Neuronal differentiation of transplanted embryonic stem cell-derived precursors in stroke lesions of adult rats

Claudia Bühnemann,1, Andreas Scholz,2, Christian Bernreuther,3 Christoph Y. Malik,2 Holger Braun,1 Melitta Schachner,3 Klaus G. Reymann1,1 and Marcel Dihné4,1

1Leibniz Institut für Neurobiologie (IFN), Neuropharmakologie, Magdeburg, 2Justus-Liebig Universität, Institut für Physiologie, Giessen, 3Zentrum für Molekulare Neurobiologie Hamburg, Universität Hamburg and 4Heinrich-Heine Universität, Neurologische Klinik, Düsseldorf, Germany

Correspondence to: Claudia Bühnemann, Neuropharmacology, Leibniz Institute for Neurobiology, Brennekestrasse 6, D-39118 Magdeburg, Germany
E-mail: claudia.buehnemann@ifn-magdeburg.de

Stroke represents one of the leading causes of death and disability in Western countries, but despite intense research, only few options exist for the treatment of stroke-related infarction of brain tissue. In experimental stroke, cell therapy can partly reverse some behavioural deficits. However, the underlying mechanisms have remained unknown as most studies revealed only little, if any, evidence for neuronal replacement and the observed behavioural improvements appeared to be related rather to a graft-derived induction of a positive response in the remaining host tissue than to cell replacement by the graft itself. The present study was performed to test a murine embryonic stem cell (ESC)-based approach in rats subjected to endothelin-induced middle cerebral artery occlusion. Efficacy of cell therapy regarding graft survival, neuronal yield and diversity, and electrophysiological features of the grafted cells were tested after transplanting ESC-derived neural precursors into the infarct core and periphery of adult rats. Here, we show that grafted cells can survive, albeit not entirely, most probably as a consequence of an ongoing immune response, within the infarct core for up to 12 weeks after transplantation and that they differentiate with high yield into immunohistochemically mature glial cells and neurons of diverse neurotransmitter-subtypes. Most importantly, transplanted cells demonstrate characteristics of electrophysiologically functional neurons with voltage-gated sodium currents that enable these cells to fire action potentials. Additionally, during the first 7 weeks after transplantation we observed spontaneous excitatory post-synaptic currents in graft-derived cells indicating synaptic input. Thus, our observations show that ESC-based regenerative approaches may be successful in an acutely necrotic cellular environment.

Keywords: stem cells; brain ischaemia; transplantation; differentiation; electrophysiology

Abbreviations: AP = action potential; EGFP = enhanced green fluorescent protein; EPSP = excitatory post-synaptic potentials; ESC = embryonic stem cell; GFAP = glial fibrillary acidic protein; MCAO = middle cerebral artery occlusion


Introduction

Neural transplantation represents an option to treat functional impairment after stroke. New therapeutic strategies are important, since pharmacologically based neuroprotection to prevent primary and/or secondary damage following stroke has so far failed in clinical studies (Dirnagl et al., 1999), and regenerative processes after stroke including enhanced neurogenesis are limited (Arvidsson et al., 2002). Transplantation of bone marrow (Chen et al., 2001a), foetal CNS (Ishibashi et al., 2004), umbilical cord blood (Chen et al., 2001b), and embryonic stem cells (ESC; Hoehn et al., 2002; Wei et al., 2005; Hayashi et al., 2006) have been used for therapy after ischaemic tissue damage in animal models. While some of these approaches have demonstrated behavioural improvements, appropriate
neuronal function of grafted cells was not shown and behavioural improvements were discussed to be related rather to a beneficial influence of the grafted cells on the host tissue (Haas et al., 2005). Our study was thus performed in the attempt to investigate if ESC-derived neural precursor cells can differentiate into mature neurons with functional characteristics after transplantation in a rodent stroke model. ESC-derived neural precursors were chosen for this study since they had been shown to function appropriately after transplantation, for instance in animal models of Parkinson’s disease (Bjorklund et al., 2002; Kim et al., 2002). In contrast to Parkinson’s disease characterized by a circumscribed area of selective degeneration of dopaminergic neurons and with limited inflammatory reactions, a cell replacement therapy in stroke, however, requires survival of grafted cells in an inhospitable environment including inflammatory reactions, tissue pan-necrosis and glial scar formation. Therapy of stroke lesions also calls for the replacement of several functionally distinct neural cell types. Here, we investigated for the first time whether murine ESC-derived neural precursors can survive and differentiate into functional neurons in the centre of a necrotic area after transplantation into young adult rats that had been subjected to experimental stroke. We show that grafts, although their size was reduced with time, can survive for up to 12 weeks in the centre of an ischaemic lesion in spite of an ongoing immune response including accumulation of macrophages/microglial cells. Furthermore, we demonstrate immunohistochemically that grafted cells differentiated into mature glial cells and neurons of several distinct mature neurotransmitter-synthesizing subtypes. Also, electrophysiological recordings from grafted cells exhibited characteristic features of neurons with synaptic input.

Material and methods

Generation of neural precursor cells from murine ESCs

An ESC line constitutively expressing enhanced green fluorescent protein (EGFP) was derived from transgenic C57BL/6j mice ubiquitously expressing EGFP under the influence of the chicken β-actin promoter (Okabe et al., 1997) as described (Abbondanzo et al., 1993; Dihné et al., 2006). Immature ESCs were subjected to the five-stage differentiation protocol with minor modifications (Okabe et al., 1996; Lee et al., 2000b; Dihné et al., 2006). Undifferentiated (Stage 1) ESCs were grown on mitomycin-treated murine embryonic fibroblasts in the presence of 1000 U/ml leukaemia inhibitory factor (LIF, Chemicon, Temecula, CA, USA) in ESC medium. After generation of embryoid bodies in ESC medium without LIF for 4 days, selection of nestin+ cells was initiated by replacing the ESC medium by ITSFn medium for 8 days (Kim et al., 2002; Stage 3) and plating dissociated cells into tissue culture dishes pre-coated with 15 μg/ml poly-L-ornithine (PLO, Sigma, Deisenhofen, Germany) and 1 μg/ml laminin (Sigma) in Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium (Invitrogen, Karlsruhe, Germany) containing 2% B-27 supplement (Invitrogen), 2 mM l-glutamine, antibiotics (Invitrogen) and 20 ng/ml fibroblast growth factor-2 (FGF-2) (PreproTech, Rocky Hill, NY, USA; early Stage 4). More than 95% of Stage 4 neural precursor cells expressed nestin and the neural cell adhesion molecule (NCAM), and proliferated in the presence of FGF-2. Removal of FGF-2 led to the differentiation of Stage 4 neural precursors within 7 days into the three principal cell types of the CNS. This stage is referred to as Stage 5 following the nomenclature of McKay and colleagues (Okabe et al., 1996). The composition of Stages 4 and 5 neural cell populations has been characterized (Dihné et al., 2006).

Animals

Male Fisher rats (Charles River, Sulzfeld, Germany) weighing 250–270 g were used. Animals were housed under a natural light–dark cycle and allowed access to food and water ad libitum.

Transient occlusion of the middle cerebral artery by endothelin-1

Induction of focal cerebral ischaemia by transient middle cerebral artery occlusion (MCAO, Sharkey and Butcher, 1995; Pringle et al., 2003) under halothane anaesthesia was performed by infusion of 375 pmol of the vasoconstrictor endothelin-1 (Sigma) over a time period of 5 min directly next to the right MCA at AP: +0.09 mm, L (right): +0.48 mm, and V: −0.75 mm according to bregma (Paxinos and Watson, 1998).

Transplantation

On the day of transplantation ESC-derived precursors (3 days in Stage 5) were dissociated and re-suspended in Hank’s balanced salt solution at a density of 50 000 viable cells per μl. Seven days after MCAO, ~100 000 cells were injected at different sites into the brain tissue. For ipsilateral transplantation, precursors were stereotaxically placed into the infarcted area (AP: +0.09; L: +0.48; V: −0.55) and at two sites next to the ipsilateral midline of the brain at AP: +0.09; L: +0.2; V: −0.21 into the cerebral cortex and at AP: +0.09; L: +0.2; V: −0.38 into the striatum (Fig. 1A). For contralateral transplantation, cells were grafted into the striatum at AP: +0.09; L: −0.3; V: −0.4 (Fig. 1B). To prevent graft rejection, animals received daily injections of cyclosporin A (Novartis, Nürnberg, Germany, 10 mg/kg, i.p.) throughout the experiment, commencing the day before transplantation. Four weeks after transplantation animals received cyclosporin A every second day.

Histology and immunohistochemistry

Following final anaesthesia animals were perfused with 4% paraformaldehyde at 1, 4 and 12 weeks after transplantation. After post-fixation overnight, brains were cryoprotected and frozen in methylbutane at −80°C. Coronal sections (20-μm thick) were prepared with a cryotome (Leica, Nussloch, Germany). For immunohistochemistry, sections were treated with 10% non-immune donkey serum (Sigma). Primary antibodies, applied at 4°C overnight, were monoclonal mouse antibodies to neuronal nuclear antigen (NeuN, 1:200, Chemicon), glial fibrillary acidic protein (GFAP, 1:200, Chemicon), nestin (1:200, BD Transduction Laboratories, San Jose, CA, USA), βIII tubulin (1:1000, Promega, Mannheim, Germany), CNPase (1:1000, Abcam, Cambridge, UK), SV2 (1:200, Developmental Hybridoma Bank, Iowa City, IA, USA) and ED1 (1:200, Chemicon), monoclonal rabbit antibody to caspase 3A (1:1000, BD Pharmingen, San Diego, CA, USA), polyclonal goat antibodies to doublecortin (DCX, 1:300, Santa Cruz...
For control experiments, at room temperature (RT). Nuclei were counterstained with DAPI and donkey anti-rabbit (1:500, Dianova, Hamburg, Germany) for 2 h secondary antibodies, e.g. donkey anti-mouse, donkey anti-goat bodies were visualized using corresponding Cy3-conjugated (1:500, Alomone Laboratories, Jerusalem, Israel). Primary anti-

The occlusion of the middle cerebral artery (red mark in Fig. 1A) and a cortico-striatal infarct shown as grey area. EGFP<sup>+</sup> ESC-derived neural precursors were transplanted into the necrotic focus (lateral green arrow) and at two sites next to the ipsilateral midline (medial green arrows) of the brain. (B) In a contralateral transplantation, EGFP<sup>+</sup> ESC-derived precursors were transplanted into the non-lesioned striatum. (C) Graft overview and detection of transplanted ESCs by EGFP labelling 4 and 12 weeks after transplantation (arrows). Representative lateral graft in the necrotic area, 4 weeks after transplantation, demonstrated by Nissl staining (C) and corresponding EGFP-fluorescence (E). An infarct without transplantation, 4 weeks after MCAO, is shown in D. (F) EGFP<sup>+</sup> lateral graft, 12 weeks after transplantation. The enlargement of the ventricles in this image is not representative as it was variable and did not correlate with graft size or experimental group. (G) Inset shows the accumulation of ED1<sup>+</sup> macrophages/microglial cells (red) around the EGFP<sup>+</sup> graft (green) 12 weeks after transplantation. (H, I) Percentage of cells with cell type-specific marker expression (NeuN, GFAP) in the total population of EGFP<sup>+</sup> cells 4 and 12 weeks after transplantation. Scale bars: C–F 500 μm; G, 200 μm; Cx = cortex; S = striatum; LV = lateral ventricle.

Electrophysiological recordings

Experiments were performed by means of the patch-clamp technique (Hamill et al., 1981; Scholz and Vogel, 2000) on visually identified grafted EGFP<sup>+</sup> cells in 150–200 μm slices prepared on a vibrisclicer (Leica VT 1000S) from animals at 4–7 weeks after transplantation. Slices were prepared in ice-cold extracellular solution (2–6°C) with modifications (Takahashi, 1990; Scholz et al., 1998) and embedded in 2% (w/v) agar (at 40°C). Coronal slices were stored at RT (22–24°C) for up to 12 h. All concentrations are given in mM. Extracellular solution contained NaCl 115, KCl 5.6, MgCl<sub>2</sub> 1, glucose 11, NaH<sub>2</sub>P<sub>4</sub> 1, NaHCO<sub>3</sub> 25, CaCl<sub>2</sub> 2.2, tetrodotoxin (TTX, Latoxan, Valence, France) 0.0001, and ethylene glycol-bis(β-aminoethyl ether) N,N',N',N'-tetraacetic acid (EGTA) 10, HEPES 10 adjusted with NaOH to pH 7.3. Patch-clamp pipettes (final resistances of 2.5–5.0 MΩ) were fabricated from borosilicate glass tubing (GC 150, Clark Electromedical Instruments, Endenbridge, Kent, UK). Whole-cell recordings were obtained using an Axopatch 200A (Axon Instruments, Sunnyvale, CA, USA) patch-clamp amplifier (Hamill et al., 1981; Scholz et al., 1998; Scholz and Vogel, 2000) at RT (22–24°C). Data points from activation and inactivation curves were fitted with a Boltzmann distribution (Scholz et al., 1998). Statistical significance (defined as P < 0.05) was determined by Student’s t test, and data are expressed as means ± standard error of the mean (SEM).
Results
Survival and migration of grafted cells

In vitro pre-differentiated neural precursors marked with EGFP were transplanted 1 week after MCAO into the infarct core (lateral graft, 100,000 cells, Fig. 1A) and at two different sites next to the ipsilateral midline within the cortex and striatum (medial graft, 200,000 cells in total, Fig. 1A). For evaluation of cell migration, some animals subjected to MCAO received solely contralateral striatal grafts (Fig. 1B).

In the ipsilateral transplantation paradigm, grafted cells were found in 10 of 11 animals, 4 weeks after grafting, and in 4 of 10 animals 12 weeks after grafting. Notably, in 8 of 11 transplanted animals, grafted cells were found in the necrotic area after 4 weeks (designated as lateral graft; Fig. 1C, E) and in 2 of 10 animals 12 weeks after grafting (Fig. 1F). Medial grafts were detected in 10 of 11 animals after 4 weeks and in 2 of 10 animals after 12 weeks. Remarkably, in some animals either the medial or the lateral grafts survived. The sizes of the surviving lateral grafts decreased from on average 7 mm³ (100,000 transplanted cells; n = 10) 4 weeks after transplantation to 0.18 mm³ or 0.56 mm³ (n = 2) 12 weeks after transplantation. The size of the surviving medial grafts decreased from on average 12 mm³ (200,000 transplanted cells, n = 10) 4 weeks after transplantation to 0.08 mm³ or 0.05 mm³ (n = 2) 12 weeks after transplantation. These data show that reduction of graft size occurs mainly between 1 and 3 months after transplantation and not in the acute period after stroke. To investigate if apoptotic cell death plays a role in graft size reduction over time, we performed caspase 3 immunohistochemistry. At 1, 4 and 12 weeks after transplantation only very few (<5%) caspase 3+/EGFP+ cells could be detected suggesting that apoptotic cell death is not the most prominent cause of the pronounced reduction in graft size (Supplementary Fig. 1A–F is available at Brain Online). Thus, we investigated whether an ongoing immune response characterized by macrophages/microglial cells could be observed after transplantation. Indeed, ED1+ macrophages/microglial cells surrounding and infiltrating grafted cells were detected at 1, 4 and 12 weeks after transplantation in both, infarcted and non-infarcted animals (Fig. 1G), suggesting that graft rejection occurs in spite of cyclosporin A treatment. Average migration of ipsilaterally transplanted cells was 300 μm away from the graft–host border zone (data not shown). Contralaterally grafted neural precursors did not reach the ipsilateral lesion side, but some cells reached the corpus callosum or lateral ventricle within a distance of 100 and 200 μm away from the graft core.

Proliferation and differentiation of grafted cells

One week after transplantation many of the EGFP+ grafted cells were also Ki-67+, thus demonstrating high proliferative activity (Supplementary Fig. 2A–C is available at Brain Online). At this time point, most of the grafted cells were negative for mature neuronal markers, such as NeuN and NF-200, or mature glial markers, such as GFAP for astrocytes and CNPase for oligodendrocytes (data not shown).

The number of Ki-67+/EGFP+ cells had decreased, 4 weeks after transplantation (Supplementary Fig. 2D–F is available at Brain Online), whereas the number of NeuN+/EGFP+ and GFAP+/EGFP+ cells had increased. Of the grafted cells ~30% now expressed NeuN, demonstrating immunohistochemically a progressive maturation into neurons (Figs 1H and I–L) and ~7% had differentiated into GFAP+ astrocytes (Figs 1I and 2P). A small number (<1%) of grafted cells had differentiated into oligodendrocytes as indicated by expression of CNPase (Fig. 2M–O). To verify the total neuronal yield of grafted cells, we used additional
pan-neuronal markers, such as DCX (Fig. 2A–C), βIII tubulin (Fig. 2D–F) and NF-200 (Fig. 2G–I). Although quantification of single cells expressing these neuronal markers was not possible, as immunoreactive cellular processes could not always be allocated to the corresponding cell somata, those markers appeared to detect a similar neuronal yield in comparison to NeuN. Some NF-200+ neurites emerging from EGFP+ cells were seen to project into the surrounding host tissue (Fig. 3). The transplantation site did not appear to influence the neuronal or astrocytic yield, as the proportion of neurons and astrocytes was not different between medial and lateral grafts. We detected neurons of several different neurotransmitter-subtypes including cholinergic (1.4% of all grafted cells; Fig. 4A–C), serotonergic (1.8%; Fig. 4G) and GABAergic neurons (Fig. 4D–F) as well as striatal neurons expressing substance P (1.4%; Fig. 4H) or DARPP32 (6.4%; Fig. 4I). Similar to βIII tubulin and NF-200, the quantification of GABAergic neurons was also difficult because it was not possible to allocate the dense GABA+ network of cellular processes to cell bodies. However, the abundant expression of GABA within the graft in comparison to the above mentioned neurotransmitter-subtypes suggests that GABAergic neurons constitute the majority of graft-derived neurons.

After 12 weeks post-transplantation the proportion of NeuN+ neurons or GFAP+ astrocytes of all transplanted cells had not changed substantially and amounted to 25 or 8% of all transplanted cells, respectively (Fig. 1H, I). As GFAP antibodies used for quantification of astrocytes only detect subpopulations of this cell type (Eng et al., 2000) the total number of graft-derived astrocytes could not be visualized...
Neuronal differentiation in stroke lesions

immunohistochemically. We did not note any signs of tumour growth between 4 and 12 weeks after grafting as the percentage of Ki-67+/EGFP+ grafted cells was low (<5%) and the graft volume decreased (see above). To detect possible synaptic input onto grafted cells immunohistochemically, we used the marker SV2 that specifically labels presynaptic vesicles. Indeed, 4 and 12 weeks after transplantation many EGFP+ grafted cells were surrounded by punctate SV2+ structures (Fig. 2R).

Electrophysiological properties of transplanted cells

Patch-clamp recordings were performed on transplanted EGFP+ cells in the centre of the graft as well as in the periphery between 4 and 7 weeks after transplantation. The diffusion of EGFP into the patch pipette confirmed the recording from grafted cells. The presence of inward Na+ currents was demonstrated in voltage-clamp configuration. Typically, a large voltage dependent inward current with fast inactivation kinetics was observed in 38 grafted cells from a total of 137 investigated grafted cells (27.7%, Fig. 4M). In 16 out of 38 cells the recorded I/V curve revealed a threshold of approximately −50 mV and a reversal potential of −61 mV that is close to the Nernst potential for Na+ of 58.7 mV (Fig. 4N). The calculated half-maximal (E50) activation of −33.2 ± 0.6 mV (n = 16) with a steepness of 5.1 ± 0.6 mV per e-fold was derived from a fitted activation curve (Boltzmann distribution, Fig. 4O). The inactivation curve was calculated from peak inward currents at 20 mV preceded by a 100 ms pre-pulse to varying potentials. The E50 was −33.6 ± 0.5 mV (n = 7) with a steepness of 5.1 ± 0.5 mV per e-fold (Fig. 4O). Furthermore, voltage-gated Na+ currents were tested by application of 100 nM TTX which blocked the maximal inward current from 1.2 pA to 0.2 nA (n = 8, Fig. 5A). The expression of Na1.1, a protein specifying a subtype type of Na+ channel, was confirmed by immunohistochemistry, 4 weeks after transplantation (Fig. 5A). Na1.2, an additional specific Na+ channel type, was also detected in grafted cells (data not shown), 12 weeks after transplantation. Outward currents in these graft-derived neurons showed characteristics of slow inactivation with varying time constants >200 ms (Fig. 5B), and their thresholds were around −40 mV (Fig. 4P). Additionally, outward currents could be blocked by 10 mM TEA from 2.3 to 0.4 nA, which results in the inability to generate APs (n = 5, Fig. 5C) in parallel to a strong depolarization of the resting potential (Erest, Fig. 5C). Interestingly, the duration of APs of grafted cells was shorter than 6 weeks in comparison to 3 weeks, indicating that they had evolved along the neuronal differentiation pathway (Fig. 6A). Transplanted cells generated repetitive firing at sustained current injections at native Er (Fig. 6B), which was seen increasingly in 2 out of 13 cells or in 6 out of 8 cells, 4 or 7 weeks after transplantation, respectively. There was no difference in the ability of repetitive firing neither at native nor at fixed resting potentials. The AP duration (APD) was comparatively long at 4 weeks after transplantation with 6.5 ± 0.8 ms (n = 7) measured at half-maximal AP amplitude and shortened 7 weeks after transplantation to 2.2 ± 0.2 ms (n = 6, Fig. 6C) at native Er. Interestingly, the APD was shortened with a fixed Er at −80 mV compared with the control at native Er, except after transplantation times of >6 weeks. However, the AP amplitude was small under native Er with 68.2 ± 9.6 mV (n = 7, 4 weeks) which increased to 95.9 ± 13.7 mV (n = 6, Fig. 6C) at 7 weeks. A fixed Er of −80 mV resulted in comparable AP amplitudes of 118–125 mV at all time points studied (Fig. 6C). The native Er was at −25.8 ± 9.0 mV, 3 weeks after transplantation, whereas a more negative Er of −54.5 ± 4.3 mV was observed at 7 weeks after transplantation (Fig. 6C). In summary, AP and Er characteristics of graft-derived cells were typical for electrophysiologically competent mature neurons.

Another type of K+ current was revealed by superfusion with external low Ca2+ solution that led to a reversible reduction of total outward currents pointing to Ca2+-dependent K+ currents (Fig. 7A, B). This reduction of Ca2+-dependent K+ currents, due to diminished Ca2+ inward currents, resulted in a prolonged AP duration (Fig. 7C).
Between 5 and 7 weeks after transplantation we noticed spontaneous inward currents in graft-derived neurons that desensitized within 2–4 ms at $E = -60$ mV (Fig. 6D). We analysed with event-based templates continuous voltage-clamp recordings to determine the peak amplitude of each event which revealed a minimal event amplitude at 11.6 ± 0.6 pA (Fig. 6E). In current-clamp recordings we observed fast spontaneous depolarizations of different voltages, reflecting different amplitudes of excitatory post-synaptic currents (EPSCs) in graft-derived neurons (Fig. 6F). A functional effect of excitatory post-synaptic potentials (EPSPs) was the generation of APs in 7 out of 13 cells. Grafted cells that generated spontaneous AP activity revealed spontaneous EPSPs in all cases (7 of 7) at frequencies of 1–18.5 Hz (Fig. 6G). At low Ca$^{2+}$ conditions we measured a reduced frequency in occurrence and size of the EPSC-amplitudes and, similarly, a reduction but not complete suppression of EPSPs and spontaneous APs.

In addition to cells that showed electrophysiological characteristics of neurons, we also detected graft-derived cells with typical features of astrocytes ($n = 99, 72.3%$ of all investigated cells). In these ramified cells with astroglial morphology, we could elicit outward currents with faster inactivation kinetics similar to A-type K$^+$ currents, in comparison to graft-derived neurons (Fig. 8A and B). The threshold of activation was around $-30$ mV and $E_{so}$ of...
Neuronal differentiation in stroke lesions

Brain (2006), 129, 3238–3248 3245

activation was 2.1 ± 0.7 mV with a slope factor of 18.1 ± 0.7 per e-fold (Fig. 8C). A three times smaller peak amplitude of outward currents (0.9 ± 0.2 nA) was measured in these cells compared with graft-derived neurons (Fig. 8D). Their input resistance was at 2.7 ± 1.2 GΩ and their native $E_r$ was with −38 mV more positive than the $E_r$ of all graft-derived neurons (Fig. 8E).

Discussion

A cell-based therapy for stroke lesions faces predominantly two major problems: first, grafts have to survive in an inhospitable environment, and second, grafts have to substitute or rescue neural cells with many different phenotypes. This demand is more pronounced when compared with Parkinson’s (Lee et al., 2000a; Freed et al., 2001; Bjorklund et al., 2002; Kim et al., 2002) or Huntington’s (Fricker-Gates et al., 2004) diseases that are known for a more selective degeneration of a particular neuronal cell type.

The few stem cell transplantation studies that have been carried out in stroke models in rodents have shown improvements in locomotor behaviour (Borlongan et al., 1998; Chen et al., 2001b; Li et al., 2001; Zhao et al., 2002). However, in most of these studies long-term graft survival and neuronal yield was poor and, so far, studies on adequate neuronal and astrocytic function in terms of electrophysiological activity of the grafted cells in the adult lesioned brain have not been reported. Thus, beneficial effects could have been due, for instance, to graft-derived rescue of host cells rather than to graft-derived neuronal substitution (Mahmood et al., 2004). Till present, only one study that demonstrated considerable behavioural improvement after ESC transplantation in an animal model for stroke has described immunohistochemically the generation of ESC-derived neurons (Wei et al., 2005). This was interpreted as a possible link between behavioural improvement and activity of graft-derived neurons. However, grafts were only observed for 2 weeks after transplantation and, as in all other previous studies, electrophysiological investigations of grafted cells were not carried out. Our goal, thus, was to demonstrate by morphological and electrophysiological criteria that ESC-derived cells can differentiate into functional neurons and astrocytes in a rodent model of stroke. We show that ESC-derived neuronal cells survived at least partly for up to 12 weeks after transplantation into the ischaemic focus. The grafted cells differentiated into several types of mature neurons and astrocytes, and extended immunohistochemically identifiable neuronal processes into the host tissue. Electrophysiological measurements indicated appropriate functional activity of graft-derived neurons and glial cells.

While 1 week after transplantation ESC-derived neural precursors showed high proliferative activity, 4 weeks after transplantation proliferation was reduced. This agrees with observations by Deacon et al. (1998) and suggests that transplanted precursor cells first proliferate after grafting before they differentiated. Thus, neurally pre-differentiated ESC-derived precursors of which ~16% show proliferative activity in vitro at the time of grafting (Dihné et al., 2006) proliferated after transplantation, but did not show subsequent tumour formation. The pronounced decline of graft size between 4 and 12 weeks was possibly derived from the immunological rejection in the xenogeneic transplantation paradigm through ED1$^+$ macrophages/microglial cells that we and others observed around and within grafts even 12 weeks after transplantation, although we used the immunosuppressant cyclosporin A (Poltorak and Freed, 1989; Finsen et al., 1991; Bjorklund et al., 2003). Reduction in graft size is most probably not primarily due to ischaemia-related processes, such as secondary inflammation that is usually attenuated by 5 weeks after MCAO (Stoll et al., 1998). Graft apoptosis due to insufficient trophic support, for instance, also does not appear to play a major role in the reduction of graft size, since numbers of caspase 3$^+$/EGFP$^+$ grafted cells were small at all survival time points investigated in this study. In summary, our results show that ESC-derived grafts transplanted into the centre of a necrotic lesion partially survive up to 12 weeks. However, since the decline in graft size after transplantation is likely to continue beyond 12 weeks, measures have to be taken to improve survival, for instance, by more effective immunosuppression, syngeneic transplantation strategies and/or beneficial molecular engineering of the transplanted cells.

While Hoehn et al. (2002) described extensive cell migration into the focal ischaemic brain lesion after transplanting immature ESCs contralaterally, we only found very limited migration when cells were transplanted contralaterally or ipsilaterally to the lesion. It is therefore possible that the migratory potential of immature ESCs is higher than that of pre-differentiated neural precursors, the cells that were used in the present study. This observation calls for experimental approaches to enhance the migratory potential of the grafted cells, preferably in their less differentiated state.

Grafted cells differentiated well as seen by their time-dependent increase in mature neuronal and glial markers after transplantation. While mature neuronal phenotypes were hardly detectable at 1 week after transplantation, at 4 and 12 weeks after transplantation ~30 or 25% of all grafted cells, respectively, expressed the pan-neuronal marker NeuN, in agreement with our electrophysiological recordings showing that ~28% of the investigated cells are neurons. This neuronal yield is supported by the values of immunoreactivity for doublecortin, NF-200, and βIII tubulin. Interestingly, after differentiation of ESC-derived neural precursors in vitro, only ~13% of cells expressed neuronal markers suggesting that host cues positively influence differentiation of precursors into neurons. The remaining part of grafted cells largely consists of astrocytes whose proportion could not be fully detected by the GFAP marker since it mostly labels cell processes and not cell
bodies (7–8% of all grafted cells), but amounted to higher values by electrophysiological characterizations (72% of all grafted cells). The extent of neuronal yield is in accordance with data presented by Wei et al. (2005) who detected ~34% ESC-derived neurons. However, graft survival and neuronal yield in the latter study was determined only for up to 2 weeks after transplantation.

In addition, our study shows that ESC-derived neural precursors transplanted into a stroke lesion differentiate into diverse neuronal subtypes, such as cholinergic, serotonergic and GABAergic neurons, as well as into striatal neurons expressing substance P and DARPP32. These results are similar to those recently published by Hayashi et al. (2006) and illustrate that ESC-derived grafts can differentiate into diverse neuronal cell types, a prerequisite to repopulate necrotic brain regions consisting of distinct types of neurons prior to the insult. The expression of the presynaptic vesicle marker SV2 by the grafted cells suggested their functional maturation. Whether SV2+ presynaptic vesicles originate from grafted cells and/or from host cells could not be determined due to limited microscopic resolution.

To further elucidate the functional properties of graft-derived neurons within the host tissue we investigated the electrophysiological features of transplanted cells at different time points after grafting. While voltage-gated inward and outward currents as well as expression of functional neurotransmitter receptors and synaptic contacts had been described for ESC-derived neurons in vitro (Bain et al., 1995; Strubing et al., 1995; Finley et al., 1996), we did not find any report on their electrophysiological features after transplantation into a stroke lesion of an adult animal. Only few functional in vivo data are available for ESC-derived neurons, e.g. when transplanted into the embryonic brain or into an animal model for Parkinson’s disease (Kim et al., 2002; Wernig et al., 2004). Also one in vitro study of hippocampal slice cultures has described some electrophysiological characteristics of ESC-derived neurons with the indication that it needs to be followed up by in vivo studies (Benninger et al., 2003).

Although patch-clamp recordings have been obtained in vitro from slices of brains of older rodents (>P18), these recordings were less successful than in younger animals. To succeed, our recordings were thus obtained mostly from superficially situated cells in slices containing transplants. In 27.7% of the transplanted cells we recorded APs and voltage-gated Na+ and K+ currents confirming their neuronal phenotype. Inward currents of transplanted cells were blocked largely by 100 nM TTX, classifying them as TTX-sensitive Na+ currents (Scholz and Vogel, 2000). The remaining inward currents exhibited kinetics typical for Ca2+ currents, being slower in comparison to those of voltage gated Na+ currents. The slow inactivation kinetics of outward currents and their block by 10 mM TEA, identified them as delayed rectifier K+ currents which not only contribute to the re-polarization phase of an AP, but also to the resting potential (RP). The calcium-dependency of these K+ currents was indicated by a reduction of total outward current in the presence of low Ca2+ concentrations and an increase in total outward current in the presence of high Ca2+ concentrations. Ca2+ activated K+ currents in graft-derived neurons contribute to the modulation of AP fired by rodent neurons (Scholz et al., 1998). These observations further support the notion that the graft-derived neurons have been functionally differentiated within the host tissue. The kinetic parameters of graft-derived Na+ currents matched most closely those of NaA.1 channels that are the most abundant channels in the CNS (Goldin et al., 2000), although its E50 of activation was 15 mV more negative than is described for this channel. The presence of this specific subtype of Na+ channel in grafted cells, 4 weeks after transplantation, was further supported by NaA.1.1 immunohistochecmy. At 12 weeks after transplantation, also NaA.1.2 channels were detected on the processes of grafted cells. Since NaA.1.2 channels are known to be expressed later than NaA.1.1 channels during CNS development (Spampanato et al., 2001), our data reveal a similar progressive maturation of graft-derived neurons and neurons in situ. Also our electrophysiological data illustrate a progressive neuronal maturation of graft-derived cells as they developed the ability to fire trains of APs. The increased AP amplitudes at fixed Erest of -80 mV can be explained by an increased availability of Na+ currents, as inactivation curves revealed substantial inactivation at resting potentials of -30 to -45 mV. Graft-derived glial cells showed a distinct current signature in comparison to graft-derived neurons (with -2.2 ± 0.4 nA Na+ currents), as they lacked Na+ currents and the ability to generate APs. Additionally, outward currents of graft-derived glial cells showed different kinetics compared with graft-derived neurons. Finally, graft-derived glial cells showed three times smaller outward current amplitudes of the A-type-like K+ current compared with the delayed rectifier K+ current type in graft-derived neurons.

Surprisingly, we noticed spontaneous EPSPs and EPSCs in graft-derived neurons between 4 and 7 weeks after transplantation, showing that they had received synaptic input. These spontaneous synaptic inputs to transplanted cells result in a sustained firing of APs with varying frequencies, being typical for cortical neuronal networks (Antkowiak, 2002). Connectivity in grafts was also observed by Wernig et al. (2004), although an extrinsic stimulation was necessary to evoke EPSPs. The origin of this input in our preparations, be it host- or graft-derived, has to be clarified by further experiments. However, as the vast majority of the investigated graft-derived cells with spontaneous EPSPs was situated within the host parenchyma, several hundreds of micrometres away from the graft core, it is likely that synaptic input onto graft-derived neurons originated from the host tissue.

In summary, our data demonstrate that ESC-derived neural precursors can survive in an adult stroke lesion paradigm for up to 12 weeks, the longest time period tested.
after transplantation. However, as survival was poor, most probably due to an ongoing immune response that may not have been fully suppressed by the immunosuppressant cyclosporin A, improved immunosuppressive strategies or generation of syngeneic and thus immunologically tolerated neural precursor cells must be found. Nevertheless, our data show that ESC-derived neural precursors differentiate into several types of neural cells. The presence of immuno-histochemically and electrophysiologically identified mature neurons and astrocytes indicate a previously not substantiated potential of ESCs to develop functional characteristics in a highly disturbed, large and inhospitable brain lesion that may be accessible for cell therapy. Further experiments are needed to show that the grafted cells effectively signal to the host and vice versa by electrophysiological demonstration of, for instance, paired recordings as the most stringent criteria for functional integration and cross-talk between host and graft. Also, genetic engineering of ESCs may provide a way to beneficially influence these cells to provide improved functional recovery.

**Supplementary material**

Supplementary data are available at *Brain* Online.

**Acknowledgements**

This project was supported by the Land Sachsen-Anhalt (FKZ-Nr.3521E/0703M to C.B., H.B. and K.G.R.), Deutsche Forschungsgemeinschaft (Projekt-Nr. Di 881/1-1 to M.D., Ch.B., M.S. and VO20-2/3 to A.S.), and the VW Stiftung (FKZ-Nr.3521E/0703M to C.B., H.B. and K.G.R.), Deutsche Forschungsgemeinschaft (Projekt-Nr. Di 881/1-1 to M.D., Ch.B., M.S. and VO20-2/3 to A.S.), and the VW Stiftung (FKZ-Nr.3521E/0703M to C.B., H.B. and K.G.R.).

**References**

D. Trceczak, B. Agari and O. Becker.


Poltorak M, Freed WJ. Immunological reactions induced by intracerebral transplantation: evidence that host microglia but not astroglia are the antigen-presenting cells. Exp Neurol 1989; 103: 222–33.


