Transplantation of placenta-derived mesenchymal stromal cells upon experimental stroke in rats

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ABSTRACT

The beneficial effects of bone marrow-derived mesenchymal stromal cell (MSC) administration following experimental stroke have already been described. Despite several promising characteristics, placenta-derived MSC have not been used in models of focal ischemia. The aim of the current study is to investigate the impact of intravenously transplanted placenta-derived MSC on post-stroke recovery. Permanent occlusion of the middle cerebral artery was induced in spontaneously hypertensive rats. MSC were obtained from the human maternal or fetal placenta and intravenously administered after 24 h (single transplantation) or after 8 h and 24 h (dual transplantation). Sensorimotor deficits were quantified for 60 days using the beam walk test and the modified Neurological Severity Score system. Infarct volume was determined in vivo by means of magnetic resonance imaging on days 1, 8, 29 and 60. Astroglial reactivity was semiquantitatively ascertained within a small and a broad region adjacent to the lesion border. The double infusion of placental MSC was superior to single transplantation in the functional tests. However, a significant difference to the control group in all outcome parameters was observed only for maternally derived MSC. These findings suggest that placental tissue constitutes a promising source for experimental stroke therapies.

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Abbreviations: bmMSC, bone marrow-derived MSC; fpMSC, placenta-derived MSC of fetal origin; GFAP, glial fibrillary acidic protein; MLC, mixed lymphocyte culture; mNSS, modified Neurological Severity Scores; MSC, mesenchymal stromal cells; mpMSC, placenta-derived MSC of maternal origin; PBMNC, peripheral blood mononuclear cells; pMSC, placenta-derived MSC; ROI, region of interest

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1. Introduction

Efficacy of cell-based therapies has been proven for several acute neurological diseases such as ischemic stroke, intracerebral hemorrhage, traumatic brain damage and spinal cord injury. Several lines of incidence indicate the potential of miscellaneous cell types constituting a therapeutic option for restricted lysis therapy (Bliss et al., 2007). Thus, an enlargement of the therapeutic time window up to at least 24 h might include a better part of the acute stroke patients (Chopp and Li, 2002). However, logistical, monetary and ethical difficulties have decelerated the translation of promising preclinical results to clinical reality so far. Fetal and neoplastic origin of applied cells may constitute an ethical obstacle, and invasive extraction processes within autogenous approaches (e.g. iliac crest puncture for bone marrow aspiration) or complex transplantation routes (e.g. intracerebral transplantation) might imply an incriminatory intervention for the already ill stroke patient. For several reasons, the placenta might be an interesting cell source for treatment of acute neurological diseases. Placental cells are easy and ethically unproblematic to obtain and are further available for allogeneic approaches due to low immunogenic properties (Le Blank, 2003). Chang et al. (2006) demonstrated a significant immunosuppressive effect by placental-derived cells which was even stronger than the one proven for bone marrow-derived mesenchymal stem cells. Placenta cell immunological properties have been previously described in the treatment of critical limb ischemia (Prather et al., 2009). Furthermore, placental cells feature multi-lineage differentiation and high expansion capacity (In 't Anker et al., 2004; Yen et al., 2005). These auspicious characteristics notwithstanding, there have been only few publications describing the administration of placenta-derived cells in models of acute neurological diseases. Okawa et al. (2001) observed survival, migration and neuronal differentiation of rat amniotic epithelial cells transplanted into the ischemic gerbil hippocampus. However, the administration of placenta-derived cells in a stroke model with functional read-out parameters has not been accomplished yet. The present study is aimed to investigate the influence of placenta-derived cells on functional outcome, infarct volume and glial reactivity following experimental brain ischemia in rats.

![In vitro characterization of pMSC used.](image)

Fig. 1 - (A–C) In vitro characterization of pMSC used. (A) Surface marker analysis of mpMSC and fpMSC displayed a typical pattern for MSC; however, the adhesion molecules (CD44, CD54, CD106, and CD166) were pronounced on mpMSC. (B) The investigation for immunogenicity displayed a significantly increased proliferation of responder PBMNC (a) by irradiated allogeneic stimulator PBMNC (b), whereas the responder cells remained unaffected by fpMSC and mpMSC. (C) Placenta-derived cells of maternal and fetal origin showed a significant decrease of allogeneic-induced proliferation of PBMNC in a dose-dependent manner. The immunosuppressive effect of fetal pMSC was superior to maternal pMSC (*p < 0.05). The lowercase letters indicate the responder (a) and stimulator (b) peripheral blood cells.
2. Results

2.1. Phenotype of pMSC

Typical MSC surface markers such as CD73, CD90 and CD29 were highly expressed by all cell populations. However, the mesenchymal stem cell marker CD271 could not be detected on placenta-derived cells (Fig. 1A). Markers for haematopoietic (CD45, CD34), endothelial (CD31) or dendritic cells (costimulatory molecules CD80 [B7-1], CD86 [B7-2]) were sporadically observed (Fig. 1A). Placental cells showed high expression of ICAM-1 (CD54) and moderate expression of VCAM-1 (CD106). However, the expression of adhesion molecules was depending on the cell source. Generally, placenta-derived MSC of maternal origin (mpMSC) showed higher levels of adhesion molecules compared to fetal placental MSC (fpMSC; Fig. 1A).

2.2. Immunogenicity and immunosuppressive potential of pMSC

Co-cultivation of allogeneic irradiated peripheral blood mononuclear cells (PBMC) with responder PBMC caused significantly increased incorporation of \(^{3}\)Thymidine which connoted a proliferation of responder lymphocytes. However, neither co-cultivation with maternal pMSC nor with fetal pMSC produced any increase of lymphocyte proliferation (Fig. 1B). Further co-cultivation of irradiated stimulator PBMC with responder PBMC and pMSC displayed clear reduction of lymphocyte proliferation by pMSC (Fig. 1C). However, this effect was evident exclusively for 10\% pMSC. Furthermore, the addition of fetal pMSC suppressed lymphocyte proliferation significantly more than maternal pMSC did.

2.3. Sensorimotor deficits

All experimental groups showed a significant reduction of beam walk categories during the course of the experiment. Compared to control group, fpMSC(1×)-treated group showed a lower recovery, while fpMSC(2×) and mpMSC(2×) treatment resulted in significantly increased functional recovery. Dual transplantation of pMSC produced significantly better results than the single treatment approach, irrespective of maternal or fetal belonging (Fig. 2A). Analysis of modified Neurological Severity Scores (mNSS) showed, analogously to the beam walk test, a distinct reduction of functional deficits during the experimental course. Compared to the control group, the reduction of mNSS points was significantly improved by mpMSC(2×) treated animals but not by fpMSC(2×) treatment. However, double administration of cells was superior to the single-treatment approach, again, independent from the cell source (Fig. 2B).

2.4. Lesion development

Examination for lesion development by magnetic resonance investigation showed a distinct reduction of lesion size between days 1 and 8 demonstrated by a decrease of infarct ratio (control group: \(-44.2\% \pm 3.7\%\); Fig. 3A). However, there was no evidence for statistically significant differences between the experimental groups on days 8 and 29 (Fig. 3B and C). On day 60 the infarct ratio of mpMSC(2×)-treated subjects was significantly reduced compared to the control group (Fig. 3D).

2.5. Glial reactivity

Investigation for glial fibrillary acidic protein (GFAP)-immunoreactive (ir) area in the grey matter adjacent to the lesion

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![Fig. 2](A–B) Development of sensorimotor deficits measured by the beam walk test (A) and modified Neurological Severity Scoring (B; mNSS). The successive decline of beam walk and mNSS data was modeled as a linear function of time. The estimated slopes were presented for each experimental group. (A) Consolidated statistical analysis displayed successive reduction of beam walk categories over the entire experiment illustrated by the following ranking: fpMSC(2×), mpMSC(2×), control, mpMSC(1×) and fpMSC(1×). Statistically significant differences were evident for the comparison of fpMSC(1×) and fpMSC(2×) \((p < 0.0001)\), mpMSC(1×) and mpMSC(2×) \((p = 0.0002)\), fpMSC(2×) and control \((p = 0.0001)\) and mpMSC(2×) and control \((p = 0.0042)\). (B) The corresponding analysis for the mNSS illustrated the ranking within the experimental groups: mpMSC(2×), fpMSC(2×), mpMSC(1×), control and fpMSC(1×). Significant differences were observed for the comparison of fpMSC(1×) and fpMSC(2×) \((p = 0.0006)\), mpMSC(1×) and mpMSC(2×) \((p = 0.0192)\), mpMSC(2×) and control \((p = 0.0058)\).
border showed a compact meshwork of GFAP-ir cell bodies and processes. The density of GFAP-ir objects was significantly increased within the small region of interest (ROI) compared to the large ROI (control group: 32.1% ± 4.4% versus 8.5% ± 0.9%, Figs. 4A and B). There was no evidence for statistically significant differences between the experimental groups within the small ROI. However, the mpMSC(2×)-treated subjects displayed a significantly increased GFAP-positive area compared to the control group in the large ROI (Fig. 4B).

3. Discussion

Bone marrow-derived MSC (bmMSC) have been successfully investigated in several preclinical studies of stroke (Chopp and Li, 2002), whereas the implementation of placental MSC (pMSC) has not been realized yet. The placenta provides several advantages as a source for MSC. As a by-product of childbirth, the placenta makes available virtually unlimited quantities of MSC without ethical considerations. According to previously published data, we evidenced that its low immunogenic properties make pMSC perfect candidates for transplantations without HLA-matching (Le Blank, 2003; Prather et al., 2009). The detected immunosuppressive characteristics of pMSC render this cell source as a promising candidate for stroke therapy, given that stroke outcome is significantly determined by immunoreactions (Strachan et al., 1992). The suppression of allogeneic T-cell proliferation is mediated by the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO), a protein expressed by bmMSC and pMSC (Jones et al., 2007). However, the immunosuppressive effect is more pronounced in pMSC, potentially due to the expression of HLA-G (Chang et al., 2006). The immunomodulating effect of pMSC has not been proven in vivo. However, it is imaginable that intravenous transplantation of pMSC attenuates the endogenous lymphocyte reaction. Splenectomy prior to

Fig. 3 – (A–D) In vivo investigation of lesion development during the experimental period using MRI. (A) Representative illustration of infarct development of one control animal on days 1, 8, 29 and 60 and one mpMSC (2×) treated animal on day 60. On day 1, all subjects showed a distinct T2-hyperintense lesion within the right middle cerebral artery territory without significant differences between the experimental groups. The ischemic lesion on day 1 was characterized by a distinct brain edema recognizable by a narrowed right ventricle and midline shift. However, on day 8 the brain edema had almost vanished, and we observed an increasing organization of the lesion until the end of the observation period. The lesions on days 29 and 60 were characterized by almost complete loss of the right cortex and development of a large liquor-filled cavity which was connected with the associated side ventricle. (B–D) The lesion volumes on days 8, 29 and 60 were displayed as percentage of day 1 lesion. There was no significant difference between the treatment groups on days 8 and 29, however, we found a statistically significant decreased lesion in mpMSC (2×)-treated subjects compared to the control group (p=0.008) at day 60.
experimental stroke caused significantly decreased neurodegeneration and less infiltration of inflammatory cells into the affected brain tissue, supposedly by removing a lymphocyte-containing compartment (Ajmo et al., 2008). Furthermore, immunodeficient SCID mice lacking T- and B-cells exhibited a markedly reduced infarct volume compared to wild-type mice (Hurn et al., 2007). Hence, the modulation of peripheral and local immunoreactions due to the transplantation of pMSC could at least partially explain the beneficial effects observed in the current study.

The functional data obtained in the current study was evaluated critically, given that behavioral tests are typically exposed to noticeable variations due to intra-individual differences, external stressors and adjustment reactions. Thus, only the repeated advancement in different behavioral tests was stated as effective improvement of functional recovery. We found statistically significant superiority of dual transplantation approaches compared to single administrations suggesting that beneficial effects of placenta-derived MSC were affected by the therapeutic window and cell dose. The cell dose might play a decisive role since we observed dose-dependent immunosuppressive effects in vitro. The time window for effective MSC treatment appeared to be less relevant, since effective MSC administration in terms of reduced infarct volume and improved functional recovery was described for transplantation time points from 2 h to 1 month upon stroke (Shen et al., 2007; Zhao et al., 2006). However, the discrimination between the effectors time window and cell dose needs to be performed in further experiments. All things considered, only dual transplantation of maternal pMSC showed a significant improvement of functional recovery compared to the control group in both functional tests. These results were further supported by the examination of infarct volume using magnetic resonance

Fig. 4 – (A–D) Assessment of glial reactivity on day 60 upon stroke onset. Density of GFAP immunoreactivity was used as a surrogate for glial activation. As illustrated in the overview (D), GFAP density was measured in a small (dark grey) and a large (pale grey) region of interest (ROI) adjacent to the infarct border. The GFAP immunoreactivity was homogenously activated in the small ROI (A), whereas the mpMSC(2×)-treated subjects exhibited a significantly increased GFAP immunoreactivity in the large ROI ($p$=0.015) (B). An exemplary illustration of glial reactivity (C) showed a dense GFAP immunoreactive network adjacent to the cavity, whereas interior tissue regions were characterized by homogenously distributed GFAP-positive stellar-shaped cells.
 imaging. Dual transplantation of mpMSC was again the only approach resulting in a significant change of infarct volume. The discrepancy between profound improvements after dual maternal pMSC transplantation and only slight effects due to dual fetal pMSC administration was astonishing since fetal MSC exhibited a significantly increased immunosuppressive effect compared to maternal MSC, as shown by us and other investigators (Roelen et al., 2009). However, hitherto unknown differences between maternal and fetal pMSC might have affected the diversity of outcome observed by us. Interestingly, we found a proportionally high expression of adhesion molecules in maternal pMSC compared to those of fetal origin. One could assume that different expression levels of adhesion molecules affect the trafficking behaviour of pMSC. Thus, the interaction between extracellularly released hyaluronic acid and CD44 receptors seemed to be determining for homing of MSC into damaged areas (Zhu et al., 2006). Homing of exogenous and endogenous MSC toward the damaged brain regions appeared to be essential for the evolvement of beneficial effects since positive effects were, at least partly, dependent on the secretion of soluble factors and consequently linked to an accumulation of MSC within the affected tissue.

Dual transplantation of mpMSC produced a significant decrease of infarct volume on day 60 in combination with functional improvement. However, effective cell transplantation had no effect on acute lesion development until day 8. Thus, it is unlikely that beneficial effects of transplanted cells were exclusively mediated by neuroprotection. It is rather imaginable that cell transplantation produced neuroregenerative effects causing slow improvements within the sub-chronic and chronic phase of stroke recovery. Chen et al. (2003) transplanted bone marrow-derived MSC intravenously upon experimental stroke and observed functional recovery along with increased subventricular zone (SVZ) cell proliferation and reduced apoptosis. Furthermore, it was shown that intravenous transplantation of human MSC significantly increased the expression of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) within the damaged hemisphere, once more associated with significantly improved functional recovery (Li et al., 2002). Intracerebral transplantation of BDNF after stroke resulted in improved functional recovery and significant neuronal remodelling without any effects on the infarct volume (Schabitz et al., 2004). Furthermore, it was shown that placental MSC produced biologically active vascular endothelial growth factor (VEGF) and transplantation of these cells into ischemic-injured limbs effectuated significantly increased blood flow and enhanced angiogenesis (Nishishita et al., 2004; Prather et al., 2009). Intravenous transplantation of VEGF-transfected human MSC after stroke caused considerably more micro-vessels in relevant areas with concurrent functional improvement (Toyama et al., 2009). Taking these findings together, we hypothesize that systematically transplanted mpMSC migrate toward the ischemic brain and secrete soluble factors with considerable effects on apoptotic processes, endogenous neurogenesis, angiogenesis and neuronal remodelling.

In the current study, improvement of functional recovery and reduction of lesion volume was accompanied by significantly increased density of GFAP-positive cells within a 600-μm wide area adjacent to the infarct border. Astroglial reactivity following experimental brain ischemia is well described, nevertheless, it is difficult to classify all characteristics of reactive astrocytes into terms of “friendly” or “adverse.” Astrocytes express several proinflammatory cytokines, chemokines and the inducible nitric oxide synthase (iNOS) causing neuronal cell death by NO production (Dong and Benveniste, 2001; Hu et al., 1997). The astrocytic gap-junction network may further deteriorate the ischemic lesion by dispersion of apoptotic substances, removal of energy-rich phosphates from the penumbral zone to the infarct core and by relaying spreading depressions responsible for secondary infarct growth (Trendelenburg and Dirnagl, 2005). On the other hand, astrocytes display numerous tissue-protective and regenerative properties within the first days after stroke, partly outweighing the adversarial effects. This was eloquently shown by Faulkner et al. (2004) using a sophisticated technique to eliminate reactive astrocytes selectively. Ablation of reactive astrocytes following spinal cord injury resulted in the augmentation of lesion volume and inflammatory response. Moreover, activated astrocytes may support post-stroke recovery by secretion of neurotrophic factors like VEGF, BDNF, NGF, basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) (Himeda et al., 2007; Lee et al., 1997; Marti and Risau., 1998; Wei et al., 2000). Liberto et al. (2004) reviewed strong evidence for several pro-regenerative properties of cytokine-activated astrocytes including the reconstitution of the blood–brain barrier, remyelination, neurogenesis, synaptogenesis and angiogenesis. The increased amount of GFAP-positive cells after dual mpMSC transplantation might represent an augmented availability of pro-regenerative astrocytes. The causal linkage between pMSC transplantation and increased astrogliosis is unclear. However, Gao et al. (2005) showed that survival of hypoxic-damaged astrocytes increased when co-cultured with bmMSC. These observations support the hypothesis that mpMSC transplantation following stroke might increase survival rates of pro-regenerative astrocytes. Interestingly, we found no difference within the small region of interest adjacent to the infarct border. Here, astrocytes formed a tight network of elongated and hypertrophied processes within a narrow margin next to the lesion cavity. This structure, also referred to as glial scar, plays a decisive role in excluding the lesion from healthy tissue but is also assumed to prevent axonal regrowth (Silver and Miller, 2004). However, the development of the glial scar was unaffected by mpMSC transplantation.

4. Conclusion

Placental tissue appeared to be a promising source for experimental stroke therapies. The observed improvement of functional recovery and lesion volume was potentially mediated by modulation of immunoreactions, local release of soluble factors and adjustment of astroglial reactivity. However, further studies are needed to confirm the beneficial effects of pMSC and to elucidate the determining factors of cell dose and therapeutic time window.
5. Experimental procedures

5.1. Preparation of pMSC

Placental MSC were obtained from Pluristem Therapeutics, Ltd. (Haifa, Israel) a company specialized in commercialization of allogeneic cell therapy products (Prather et al., 2008). Briefly, term human placentas were derived from healthy donor mothers following informed consent. Placental stromal cells were recovered from the decidua (maternal part) and the chorionic villi (fetal part) and grown as 2D cultures and, subsequently, stored as 2D-cell stocks tested for sterility, viability and immunophenotype. 2D-cell stocks were further expanded in the presence of FCS without any additional supplements in a 3D bioreactor based on a fibrous matrix. After one to two weeks of growth, cells were harvested and cryopreserved in liquid nitrogen. Upon administration, the cryopreserved cells were thawed and prepared for injection.

5.2. Flow cytometric analysis

Aliquots of cells were labeled with the following conjugated anti-human antibodies: CD29–FITC, CD34–APC, CD45–FITC, CD54 (ICAM-1)–PE, CD86 (B7-2)–PE, CD90–PE (Immunotech); CD31 (PECAM-1)–FITC (ImmunoTools); CD58 (LFA-3)–PE, CD166 (ALCAM)–FITC (Serotech); CD44 (HCAM)–FITC, CD80 (B7-1)–PE (BD Pharmingen); and CD51 (Vitronectin receptor)–FITC, CD102 (ICAM-2)–FITC, CD105 (VCAM)–PE (Southern Biotech). The monoclonal antibodies SH3 (CD73) and SH4 were labelled with secondary goat anti-mouse-FITC or -PE antibodies (DakoCytomation). Labelling of 1 × 10^6 cells was carried out using mAbs at room temperature for 20 min. After washing twice with PBS/1% FCS (250 × g, 5 min), cells were fixed with 3% formaldehyde. Isotype-identical mAbs served as controls. Analysis was performed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

5.3. Immunogenic profile of pMSC

The immunogenic potential of pMSC was tested using a mixed lymphocyte culture (MLC). Briefly, untreated peripheral blood mononuclear cells (10E5 PBMC, as responder) were co-cultured with irradiated stimulator cells (30 Gy; 10E5 fpMSC, 10E5 mpMSC or 10E5 allogeneic PBMC) in triplicates. Six days later, the proliferative response of PBMC was assessed by 3H-Thymidine incorporation.

5.4. Immunosuppressive potential of pMSC

To determine the ability of pMSC to suppress the activity of PBMC responder cells, co-cultures of untreated PBMC (10E5, as responders) plus irradiated allogeneic PBMC (30 Gy; 10E5, as stimulus) were treated with fpMSC or mpMSC at concentrations of 0.1%, 1% and 10% on day 0. On day 6 the proliferative response of responder cells was assessed by 3H-Thymidine incorporation.

5.5. Experimental stroke and cell transplantation

All experimental procedures were performed in accordance with the guidelines on care and use of experimental animals set by the governmental authorities (reference number TVV18/07). Altogether, 40 male spontaneously hypertensive rats (Charles River Inc.) weighing approximately 250 g were randomly assigned to one of five experimental groups (n=8 each): (a) single and (b) dual injection of fpMSC, (c) single and (d) dual injection of mpMSC and infusion of vehicle solution (e). Single treatment approaches were accomplished 24 h following stroke, whereas dual transplantations were performed 8 h and 24 h upon stroke onset. The animals were kept under constant living conditions and had free access to food and water. Experimental brain ischemia was induced by permanent occlusion of the middle cerebral artery (MCAO), as previously described (Tamura et al., 1981). Briefly, animals were anaesthetized with Ketamine Hydrochloride (100 mg/kg, Merial Ltd.), Xylacin (10 mg/kg Bayer Inc.) and Atropine (0.1 mg/kg, Ratiopharm Inc.) given as intraperitoneal injection. Core body temperature was maintained at 37.5 °C using a feedback controlled heating pad connected to a rectal probe. After sub-temporal craniotomy, the middle cerebral artery was permanently occluded by thermocoagulation. One animal of group (d) died primarily because of anaesthesia. The administration of 10E6 cells per transplantation time point was given via a 26-gauge catheter inserted in a tail vein. The transplantation procedures were carried out under a short isoflurane anesthesia (of approximately 30 s).

5.6. Behavioral tests

Quantification of functional deficits was performed using the beam walk test, as previously described (Kundrotiene et al., 2002). Briefly, rats had to cross a narrow beam (14 mm in diameter, 1 m in length) at the end of which the animal’s home cage was placed. The time needed by the rat to cross the bar was documented and translated into the following categorical values: 0–5 s: category (0), 5–10 s: category (1), 10–15 s: category (2), 15–20 s: category (3), hanging at the beam for more than 20 s: category (4), falling from the beam: category (5). A total of five categorical values was recorded for each rat at each time point. The resulting five categories per animal were averaged to obtain a summary measure for the performance of the animal at the corresponding time point. Animals were trained for seven days until the recording of the baseline data one day before stroke induction. A predefined exclusion criterion was a daily average of more than one category at baseline ascertainment. During the experiments, subjects were examined on days 1, 4, 5, 14, 21, 28, 35, 42, 49 and 56 upon stroke onset. The modified Neurological Severity Score (mNSS) (Chen et al., 2001) was collected on the corresponding days subsequent to the beam walk test. All behavioral tests were accomplished by one investigator blinded to the group allocation.

5.7. Magnetic resonance imaging

Lesion development was examined using magnetic resonance imaging (MRI) on days 1, 8, 29 and 60. Subjects were anesthetized according to the protocol used during MCAO. MRI was performed using a 1.5 T scanner (Gyroscan Intera human whole-body spectrometer, Philips) equipped with a small loop RF-Coil (47 mm Microscopy Coil, Philips). T2-weighted sequences (T2-TSE) were performed at each MRI...
session consisting of 20 transverse slices (matrix: 224×224; field of view: 50 mm; slice thickness: 1 mm). All sequences were measured with a turbo-spin echo sequence. For further processing, MRI sequences were exported as DICOM sequences. Volumes of ischemic lesion as well as ipsilateral and contralateral side ventricles were determined by a blinded investigator using the open source image processing software ImageJ. Due to optical fusion of the ischemic lesion and the ipsilateral side ventricle on days 29 and 60 (Fig. 3A) we decided to measure the corrected infarct volume using the following formula: lesion volume plus the ipsilateral ventricle volume minus the contralateral ventricle volume. Subsequently, each calculated volume was displayed as the percentage of the infarct volume on day 1.

5.8. Analysis for astroglial reactivity

After the final behavioral test, animals were deeply anaesthetized with carbon dioxide and transcardially perfused with 250 mL of phosphate buffered solution followed by perfusion with 200 mL of 4% formalin solution. Brains were removed, post-fixed in 4% formalin solution for further 24 h, drained in ascending concentrations of sucrose for three days (10%, 20% and 30%) and cryopreserved at –80°C. Subsequently, the brains were cut with a cryostat into 30-μm-thick coronal sections. Immunohistochemical staining of reactive astrocytes was performed using a 200-fold diluted Alexa Fluor 488-conjugated anti-GFAP antibody (Milipore). Every 10th brain slide within the fronto-occipital extend of ischemic brain lesion was selected for immunohistochemical quantification of the GFAP-immunoreactive area. An investigator blinded to the group allocation created a small (200 μm deep) and a large (650 μm deep) region of interest adjacent to the lesion border (see Fig. 4). Both regions of interest were cut out and binarized using the Otsu threshold (ImageJ). The percentage of the GFAP-immunoreactive area to the total area was calculated for each brain slide and summarized for each brain.

5.9. Statistical analysis

For behavioral data magnitude of effects and levels of significance were evaluated using a linear mixed model. Initial performance and animal number were treated as random factors, while the long term linear time trend within experimental groups was estimated as fixed effect. In case of random factors, while the long term linear time trend within experimental groups was estimated as fixed effect. Initial performance and animal number were treated as random factors. Lesion development and global significance, contrasts of the time effect were calculated separately. Contrasts of interventional groups compared to control group were calculated only in case of global significance of ANOVA. p-values of these post-hoc tests were adjusted with Bonferroni correction. ANOVA analysis was performed using SPSS 15.0.1 (SPSS Inc., Chicago, IL, USA). Mixed model analysis was performed using the statistical software package SAS 9.1 (SAS Institute Inc., Cary, NC, USA). p-values of 0.05 or less were considered as statistically significant. Data were presented as mean ± standard deviation.

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