous EE increased the percentage of BrdU+/GFAP+ cells when compared with TBI + STD ($p < 0.05$). No effect of housing was observed in the percentage of undifferentiated (BrdU+/GFAP-/NeuN-) cells that survived 10 days after injection (Fig. 5C).

Discussion

Numerous studies have shown unequivocally that EE, a rodent analogue of rehabilitation, confers significant motor, cognitive, and histological benefits, relative to STD-housing, after experimental TBI.^{8–11} While most of the EE studies have focused on the traditional model of providing enrichment immediately and continuously after TBI, our group has begun refining the EE paradigm to

FIG. 5. Rats were subjected to either a controlled cortical impact or sham injury and randomly assigned to standard (STD) housing, early + continuous environmental enrichment (EE), or delayed + abbreviated EE. On post-operative Days 12–14 for the STD and early + continuous EE groups and Days 15–17 for the delayed + abbreviated EE groups, the rats were injected twice a day with BrdU (500 mg/kg) and further tested on a Morris water maze task. Rats were sacrificed 48 h after the probe trial (i.e., memory retention) and the phenotype of BrdU+ cells was evaluated by confocal microscopy. **(A)** Representative images of confocal imaging z-stack orthogonal reconstruction of triple immunofluorescent staining for BrdU (red), GFAP (green), and NeuN (blue). Inserts show higher magnification of cells depicted by white arrows (scale bar = 200 μm). **(B)** Percentage of BrdU+ nuclei co-localized with the different cell markers. Mean ± SEM (standard error of the mean) ** $p < 0.01$ and * $p < 0.05$ vs. TBI + STD. TBI + STD, $n = 7$; TBI + continuous EE, $n = 7$; TBI + abbreviated EE, $n = 7$.

mimic real neurorehabilitation practice, where physiotherapy does not commence until after a delayed period, usually after discharged from critical care, and then typically limited to only a few hours a day.^{50–53} In accordance with previously published findings, the current data showed that both EE groups performed significantly better than the STD-housed controls on the beam-balance motor task. Additionally, both EE paradigms significantly reduced the time to reach the escape platform and increased the time in the target quadrant during the probe trial relative to the STD-housed controls, indicating facilitated spatial learning and enhanced memory. Importantly, from the perspective of model refinement, there were no differences between the two EE conditions, which lends credence to the delayed + abbreviated EE paradigm as a relevant pre-clinical model of neurorehabilitation. By manipulating the onset and duration of EE exposure and quantifying newly generated cells that survive after training in the MWM spatial learning task, we demonstrated that even though both early + continuous EE and delayed + abbreviated EE promote recovery after TBI, only continuous EE lead to a significant increase in the number of DCX+ cells in the hippocampal dentate gyrus relative to STD controls.

The effects of EE on hippocampal plasticity have been widely studied.^{8,54,55} In non-injured animals, it has been shown that hippocampal neurogenesis is not required for the behavioral effects observed after EE^{33,56}; however, this was not necessarily true for the injured brain as demonstrated by Grégoire and colleagues³⁴ who studied differential settings of EE cages and demonstrated that running, but not the complex environment per se, stimulates hippocampal neurogenesis in healthy animals. Therefore, to test whether EE-induced hippocampal neurogenesis is related to recovery after TBI, we evaluated the survival of newly generated cells after training in a spatial learning task. We demonstrated that an EE setting without the running wheel is robust enough to promote behavioral recovery and to increase neurogenesis after TBI beyond that of STD-housed controls without causing any effects in

non-injured sham controls, suggesting that the injured brain's response to EE differs substantially than that of healthy animals. In concordance with our findings, it has been observed that under normal conditions NSCs do not migrate away from their traditional GCL fate, but they do respond to neural damage,^{57,58} even if the hippocampus is not directly affected by damage, such as in mild cortical impact models.⁵⁸ Moreover, the effect of early and continuous EE on DCX+ cell density was selective to the more rostral part of the dorsal hippocampus, without affecting the ventral pole. In rodents, hippocampal connectivity⁵⁹ and gene expression⁶⁰ vary along the dorso-ventral axis, with a more robust correlation between the dorsal pole and cognition,^{60,61} thus suggesting that this gradient could also influence the response of the neurogenic niche.

The effects of TBI on the neurogenic niche during the early post-injury period are still controversial. Many studies have observed that shortly after TBI there is a transient increase in the number of proliferating cells (BrdU+)⁶² and immature neurons (DCX+) in the SGZ and that the proliferative rate stabilizes within 14 days.^{63,64} However, contrasting evidence shows that DCX+ cells are highly vulnerable to TBI and die within 24–72 h after injury.⁶⁵ Additionally, dendrites of the surviving DCX+ in the ipsilateral hippocampus were found to be short and congruent with what could be considered an immature morphology.⁶⁵ Moreover, recent work has shown that this transient increase in neurogenesis after TBI is followed by a long-term decline in the proliferative capacity of the neurogenic niche 30 and 90 days after injury.⁶⁶ In order to clarify the possible role of learning-induced hippocampal neurogenesis on functional recovery induced by EE without the possible confounding effects of the biphasic effects of TBI, we chose to evaluate neurogenesis after the immediate post-injury effects on proliferation begin to disappear (post-injury Days 12–14 for the early + continuous EE group and Days 15–17 for the delayed and abbreviated EE group), but before the TBI-induced exhaustion of the neurogenic niche is observed. In accordance with this idea, the present work showed that 3 weeks after injury, there was no effect

of TBI on the survival rate of NPCs that were proliferating 12–14 or 15–17 days after injury (BrdU+), or the number of immature neurons (DCX+).

To produce new neurons, NSCs must go through a process of proliferation, migration, differentiation, and integration into the existing circuitry in the brain. Adult newborn cells progress through several stages of development before finally acquiring a mature phenotype. These stages can be differentiated by the temporal expression of different proteins. Astrocyte-like precursor cells type-1 give rise to transiently amplifying neuronal progenitor cells type-2b and type-3 that express DCX. Finally, newborn cells become DCX+ post-mitotic immature neurons and later mature into dentate granule cells that express NeuN. Type-2b cells are characterized by a small soma, irregular shaped nucleus, and short horizontally orientated processes. Type-3 stage is a transition phase from the slowly proliferating neuroblasts to the post-mitotic immature neuron. Under normal conditions, type-3 cells display little proliferative activity, but under pathophysiological conditions (e.g., seizures), they can dramatically increase their proliferative activity.⁶⁷ Our results show that only early + continuous EE decreases the proportion of immature DCX+ cells while it increases early post-mitotic cells.

One of the limitations of our study is that because we did not inject BrdU immediately after injury, it is not possible to determine if the increase in the proportion of post-mitotic DCX+ cells induced by early + continuous EE is due to enhanced survival of cells generated during the early post-injury period or an accelerated maturation rate. In our paradigm, delayed + abbreviated EE was initiated after the immediate rise in neurogenesis caused by TBI. However, evaluating the effects of EE on these early generated cells was not within the scope of our study, because only immature cells are related to learning. In support of this, we observed a negative correlation between the probe trial and the number of DCX+ cells.

Under normal physiological conditions, the adult SGZ neurogenic niche gives rise mainly to dentate granule cells that, when mature, express NeuN. However, after brain injury, NSCs have the capacity to respond to the insult and produce neurons as well as astrocytes and oligodendrocytes.²⁹ Moreover, it has been shown that TBI induces gliogenesis (BrdU+/GFAP+) ipsilateral to the lesion,⁶³ suggesting that brain trauma can alter the balance between neurogenesis and gliogenesis. In this study, we observed that while there was no effect on the proportion of BrdU+ cells that differentiate into neuronal phenotype, early + continuous EE increases the percentage of BrdU+ cells that differentiate into GFAP+ phenotype, which suggests an increase in gliogenesis.

The effect of abbreviated EE and TBI on hippocampal neurogenesis has been previously studied by Gaulke and colleagues,⁴¹ who exposed rats to 1 h of EE daily for three weeks and labeled NPCs that were proliferating 24 h later. Their results showed that limited EE increased NPCs survival, but not proliferation; however, they were unable to directly correlate these effects with behavioral recovery as neurogenesis and cognitive performance were evaluated in separate groups of rats. Moreover, as we have previously discussed, TBI causes an immediate rise in neurogenesis that decreases as recovery progresses.⁶³ This immediate neurogenic effect is not necessarily related to improved performance in the MWM as only the early stages of newly generated neurons are the ones that have been shown to be necessary for learning and memory.^{24,68,69}

In the current study, we quantified the number and density of BrdU+ cells labeled before training. This approach was previously reported by Ambrogini and colleagues,²⁶ who studied the effect of learning on newborn cell survival by injecting BrdU twice a day

over 3 days before training in a spatial learning task. They observed that the survival of newborn cells at an early stage of differentiation was enhanced by learning and that there was a correlation between the actual individual learning and newborn cell survival. Similar experimental designs aimed to study the effect of learning on BrdU+ cells labeled before training have been reported with variations in the injection time in rats^{24,70} and mice.⁷¹ However, because it has been suggested that training in the MWM decreases survival of BrdU+ cells that were labeled 8–10 days after training,⁷¹ in the present work BrdU injections were performed 3 days before testing in the MWM. This approach afforded the opportunity to evaluate if EE modifies the well-known effects of learning on the neurogenic niche and exclude possible confounding effects of training on the survival of earlier generated cells (i.e., cells generated during the immediate post injury period). We observed that 3 weeks after TBI, only early + continuous exposure to EE increased neurogenesis beyond that of STD-housed controls, while both early + continuous EE and delayed + abbreviated EE promoted similar recovery. By labeling NPCs before training on a spatial learning task, we correlated cognitive performance and neurogenesis and found that the increase in the survival and differentiation of newly generated neurons does not appear to be critical for EE-mediated improvement of cognitive performance after TBI.

Conclusions

Two major conclusions emerged from the present work. First, we demonstrated that both early + continuous EE and delayed + abbreviated EE provided cognitive recovery after TBI, but did not differ from one another. This novel finding suggests that the latter, rehabilitation-relevant model may be useful for further evaluation of TBI and other CNS diseases such that successful translation from the bench-to-bedside may be facilitated. Second, the data indicate that the EE-mediated functional recovery after brain trauma is not contingent on increased neurogenesis as both EE groups enhanced behavioral performance equally, but only the early + continuous group increased neurogenesis relative to STD controls. Ongoing studies in our laboratory are elucidating alternate viable mechanisms mediating the benefits induced by EE.

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No competing financial interests exist.

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