

## **Long-term Effects of Enriched Environment on Neurofunctional Outcome and CNS lesion volume after traumatic brain injury in rats**

Marc Maegele<sup>1,2</sup>, Maximilian Braun<sup>1</sup>, Arasch Wafaisade<sup>1,2</sup>, Nadine Schäfer<sup>1</sup>, Marcela Lippert-Gruener<sup>3</sup>, Christian Kreipke<sup>4</sup>, Jose Rafols<sup>4</sup>, Ute Schäfer<sup>5</sup>, Doychin N. Angelov<sup>6</sup>, Ewa Klara Stuermer<sup>1</sup>

<sup>1</sup>Institute for Research in Operative Medicine (IFOM), University of Witten-Herdecke (Campus Cologne-Merheim), Cologne (Germany)

<sup>2</sup>Department for Traumatology and Orthopedic Surgery, Cologne-Merheim Medical Center (CMMC), University Witten-Herdecke (Campus Cologne-Merheim), Cologne (Germany)

<sup>3</sup>Department for Neurosurgery, University of Cologne Medical Center, Cologne (Germany)

<sup>4</sup>Department of Anatomy, Wayne State University School of Medicine, Detroit, Michigan (USA)

<sup>5</sup>Experimental Neurotraumatology, University Hospital for Neurosurgery, Medical University Graz, Graz (Austria)

<sup>6</sup>Institut I for Anatomy, University of Cologne, Cologne (Germany)

### **Correspondence to:**

Marc Maegele

Department of Traumatology and Orthopedic Surgery

University of Witten/Herdecke

Cologne-Merheim Medical Center (CMMC)

Institute for Research in Operative Medicine (IFOM)

Ostmerheimerstr. 200, D-51109 Cologne, Germany

Telephone: +49-221 8907 13614; FAX: +49 221 989 5721

E-Mail: Marc.Maegele@t-online.de

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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) versus standard housing (SH). Male Sprague Dawley rats (300-350g, 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 versus 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.

## **Keywords**

Traumatic brain injury; enriched environment; controlled cortical impact; neurobehavioral; functional recovery

## **Introduction**

Traumatic brain injury (TBI) continues to be the leading cause of death and long-term disability world-wide (Waxweiler 1995; Bruns 2003; Langlois 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 1996; Bruns 2003). Only 1/4 patients of the total may reach good recovery with no or only minimal deficits. Thus, TBI represents a highly relevant medical and socioeconomic burden for modern societies (Murray 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 (Dixon 1987; McIntosh 1989; Dietrich 1996; Pierce 1996), numerous investigations describe the major histopathological consequences to trauma: lesion-induced vascular perturbations (Cortez 1989; Schmidt 1993; Fukida 1995), glial hypertrophy and proliferation (Hill 1996), and neuronal necrosis (Soares 1992, 1995; Dixon 1987; Dietrich 1994 a,b,c; Hicks 1996; McIntosh 1989). These reports also confirm that, causing profound cell death (necrotic and apoptotic) and axonal degeneration throughout the brain (Cortez 1989; Dietrich 1994a; Soares 1995; Hicks 1996; Conti 1998), the LFP model is the one that most closely mirrors postlesional events associated with TBI in humans (Dietrich 1996; McIntosh 1989; Pierce 1996; Graham 2000).

Voluminous experimental work has been conducted to characterize new neurobiological events after TBI (Saatman 2001; Stein 2002), unknown effect(s) of different pharmacological trials (Wahl 2000; Belayev 2001; Bentzer 2001; LaPlaca 2001; Marklund 2001; Alessandri et al., 2002; Faden et al., 2001, 2003), and various post-traumatic treatments (Dietrich et al., 1994a; Bramlett et al., 1997; Philips et al., 2001; Knoblich and Faden, 2002; Hicks et al., 2002a and b; 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 et al. (1964), Rosenzweig (1966) and Dobbing (1970). To date, a series of behavioural, 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 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 1992; Kempermann 1997; Nilsson 1999). At the behavioural level, EE enhances learning and memory (Mysliveček 1987; Moser 1997; Rampon 2000; Tang 2001; Schreijver 2002; Lee 2003), reduces memory decline (Bennet 2006), decreases anxiety, and increases exploratory activity (Chapillon 1999; Roy 2001; Benaroya-Milshtein 2004; Friske 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 sequelae 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 (see 2.1.4.; Nithianantharajah 2006), the present research proposal aimed to further investigate the benefits associated with EE, that have been observed up to 30 days post-injury (Maegle 2005a,b; Lippert-Gruener 2006a,b, 2007), 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 2000, 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 priori 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 2000) and has been described previously (Dixon 1987; McIntosh 1989). In brief, under anesthesia with sodium pentobarbital (60mg/kg BW i.p.), animals were fixated in a stereotaxic frame (Kopf Instruments, Tujunga, CA). After incision of the scalp, the temporal muscles were reflected and a 4.8mm craniotomy, 2.5mm lateral to the sagittal 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 skull, the incision closed by interrupted 4.0 silk sutures and the animals placed onto a heated pad for 1h 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 (425x266x185 mm<sup>3</sup>; 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, 610x435x215 mm<sup>3</sup> 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 12h 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 1992; Sinson 1995) and results correlate with injury severity (Sullivan 1976; McIntosh 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 24h 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 24h 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 1957; Jones 1968). For the test a series of three trials with at least 5 minutes 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 seconds. 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 sec. The mean duration (in sec), 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 minutes before a new trial was started. Baseline values were recorded 24h prior to injury. The extent of sensorimotor impairment was surveyed at 24h 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 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 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 122cm in diameter, with 18 evenly spaced holes, 7cm 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 (Bastian 2006). 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 sec) 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 asymmetry test, Cylinder test)*

Experimental LFP brain injury is known to be associated with reduced contralateral forelimb motor function and coordination (McIntosh 1989; Pierce 1998; Schallert 2000; DeBow 2003; Grow 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 minutes while exploring a transparent glass cylinder (25cm 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 24h prior and 24h 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 seconds 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 pre-cut 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 - 0.3mm) the caudal cut was made approximately 8mm 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  $-80^{\circ}\text{C}$ ,  $40\mu\text{m}$ -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  $40\mu\text{m}$ -thick, free-floating sections. After sections were washed in 1xPBS quenching of endogenous peroxidase was performed using  $500\mu\text{l}$  per section of 3%  $\text{H}_2\text{O}_2$  in water (Sigma-Aldrich CO, St. Louis, MO, USA) for 5 minutes, followed by thorough washing in 1xPBS. Sections were then incubated in  $500\mu\text{l}$  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 24h. 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 minutes each. Visualization was performed with 3.3'-DAB (Sigmafast<sup>TM</sup>, 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<sup>TM</sup> slides (Fisher Scientific, Pittsburgh, PA, USA), air-dried, cleaned in Xylenes and coverslipped using Permount<sup>®</sup> (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 1989; Hicks 1996; Smith 1997). In accordance to the fractionators sampling strategy we stained every 20<sup>th</sup> 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 1988), multiplying the total lesioned area

( $\mu\text{m}^2$ ) by the mean section thickness (40  $\mu\text{m}$ ). 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:  $V = A d = Pa(p) d$ . 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  $\mu\text{m}$  to estimate the total lesion area in  $\mu\text{m}^2$ . Image acquisition for quantification of cortical thickness was performed using a Nikon D3 with AF-S Micro Nikkor 60mm 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 24h 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:  $28 \pm 0.0$ ; SH:  $12.2 \pm 1.0$ ; EE:  $11.7 \pm 1.1$ ; 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) (Figure 1). Within the further sequelae 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 sensorimotoric test (dpi-1: 2.49±1.42m, 23.07±447rpm, 49.02±14.9s; dpi +90: 3.04±1.5m, 26.74±4.99rpm, 55.80±15.51s). In the Rota-Rod test at 24h 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±7.51s). Recovery in EE animals was more pronounced and statistically significant ( $p=0.04$ ) for distance (EE: 1.08m; SH: 0.59m) and time (EE: 31.54±7.66s; SH: 21.61±8.85s) at day 15 post injury while the amount of recovery in SH animals was not (Figure 2). Both injured groups showed stagnating sensorimotor coordination in the RotaRod test from day 7 to day 15 and exhibited diminishing performance in all 3 dimensions at day 90 (EE: 0.71±0.36m, 12.82±3.84rpm, 24.46±7.85s; SH 0.66±0.61m, 11,81±5.57rpm, 22.36±11.51s) 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±0.67m, 25.09±3.38rpm, 50.33±7.51s; SHAM+EE: 2.72±0.82m, 28.76±1rpm.35, 52.72±7.79s).

#### *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 seconds while animals in SH animals after 158.8 ±92.0 seconds. Sham operated animals had located the escape box after 129.3 ±113.7 seconds 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 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 (Figure 3). At BCM day 5 EE animals and sham operated animals showed similar latencies and errors ( $8.7\pm 6.1$  sec. vs.  $13.2\pm 8.2$  sec. and  $1.9\pm 2.4$  vs.  $2.2\pm 1.4$  errors). Animals in the SH-group performed notably worse on day 5 of the BCM with respect to latency ( $27.5\pm 14.4$  seconds) and errors ( $3.1\pm 1.8$ ). 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:  $9.33\pm 5.81$ s,  $2.25\pm 2.07$  errors; SHAM+SH:  $11.1\pm 2.68$ s,  $2.25\pm 1.51$  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% (Figure 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:  $39.5\pm 0.24\%$  ; EE:  $38.0\pm 28\%$ ). 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:  $33.7\pm 26\%$ ; EE:  $43.4\pm 18\%$ ). 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:  $48.1\pm 0.7\%$ ; SHAM+EE:  $49.5\pm 0.9\%$ ). 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  $87.5\pm 5.5$  mm<sup>3</sup> while animals in EE had a mean cortical volume of  $104.1\pm 4.1$ mm<sup>3</sup> ( $p = 0,003$ ) on day 90 post injury (Figure 5D). Representative sections at Bregma level  $-3.14$  for one animal from each experimental group were shown (Figure 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.

## **Discussion**

Environmental enrichment has been shown to have numerous beneficial effects on brain and behaviour. 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 1997; Pierce 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 2005; Lippert-Gruener 2007). The present study investigating the potential of long-term effects associated with EE after LFP brain injury in rats underlines the findings of Kovesdi (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 sensori-/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 1996; Maegele 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. Histomorphologically, 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 1998; Lippert-Gruener 2007). 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 2001; Stein 2002), unknown effect(s) of different pharmacological trials (Wahl 2000; Belayev 2001; Bentzer 2001; LaPlaca 2001; Marklund 2001; Alessandri 2002; Faden 2001, 2003), and various post-traumatic treatments (Dietrich 1994a; Bramlett 1997; Philips 2001; Knobloch 2002; Hicks 2002a& b; Rice 2002). To date, a series of behavioural, cellular and molecular studies have revealed significant effects of EE on rodents and other species, and provided new insights into the mechanisms of experience-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 2013).

At the behavioural level, EE enhances learning and memory (Moser 1997; Rampon 2000; Tang 2001; Schreijver 2002; Lee 2003), reduces memory decline (Bennet 2006), decreases anxiety, and increases exploratory activity (Chapillon 1999; Roy 2001; Benaroya-Milshtein 2004; Fiske 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 1976; Whishaw 1984; Kolb 1991; Hamm 1996; van Rijzingen 1997; Passineau 2001; Wagner 2002; Kozlowski 2004), in which early, but not immediate onset seemed to be of advantage (Matter 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 2013).

Early experiments in wild-type rodents investigating the effects of differential housing showed that EE altered cortical weight and thickness (Bennet 1969; Diamond 1972 and 1976; van Praag 2000).

Enrichment following TBI has been shown to have beneficial effects on the brain, such as preservation of tissue integrity, decreasing lesion size (Passineau 2001), enhancing dendritic branching and the size of synapses (Greenough 1973, 1985; Conner 1982; Turner and 1985; Kolb 1991; Rampon 2000; Faherty 2003; Leggio 2005), promoting the survival of progenitor cells (Gaulke 2005), increasing neurotrophins (BDNF, NGF)-that both play integral roles in neuronal signalling (Torasdotter 1998; Pham 1999; Ickes 2000; Chen 2005), and decreasing DAT levels (Wagner 2005). Furthermore, EE has been associated with increased neurogenesis and integration of these newly born cells into functional circuits (Kempermann 1997, 1998a&b, 2002; van Praag 2000; Bruel-Jungermann 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 1996; Bramlett 1997; Passineau 2001; Hicks 2002). It enhanced the regenerative plasticity responses of the brain to injury and thus promoted not only functional but also histomorphological recovery (Nilsson et al. 1999; Passineau et al. 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.



## Figure Legends

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

**Figure 2:** RotaRod test to evaluate sensorimotor coordination and recovery at baseline, 24h 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  $\pm$  SD for time (seconds), distance traveled (meter) and speed (rounds per minute, rpm). \*  $p < 0.05$  vs. SH

**Figure 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  $\pm$  SD. \*  $p < 0.05$  vs. SH; # $p < 0.05$  vs. SHAM.

**Figure 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 limb 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).

**Figure 5:** Rat brain sections at Bregma -3.14 from a representative sham-operated animal (A), a SH (B) and a 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  $\text{mm}^3$  for animals housed in SH and EE (n=4 per group) calculated according to the Cavalieri method (Gundersen et al., 1988). Boxplots show median  $\pm$  SD; whiskers show extrema. \*  $p < 0.05$  vs. SH

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Figure 1

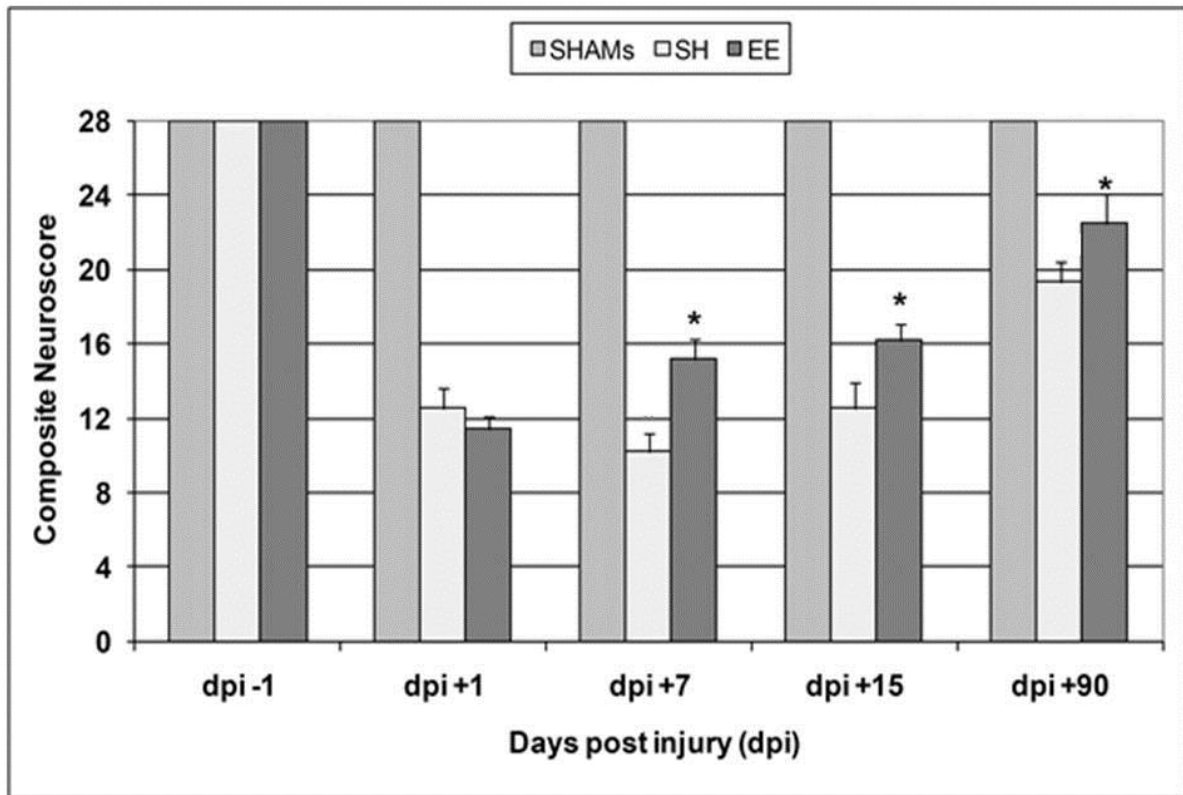


Figure 2

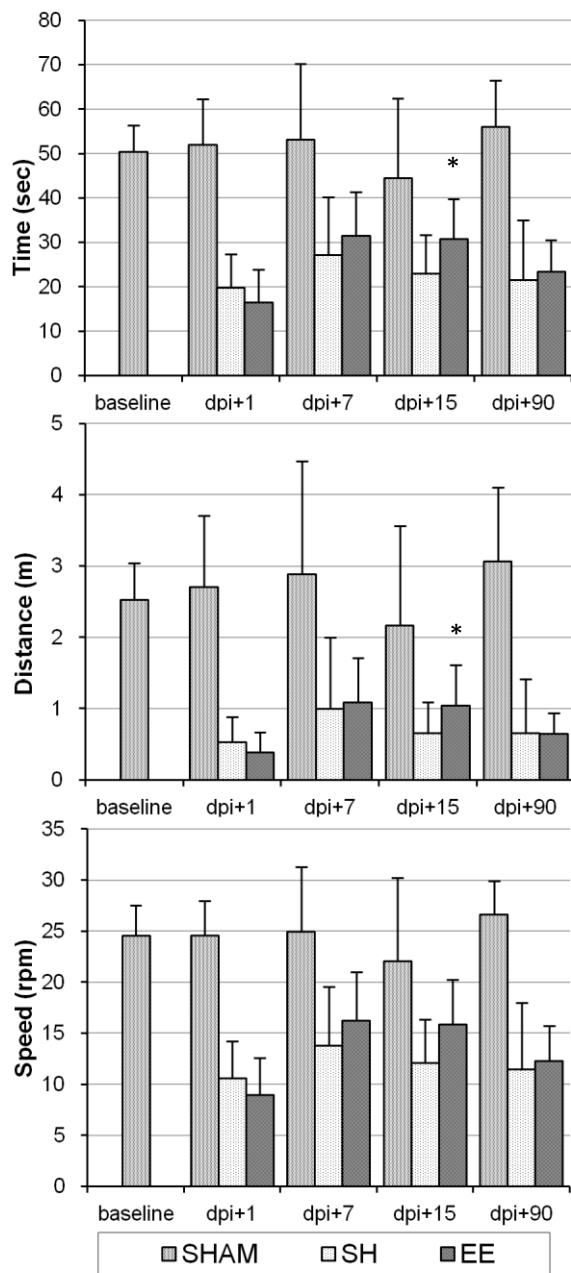


Figure 3

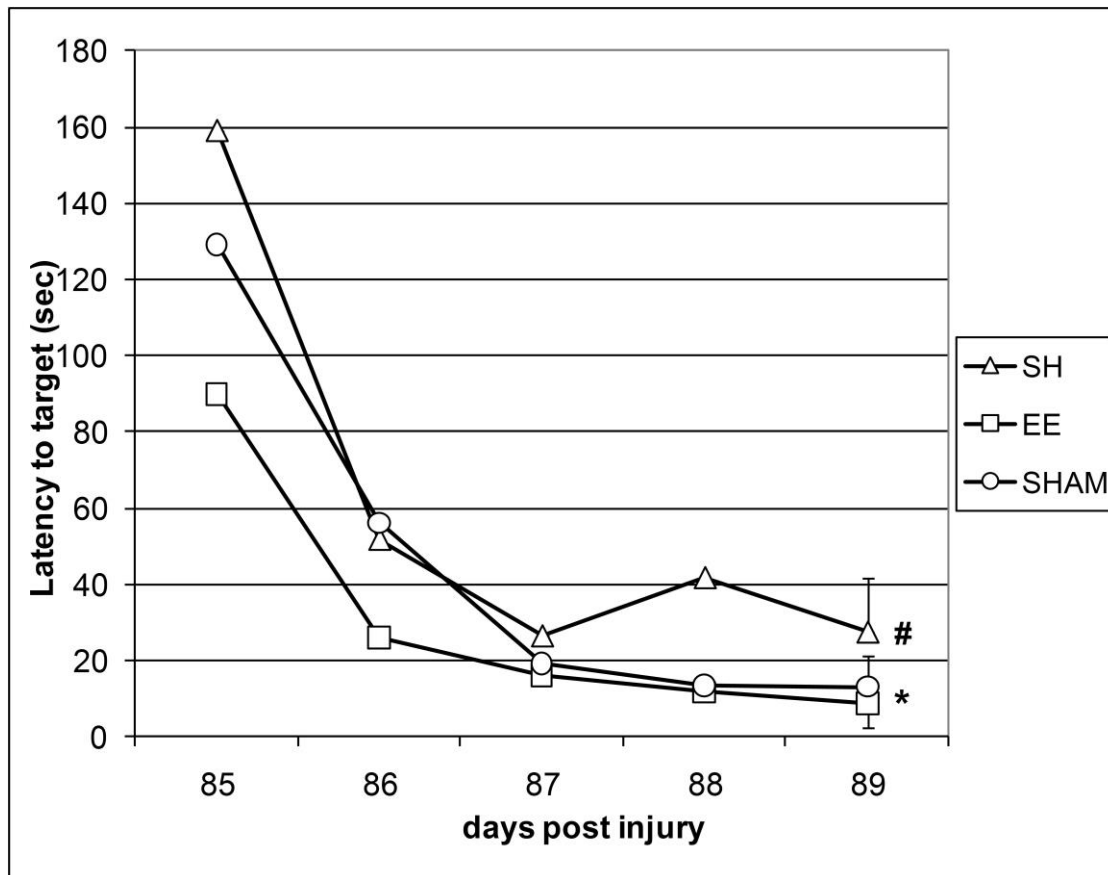


Figure 4

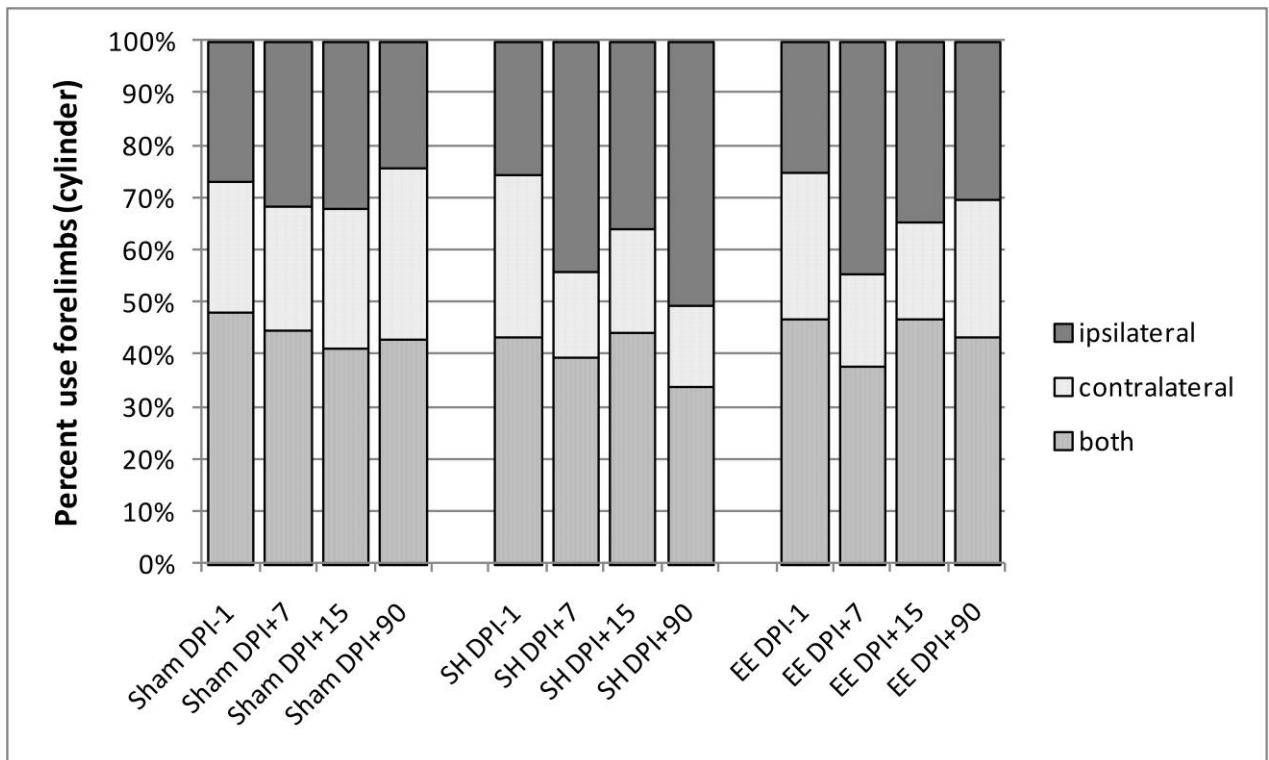


Figure 5

