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Extract Derived from Rat Brains in the Acute Phase Following Traumatic Brain Injury Impairs Survival of Undifferentiated Stem Cells and Induces Rapid Differentiation of Surviving Cells

Kristine Bentz[1*], Marek Molcanyi[2*], Annette Schneider[3], Peter Riess[4], Marc Maegele[5], Bert Bosche[2], Jürgen A. Hampl[2], Jürgen Hescheler[6], Silke Patz[7] and Ute Schäfer[7]

[1]Videnregion, Syddanskuniversitet, [2]Clinic of Neurosurgery, University of Cologne, [3]Institute for Research in the Operative Medicine, University of Witten/Herdecke, [4]Waldklinik, Department of Trauma and Orthopaedic Surgery, Bonn, [5]Clinic of Trauma and Orthopaedic Surgery, University of Witten/Herdecke, [6]Dept. of Neurophysiology, Medical Faculty, University of Cologne, [7]Research Unit Experimental Neurotraumatology, Medical University Graz, *both authors contributed equally

Key Words
TBI • Stem cell • Differentiation • Inhibition • Oct-4 • Nestin • Fgf5

Abstract
Dramatic cerebral responses following brain injury (TBI) comprise inflammation, cell death, and modulation of trophic factor release. These cerebral modulations might induce and /or attenuate acute neuronal damage. Here, we investigated the effect of tissue extract derived from healthy (HBE) or injured rat brain (TBE) on the differentiation of cultured embryonic stem cells *in vitro*. Rats were sacrificed at t = 45 minutes following lateral fluid-percussion injury and extracts of cerebral tissue were prepared from 4-6 healthy or injured rat brain hemispheres. Murine embryonic stem cells (CGR8) cultured in serum-free medium were then conditioned for a week with HBE or TBE. Omission of serum from the culture medium induced neural differentiation of CGR8 stem cells, as indicated by a significant time dependent down-regulation of *oct-4* with a concomitant upregulation of *nestin* after 7 days. In parallel cell loss was observed that seemed to be largely due to apoptotic cell death. In TBE treated cells, on the other hand, a significant amplification of apoptotic cell death, enhancement of *nestin and MAP2* expression and marked morphological changes such as axonal-like outgrowth was observed within 3 days of conditioning. Treatment of stem cells with HBE resulted in less pronounced neuronal differentiation processes. Axonal-like outgrowth was not observed. Our data suggest that during the early acute phase of traumatic injury the cerebral environment is disposed to detrimental as well as potent protective signals that seem to rapidly induce neurogenic processes.

Introduction

Cell replacement strategies have been demonstrated to be a promising therapeutic approach in the treatment

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Univ.-Prof. Dr. Ute Schäfer
Research Unit Experimental Neurotraumatology, Medical University Graz
Auenbrugger Platz 2, 8036 Graz (Austria)
Tel. 0043 316 38580038; Fax 0043 316 38572085
E-Mail ute.schaefer@medunigraz.at

of experimental traumatic brain injury (TBI). Migration and differentiation of stem cell derived precursors or progenitors [1-5] seem to be accompanied by an improvement of neurological motor function [4, 6, 7] as well as sensorimotor functional recovery [2] in a variety of experimental TBI models.

Despite these promising reports, it has to be noted that in most cases the number of surviving and differentiating cells post-implantation is insignificant and cannot always explain the functional improvement reported. Stem cell differentiation seems to be not just defined but also restricted by the host environment. This assumption is underlined by reports describing the post-traumatic brain as a hostile environment due to the onset of an acute inflammatory response [8, 9]. The post-traumatic inflammatory response is associated with an activation of immunocompetent cells, the release of immune mediators, the breakdown of the blood-brain barrier as well as the infiltration of peripheral blood cells and neurotoxic components [10-12]. The cellular immune responses, particularly the recruitment of macrophages has been shown to be responsible for loss of cells due to extensive phagocytosis of the implanted cells [13]. Post-traumatic oxidative stress is considered another obstacle and major cause of cell death. Oxidative stress might ultimately prevent appropriate migration and differentiation of transplanted cells due to a lack of trophic support and an energy substrate deprivation [14-16]. The potential detrimental effect of the post traumatic brain environment was also shown *in vitro*. Inflammatory cytokines released following neural trauma such as interferon-gamma (IFN gamma) or tumour necrosis factor-alpha (TNFalpha) seem to inhibit the generation of neurospheres and to exhibit cytotoxic effects on neural stem cells [17].

Hence, pathophysiological changes associated with TBI may affect the survival, migration and differentiation of transplanted cells to a great extent and may thereby impair the ability of stem cells to replace brain derived cells and restore trauma induced damage to brain tissue.

The aim of the present study was to examine the effect of trauma associated environmental alterations on stem cell survival and differentiation in more detail. Therefore, enriched extract of fluid percussion injured [18] or healthy rat brains was added to undifferentiated murine embryonic stem cells (CGR8) cultured in feeder-free conditions. Time-dependent survival, proliferation and differentiation of murine stem cells were examined over a period of seven days. Time dependent expression of specific markers such as *oct-4* (stem cell marker), *gata6*, *brachyury* and *fgf5* (markers of the three embryonic germ layers endoderm, mesoderm and ectoderm, respectively), *nestin* (marker of early neural differentiation) and *map2* (expressed in post-mitotic neurons) were evaluated in order to determine the differentiation status of cells. Differentiation dependent morphological alterations were also taken into account.

Addition of extract derived from traumatized or healthy rat brains resulted in a massive loss of cultivated cells. Differentiation of surviving cells induced by the omission of FCS was modulated by the addition of extract derived from either traumatized or healthy rat brains as determined by expression of differentiation markers. Furthermore, addition of brain extract derived from traumatized rat brains seemed to induce rapid neuronal development since a significant induction of nestin and Map2 expression was observed within 3 days paralleled by morphological changes such as extensive axonal outgrowth.

Materials and Methods

Cell Culture

Murine embryonic stem cells, CGR8, were cultured at 1 x 10^5 cells/well on gelatine-coated 6 well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 u/mL leukemia-inhibiting factor (lif) and 50µM β-2-mercaptoethanol in gelatine--coated cell culture flask at 37°C in a humidified 5% CO_2 atmosphere.

In order to analyze brain microenvironment dependent modifications of stem cells, cells were cultured at 1 x 10^5 cells / well in 6-well plates and cultured in serum-free DMEM/Ham's F12 (stable glutamine, 5 mM sodium pyruvate). Serum was substituted by 10% Knockout™SR (Invitrogen). Subsequently 20% cerebral tissue extract supernatant was added. Cells were cultured for up to 7 days. Survival, proliferation and differentiation of cells were analysed at day 3, 5, and 7.

Lateral fluid percussion brain injury and brain tissue extract production

All procedures were carried out in accordance with animal protection guidelines, and were approved by the local government authorities of North-Rhine Westphalia. Male Sprague-Dawley rats were subjected to lateral fluid percussion traumatic brain injury (TBI) as described earlier [18, 19]. In brief, the animals were anaesthetised with 60mg/kg body weight pentobarbital intraperitoneally. Animals were placed in a stereotaxic frame, and an opening, 5.0 mm in diameter, was made over the left parietal cortex. The fluid percussion (FP) device was attached via a male Leur-lock which coupled with a

female Leur-lock implanted over the exposed dura of the rat. FP injury of moderate severity (2.4 atm) was induced via a pressure pulse as previously described by McIntosh et al. [18]. Animals were sacrificed 45 minutes after FP injury. For the production of normal brain tissue extract animals underwent craniotomy but received no injury.

Brain extracts were derived by pooling and homogenising the respective cerebral hemispheres of traumatised or healthy rats as described by Chen et al. [20]. Briefly, brain hemispheres were placed on ice, and the wet weight in grams was rapidly measured. Subsequently, the tissue pieces were homogenized by adding DMEM (150 mg tissue / ml DMEM) and were incubated on ice for 10 min. The homogenate was centrifuged for 10 min at 10 000 x g at 4°C. The supernatant was collected and stored at –80°C. Just before cell treatment, the homogenate was diluted in DMEM/Ham's F12 medium (1:2).

RNA Isolation and cDNA Amplification

Total RNA was isolated from CGR8 cells with Quiagen RNA Isolation Kit according to manufacturer's instructions. RNA was quantified spectrophotometrically, and its quality was determined by agarose gel electrophoresis and ethidium bromide staining. Only samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for real-time RT-PCR. cDNA amplification was performed using the Advantage RT-for-PCR Kit (BD Biosciences) according to manufacturer's instructions. In brief, total RNA (1µg) was reverse transcribed in a total volume of 20 mL containing 1x reaction buffer, 200 U of Moloney-murine leukemia virus reverse transcriptase, 1 U/µL of RNase inhibitor, 20 pmol random hexamer primer and 0,5 mM of each deoxynucleotide. The reaction was performed for 60 minutes at 42°C and for 5 minutes at 94°C to stop any cDNA synthesis reaction and to destroy any DNase activity. Prior to reverse transcription, all RNA samples were denatured for 5 minutes at 70°C to melt secondary structure with the template and cooled immediately on ice for 5 minutes to prevent secondary structure from reforming. The cDNA was stored at -70°C until use.

Quantitative real-time RT-PCR

For the RT-PCR reaction, the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) containing 300 nM forward primer, 900 mM reverse primer, and 200 nM probe was used at 50 µL/tube. Amplification and detection was performed using an ABI Prism 7700 Sequence Detector System (Applied Biosystems) with the following profile: 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, and 40 cycles each at 95°C for 15 seconds and at 60°C for 1 minute.

Pre-designed Asays-on-Demand TaqMan probes and primer pairs for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (NM_008084), POU5F1 (*oct-4*) (NM_013633), *nestin* (NM_016701) and microtubule-associated protein 2 (*map2*) (NM_008632) were obtained from Applied Biosystems, Inc. In all PCR reactions expression of the house keeping gene *gapdh* was used as internal control. Equal amounts of input cDNA were used for all qRT-PCR reactions and studies were conducted in triplicates. Data are expressed as the ratio of target mRNA to *gapdh* mRNA and are shown in mean values.

Semi-quantitative PCR

For PCR analysis 1 µL of cDNA was included in a total reaction volume of 50 µL. The PCR reaction mixture contained 1x PCR buffer, 0.2 mM each dNTP (PeqLab), sense and antisense primers, 0.2 mM each and 1 unit of Platinum® Taq DNA Polymerase (Invitrogen). The sequences of primers used in this study were as follows: *gapdh* (182 bp) sense 5'-AGA ACA TCA TCC CTG CAT CC-3', antisense 5'-CCT GTC TCA CCA CCT TCT TG-3'; *gata6* (573 bp) sense 5'-GCA ATG CAT GCG GTC TCT AC-3'; antisense 5'-CTC TTG GTA GCA CCA GCT CA-3'; *brachyury* (942 bp) sense 5'-TGC TGC CTG TGA GTC ATA AC-3', antisense 5'-TCC AGG TGC TAT ATA TTG CC-3'; *fgf5* (464 bp) sense 5'-AAA GTC AAT GGC TCC CAC GAA-3', antisense 5'-CTT CAG TCT GTA CTT CAC TGG-3'; *oct-4* (592 bp) sense 5'-AGA GGG AAC CTC CTC TGA GC-3', antisense 5'-CTG GGA AAG GTG TCC CTG TA-3'; *nestin* (432 bp) sense 5'-CTC GGG AGA GTC GCT TAG AG-3', antisense 5'-ATT AGG CAA GGG GGA AGA GA-3'. Amplification was performed using an T gradient thermal cycler (Biometra) and the cycling parameters consisted of an initial step at 94°C for 5 minutes, followed by 37, 40, 37, 42, 42 and 42 cycles of amplification for *gapdh*, *brachyury*, *gata6*, *fgf5*, *oct-4* and *nestin*, respectively. Each cycle included 30 seconds at 94°C for denaturation, followed by an annealing step of 56°C, 53-62°C, 58°C, 58°C, 57°C and 57°C for 1 minutes for *gapdh*, *brachyury*, *gata6*, *fgf5*, *oct-4* and *nestin*, respectively; followed by 72°C for 1 minute. For all genes a final extension step was performed at 72°C for 5 minutes. PCR products were run on 2% agarose gels and stained with ethidium bromide. The resulting gels were photographed and signals were semi-quantified using Gel-Pro® Analyzer imaging software (Media Cybernectics). In all PCR reactions expression of the house keeping gene *gapdh* was used as internal control. Equal amounts of input cDNA were used for all PCR reactions and studies were conducted in duplicate. Data are expressed as the ratio of target mRNA to *gapdh* mRNA and are shown in mean values ± standard error of the mean.

Cell proliferation assay

The proliferation activities of the CGR8 cell line was analyzed by a BrdU based flow cytometry kit (BD Pharmingen) according to manufacturer's instructions. In brief, suspensions of the cell line were seeded in gelatine-coated 12-well plates at 4 x 10^4 cells / well. Cells were incubated at 37°C in a humidified 5% CO2 atmosphere and treated with brain extracts for 3, 5 and 7 days. 24 *hours* prior to flow cytometric analysis the cells were exposed to 10 µL M 5-bromo-2'-deoxyuridine (BrdU) solution. The next day cells were fixed and permeabilized with BD Cytofix/Cytoperm Buffer, followed by DNase treatment to expose the incorporated BrdU and eventually, the BrdU was stained with FITC-conjugated antibody. Flow cytometric analysis was conducted on a FACScan (BD Biosciences).

Trauma-related changes in cell morphology and proliferation capacity were monitored with a Nikon Eclipse TS100 inverted microscope and photographs were taken with a Nikon Coolpix 5000 digital camera.

Immunocytochemistry and quantification of fluorescence signals

Following differentiation of embryonic stem cells into cells of neuronal cell lineage cells were fixed for 5 min. in 4% paraformaldehyde/0.15 % picric acid. Subsequently, cells were incubated with primary antibodies diluted in PBS/1% BSA for 16 h at 4°C. Slides were rinsed in PBS and then incubated with secondary antibodies diluted in PBS/BSA for 1 h at room temperature. Primary antibodies used were anti nestin antibodies (1:100, Acris, Hiddenhausen, Germany), and anti-Map2 antibodies (1:1000, Abcam, Cambridge, UK). Secondary antibodies: anti-chicken IgG Alexa 555 conjugated (1:300, Invitrogen) and anti rabbit IgG Chromeo 642 conjugated (1:1250, Abcam).

The labeled cells were subsequently viewed with the Olympus Fluorescent Microscope of CellCelector (Aviso). Quantification of optical density was evaluated with the CellCelector-Analysis Software. Fixed settings were chosen both for either green or red fluorescence. Values are as follows:

Map2: Exp. Time: 517ms; Gamma 0,92; Gamma 1,0x, Color 511nm; Nestin: Exp. Time: 727 ms; Gamma: 0,76; Gain 1,4x; Color 708 nm.

A minimum of 50 cells per picture were measured. All experiments were repeated three times.

Apoptosis

DNA fragementation was quantified using the Cell Death Detection ELISAPlus“ Kit by Roche.

1 x 10^4 cells (passage 3-5) were cultivated in 100 µl medium without FCS or lif and prelabelded with BrDU according to the manufacturers instructions. Subsequently 20% HBE or 20% TBE was added to the cells and incubated for up to 7 days. Subsequently cells were centrifuges and the cellular lysate was analysed for apoptosis by ELISA.

Data Analysis

Two-tailed student’s t-test was used to evaluate differences between experimental samples and respective controls. For multiple comparisons statistical significance was evaluated using ANOVA analysis. Tests were two-sided and only p-values <0.01 were considered significant unless stated otherwise. All values are expressed as mean ± SEM and are derived from at least 3 independent experiments.

Results

Brain-associated factors influence CGR8 stem cell survival and proliferation

The proliferative activity of undifferentiated embryonic stem cells was determined under standard condi-

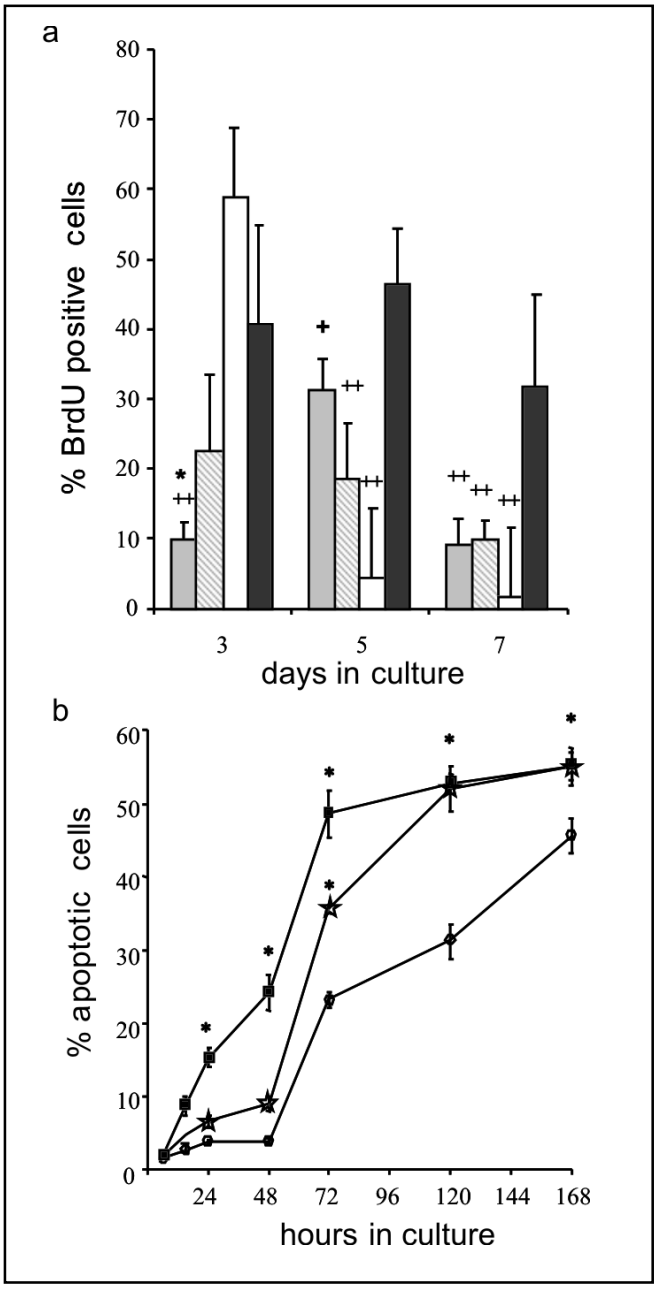

Fig. 1. Proliferation and apoptosis of conditioned and unconditioned CGR8 cells. CGR8 stem cells were conditioned with brain extract derived from traumatized rats (TBE, grey bars) or brain extract from healthy animals (HBE, striped bars). Alternatively, cells were grown in serum-free medium (differentiation control, white bars) or under normal/standard conditions (+FCS, + lif, black bars). A. Cell proliferation measured by BrdU incoporation in CGR8 stem cells over a 7 day period. Data are means ± s.e.m. presented as percentage of BrdU positive cells.. Significant difference TBE vs HBE (* = p<0,05;). $^{+}$Significant difference from standard ($^{+}$ = p<0,01; $^{++}$ = p<0,001).B. Apoptosis of conditioned (TBE: black square; HBE: open star) and unconditioned/control (-FCS, -lif: open circle) CGR8 cells. Apoptosis was quantified by ELISA analysis of DNA fragmentation. Significant difference from control (* = p<0,001).

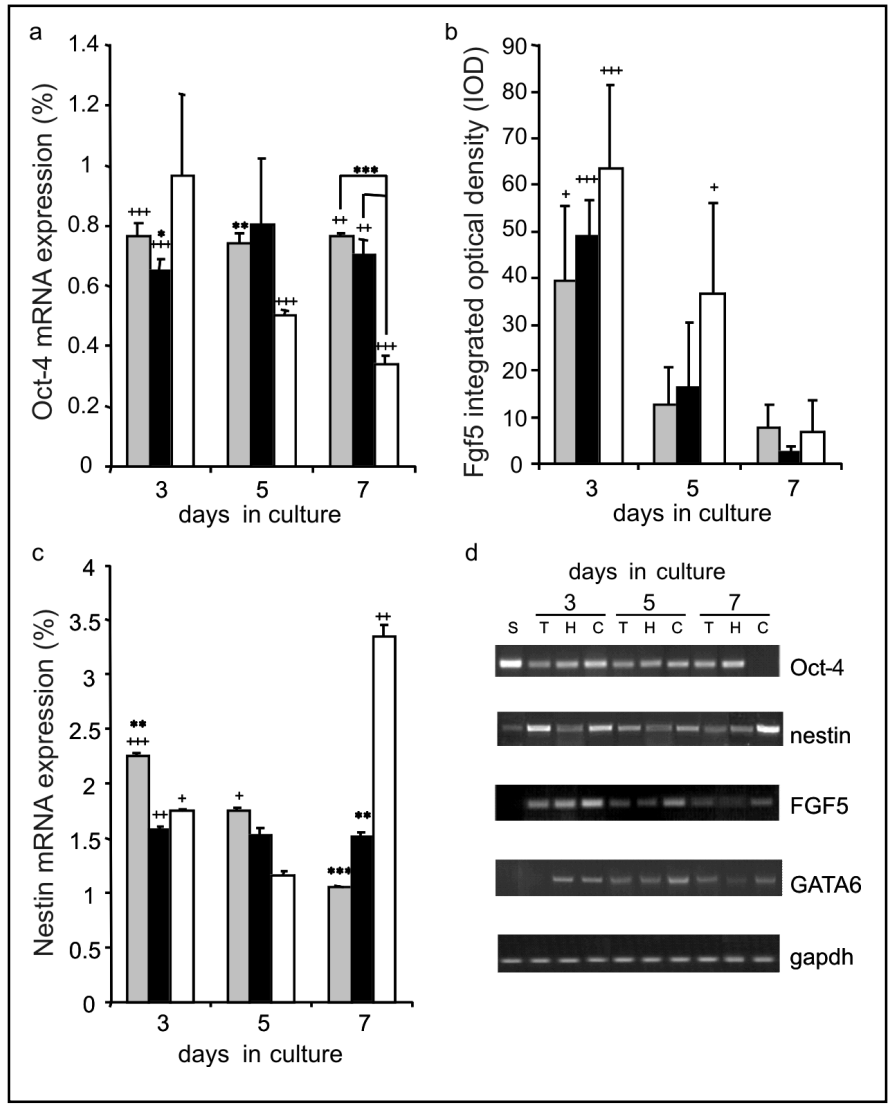

Fig. 2. Brain-extract induced modulation of stem cell differentiation. CGR8 stem cells were conditioned with brain extract derived from traumatized rats (TBE, grey bars) or brain extract from healthy animals (HBE, striped bars). Alternatively, cells were grown in serum-free medium (differentiation control, white bars) or under normal/standard conditions (+FCS, + lif). Expression of mRNA for *oct-4* (a), *fgf5* (b) and *nestin* (c) was assessed by PCR. In (d) representative gel electrophoresis of PCR products for all used differentiation markers are depicted. The *gapdh* gene expression was used as internal control. Abbreviations - S: Standard conditions (medium supplemented with 20% FCS, leukaemia-inhibiting factor), T: brain extract derived from traumatized rat brains, H: brain extract derived from healthy rat brains, C: control conditions (omission of FCS and lif from medium) Data are means ± s.e.m. presented as multiples of standard in a and c and as raw data in b (no detectable expression of *fgf5* was seen in standard cells), respectively. *Significant difference from control (* = p<0,05; ** = p<0,01; *** = p<0,001). $^{+}$Significant difference from standard ($^{+}$ = p<0,05; $^{++}$ = p<0,01; $^{+++}$ = p<0,001).

tions (medium supplemented with 20% FCS and leukaemia-inhibiting factor/ lif), control conditions (omission of FCS and lif) and when cells were conditioned with 20% extract derived from traumatized rat brains (TBE-traumatic brain extract) or 20% extract from healthy rat brains (HBE- healthy brain extract). Omission of FCS and lif from culture medium resulted after an initial burst (day 3: 58.9% ± 9.3% BrdU positive cells) in a significant drop in the percentage of proliferating cells at day 5 and 7 (day 5: 4.3% ± 1.71%) (Fig. 1A) as compared to cells grown under standard conditions. Addition of TBE or HBE rescued the proliferative activity of cells grown under serum free conditions. At day 5 a significant increase in proliferative activity was observed in cells treated with TBE or HBE as compared to untreated serum free cells, probably due to growth inducing factors in the brain extract. However, the proliferative activity of treated cells was at no time point comparable to the proliferative activity of cells cultured under standard conditions. The activation of proliferation was paralleled by a massive loss of cells that seemed to be mediated by apoptosis. Independent of the extract source cells displayed a significant increase in the percentage of apoptotic cells within 24 hours (Fig. 1B). Three days after treatment with TBE or HBE 50% of the cells were apoptotic. The significant increase in the percentage of apoptotic cells and thereby overall loss of cell numbers had to be taken into consideration when interpreting the data concerning proliferation or induction of cell differentiation.

Brain extract induces the differentiation of CGR8 stem cells into the neuroectodermal lineage

Real time PCR results revealed that omission of FCS in CGR8 cell cultures lead to a rapid and significant down-regulation of *oct-4*, usually expressed in pluripotent cells,

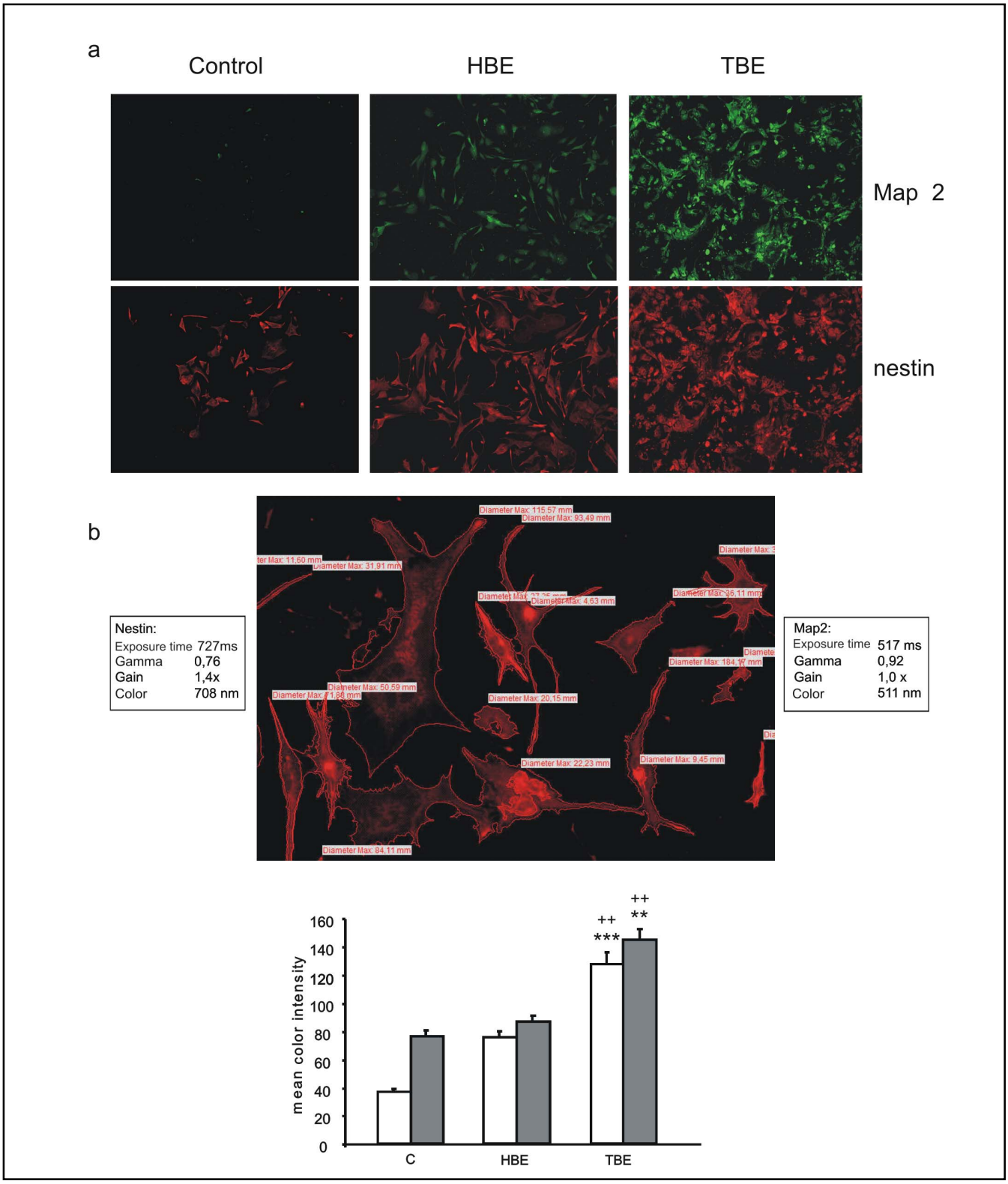

Fig. 3. Brain-extract modulation of stem cell differentiation at day three following conditioning. CGR8 stem cells were conditioned with brain extract derived from traumatized rats (TBE) or brain extract from healthy animals (HBE). Alternatively, cells were grown in serum-free medium (differentiation control) or under normal/standard conditions (+FCS, +lif). A. Expression of *nestin* (red), and Map2 (green) was assessed by immunocytochemistry. B. Quantification of optical density of nestin (white bars) or MAP2 (grey bars) signals were evaluated. Data are presented as mean ± s.e.m of raw data. Significant difference from control (** = $p<0,01$; *** = $p<0,001$) or HBE conditioned cells ($^{++}$ = $p<0,01$).

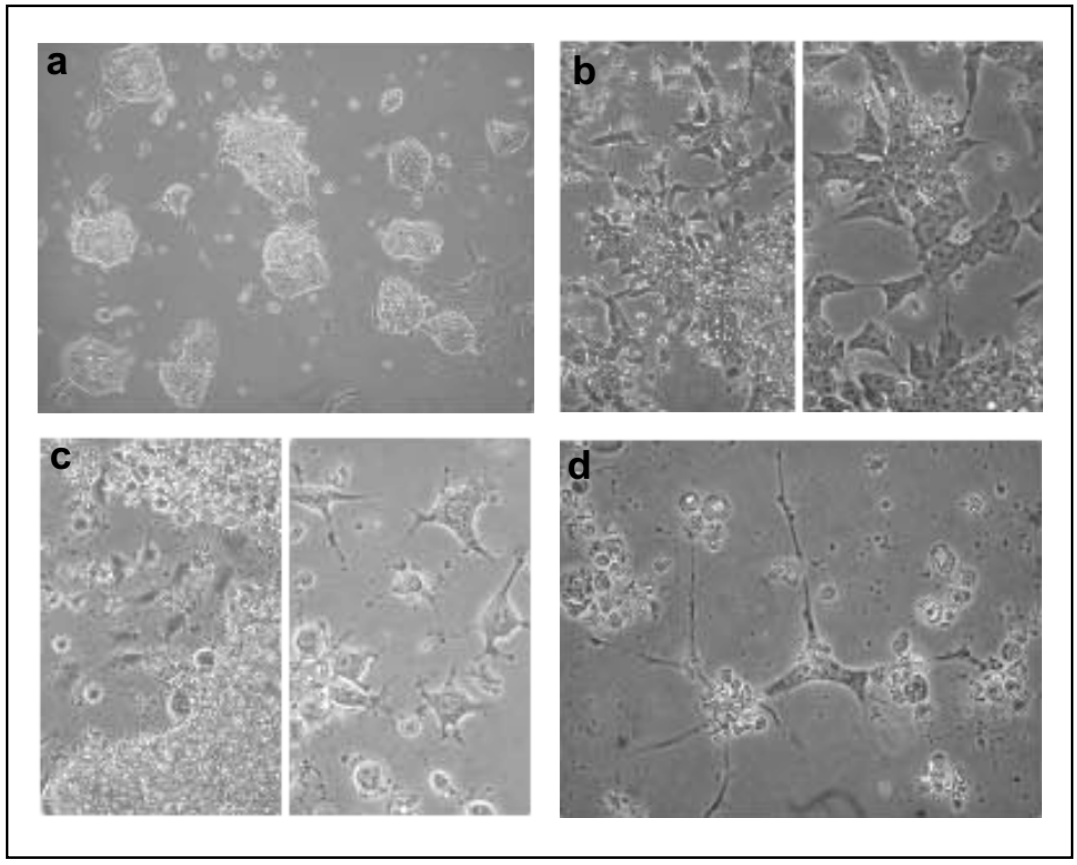

Fig. 4. Brain extract induced modulation of stem cell morphology at day three following conditioning. a. Cells were grown under normal/standard conditions (+FCS, + lif) or b. in serum-free medium (differentiation control). c. CGR8 stem cells were conditioned with brain extract derived from healthy animals (HBE) or d. brain extract from traumatized rats (TBE). Phase contrast microscopy was carried out at a magnification of 10x or 40x.

after 5 and 7 days (50,0% ± 1,9%; p<0,001; 33,8% ± 4,9%; p<0,001, respectively), indicating the onset of differentiation at these time points (Fig. 2A). mRNA levels of *oct-4* in CGR8 cells incubated with either TBE or HBE were down-regulated within 3 days (TBE: 76,8% ± 4,1%, p < 0.01; NBE: 64,8% ± 4.2%, p<0.01) as compared to untreated cells. Following the initial down-regulation of *oct-4* expression, levels remained fairly constant in these groups during the observation period. Differences in *oct-4* expression induced by TBE or HBE were negligible. Results are presented as percentage *oct-4* expression of cell grown in standard conditions.

Development into the neuroectodermal, endodermal and mesodermal lineage was examined by expression analysis of fgf5, gata6 and brachyury, respectively.

fgf5 was not expressed in CGR8 cells grown under standard conditions. A marked increase in *fgf5* expression was observed within 3 days when CGR8 cells where grown under serum-free conditions (63.3 ± 18.3 IOD). Thereafter *fgf5* expression decreased. After 7 days *fgf5* expression was negligible. Similar but less pronounced time-dependent patterns of *fgf5* expression were observed after addition of brain extract derived from traumatized and healthy brains (Fig. 2B).

Expression of *brachyury* was not detectable in any group. Although expression of the endodermal lineage marker gata6 was observed in all groups, expression was partly at the limit of detection. Moreover, expression of *gata6* was randomly distributed in all experimental groups and varied within different experiments. Since a time-dependent or experimental group associated pattern was not observed statistical evaluation has been waived (results not shown).

The analysis of cell lineage determination in conditioned and unconditioned cells has indicated a tendency of cell differentiation towards a neuroectodermal lineage. We have therefore examined early neuronal development in more detail.

Brain extract derived from brain injured rats induces a rapid neuronal differentiation of CGR8 stem cells

The expression of *nestin*, a marker for neural progenitor cells, was significantly up-regulated within 3 days in cells treated with TBE (225% ± 12.8%, p < 0.001) as compared to untreated cells, indicating TBE to induce a comparatively rapid induction of early

differentiation events (Fig. 2C). After 7 days nestin expression in TBE treated cells was comparable to cells grown under standard conditions. Nestin expression in HBE treated cells was slightly elevated by three days of incubation. The level of expression did not vary during the incubation period. On the other hand, cells grown under serum-free conditions displayed a massive increase in nestin expression only after 7 days in culture (334.8% ± 96.2%, $p<0.001$), indicating the induction of neuronal differentiation by default to be time delayed as compared to stem cells treated with TBE. This is in accordance with the pronounced reduction of oct-4 and Fgf-5 expression after 7 days in stem cells cultured without FCS and lif.

The results obtained by real-time RT-PCR were confirmed in immunocytochemical analysis. A pronounced expression of nestin was observed in cells following three days of incubation with TBE (Fig. 3A). Furthermore TBE treated cells also expressed Map2. Cells treated with HBE or untreated cells also expressed nestin and Map2. However, analysis of fluorescence intensity revealed significant more intensive nestin and Map2 signals in TBE treated cells than in untreated cells or cells incubated with HBE (Fig. 3B). The expression of nestin and Map2 confirmed the observed tendency of cells, in particular of TBE treated cells, towards neuronal development.

Microscopic analysis also revealed a change in the morphology of untreated and treated cells. In untreated cells cultured without FCS or lif for three days flattening of the cell body was observed which was accompanied by the separation of single cells from the aggregate (Fig. 4B). HBE treated cells developed minor cellular outgrowth (Fig. 4C). In CGR8 cells treated with TBE sporadic but extensive axonal like sprouting was observed in surviving cells, indicating a more accentuated induction of early neurogenesis by brain extract from injured animals (Fig. 4D). Staining of these cells with NeuN antibodies resulted in signals that were hardly detectable and unconvincing (results not shown), indicating that the incubation of TBE did induce a pronounced induction of early neurogenesis within three days resulting in neuronal precursor cells rather than differentiated neurons.

Discussion

In the present study, cultured embryonic stem cells (feeder cell independent CGR8) treated with brain-derived extract from traumatized rat brains were stimulated to undergo accelerated early differentiation processes when compared to spontaneously differentiating stem cells cultured without FCS or lif. Within three days of TBE conditioning a significant down-regulation of *oct-4* was accompanied by the upregulation of *nestin* mRNA and protein as well as Map2 protein. In parallel we observed a massive loss of cells which seemed to be largely due to apoptotic cell death. Apoptotic cell loss and expression of early neuronal differentiation markers was significantly less pronounced when embryonic stem cells were incubated with extract from healthy rat brains. This data suggests that even during the acute phase following TBI, detrimental effects are accompanied by protective responses that can potentially induce early differentiation events.

An induction of neuronal differentiation by soluble brain derived factors has been demonstrated for mesenchymal stem cells. Soluble fraction of hippocampus, cerebellum or cortex of healthy rats resulted in bipolar and mulipolar phenotypes. The diffusible factor activity was particularly concentrated in a neurogenic structure, i.e. the hippocampus [21]. Interestingly, although expression of neuronal markers NF-H and GAP-43 was induced in MSCs conditioned with soluble factors from hippocampus, it seemed that induction of NeuN expression needed an auxiliary stimulus. Furthermore induction of differentiation of MSCs by soluble factors from brain seemed to be independent of NGF or BDNF.

Moreover, our own previously reported results demonstrate that even injury induced differentiation is BDNF independent. Conditioning of different embryonic stem cell lines, including the cell line CGR8, with extract from healthy and injured rat brains resulted in a pronounced release of BDNF [22]. However, there was no significant difference in the amount of BDNF release when cells were incubated with TBE or HBE. An autocrine BDNF mediated induction of neuronal differentiation by TBE stimulated cells could thereby be excluded. In addition the concentration of BDNF in extract derived from injured or healthy rat brains was unsubstantial.

Further evidence that BDNF on its own is not sufficient to induce neuronal differentiation was provided by a study analysing in vitro differentiation of adult hippocampus derived stem cells [23]. In this study neuronal differentiation of adult hippocampus derived stem cells, i.e. extensive dendritic processes, Map2 expression, formation of synaptic connections, was only induced by the induction of neuronal injury or cell death in nearby cul-

tured neurons or by conditioned medium from injured neuronal cultures [23]. These findings comfirm our observations and indicate that conversion of endogenous pluripotent precursor cells or embryonic stem cells into a neuronal lineage in response to local brain injury might be an important component of central nervous system homeostasis.

We would like to point out that similar to Rivera and co-workers we did not observe a compelling NeuN signal that might indicate the differentiation of surviving cells in post mitotic neurons. It has to be taken into consideration that the majority of cells survived for 3 to 5 days. This time span might be too short for cells to differentiate into mature neurons. Moreover the extract was derived from rat brains during the acute phase of traumatic injury (t=45min). Differentiation and integration of mature neurons might be promoted at a later time point in the sequel of traumatic brain injury.

However, injury induced *in vivo* neurogenesis, i.e. the induction of neuronal differentiation of cerebral precursor cells, has been shown to be accompanied by an increase of nestin positive precursor cells within 24 hrs following injury, lasting up to 7 days [24]. These results are in accordance with our own in vitro results showing a rapid burst of differentiation processes inducible by brain extract derived during the acute phase of traumatic injury. In transplantation studies the differentiation of more mature cell types from transplanted stem cells were observed up to three weeks after injection of stem cells [4]. Currently quiet some effort is assigned to the elucidation of the time dependent sequel of injury induced neuronal differentiation, i.e. neurogenesis, and the identification of endogenous factors that might be involved in protective and/or detrimental mechanism following brain injury *in vitro* and *in vivo*.

Embryonic stem cells provide both a powerful assay system for, and a potential source of, regulatory factors involved in differentiation [21, 25-27]. However, a significant disadvantage to the use of ES cells has been the requirement to employ feeder layers of embryonic fibroblasts to support the isolation and maintenance of pluripotential stem cells. The stem cell line CGR8 has already been demonstrated to exhibit similar response pattern as a feeder cell dependent stem cell line [22]. The use of the stem cell line CGR8 thereby allows an unambiguous examination of the effect of the cerebral environment on differentiation *in vitro* as underlined by the findings reported in this paper.

We will further utilize this system in order to identify and characterize the nature of the cerebral promoters that induce cell death and/or differentiation during acute traumatic injury.

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