Combination of growth factor treatment and scaffold deposition following traumatic brain injury has only a temporary effect on regeneration

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Abstract

The recovery after traumatic brain injury (TBI) is hampered by the poor regenerative capacity of the brain. Today there is no treatment available that effectively restores lost brain tissue, but much research is focused on the stimulation of endogenous neural stem cells to viably and functionally repopulate the injured parenchyma. It is crucial that the therapies have a proven long-term effect on both regeneration and functional recovery to be clinically interesting. Here we have studied the induction of stem cell activation in rats at three weeks and six weeks after inducing TBI using controlled cortical impact model at a severe setting. We combined intracerebroventricular growth factor and scaffold treatment in order to accomplish an optimal effect on the stem cell regeneration. Immediately after TBI epidermal growth factor infusion with osmotic minipumps was started and continued for seven days. The pumps were removed and an extracellular matrix scaffold containing vascular endothelial growth factor was deposited into the cortical cavity. Three weeks after injury there was a positive effect of the treatment with a significant increase in neuronal and astrocytic regeneration. However, after six weeks there was no difference in the number of newly generated neurons and astrocytes in treated or untreated rats. Evaluation of tissue loss and spatial learning in the Morris water maze corroborated that the treatment had no effect at the later time point. Our results highlight the importance of long-term studies to ensure that a promising effect on tissue regeneration and functional outcome is not only temporary.

1. Introduction

Traumatic brain injury (TBI), caused by e.g., motor vehicle accidents and falls, is the leading cause of death and disabilities in young adults in the industrialized world. Many of the survivors suffer from severe disabilities for the rest of their lives, including motor deficits, impaired cognitive function and memory loss. Today there is no pharmacological treatment available to protect or restore the injured brain tissue after TBI, but there is hope in being able to use stem cell therapies to promote regeneration in the future. However, to be realized in a clinical setting, neural stem cell
therapies need to have a proven long-term effect on neuronal regeneration and functional outcome.

It is now well established that neurogenesis in the adult brain persists throughout the entire life in the regions of the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus (Yao et al., 2012). Furthermore, it has been shown that the amount of newly generated cells can be modulated by neuropathological conditions, for example, enhanced cell proliferation has been demonstrated after experimental TBI (Blizzard et al., 2011; Chirumamilla et al., 2002; Ramaswamy et al., 2005). However, it is not clear whether this activation leads to a stable and productive neurogenesis as probably only a very limited number of surviving neurons integrate properly after injury (Kernie and Parent, 2010).

Much research has been dedicated to identifying and evaluating the effects of growth factors and other substances on endogenous stem cell proliferation and how these effects could produce functional neurons that would repair tissue and improve function. For example intracerebroventricular (i.c.v.) infusion of epidermal growth factor (EGF) has been shown to increase the number of bromodeoxyuridine (BrdU) positive cells in the SVZ and the DG one week after TBI in rats. However, four weeks after trauma the authors did not find any difference in the number of BrdU positive cells compared to untreated controls. The EGF-treatment also resulted in improved functional outcome three weeks after trauma, but no experiments were performed at later time points (Sun et al., 2010). Similar to EGF, vascular endothelial growth factor (VEGF) has been reported to stimulate adult neurogenesis and it has also been shown to act as a chemoattractant for neural stem cells in vitro (Jin et al., 2002; Zhang et al., 2003). Moreover, VEGF is known to be the key factor in the regulation of endothelial cells and vessel formation (Karamysheva, 2008). Inducing over-expression of VEGF-A using adenoviral delivery of a specific promotor was recently shown to decrease motor deficits and reduce cell death after TBI in rats (Siddiq et al., 2012). These previous investigations clearly indicate that both EGF and VEGF are

Fig. 1 – The rats were sacrificed for tissue analysis at either day 21 or day 42 post-injury (Perfusion). In order to label dividing stem and precursor cells the rats received daily BrdU injections during the first week following CCI (BrdU). To test the spatial learning Morris water maze (MWM) experiments were performed days 36–39 post-injury (A). In GF-treated rats an osmotic mini pump with EGF was connected to the contralateral lateral ventricle directly after the CCI. At day 7 the EGF-pump was removed and the cortical cavity was filled with an ECM-scaffold supplemented with VEGF. (B) TBI control rats had their mini-pumps filled with saline and received no scaffold at day 7. (C) Sham operated controls received the surgery, but neither pump nor scaffold. (D) Selected fields around the injury site were photographed and analyzed.
interesting as potential neuroprotectors and stem cell activators after acute brain injury.

Moderate to severe focal injury results in the formation of a cavity (Marklund et al., 2001), that continues to expand up to a year after TBI in rats (Dixon et al., 1999). Placing an extracellular matrix scaffold into the cavity may support the surrounding brain tissue, function as a substrate for cell growth, axon regeneration and neurite formation and allow cell infiltration (Orive et al., 2009). Extracellular matrix (ECM) supplies critical chemical and physical signals to initiate or sustain cellular functions and many novel biomaterials are based on various ECM components.

In the present study we have investigated the capacity of endogenous neural stem cells to form new neurons and glia after TBI under favorable conditions by combining growth factor treatment and deposition of a scaffold to the injury site. The scaffold we used is a cell produced ECM gel that resembles the ECM of the brain and should be favorable for the infiltrating cells. The major aim with the study was to elucidate whether increased neurogenesis during the first weeks after injury also result in a long-lasting effect on tissue regeneration and functional recovery after TBI.

2. Results

In this study we wanted to investigate the effects of a combined therapy of growth factors and an ECM scaffold on

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Fig. 2 – Immunofluorescence stainings with specific antibodies to BrdU (green) and the neuronal marker NeuN (red) were performed to identify the newly formed neurons at day 21 (A–F) and day 42 (G–L) after CCI. At day 21, BrdU positive neurons (white arrow heads) are present in the injured cortex in both the injured controls (A–C) and GF-treated rats (D–F). At day 42 no or few BrdU positive neurons were found (G–L). Scale bar = 10 μm.
the capacity of endogenous neural stem cells to regenerate lost brain tissue after experimental TBI in male rats. Directly after injury an osmotic mini pump was connected to the contralateral lateral ventricle and an infusion of EGF was started. At day 7 we removed the pumps and filled the cortical cavity with an ECM-gel supplemented with VEGF, hereafter called GF-treated animals (Fig. 1A). TBI control rats had their mini-pumps filled with saline and received no scaffold at day 7, hereafter called injured controls (Fig. 1B). Sham operated controls received the craniotomy surgery, but not CCI, pump or scaffold, hereafter called sham animals (Fig. 1C). In order to follow regeneration of neurons and glia by immunohistological analyses, the animals received daily BrdU injections the week after CCI and were sacrificed by immunohistological analyses, the animals received daily BrdU injections the week after CCI and were sacrificed by immunohistological analyses, the animals received daily BrdU injections the week after CCI and were sacrificed by immunohistological analyses, the animals received daily BrdU injections the week after CCI and were sacrificed by immunohistological analyses, the animals received daily BrdU injections the week after CCI and were sacrificed by immunohistological analyses.

2.1. Scaffold and GF treatment causes an initial increase in neurogenesis after TBI

Immunofluorescence stainings with specific antibodies to BrdU and NeuN were performed to quantify the generation of neurons after TBI around the injury site (Fig. 1D). Representative high-magnification pictures (Fig. 2A–F) show BrdU positive neurons present in the injured cortex three weeks after TBI, in both the GF-treated and injured control animals. Low-magnification images of the BrdU/NeuN stainings are shown in Supplementary Fig. 1. Counting NeuN/BrdU double positive cells revealed a threefold increase in the number of new neurons in the GF-treated rats compared to the untreated rats (Fig. 3). Interestingly, the same experiments in sections from rats sacrificed 6 weeks after TBI show a very different result, with no or few BrdU positive neurons present (Fig. 2G–I). The cell counts revealed that there are no BrdU positive neurons left in the tissue, showing that the new neurons that are generated during the first weeks after injury do not survive once the scaffold has been degraded (Fig. 3). Moreover, the number of doublecortin (DCX) neuroblasts in the GF-treated rats and injured controls 6 weeks after injury showed no significant difference between the groups. In control animals there was 5.9±3.1 and in GF-treated rats there was 3.1±2.4 positive cells/field

(p=0.417). Quantification of the total number of neurons around the injury site indicates that the treatment have a sparse protective effect on the neurons at the injury site. At day 21 we did not find any significant difference between the treated and the untreated animals (63.5±29.2 compared to 60.5±23.3 neurons/field, p=0.351), but at day 42 we found slightly more neurons in the treated animals than in the untreated controls (66.8±23.0 and 51.4±25.1 neurons/field respectively, p=0.020) (Fig. 4). VEGF is known to be the key factor in the regulation of endothelial cells and vessel formation (Karamysheva, 2008), but brain sections immunostained for the blood vessel marker RECA-1 did not show any differences in the blood vessels in the injury zone six weeks post-injury (Supplementary Fig. 2).

2.2. GF treatment causes no long-term increase in glial cell generation

Tissue sections from three or six weeks after TBI were double labeled with antibodies to BrdU and GFAP. Representative pictures show that there are many BrdU positive astrocytes in both treated and untreated animals at three weeks after TBI (Fig. 5A–F). Animals sacrificed at six weeks after injury have fewer double positive cells compared to injured control animals sacrificed after three weeks (Fig. 5G–I). Low-magnification images of the BrdU/GFAP stainings are shown in Supplementary Fig. 3. Quantification of the number of BrdU+/GFAP+ cells show that there is a significant increase in new astrocytes three weeks after TBI in the GF-treated group compared to the injured controls (Fig. 6). After six weeks there is a significant decrease in double positive cells in both groups compared to the situation at three weeks after injury (Fig. 6). The decrease over time in the GF-treated animals is more dramatic than for the injured controls and at six weeks there is no longer a significant difference in the number of BrdU+ astrocytes between the two treatment groups (Fig. 6). These results demonstrate that the treatment only has a temporary effect on glial regeneration/proliferation after TBI, and that the astrocytes that are formed during the first weeks after injury

Fig. 3 – Quantification of the number of NeuN/BrdU double positive cells revealed a threefold increase in the number of new neurons in the GF-treated rats compared to the injured control rats at day 21 post-TBI. At day 42 after injury there are however no BrdU positive neurons left.

Fig. 4 – Quantification of the total number of NeuN positive cells indicates that the treatment have a sparse protective effect on the neurons at the injury site. At day 21 there were no significant difference between the treated and the untreated animals, but at day 42 we found slightly more neurons in the treated animals than in the untreated controls.

Fig. 5 – Quantification of the number of GFAP positive cells in the GF-treated and injured control animals at three weeks after TBI. At day 21 after injury there are similar number of GFAP+ cells in both groups, but at day 42 we found slightly more GFAP+ cells in the GF-treated animals.
have a better survival rate than the new neurons. Immunofluorescence stainings with specific antibodies to OX42 show that GF-treatment has no clear effect on the microglia/macrophages activation following TBI (Supplementary Fig. 4).

2.3. GF-treatment attenuates tissue loss three weeks after TBI but has no long-term effect

In order to determine if the treatment reduces the tissue loss after CCI, we measured the volume of the injured hemisphere and compared it to the uninjured hemisphere at three and six weeks after CCI. In GF-treated rats there was a reduction of tissue loss compared to injured control rats at three weeks (12.5 ± 5.2% vs. 21.2 ± 3.7%), but it failed to reach significance (p=0.15). At six weeks after trauma the tissue loss was actually higher in the GF-treated group, 27.7 ± 2.3% compared to the injured controls 24.4 ± 3.0%, but again with no statistically significant difference present (p=0.38) (Fig. 7). The lesion volume was significantly larger at six weeks compared to three weeks for both the GF-treated and injured control groups (p<0.05) (Fig. 7). This demonstrates that the initial increase in BrdU positive astrocytes did not result in any long-term tissue regeneration.

2.4. GF-treatment improves the functional outcome early, but not late after injury

Results from previous studies suggest that EGF, VEGF or scaffold treatment following TBI have a positive effect on the functional outcome. In all these studies the functionality
tests were performed from two to four weeks after injury, which could be considered quite early. Here we tested the outcome of GF-treatment on spatial learning after TBI by Morris water maze (MWM) analysis at two different time points, 16–19 days post-injury (for rats with 21 days survival) and 36–39 days post-injury (for rats with 42 days survival). In the early MWM experiments, we found a significant decrease in the latency to find the platform ($p<0.05$) in GF-treated animals compared to injured controls at day 17–19 (Fig. 8A). The sham injured animals had shorter latencies than both trauma groups at days 16 and 17 ($p<0.05$), but only significantly different from the injured controls at days 18 and 19 ($p<0.01$).

In the late MWM trial, there was a robust injury effect on the latency to find the platform on days 37 to 39, when comparing both trauma groups to the sham injured controls ($p<0.01$; Fig. 8B). All treatment groups showed a significant improvement ($p<0.05$) in mean latency to find the platform from day 36 to day 37. From day 37 and forward no further improvement was found to be statistically significant. GF-treated animals did not differ from injured controls at any time point of the learning trials, demonstrating that the combined GF and scaffold-treatment had no effect on functional recovery after 6 weeks (Fig. 8B). No motor deficits were noticed as no difference in swim speed between the treatment groups was found on any of the days in the two timepoints of the experiments (data not shown).

3. Discussion

At the time of impact, TBI results in death of neurons and glial cells, capillary ruptures and widespread axonal damage. This primary injury is then followed by a complex secondary injury cascade that progresses for weeks post-ictus, leaving the patient with a much larger brain lesion (Maas et al., 2008). Endogenous repair mechanisms based on the proliferation of neural stem cells have been identified in the injured brain (Kernie and Parent, 2010). Unfortunately, this spontaneous neurogenesis is not sufficient to induce clinically significant recovery. Increasing the physiological neurogenesis by administration of various growth factors has been demonstrated to improve functional outcome during the first couple of weeks post-TBI (Kernie and Parent, 2010; Sun et al., 2009, 2010). Although, the long-term effects of drug-induced regeneration remains unclear.

In the present study we evaluated the effect of growth factor and scaffold treatment three and six weeks after CCI in rats, with the aim to compare the results from the two time points. By combining growth factor infusion and the deposition of ECM-scaffold to the injury site our intention was to accomplish a synergistic effect on endogenous stem cells resulting in improved regeneration, differentiation and cell survival. EGF is known to be a potent mitogen for adult neural stem cells, and i.c.v. infusion for one week will greatly expand the stem cell pool around the ventricles (Craig et al., 1996). Based on the results of others we hypothesized that VEGF would induce migration of the expanded stem cell population to the injury site (Jin et al., 2002; Siddiq et al., 2012; Zhang et al., 2003), where differentiation and survival of the stem cells into neurons and glia would be supported by the ECM-scaffold. The ECM-scaffold has previously been extensively used to promote stem cell adhesion and differentiation.
in vitro (Uemura et al., 2010). Moreover, many factors in the ECM are known to be important for cell survival, for example it has been demonstrated that both fibronectin and laminin have a clear anti-apoptotic effect on neuronal cells in vitro (Gibson et al., 2005; Hall et al., 2008).

A number of studies have been published using different scaffolds based on gelatin, hyaluronic acid, collagen or novel biomaterials (Orive et al., 2009). However, most of the studies have used cortical excision to create a cavity to deposit the scaffold into (Guo et al., 2009; Wong et al., 2007, 2008), creating a situation that is very different from the cavity formation seen after focal TBI. A scaffold made of a hyaluronic acid hydrogel modified with laminin was suggested to reduce the glial scar when it was implanted directly after cortical excision in rats (Hou et al., 2005). In a well characterized model of penetrating brain injury (PBI) Elias and Spector used a type 1 collagen scaffold with or without soluble Nogo receptor 1 (sNgR) (Elias and Spector, 2012), showing a clear infiltration of glia and endothelium into the injury site in the rats receiving the scaffold with sNgR. In another study, experiments with collagen scaffolds showed no reduction of the lesion size or improvement in functional outcome, unless they were seeded with mesenchymal stromal cells (Lu et al., 2007). Several studies have used scaffolds containing different types of progenitor cells in order to increase transplantation efficiency and it has been shown that the survival of transplanted neural stem cells was significantly higher when delivered within a collagen–laminin or collagen–fibronectin scaffold (Tate et al., 2009). Furthermore, transplantation of human bone marrow stromal cells with collagen scaffolds was shown to have an effect on functional outcome during the first two weeks after TBI while transplantation without scaffold had no effect (Mahmood et al., 2011; Qu et al., 2011).

In line with a previous investigation on the effect of EGF treatment after TBI (Sun et al., 2010), we found a significant increase in regeneration of neurons and astrocytes three weeks after injury, which correlated with an improvement in functional outcome as measured by the Morris water maze. However, in contrast to our encouraging findings at three weeks after injury the result from six weeks after injury was discouraging. At this time point almost no BrdU positive neurons and very few BrdU positive astrocytes were present and there was no longer any difference between the GF-treated group and the injured controls regarding the number of new neurons and astrocytes.

In our tissue loss analyses we found a trend towards a decrease in lesion tissue loss in GF treated animals compared to injured controls at three weeks post-injury, but the reduction was not statistically significant. Quantification of the total number of NeuN positive cells, revealed a slight, but significant increase in the number of neurons around the injury site after 6 weeks, indicating that the combination treatment might have a modest, positive effect on neuronal survival. The increased number of neurons did however not result in an overall reduction of tissue loss.

In order to test if the treatment had any effect on functional outcome after TBI we used the spatial learning test Morris water maze (Morris, 1981). The test is commonly used to evaluate the effect of TBI and potential treatment strategies. Previous studies using growth factor treatment after TBI have shown beneficial effects on MWM performance (Lu et al., 2005; Sinson et al., 1995). The positive effect of EGF infusion and VEGF scaffold that we found in the 16–19 days MWM trial was in accordance with a previous investigation showing that EGF-infusion seven days after TBI in rats had a robust effect on MWM performance at days 21–25 post-injury (Sun et al., 2010). The improvement found by Sun et al. in MWM performance could at least partly be explained by more surviving neurons in the CA3 and hilus of the dentate gyrus in the hippocampus, in GF-treated animals (Sun et al., 2010), however, the authors did not see any increase in neurogenesis 28 days after injury and no MWM experiments

**Fig. 8** - At the first two days the MWM experiment for the 21 days survival animals (day 16 and 17) the sham animals had a significantly shorter latency to find the hidden platform than both injured groups (p<0.05; denoted with a small cross). At day 18 and 19, the sham animals were faster than the injured control group (p<0.01; denoted with two small crosses), but did not differ significantly to the GF-treated animals. At day 17 to 19 the GF-treated animals found the platform significantly quicker than the injured controls (p<0.05; denoted with an asterisk)(A). At the first day of learning trials (day 36) for the 42 days survival animals there were no differences between the treatment groups. On day 37 to day 39 post injury the sham operated animals had a significantly shorter latency to find the hidden platform (p<0.05; denoted with an asterisk). There was no difference between the GF-treated and the injured controls at any time point (B).
were performed at later time points. In a recent study it was demonstrated that upregulation of VEGF following TBI in rats reduced the number of TUNEL positive cells in hippocampus at 72 h and 7 days, but had no positive effect on MWM performance from 10 to 30 days post-trauma (Siddiq et al., 2012). While we found a significant effect of the trauma on MWM performance days 36–39 after injury, we could not see any effect of the treatment at this time point. The injury to the ipsilateral hippocampus was substantial in all animals subjected to TBI, which contributes to a significant part of the learning deficits, as we have shown after a similar injury in rats (Clausen et al., 2005), though an equally large part was dependent on the cortical lesion. As there was no difference in cortical lesion in the trauma groups it was not entirely surprising not to find any difference in MWM performance.

4. Conclusion

Activation and recruitment of endogenous stem cells to the injury site has been suggested as a promising and realistic strategy to create new brain tissue, since it circumvents many ethical issues associated with e.g. embryonic stem cells and also eliminates the potential for immunological incompatibility (Erlandsson and Morshhead, 2006). In the present study we show that combined intracerebroventricular growth factor and scaffold treatment has a positive effect on neuronal and astrocytic regeneration three weeks after traumatic brain injury. However, six weeks after trauma there was no longer a difference in the number of newly generated neurons and astrocytes in treated and untreated rats. Our results highlights the importance of investigating regeneration over longer survival times, since initial positive effects of a treatment on tissue regeneration and functional outcome not necessary indicate that the treatment have a positive long-term effect.

5. Experimental procedures

5.1. Animals

Sprague-Dawley male rats (n=41, weight 330–400 g) were purchased from Taconic M&B laboratory (Ry, Denmark). The animals were allowed free access to water and food and were housed in an animal facility with a 12-hour light/dark cycle. All procedures involving animals were approved by the local ethics committee for animal research and all animals were treated according to guidelines from The National Board for Agriculture.

5.2. Controlled cortical impact

Controlled cortical impact (CCI) was performed as previously described (Dixon et al., 1991; Lighthall et al., 1990). Briefly, isoflurane (4% in air) was used to induce anesthesia. The rats were moved to a stereotaxic frame connected by a rectal probe (CMA150, CMA, Stockholm, Sweden). A midline incision on the scalp was made after a sub-coetaneous injection of bupivacaine (Marcain®, AstraZeneca, Sweden). A craniotomy, 5 × 6 mm², was created 3 mm posterior to bregma and 2 mm lateral to the midline, over the right parietal cortex extended laterally towards the temporal crista. The stereotaxic frame was moved to the CCI-device (VCU Biomedical Engineering Facility, Richmond, Virginia, USA) with a 4.0 mm diameter piston. The injury was produced by a 2.0 mm compression of the brain at a speed of 3 m/s. After the injury, the bone flap was replaced and the wound was closed up with interrupted sutures. Sham-operated animals underwent identical surgery and anesthesia but did not receive the CCI-injury. The weight and wound healing were controlled daily post-surgery until a stable recovery was observed.

5.3. EGF-infusion

Directly after the CCI, EGF treated rats had an infusion cannula (brain infusion kit 2, Alzet) implanted in the lateral ventricle, contralateral to the CCI-injured hemisphere (1 mm posterior and 2 mm lateral from bregma). The cannula was connected to an osmotic mini-pump (Alzet Model 2001, infusion rate 1 μl/h for 7 days) containing recombinant human EGF (10 μg/ml) (BD). The pump was placed subcutaneously on the neck.

5.4. Application of VEGF containing scaffold

One week post-CCI, anesthesia was induced both systemically and locally, as described above. The wound was opened and cleaned and the bone flap was removed. 50 μl ECM gel (Sigma-Aldrich) containing 1 μg/ml VEGF (Sigma-Aldrich) was applied to fill the cavity, after which the bone flap was replaced. The osmotic mini-pump was removed and the wound was sutured. The weight and wound healing were controlled daily post-surgery until a stable recovery was observed.

5.5. BrdU-injections, perfusion and preparation of tissue sections

Both GF treated, untreated and sham rats received 7 bromodeoxyuridine (BrdU) injections (Sigma-Aldrich, 60 mg/kg, i.p.), one every day during the first week post-injury. Animals were sacrificed 21 days (GF-treated rats, n=5 and control rats n=4) and 42 days (GF- treated rats, n=7 and control rats n=7) post-injury by an overdose of pentobarbital sodium (600 mg/kg) and transcardially perfused with PBS, followed by 4% phosphate-buffered formaldehyde (Histolab AB, Gothenburg, Sweden). The brains were frozen and stored at −70 °C before being sectioned coronally at a thickness of 14 μm in a cryostat.

5.6. Calculation of the tissue loss

Eight sections mounted on Superfrost slides and spaced from bregma 1 mm to −6 mm were selected for and stained for hematoxylin and eosin. Cover slips were mounted with Pertex (Histolab Products AB). The sections were digitally
photographed under a stereomicroscope (Stemi 2000, Zeiss GmbH) connected to a computer with the Axiovision 4.8 software (Zeiss GmbH). The sections were measured using the free software ImageJ (NIH). To calculate the volume we used the formula \( V = 1 - \frac{8}{3} \sum (A_{n} + A_{n+1}) / d^2 \), where \( A \) is the area and \( d \) is the distance between sections. Hemispheric tissue loss was calculated as the percentage of the contralateral (uninjured) hemisphere volume \( (V_c) \) using the following formula: \( [(V_c - V_i) / (V_c)]100 \), where \( V_i \) represents the volume of the ipsilateral (injured) hemisphere (Zhang et al., 1998).

5.7. Immunohistochemistry

The primary antibodies used in this study were monoclonal mouse NeuN (1:100, Millipore), polyclonal rabbit-anti-glia fibrillary acidic protein (GFAP) (Dako, 1:200), monoclonal mouse OX42/CD11b (Millipore, 1:200), monoclonal guinea pig-anti-doublecortin (DCX) (Millipore, 1:1000), monoclonal mouse anti-rat RECA-1 (SeroTec, 1:40) and monoclonal rat anti BrdU (Abcam, 1:100). The secondary antibodies were Alexa Fluor 488-goat-anti-guinea pig (Invtrogen, 1:200), Cy3-goat-anti-rabbitIgG (Sigma-Aldrich, 1:200), Cy3-goat-anti-mouse Igg (Sigma-Aldrich, 1:200) and FITC goat-anti-rat IgG (ZyMax, 1:200). Sections used for BrdU-detection, where incubated at 37°C for 30 min with 2 M HCl and washed 3 times in PBS prior to the staining procedure. Permeabilization and blocking was performed for 1 h in RT in 0.3% Triton X-100 in PBS (0.3% Triton/PBS) and 5% NGS. Primary antibodies were diluted in 0.3% Triton/PBS with 0.5% NGS and incubated O/N in 4°C. Sections were thoroughly washed in PBS and incubated 37°C with secondary antibodies (2′ ab) for 1 h and washed in PBS. Following the final staining step the sections were mounted on glass with Vectashield (Vector). Four regions of interest (450 μm x 330 μm, Fig. 1D) in the cerebral cortex around the cavity was photographed using a fluorescence microscope (Zeiss) (40 x) at three different levels/animal, bregma −1, −3 and −5.

5.8. Morris water maze

The rats were handled by the investigator daily for a week prior to the test in order to habituate the animals. Morris water maze was used to evaluate the spatial learning. The training trials were made at days 16–19 after trauma in the animals with 21 days survival time, sham-operation \((n = 7)\), injured controls \((n = 7)\) and GF-treated animals \((n = 7)\). Animals with 42 days survival time were subjected to training trials at days 36–39 after injury, sham-operation \((n = 6)\), injured controls \((n = 7)\) and GF-treated animals \((n = 7)\). The test is designed to train a rodent to find a submerged platform (diameter of 15 cm) in a dark, water-filled pool (diameter 120 cm) surrounded by four drapes with different visual cues. Four training trials of 120 s each were performed per rat and day, with a 30 min inter-trial interval, for four consecutive days. The rat was placed in the pool facing the wall, at four different positions (north, east, south and west). A cc-camera mounted above the pool and connected to a computer was used to digitally record the movement of the rat during the trial. When the rat found the platform and mounted it the trial was over and the rat was allowed to remain there for 15 s in order to learn the location of the platform from the visual cues surrounding the pool. The data collected were stored and processed by a computer with tracking software (HVS Image Ltd.).

5.9. Statistical analyses

The quantifications of the immunostainings and the measurement of tissue loss are presented as mean value ± standard error of the mean (SEM). The levels of significance were determined by unpaired Student’s t-test \( (p < 0.05) \) was designated with one asterisk; \( p < 0.01 \) was designated with two asterisks and \( p < 0.001 \) designated with three asterisks). The data from Morris water maze analyzed using two ways ANOVA for treatment and trial day. Differences between days and groups were further analyzed using Bonferroni’s post hoc test.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2014.08.043.

References


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