Long-Term Effects of Enriched Environment on Neurofunctional Outcome and CNS Lesion Volume After Traumatic Brain Injury in Rats

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Summary

To determine whether the exposure to long term enriched environment (EE) would result in a continuous improvement of neurological recovery and ameliorate the loss of brain tissue after traumatic brain injury (TBI) vs. standard housing (SH). Male Sprague-Dawley rats (300-350 g, n=28) underwent lateral fluid percussion brain injury or SHAM operation. One TBI group was held under complex EE for 90 days, the other under SH. Neuromotor and sensorimotor dysfunction and recovery were assessed after injury and at days 7, 15, and 90 *via* Composite Neuroscore (NS), RotaRod test, and Barnes Circular Maze (BCM). Cortical tissue loss was assessed using serial brain sections. After day 7 EE animals showed similar latencies and errors as SHAM in the BCM. SH animals performed notably worse with differences still significant on day 90 ($p<0.001$). RotaRod test and NS revealed superior results for EE animals after day 7. The mean cortical volume was significantly higher in EE vs. SH animals ($p=0.003$). In summary, EE animals after lateral fluid percussion (LFP) brain injury performed significantly better than SH animals after 90 days of recovery. The window of opportunity may be wide and also lends further credibility to the importance of long term interventions in patients suffering from TBI.

Key words

Traumatic brain injury • Enriched environment • Controlled cortical impact • Neurobehavioral • Functional recovery

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Introduction

Traumatic brain injury (TBI) continues to be the leading cause of death and long-term disability world-wide (Waxweiler and Thurman 1995, Bruns and Hauser 2003, Langlois and Rutland-Brown 2006). In the United States an estimated number of 1.6 million persons sustain a TBI each year with 52,000 deaths and 80,000 patients suffering from permanent neurological impairment (Sosin *et al.* 1991, Bruns and Hauser 2003). Only 1/4 patients of the total may reach good recovery with no or only

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minimal deficits. Thus, TBI represents a highly relevant medical and socioeconomic burden for modern societies (Murray and Lopez 1997, Ghajar 2000).

The main features of the central nervous system (CNS) response to traumatic brain injury (TBI) have been principally elucidated. Using the lateral fluid percussion (LFP) model in rats (McIntosh and Vink 1989, Dietrich *et al.* 1996, Pierce and Trojanowski 1996), numerous investigations describe the major histopathological consequences to trauma: lesion-induced vascular perturbations (Cortez and McIntosh 1989, Schmidt and Grady 1993, Fukida *et al.* 1995), glial hypertrophy and proliferation (Hill and Barbarese 1996), and neuronal necrosis (Soares *et al.* 1992, 1995, Dietrich *et al.* 1994a,b, Hicks and Soares 1996, McIntosh and Vink 1989). These reports also confirm that, causing profound cell death (necrotic and apoptotic) and axonal degeneration throughout the brain (Cortez and McIntosh 1989, Dietrich *et al.* 1994a, Soares *et al.* 1995, Hicks and Soares 1996, Conti *et al.* 1998), the LFP model is the one that most closely mirrors postlesional events associated with TBI in humans (McIntosh and Vink 1989, Dietrich *et al.* 1996, Pierce and Trojanowski 1996, Graham *et al.* 2000).

Voluminous experimental work has been conducted to characterize new neurobiological events after TBI (Saatman *et al.* 2001, Stein *et al.* 2002), unknown effect(s) of different pharmacological trials (Wahl *et al.* 2000, Belayev *et al.* 2001, Bentzer *et al.* 2001, Faden *et al.* 2001, 2003, LaPlaca *et al.* 2001, Marklund *et al.* 2001, Alessandri *et al.* 2002), and various post-traumatic treatments (Dietrich *et al.* 1994a, Bramlett and Dietrich 1997, Philips *et al.* 2001, Knoblach and Faden 2002, Hicks and Zhang 2002, Rice *et al.* 2002). Only some studies have focused on the concept of environmental enrichment (EE) after TBI, first described by Hepp *et al.* (1947), and then further developed by Diamond and Krech (1964), Rosenzweig (1966) and Dobbing (1970). To date, a series of behavioral, cellular, and molecular studies have revealed significant effects of EE on rodents and other species, and provided new insights into the mechanisms of experienced-dependent plasticity, including adult neurogenesis and synaptic plasticity (Nithianantharajah and Hannan 2006). EE has been reported to lead to enhanced expression of trophic factors and neurogenesis and to increase the number of dendrites, synapses, glia cells and blood vessels (Falkenberg and Mohammed 1992, Kempermann and Kuhn 1997, Nilsson and Perfilieva 1999). At the behavioral level, EE enhances learning and memory (Myslivecek and Hassmannova 1987, Moser *et al.* 1997, Rampon *et al.* 2000a,b, Tang *et al.* 2001, Schrijver *et al.* 2002, Lee *et al.* 2003), reduces memory decline (Bennett *et al.* 2006), decreases anxiety, and increases exploratory activity (Chapillon *et al.* 1999, Roy *et al.* 2001, Benaroya-Milshtein 2004, Friske and Gammie 2005).

To date, the maximum observation periods after TBI and exposure to EE have been restricted to four to eight weeks following impact only. Thus, the regeneration potential of the experimentally lesioned brain beyond this time window including ways to trigger these potentials for improved outcome is still scarce. Further, it is not known whether there exist specific time windows within the post-injury sequalae beyond traditional survival times in which the lesioned brain is more receptive for external clues that may be translated into central reorganization and improved function. In considering a standardized experimental approach and the key aspects of EE, i.e. environmental complexity and novelty (Nithianantharajah and Hannan 2006), the present research proposal aimed to further investigate the benefits associated with EE, that have been observed up to 30 days post-injury (Maegele and Lippert-Gruener 2005a,b, Lippert-Gruener *et al.* 2006a,b), but now on an extended time scale up to three months after injury. Together with previous findings results may be translated into optimizing current clinical stimulation concepts in the rehabilitation of brain trauma patients (Lippert-Gruener and Terhaag 2000, Lippert-Gruener *et al.* 2002, 2003).

Materials and Methods

Overview of experimental animal groups

Male Sprague-Dawley rats (300-350 g, n=28) were obtained from Harlan-Winkelmann (Borchen, Germany) and housed in individual cages. After acclimatization, animals were randomized into the two housing paradigms: in standard housing (SH) or in an EE for a total of 90 days after surgery. Each group consisted of 14 animals, 4 sham-operated animals as well as 10 traumatized animals. For analysis SHAM animals from both housing paradigms were pooled into one group (n=8) a priory as we assumed no significant effect of housing paradigm on our outcome parameters. Furthermore, this group size increased the statistical power. All experimental procedures confirmed with the guidelines of the Witten-Herdecke University and the

Local Animal Ethics Committee.

Operative procedures and lateral fluid percussion brain injury (LFP)

The LFP brain injury model is one of the most widely used and well characterized models of experimental traumatic brain injury (Laurer and Lenzlinger 2000) and has been described previously (McIntosh and Vink 1989). In brief, under anesthesia with sodium pentobarbital (60 mg/kg BW i.p.), animals were fixated in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). After incision of the scalp, the temporal muscles were reflected and a 4.8 mm craniotomy, 2.5 mm lateral to the sagital sinus and centered between bregma and lambda was drilled, keeping the dura mater intact. To this a hollow female Luer-Lok fitting was fixated using dental cement. TBI was induced with the fluid percussion device. Prior to induction of trauma a connection between the female Luer-Lok, anchored in the rats skull and the male one on the fluid percussion device was made, creating a closed system filled with isotonic saline in connection with the dura. For the induction of trauma, a metal pendulum was released from a pre-selected height, striking the other end of the Plexiglas piston. The induced rapid injection of saline into the closed cranial cavity thus created a pulse of increased intracranial pressure of 21-23 ms duration. This caused a brief displacement and deformation of neural tissue. The pressure pulse was recorded using a computer oscilloscope emulation program (RC Electronics, Santa Barbara, CA, USA) *via* a transducer (Gould) housed in the injury device. The injury was induced at a moderate level (2.1 atm). Afterwards the cemented Luer-Lok was removed from the scull, the incision closed by interrupted 4.0 silk sutures and the animals placed onto a heated pad for 1 h following surgery. Sham operated animals underwent surgical procedures as described above without being subject to LFP brain injury.

Standard housing and enriched environment

After surgery rats held in the standard housing paradigm remained in standard cages (425 x 266 x 185 mm; polycarbonate, Techniplast, Buguggiate, Italy) with no specific stimulation. Rats subjected to EE were placed in specifically designed cages, experiencing group living. The EE consisted of three cages, 610 x 435 x 215 mm of size, connected in a row *via* tunnels. The EE furthermore consisted of horizontal and inclining platforms, climbing ladders, balls, tunnels, bridges, hanging ropes, bells. Objects and toys were randomly circulated as some were removed and others were added within the course of the experiment. In both housing paradigms food and water were available *ad libitum*; temperature was 22 °C and a 12 h light-dark-cycle. The sham operated animals were randomized into one of either housing paradigms. Wherever scientifically feasible their results were pooled to reduce the total number of animals used. All trials were performed by an investigator blinded to housing paradigm and injury status.

Neurofunctional evaluation (Composite Neuroscore)

Evaluation of neuromotor impairment after TBI by using a Composite Neuroscore (NS) test has been described previously (Okiyama *et al.* 1992, Sinson *et al.* 1995) and results correlate with injury severity (Sullivan *et al.* 1976, McIntosh and Vink 1989). Scoring for each animal ranged from 0 (severely impaired) to 4 (normal strength and function) for each of the following modalities: (1) left and (2) right forelimb flexion during suspension by the tail; (3) left and (4) right hind limb flexion with the forelimbs remaining on a flat surface as the hind limbs are lifted up and down by the tail; (5) ability to resist lateral pulsion to the left and (6) right; (7) ability to stand on an inclined plane in the left; (8) right and (9) vertical position. Inclined plane scoring (0-4) is determined by the animal´s ability to stand at an angle up to 45 degrees (4=45°, 3=42.5°, 2=40°, 1=37.5°, 0≤37.5°). The scores for (7), (8), and (9) were averaged, and a composite neurological motor score (0-28) was calculated for each animal from the summation of individual test scores. Baseline composite neuromotor scores were calculated 24 h prior to injury. The degree of acute neurological impairment after trauma and prior to the beginning of either SH or EE was assessed in all animals at 24 h post-injury; the recovery of neuromotor functions was evaluated blinded to the injury status at days post-injury 7, 15, and 90 days.

Sensorimotor coordination (Rota-Rod test)

Sensorimotor coordination was assessed using the Rota-Rod test (IITC Life Science, Woodland Hills, USA) (Dunham and Miya 1957, Jones and Roberts 1968). For the test a series of three trials with at least 5 min of rest was performed. All animals were placed on a cylinder which then gradually began to rotate with an increasing speed of 0 to 30 rpm within 60 s. The animals

were placed on textured drums to avoid slipping. The system provided individual timers for measuring the time the animals stayed on the rod. Animal falls were detected by light-beam sensors mounted into each compartment. Each trial was terminated if an animal fell or jumped of the cylinder or remained on it for >90 s. The mean duration (in s), distance (in m), and maximum speed (in rpm) were recorded for a series of three consecutive trials. Animals were allowed to recover in their home cages for at least 3 min before a new trial was started. Baseline values were recorded 24 h prior to injury. The extent of sensorimotor impairment was surveyed at 24 h post-injury and at days post injury 7, 15, and 90 after trauma the extent of recovery was assessed.

Spatial reference memory (Barnes Circular Maze)

The Barnes Circular Maze (Barnes 1979) has been adapted to assess spatial reference memory following TBI (Maegele *et al.* 2005). The maze represents an efficient and proven alternative to the commonly used water-maze-test with less stress to the animal, less physical demand, and fewer trials over fewer days for satisfactory training (Fox *et al.* 1998). During the Barnes Circular Maze procedure, the animals had to locate a dark escape chamber, hidden underneath one of a series of holes around the perimeter of a bright disc. This disc was illuminated by four overhead lamps to create a low-level adverse stimulus. Our maze was manufactured from white acrylic plastic to form a disk 1.5 cm thick and 122 cm in diameter, with 18 evenly spaced holes, 7 cm in diameter, at its periphery. All trials were recorded by a video camera above the maze to measure the distance covered by each animal using an electronic tracking system. Animals had to perform two trials per day for five consecutive days (day 85-89 after TBI). Trials were ended after the animal had entered the escape chamber or when a pre-determined time (300 s) had elapsed, whichever occurred first. All surfaces were cleaned before each trial to eliminate possible olfactory cues from preceding animals.

Forelimb sensorimotor function (Limb-use asymetry test, Cylinder test)

Experimental LFP brain injury is known to be associated with reduced contralateral forelimb motor function and coordination (McIntosh and Vink 1989, Pierce and Smith 1998, Schallert and Fleming 2000, DeBow *et al.* 2003, Grow and Liu 2003). When placed in the cylinder, animals will usually rear and explore the cylinder walls with their forepaws, allowing three categories of placements to be recorded: i) independent ipsilateral limb use, ii) independent contralateral limb use, and iii) both movements, when the animal uses both paws in unison or in quick succession. Symmetry of forelimb use was assessed by videotaping rats for 3 min while exploring a transparent glass cylinder (25 cm in diameter, 30 cm in height). To facilitate scoring of movements while the animal was facing away from the camera, a mirror was placed behind the cylinder. Animals were tested using a red lamp in the dark during the animals' light phase, to encourage exploratory behavior and rearing. Scoring of forelimb use was done blinded to housing and surgery status. The scored behaviors were calculated in percentage use of the forelimb used (ipsilateral, contralateral, and both) in relation to the total number of limb use observed. Values were recorded 24 h prior and 24 h post-injury. Recovery was evaluated at days 7, 15, and 90 post injury.

Tissue preparation

At day 90 post-injury, the animals were re-anesthetized and transcardially perfused with 0.9 % NaCl in distilled water for 60 s followed by fixation with 4 % paraformaldehyde (PFA) in 0.1 m phosphate buffer (pH 7.4). After removal, brains were stored in 4 % PFA until further processing. For quantification of lesion volume four animals from each group were selected at random. Brains for immunocytochemistry were placed in a cryoprotective solution for 24 h (20 % sucrose in 4 % PFA). Brains were then precut using a brain slicer matrix (Zivic Instruments, Pittsburgh, PA, USA). The rostral border of the resection was defined by the demarcation of the infundibular recess (Bregma –0.3 mm) the caudal cut was made approximately 8 mm dorsally (Bregma –6.8 mm). The resulting brain-slice was embedded in TFM™ tissue freezing medium (Triangle Biomedical Sciences, Inc., Durham, NC, USA). After freezing at –80 °C, 40 µm-thick sections were cut on a Cryostat and stored in 1x PBS (P5493, Sigma-Aldrich CO, St. Louis, MO, USA) until further processing.

Immunocytochemistry

Immunocytochemistry was performed on 40 µm-thick, free-floating sections. After sections were washed in 1xPBS quenching of endogenous peroxidase was performed using 500 µl per section of 3 % H_2O_2 in water (Sigma-Aldrich CO, St. Louis, MO, USA) for 5 min, followed by thorough washing in 1xPBS. Sections

were then incubated in 500 µl 0.6 % Triton-X 100 in 1xPBS (Sigma-Aldrich CO, St. Louis, MO, USA) containing 5 % NGS (Normal Goat Serum, Sigma-Aldrich CO, St. Louis, MO, USA) and primary antibody, GFAP (1:150, G3893, Sigma-Aldrich CO, St. Louis, MO, USA). Sections were placed on a rocker (Shaker 25, Labnet International Inc., Woodbridge, NJ, USA) for 24 h. After 3 washes in 1xPBS, sections were processed using the Vectastain Elite Kit (Vector Laboratories, Inc., Burlingame, CA, USA). Biotinylated secondary antibody was used at 1:200 in 1xPBS, A and B reagents were used at 1:66 each. The incubation time was 30 min each. Visualization was performed with 3.3'-DAB (Sigmafast™, D4293, Sigma-Aldrich CO, St. Louis, MO, USA). For controls, sections incubated with the omission of primary antibody yielded blank sections. Sections were then mounted on Fisherbrand™ slides (Fisher Scientific, Pittsburgh, PA, USA), air-dried, cleaned in Xylenes and coverslipped using Permount® (Fisher Scientific, Pittsburgh, PA, USA).

Quantification of cortical tissue loss

The assessment of differences in tissue loss between both groups was performed by measuring cortical thickness from Bregma –1.3 to Bregma –6.3. As previously demonstrated, regions with greatest cortical damage are located in between those two planes at time points between one month and one year after LFP induced TBI (Cortez and McIntosh 1989, Hicks and Soares 1996, Smith and Chen 1997). In accordance to the fractionators sampling strategy we stained every 20th coronal section throughout the pre-cut brain (a total of 6 equidistant sections) using GFAP-staining. The cortical volume for each group was calculated according to the Cavalieri method (Gundersen *et al.* 1988), multiplying the total lesioned area (µm^2) by the mean section thickness (40 µm). Detailed accounts of the principles and the procedures for calculating coefficients of error are given in Gundersen and Jensen (1987) and West and Gundersen (1990). In brief, the cortical volume (V) was estimated using an exhaustive set of parallel slices through the brain at a known mean slice separation (d). The position of the first slice must be uniform random in the interval 0-d. It is important that only one face of each section is measured. The slice areas of the appropriate faces were estimated by randomly superimposing a systematic array of test points on each face in turn. The points falling on all of the section faces, P were counted. The estimated object volume could be calculated by the formula: $V = A\ d = P a(p)\ d$. In this calculation $a(p)$ is the areal equivalent of one test point on the scale of the specimen (=brain). Previously, it was shown that efficient estimates could be obtained from few as five to six slices per object or brain, respectively (Mayhew 1991). Cortical thickness was measured in µm to estimate the total lesion area in µm^2. Image acquisition for quantification of cortical thickness was performed using a Nikon D3 with AF-S Micro Nikkor 60 mm lens (Nikon Europe B.V.). Image calibration and analysis was performed using AxioVision 4.8 software (Carl Zeiss Inc.).

Statistical analysis

All statistical analysis was performed using SPSS 13.0 software. Data surveyed from all experimental groups was tested for differences using one-way descriptive statistics and one-way ANOVA as well as *post-hoc* analysis. A level of significance of $p<0.05$ was used for all analyses.

Results

Neurofunctional evaluation (Composite Neuroscore)

In the Composite Neuroscores (NS) no differences were observed between intact animals pre-injury with respect to forelimb flexion, lateral pulsation, hind limb function and baseline angles in the angle-board test. At 24 h post-injury, all animals subjected to LFP brain injury showed a similar level of severe neurofunctional impairment compared to their uninjured SHAM counterparts irrespective of housing conditions (SHAM: 28±0.0, SH: 12.2±1.0, EE: 11.7±1.1, both $p<0.005$ vs. SHAM). Within the first week after injury, EE animals recovered significantly some neurological function (EE 15.2±1.4) while animals held under standard housing displayed a worsening with respect to their neurofunction (SH 10.2±0.9) (Fig. 1). Within the further sequalae of the experiment until the end of the observation period (day 90) both groups (SH and EE groups) recovered a considerable amount of neurofunction but with EE animals being always significantly superior ($p<0.005$) over SH animals at all time points studied (SH 19.3±1.1, EE 22.4±1.7).

Sensorimotor coordination (Rota-Rod Test)

Within 90 days of the trial SHAM rats showed excellent results in the three categories of the sensomotoric test (dpi-1: 2.49±1.42 m, 23.07±447 rpm, 49.02±14.9 s, dpi+90: 3.04±1.5 m, 26.74±4.99 rpm,

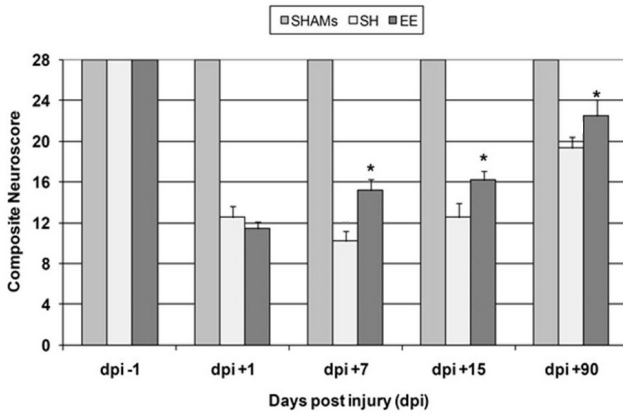

Fig. 1. Composite Neuroscore. Evaluation of neuromotor function and recovery over a 90 day period. All injured animals showed a comparable decline in neuromotor function 24 h post injury with faster recovery after EE. dpi, days post injury; SH, standard housing; EE, enriched environment. Values are shown as mean scores ± SD. * p<0.05 vs. SH.

55.80±15.51 s). In the Rota-Rod test at 24 h post-injury, all animals subjected to LFP brain injury showed a significant decline in all three dimensions of the test, i.e. time, distance and maximum speed sustained compared to their sham counterparts (p<0.005 vs. SHAM), but the difference between SH and EE animals was not significant, indicating a similar level of inflicted brain injury. The SH rats always showed the highest neurological deficit (dpi+1: 0.4±0.32 m, 9.05±3.64 rpm, 16.65±7.51 s). Recovery in EE animals was more pronounced and statistically significant (p=0.04) for distance (EE: 1.08 m, SH: 0.59 m) and time (EE: 31.54±7.66 s, SH: 21.61±8.85 s) at day 15 post injury while the amount of recovery in SH animals was not (Fig. 2). Both injured groups showed stagnating sensorimotor coordination in the RotaRod test from day 7 to day 15 and exhibited diminishing performance in all three dimensions at day 90 (EE: 0.71±0.36 m, 12.82±3.84 rpm, 24.46±7.85 s, SH: 0.66±0.61 m, 11.81±5.57 rpm, 22.36±11.51 s) compared to days 7 and 15. On day 90 post injury no influence of EE on healthy rats could be detected (SHAM+SH: 2.05±0.67 m, 25.09±3.38 rpm, 50.33±7.51 s, SHAM+EE: 2.72±0.82 m, 28.76±1 rpm, 52.72±7.79 s).

Spatial reference memory (Barnes Circular Maze)

At Barnes Circular Maze (BMC) on day 1 animals in EE had found the escape chamber after 89.8±86.1 s while animals in SH animals after 158.8±92.0 s. Sham operated animals had located the escape box after 129.3±113.7 s of search. Errors made in EE- and SH- animals were comparable (7.4±5.2 vs. 7.6±5.3), sham-operated animals made 9.8±6.1 errors on average. An apparent learning curve for spatial reference memory was evident in both injured groups as well as in sham-operated animals over the 5 day training period. Animals housed in EE performed better than SH and

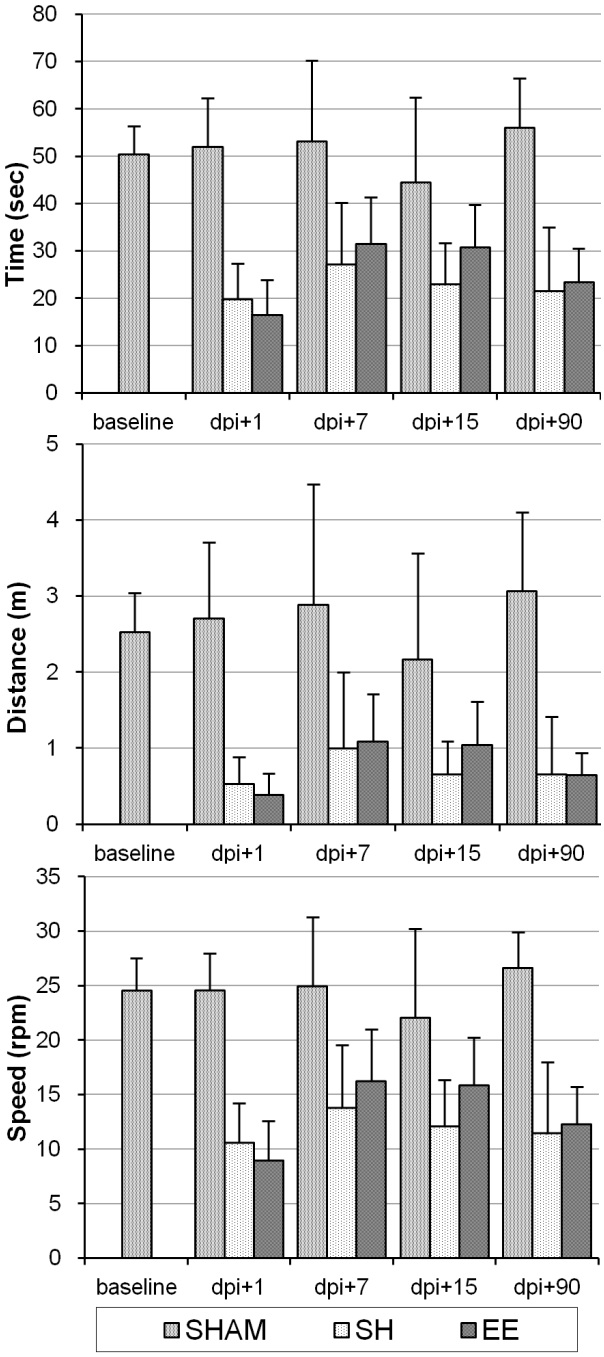

Fig. 2. RotaRod test to evaluate sensorimotor coordination and recovery at baseline, 24 h post injury (dpi 1) and dpi 7, 15, 30 and 90. Injured animals show a similar level of decline (dpi 1) compared to baseline with faster recovery after EE. Values are shown as mean ± SD for time (seconds), distance traveled (meter) and speed (rounds per minute, rpm). * p<0.05 vs. SH.

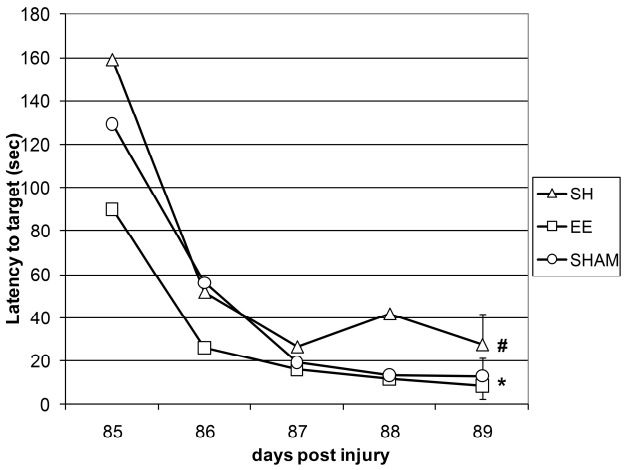

Fig. 3. Spatial reference memory tested with the Barnes Circular Maze at dpi 85-89 (day 1-5) measured by latency to target in sec. EE-stimulated animals performed like sham-operated animals from day 2. Values are shown as mean scores ± SD. * $p<0.05$ vs. SH; # $p<0.05$ vs. SHAM.

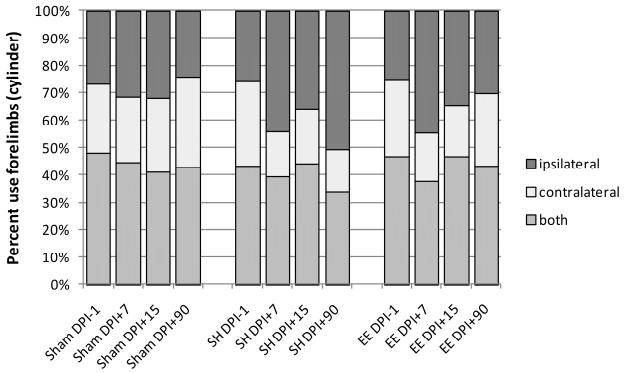

Fig. 4. Assessment of forelimb sensorimotor function during exploration of a glass cylinder before injury (DPI-1), and recovery at day 7, 15 and 90 post injury. Percentaged use of forelimb, contralateral forelimb and use of both forelimbs simultaneously were shown with regard to the site of injury with a remarkable decrease of the use of the contralateral limp on 90 DPI in the SH-group; EE-group reached sham-level of the limb at 90 DPI. DPI, days post injury; SH, standard housing; EE, enriched environment.

sham-operated animals during the duration of the trial with respect to latency. From day 3 onwards, latencies of sham animals were comparable to EE animals (Fig. 3). At BCM day 5 EE animals and sham operated animals showed similar latencies and errors (8.7±6.1 s vs. 13.2±8.2 s and 1.9±2.4 vs. 2.2±1.4 errors). Animals in the SH-group performed notably worse on day 5 of the BCM with respect to latency (27.5±14.4 s) and errors (3.1±1.8). The differences in latencies between the groups on the final BCM day were statistically significant (EE vs. SH $p<0.001$). On day 90 post injury no influence of EE on healthy rats could be observed (SHAM+EE: 9.33±5.81 s, 2.25±2.07 errors; SHAM+SH: 11.1±2.68 s, 2.25±1.51 errors).

Forelimb sensorimotor function and recovery (Limb-use asymmetry test, Cylinder Test)

At baseline, all animals showed a simultaneous use of both forelimbs of approximately 45 % (Fig. 4, dpi-1). Seven days after injury, animals under both housing paradigms showed a not significant decrease of contralateral forelimb use and a slight decline in use of both limbs simultaneously (SH: 39.5±0.24 %, EE: 38.0±28 %). Over the course of the study SH animals did show a tendency towards recovery at day 15 but declined until day 90 to comparable forelimb sensorimotor function impairment as shown on day 7 post injury (dpi+90: SH: 33.7±26 %, EE: 43.4±18 %). Animals in EE showed a more pronounced and also retained recovery over days 15 to 90 coming close to pre-injury levels. No influence of EE on healthy rats in the simultaneous use of both forelimbs could be detected on day 90 post injury (SHAM+SH: 48.1±0.7 %, SHAM+EE: 49.5±0.9 %). None of the group-differences were statistically significant.

Assessment of cortical tissue loss and gross pathological alterations

Animals that were held under SH conditions had a mean cortical volume of 87.5±5.5 mm^3 while animals in EE had a mean cortical volume of 104.1±4.1 mm^3 ($p=0.003$) on day 90 post injury (Fig. 5D). Representative sections at Bregma level –3.14 for one animal from each experimental group were shown (Fig. 5A-C). As previously calculated, a markedly reduced loss of cortical thickness was observed for EE animals. Similarly, ventricular enlargement was substantially less pronounced in EE animals compared to SH animals but was clearly discernible in all injured animals compared to sham-operated animals. We also found that loss of tissue in both internal capsule and hippocampus was more pronounced in SH animals compared to EE animals but present in both groups compared to sham operated animals. Astrocytosis throughout the ipsilateral hemisphere was present in all injured animals. It extended from the region of the cortical cavity to the ipsilateral internal capsule, along the commissural fibers and also into the ipsilateral thalamus.

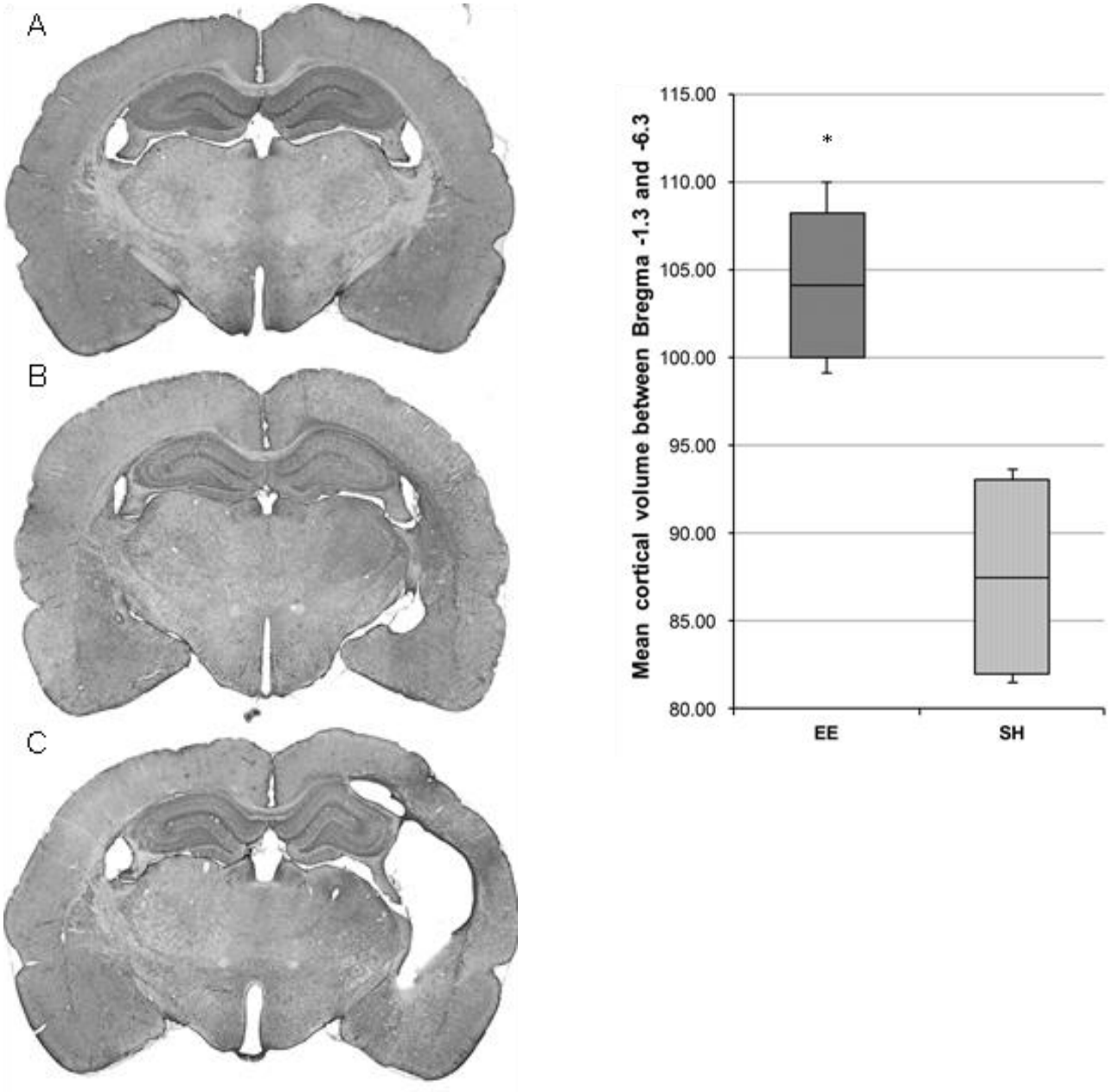

Fig. 5. Rat brain sections at Bregma –3.14 from a representative sham-operated animal **(A)**, a SH **(B)** and an EE animal **(C)** after LFP brain injury. Notably difference between SH and EE brains in structural damage of the hippocampus, the external and internal capsule on the injured side. **(D)** Mean cortical volume after LFP brain injury in mm^3 for animals housed in SH and EE (n=4 per group) calculated according to the Cavalieri method (Gundersen *et al.* 1988). Boxplots show median ± SD; whiskers show extrema. * $p<0.05$ vs. SH.

Discussion

Environmental enrichment has been shown to have numerous beneficial effects on brain and behavior. Unfortunately, current knowledge from these investigations is restricted to short-term survival periods (14-30 days) only leaving the potential mid- to long-term benefits associated with EE unclear. Because there is an ongoing loss of brain tissue and impairment of neurological functioning as assessed by the Morris Water Maze and Composite Neuroscore that seem to be progressive for up to one year after LFP brain injury (Smith and Chen 1997, Pierce and Smith 1998) it is of great interest what continuous EE can do. The results for up to 30 days post injury showed promising improvement on functional outcome and tissue integrity following TBI (Maegele *et al.* 2005). The present study investigating the potential of long-term effects associated with EE after LFP brain injury in rats underlines the findings of Kovesdi *et al.* (2011) after 71 days of EE and show a decelerated, but continuous recovery of brain function.

In general, animals after subjected to LFP brain

injury followed by exposure to EE behaved better in three out of four standardized tests to assess both sensorimotor or neuromotor function and spatial reference memory. SH animals also showed a clear learning-curve but performed worse than injured animals from EE and sham-operated animals. The advantage for EE vs. SH-housed animals with respect to spatial learning as described previously for short-term survival (Hamm and Temple 1996, Maegele *et al.* 2005) thus seems to be sustained over longer periods post injury as well. The results obtained from the cylinder test for forelimb sensorimotor function and recovery showed also a trend in favor of EE animals however without reaching statistical significance. Histo-morphologically, the results from neurofunctional testing were reflected by the preservation of cortical thickness at the lesion site. Although none of the animals in the EE group showed full recovery when compared to baseline and sham-operated animals, the pattern of recovery was similar to that with shorter survival periods as previously reported by our group (Pierce and Smith 1998). Both groups continued to improve their scores markedly from days 30 to 90 post injury.

Voluminous experimental work has been conducted to characterize new neurobiological events after TBI (Saatman *et al.* 2001, Stein *et al.* 2002), unknown effect(s) of different pharmacological trials (Wahl *et al.* 2000, Belayev *et al.* 2001, Bentzer *et al.* 2001, LaPlaca *et al.* 2001, Marklund *et al.* 2001, Faden *et al.* 2001, 2003, Alessandri *et al.* 2002), and various post-traumatic treatments (Dietrich *et al.* 1994a, Bramlett and Dietrich 1997, Philips *et al.* 2001, Knoblach and Faden 2002, Hicks and Zhang 2002, Hicks *et al.* 2002, Rice *et al.* 2002).

To date, a series of behavioral, cellular and molecular studies have revealed significant effects of EE on rodents and other species, and provided new insights into the mechanisms of experienced-dependent plasticity, including adult neurogenesis and synaptic plasticity. Interestingly, EE seemed to improve the outcome even if it is applied 15 days before TBI (Johnson *et al.* 2013).

At the behavioral level, EE enhances learning and memory (Moser *et al.* 1997, Rampon *et al.* 2000a,b, Tang *et al.* 2001, Schrijver *et al.* 2002, Lee *et al.* 2003), reduces memory decline (Bennett *et al.* 2006), decreases anxiety, and increases exploratory activity (Chapillon *et al.* 1999, Roy *et al.* 2001, Benaroya-Milshtein 2004, Friske and Gammie 2005).

Similarly, several studies have investigated the effects of EE of functional recovery after experimental models of TBI. Meanwhile, there is a body of evidence that that EE following TBI enhances functional outcome and attenuates both motor and cognitive deficits (Schwartz 1964, Will *et al.* 1976, Whishaw *et al.* 1984, Kolb and Gibb 1991, Hamm and Temple 1996, van Rijzingen *et al.* 1997, Passineau and Green 2001, Wagner *et al.* 2002, Kozlowski *et al.* 2004), in which early, but not immediate onset seemed to be of advantage (Matter *et al.* 2011). A recent study combined EE with a transplantation of murine embryonic stem cells 7 days after brain injury in rats and pointed out a further advantage compared to sole EE stimulation (Peruzzaro *et al.* 2013).

Early experiments in wild-type rodents investigating the effects of differential housing showed that EE altered cortical weight and thickness (Bennett *et al.* 1969, Diamond *et al.* 1972, 1976, van Praag *et al.* 2000). Enrichment following TBI has been shown to have beneficial effects on the brain, such as preservation of tissue integrity, decreasing lesion size (Passineau and Green 2001), enhancing dendritic branching and the size of synapses (Greenough and Volkmar 1973, Greenough *et al.* 1973, 1985, Conn**o**r *et al.* 1982, Turner and Greenough 1985, Kolb and Gibb 1991, Rampon *et al.* 2000a,b, Faherty *et al.* 2003, Leggio *et al.* 2005), promoting the survival of progenitor cells (Gaulke *et al.* 2005), increasing neurotrophins (BDNF, NGF)-that both play integral roles in neuronal signaling (Torasdotter *et al.* 1998, Pham *et al.* 1999, Ickes *et al.* 2000, Chen *et al.* 2005), and decreasing DAT levels (Wagner *et al.* 2005). Furthermore, EE has been associated with increased neurogenesis and integration of these newly born cells into functional circuits (Kempermann and Kuhn 1997, Kempermann *et al.* 1998a,b, 2002, van Praag *et al.* 2000, Bruel-Jungermann *et al.* 2005). In both experimental groups, a loss of cortical tissue was observed at 90 days post injury, but EE was comparably associated with a substantial preservation of cortical thickness at the lesion site. Animals in the SH paradigm did exhibit a markedly more pronounced loss of hippocampal tissue compared to them in EE. Possible explanations for this may be that EE attenuated the acute and delayed tissue damage having neuroprotective properties (Pierce and Trojanowski 1996, Bramlett and Dietrich 1997, Passineau and Green 2001, Hicks and Zhang 2002, Hicks *et al.* 2002). It enhanced the regenerative plasticity responses of the brain to injury and thus promoted not only functional but also histomorphological recovery (Nilsson and Perfilieva 1999, Passineau and Green 2001). The

limitation of this study is certainly the qualitative description of ventricular enlargement, astrocytosis and hippocampus tissue loss without quantifying these changes.

Conclusion

In the present study, animals after LFP brain injury stimulated by EE performed significantly better in three out of four standardized tests to assess sensorimotor and neuromotor function, as well as spatial reference memory after 90 days of recovery. Early observation time points may overestimate the amount of sustained recovery and it may be prudent to include longer survival times into future studies concerning the outcome after treatments of TBI to account for the phenomenon of progressive worsening of neurofunctional and histological outcome parameters over time. Although effective treatments in the acute setting are clearly needed, this study shows that the window of opportunity may be wide and also lends further credibility to the importance of long term interventions in patients suffering from TBI. Continuous cognitive and physical training (physiotherapy, ergotherapy, etc.) on the basis of social integration and supporting medication may to lead to a slow but continuous recovery which is even effective in long-term duration.

Conflict of Interest

There is no conflict of interest.

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