Equivalent Neurogenic Potential of Wild-Type and GFP-Labeled Fetal-Derived Neural Progenitor Cells Before and After Transplantation Into the Rodent Hippocampus

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Introduction. The hippocampal formation is a specific structure in the brain where neurogenesis occurs throughout adulthood and in which the neuronal cell loss causes various demential states. The main goal of this study was to verify whether fetal neural progenitor cells (NPCs) from transgenic rats expressing green fluorescent protein (GFP) retain the ability to differentiate into neuronal cells and to integrate into the hippocampal circuitry after transplantation.

Methods. NPCs were isolated from E14 (gestational age: 14 days postconception) transgenic-Lewis and wild-type Sprague-Dawley rat embryos. Wild-type and transgenic cells were expanded and induced to differentiate into a neuronal lineage in vitro. Immunocytochemical and electrophysiological analysis were performed in both groups. GFP-expressing cells were implanted into the hippocampus and recorded electrophysiologically 3 months thereafter. Immunohistochimical analysis confirmed neuronal differentiation, and the yield of neuronal cells was determined stereologically.

Results. NPCs derived from wild-type and transgenic animals are similar regarding their ability to generate neuronal cells in vitro. Neuronal maturity was confirmed by immunocytochemistry and electrophysiology, with demonstration of voltage-gated ionic currents, firing activity, and spontaneous synaptic currents. GFP-NPCs were also able to differentiate into mature neurons after implantation into the hippocampus, where they formed functional synaptic contacts.

Conclusions. GFP-transgenic cells represent an important tool in transplantation studies. Herein, we demonstrate their ability to generate functional neurons both in vitro and in vivo conditions. Neurons derived from fetal NPCs were able to integrate into the normal hippocampal circuitry. The high yield of mature neurons generated render these cells important candidates for restorative approaches based on cell therapy.

Keywords: Neural progenitor cells, Transplantation, Electrophysiology, Synaptic currents.

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circuits was published only in 2005 (7, 8). In this regard, because of technical difficulties in labeling transplanted cells specifically and recognizing them in living slices, few reports have described electrophysiologic maturation and integration of progenitor cells grafted in the brain. Considering fetal-derived neural progenitor cells (NPCs), there are only few reports on functional maturation after transplantation into the brain of neonatal rats (9, 10). Under these conditions, the favorable microenvironment in the developing brain, permissive to maturation of stem cells, represents a distinct situation to that observed in the adult or in diseased brain.

Recently, Kobayashi and coworkers (11) developed an inbred transgenic Lewis rat strain that express green fluorescent protein (GFP) ubiquitously. In this strain, the reporter genes were driven under a ubiquitous cytomegalovirus enhancer/chicken β-actin promoter. This rat model represents a valuable tool in the context of cell-based therapies and cellular transplantation studies. For instances, the GFP is an ideal marker because it simplifies genotyping and can be visualized directly under ultraviolet light or by immunohistochemical enhancement. However, it remains to be clarified whether the neurogenic potential of GFP-labeled NPCs is similar to that of wild-type cells, and whether GFP expression is stable through all developmental stages.

Based on the background outlined, the main scope of this study was to evaluate the neurogenic potential of expanded GFP-labeled NPCs in comparison with wild-type ones and the ability of those cells to generate functional neurons after implantation into the adult hippocampus, where they might be integrated into local neuronal circuitries.

**RESULTS**

**Differentiation of Cells Derived From GFP-Expressing Transgenic Fetal Tissue**

After expansion in medium containing fibroblast growth factor b and epithelial growth factor, aggregates of NPCs are formed, so-called neurospheres, which are strongly positive for markers of immature neuroepithelial cells such as nestin (Fig. 1A). At this stage, cultures derived from wild-type and transgenic animals were stained for nestin and glial fibrilar acidic protein (GFAP), without statistical difference (α=5%, P for nestin 0.8675 and for GFAP 0.9132, n=5 experiments). In both groups, the percentage of microtubule-associated protein 2 (MAP2)-positive cells at this stage was 0 (P=0.9119; Fig. 2A).

By cultivating the cells onto polyornithine-coated cover slips in the presence of brain-derived neurotrophic factor and 3-isobutyl-1-methylxanthin, phosphodiesterase inhibitor, cellular elongations grew from the spheres, and neuroblasts were formed, which then differentiated into neurons during their migration process away from the spheres, as described previously (Fig. 1B–D) (12). After 1 week, expression of neuronal markers was observed, such as MAP2, isoforms a and b (MAP2ab), βIII-
tubulin (which marks also young neuronal cells), neuron-specific nuclear protein (NeuN), and neurofilament 200 kDa (NF200). The comparison between wild-type and transgenic cultures revealed no statistical difference, for α of 5% (P values: MAP2, 0.5643; βIII-tubulin, 0.6782; NeuN, 0.6734; and NF200, 0.7719; Fig. 2B).

**Comparison Between Transgenic and Wild-Type Cultures Regarding Functional Maturation In Vitro**

At the seventh differentiation day in vitro, GFP-expressing and wild-type cells were compared regarding their electrophysiologic properties. Voltage-clamp recordings revealed high-amplitude Na and K currents, with voltage-dependent activation identical to that of mature neuronal cells. In Figure 3, a typical recording from a neuron derived from GFP-NPCs is shown. At this stage, we could elicit trends of action potentials in current clamp, and spontaneous synaptic currents could be recorded on both cell groups in a comparable extent. The peak amplitudes of Na and K currents were analyzed in both groups, and no statistical difference was found (Fig. 2C, P=0.4378).

**Analysis of Functional Maturity of GFP-Expressing Cells After Implantation Into the Rodent Hippocampus**

Of the surviving cells expanded in vitro, 97.4% expressed high levels of GFP just before stereotactic implantation. Three months after implantation, the hippocampus was populated by GFP-positive cells, most of which were also positive for mature neuronal markers. Figure 4 depicts examples of cells positive for MAP2ab (A–C), NF200 (D–F), and NeuN (G–I). Synthesis of neurotransmitters typically encountered in the hippocampal formation, such as γ-aminobutyric acid (GABA)-amino-transporter 1 (J–L) and glutamate (M–O), was also demonstrated by immunohistochemistry. Stereologic quantifications revealed 4514 ± 317 GFP-positive cells, and 3528 ± 219 NeuN/GFP-positive neurons derived from the implanted cells, which corresponds to 3.0% and 2.4% of the implanted cells.

The functional maturation of the implanted cells was also assessed by patch clamp (Fig. 5). The voltage-clamp protocol revealed large inward Na⁺ and outward K⁺ currents, as confirmed after pharmacologic blocking. These current-voltage relationships depicted a clear voltage dependence. We were also able to elicit trends of action potentials in current clamp mode, by injection of −20- to +70-pA current during 1000 msec from a holding potential of −80 mV (Fig. 5E). Excitatory postsynaptic potentials were recordable in current clamp mode (Fig. 5F); blockade of the GABAergic component with bicuculline was shown to reduce the frequency and amplitude of synaptic events, whereas total blockade was achieved when the glutamate α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate, glutamate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione, AMPA/kainate receptor antagonist was added to bicuculline. Taken together, our data indicate that neurons generated from grafted cells were indeed being incorporated into local host neuronal networks.

**DISCUSSION**

In this study, we report complete morphologic maturation of GFP-labeled NPCs in vitro and after implantation into normal nonlesioned hippocampus, evidenced (1) by expression of mature neuronal markers in immunohistochemical staining (NeuN, MAP2, and NF200) and (2) through synthesis of GABA and glutamate, demonstrated by immunohistochemistry. In addition, electrophysiology confirmed neuronal maturity, detecting large Na⁺ and K⁺ voltage-dependent currents, firing of trends of action potentials, and the presence of spontaneous GABAergic and glutamatergic excitatory synaptic potentials. Moreover, the functional maturation achieved by GFP-labeled cells was similar to that of wild-type cells, and GFP expression was also demonstrated by immunohistochemistry.
was stable throughout the differentiation process. The functional integration of transplanted cells reported herein supports the notion that the activity of neurons derived from the grafted cells can be regulated by host neurons. Precisely how these cells influence the overall behavioral performance in animals remain unclear and should be the focus of future investigations.

Fetal neural tissue has been used extensively for restorative purposes. These important reports provided the first evidence that primary fetal cells were able to survive within the brain of the host and generate mature neurons, which in turn grew long axons that could reach relatively distal structures, organize new synapses, and release neurotransmitters such as dopamine (6, 13–17). Several clinical trials in humans with Parkinson’s disease have been described that adult neuronal cells initially labeled both transgenic and wild-type fetal neural tissue. In addition, these studies reported similar functional properties of neurons derived from neural stem cells. In fact, the interneuron cell type seemed to predominate in our slices.

In vitro functional maturation of green fluorescent protein-labeled neural stem cells derived from rodent fetal neuronal tissue. (A) Phase contrast microscopy showing the patching process of a cell in culture. (B) Voltage clamp traces showing absolute inward and outward currents from cell patched in A; holding potential −80 mV, pulse duration 100 msec, intensities from −70 to +70 mV at 10 mV increment; leak and capacitive currents were subtracted by a p/-4 protocol (for details see text), traces represent average of five consecutive sweeps. (C) Voltage-dependent activation of Na+ currents (I–V relationship). Experimental data were fitted with a Boltzmann function multiplied with a driving force derived from the Nernst equation in the form

\[ I(V) = \frac{[(V-E)G_{\text{max}}]}{(1 + \exp[-(V-V^\text{1/2})/\text{K})]}. \]

Best-fit parameters were for the represented cell V1/2 = −8.7 ± 0.5 mV, slope = 7.6 ± 0.4 mV, gmax = 39.8 ± 1.8 nS, and ENa = 101.4 ± 2.4 mV. (D) I–V relationships for outward currents (K+ currents), fitted with the same theoretical model described for Na+. Best-fit parameters were V1/2 = 15.5 ± 0.8 mV, slope = 11.0 ± 0.3 mV, gmax = 29.8 ± 1.9 nS, and EK = −89.5 ± 10.1 mV. (E) Trends of action potentials elicited in current clamp after injection of a 70-pA current applied during 100 msec. (F) Spontaneous synaptic currents recorded from the same cell.

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with GFP do not continue expressing GFP by the end of the maturation process (25). Our data do not corroborate these previous observations but underline the value of GFP-labeling methods on studying neuronal differentiation after transplantation. Moreover, in further studies on dopaminergic differentiation of GFP-labeled NPCs from the same rat strain in a rat model of Parkinson’s disease, we could verify that up to 80% of striatal tyrosine hydroxylase-positive cells on the lesion side coexpress GFP 15 weeks after transplantation, proving a remarkable stability of the marker (26).
Another central aspect of this study investigates the generation and integration of new neuronal cells derived from implanted stem cells into the hippocampus. It has been recently shown that young granular cells in the dentate gyrus differ substantially to their older neighboring counterparts. For instance, they have high input resistance, which leads to enhanced excitability and generation of action potentials in response to even weak excitatory stimuli, in addition to being more suitable to long-term potentiation, which indicates an enhanced synaptic plasticity (27–31). In general, an adequate learning process is dependent on proliferation of local neural stem cells, survival of newborn neurons, and also apoptosis of relatively immature cells that have not established learning-related synaptic connections (32, 33). In fact, the hypothesis that inhibition of neurogenesis may cause learning impairment, while enhanced neurogenesis may improve learning, has recently been confirmed by various authors (21, 34–37). It has been demonstrated by mathematical simulations that incorporation of new neurons facilitates both clearance of old memories and storage of new information (38–40). This pattern seems to represent a complex balance where an increased number of new granule cells can facilitate epileptiform activities (41). Conversely, the role of artificially implanted stem cells in this system, with the possibility of synaptic integration and formation of new circuits, and the behavioral significance of these changes have been largely unknown and represent an interesting constellation, both from a basic science and a clinical point of view.

In summary, our study provides novel evidence that the repopulation of the adult hippocampus with new, genetically altered, implanted neuronal cells is feasible and results in a functional integration of the donor and derived neurons. This may also raise the possibility that in the future, certain diseases primarily affecting the hippocampal formation may be successfully treated by novel cell-based transplantation approaches.

**MATERIALS AND METHODS**

**Neural Progenitor Cells Isolation and Culture**

Inbred GFP-transgenic Lewis rats used in this study were kindly provided by Kobayashi. These rats previously described by Inoue et al. (11) express GFP under the control of a cytomegalovirus/β-actin promotor. Time-pregnant animals were killed by intraperitoneal lethal injection of ketamine in accordance with the national and institutional guidelines for animal experimentation, after approval of the Research Ethics Committee of the Albert-Ludwig University Freiburg, Germany. The method for extraction and isolation of NPCs from the telencephalic vesicles of E14 (gestational age: 14 days postconception) rat embryos has been described previously (42). Proliferation medium consisted of Dulbecco’s modified eagle medium/Ham’s nutrient medium at a ratio of 3:1, 1% mixture of penicillin, streptomycin and amphotericin, 2% B27 supplement (all purchased from Invitrogen, Darmstadt, Germany), and epithelial growth factor (Pepro Tech, Hamburg, Germany). For tissue culture, NPC medium consisted of Ham’s nutrient medium at a ratio of 3:1, 1% mixture of penicillin, streptomycin and amphotericin, 2% B27 supplement (all purchased from Invitrogen, Darmstadt, Germany), and epithelial growth factor (Pepro Tech, Hamburg, Germany). The medium was changed every 5 to 6 days and passages performed once a week by mechanical dissociation of the formed spheres.

**Induction of Neuronal Phenotype In Vitro**

After at least three passages, cells were plated at high densities on poly-ornithine (Sigma)-coated cover slips in a medium composed of minimum essential medium (Sigma), 1% N2 serum supplement, 2% sodium pyruvate,
mM-1-glutamine, 4 mM glucose, 0.1% bovine serum albumin, 1% mixture of penicillin, streptomycin and amphotericin (all from Invitrogen), supplemented with brain-derived neurotrophic factor 25 ng/mL (R&D Systems) and 0.5 mM 3-isobutyl-1-methylxanthine (3-isobutyl-1-methylxanthine, phosphodiesterase inhibitor, Sigma-Aldrich). Medium was changed every other day. Cells were fixed and immunostained immediately after electrophysiologic recording.

**Stereotactic Implantation**

To minimize the trauma related to the stereotactic implantation of the cell solution, we used a microtransplantation technique as described previously (43). Twenty-six-week-old female Sprague-Dawley rats (approximately weighing 190 g) were used as host animals and were therefore fixed to a stereotactic frame Cunningham (Stoelting Co., Dublin, Ireland) under general anesthesia with ketamine (Pfizer, Karlsruhe, Germany). The coordinates used for implants into the dentate gyrus of the hippocampus were anterior-posterior (stereotactic coordinate), −5.4 mm; lateral (stereotactic coordinate), +4.2; vertical (stereotactic coordinate), −6.5 to −4.5 for 10 separate deposits made 0.2 mm apart. The cells were resuspended at 150,000 cells/μL. Each deposit (10 per animal) constituted 0.2 μL of cell suspension implanted into the posterior aspect of the hippocampal formation.

**Animal Care**

All animals were immunosuppressed, commencing 1 day before transplantation, by daily intraperitoneal injections of cyclosporine A (Sandimmun, 10 mg/kg, Novartis Pharma, Nurenberg, Germany). Immunosuppression was combined with prophylactic oral administration of antibiotics (Sulldoxin and Trimethoprin, Bayer, Germany) in the drinking water. Animals were housed in a temperature-controlled enriched environment under a 12-hr light/dark cycle with access to food and water ad libitum.

**Electrophysiology**

Standard whole-cell patch clamp recording methods were used to examine the physiologic properties of implanted fetal-derived neuronal stem cells. To this end, the animals were anesthetized with isoflurane introduced to the inspiration airflow (4%–5%, Abbott, Ludwigshafen, Germany) and killed by decapitation, in accordance with national and institutional guidelines. Transverse 300-μm-thick slices were cut from the hippocampus of the implanted animals, 12 weeks after grafting, using a custom-built vibrating microtome (44). Slices were maintained at 35°C for 30 min after slicing and then stored at room temperature in sucrose-based solution containing (in millimolar): 87 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 75 sucrose, 0.5 CaCl2, 7 MgCl2, and 25 glucose, equilibrated with 95% O2 and 5% CO2. Transplanted cells were first identified by their fluorescence, excited by a 405-nm diode fluorescence lamp, and visualized with a charged coupled device camera (“charged coupled device,” Zeiss, Jena, Germany). After cell identification, a patch clamp was established under visual control using infrared differential interference contrast videomicroscopy (Zeiss). A detailed description of the recording conditions, solutions, technique, and off-line data processing was provided in a previous publication (45).

**Immunostaining**

Twelve weeks after implantation, animals were killed, and the brains were cryosectioned at 40 μm in the coronal plane. The immunohistochemical and immunocytochemical procedures were carried out as described previously (20, 45, 46). The following primary antibodies were used: anti-GFAP (polyclonal, produced in rabbits, purchased from Millipore, Temecula, CA, 1:500), anti-Glutamate (monoclonal, mouse, Millipore, Temecula, CA, 1:200), anti-NeuN (monoclonal, mouse, Millipore, Temecula, CA, 1:250), anti-NF200 (monoclonal, mouse, Millipore, Temecula, CA, 1:200), anti-GABA-amino-transporter 1 (polyclonal, rabbit, Millipore, Temecula, CA, 1:500), and antiglutamate (monoclonal, mouse, Millipore, Temecula, CA, 1:5000). The secondary antibodies were anti-rabbit, anti-mouse or anti-guinea pig AlexaFluor 594 (Molecular Probes, Karlsruhe, Germany), used at 1:200. The nuclei were stained with 4’,6-diamine-2-phenylindole dihydrochloride (Sigma, 1:10,000). The original GFP signal was not enhanced with specific antibodies. Biocytin staining was revealed by DyLight 649 Streptavidin (1:200, Vector Laboratories, Burlingame, CA).

**In Vitro and In Vivo Quantifications and Statistical Analysis**

In vitro quantifications of marker positivity in fixed cultures were performed under epifluorescence. Therefore, at least 10 cover slips from 3 consecutive experiments were analyzed. Mean comparisons between groups were performed by using the Mann-Whitney U statistical test, because distribution of the residues was not Gaussian. All data are reported as mean ± standard error of the mean, and the significance level (P) is indicated in the text. For computation, we used SPSS version 13.0 Software (SPSS Inc., Chicago, IL).

To quantify the neuronal differentiation in vivo, we used one of the five series of slices to stain with NeuN. Coexpression of NeuN and GFP was analyzed under an epifluorescence microscope BX61 (Olympus Europe, Hamburg, Germany), equipped with a high-sensitivity digital camera DP70 and stereo system computer assisted stereological toolbox (Olympus Europe, Hamburg, Germany). Cells expressing both markers were counted by scanning the slide with an automatic microcator (Heidenhain, Traunreut, Germany) at 40× magnification and with an optic frame of 50×/50 μm, taking care to focus over the entire slice thickness (40 μm) for each frame. Finally, the entire hippocampus of each animal was scanned. Pictures shown were obtained in a Leica (Munique, Germany) TCS SP2 confocal system (405-nm diode, ArKr 488 nm, Ar 594 nm, and Xn 633-nm lasers) and were digitalized at 2048×2048 pixels.

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