ARKINSON’s disease is one of the best-characterized disorders of the extrapyramidal motor system, and is distinguished by typical movement dysfunctions such as rigor, tremor, and akinesia. Although the origin of PD is a matter of debate, it is widely acknowledged that it is caused by the degeneration of dopaminergic neurons in the pars compacta of the substantia nigra. The characteristic symptoms appear when more than 70% of dopaminergic nigrostriatal neurons have degenerated. There is currently no satisfactory long-term treatment for this disease. For example, symptomatic treatment with levodopa, which crosses the blood–brain barrier, is only temporarily effective; meanwhile the degeneration of dopaminergic neurons progresses and side effects appear.19

One future approach for the treatment of PD could be the transplantation of dopaminergic neurons into the caudoputaminal brain region. Both rodent and nonhuman primate models have been developed to test these therapeutic approaches. The rat model, in which unilateral injections of 6-hydroxydopamine are delivered into the ascending nigrostriatal pathway, has been shown to be particularly suitable.29 Until now, fetal neural progenitor cells have been used in transplantation experiments. These cells have been isolated from the mesencephalon of rat and mouse embryos, and more recently also from human fetuses. However, this approach is hampered by two major problems: first, there is the limited reproducibility inherent in using human fetal material from different individuals, and second there is the limited differentiation capacity of fetal neural progenitor cells. To circumvent these problems in the present study, we examined the possibility of transplanting ESC-derived neural precursor cells into a normal rat brain.

Embryonic stem cell lines, which are derived from the inner cell mass of preimplantation mouse embryos, are
Transplantation of fluorescence-labeled embryonic stem cells potentially capable of being maintained in a pluripotent state in the presence of LIF. After withdrawal of LIF, these cells are able to differentiate into derivatives of all three germ layers. Treating ESCs in vitro with the indolent agent RA has been shown to induce differentiation of a high proportion of these cells into neurons and glia. More recently, Okabe, et al., have reported on an efficient procedure to generate proliferative neural precursor cells from ESCs. We have modified this protocol by cultivating the plated ESCs in a rat astrocyte-conditioned synthetic medium to precondition neural precursor cells for use in transplantation experiments in rats.

To identify the transplanted neural precursor cells in the recipient tissue (rat brain), we generated a stably transfected ESC clone by using a vector containing the GFP gene under the control of a β-actin promoter. This genetic method of cell labeling facilitates observation of general differentiation of all cell types after transplantation. Furthermore, the relative differentiation of neurons and glia can be evaluated by morphological and immunocytochemical methods posttransplantation. Additionally, the fate of transplanted cells and their distribution into the surrounding tissue can be observed easily with fluorescence microscopy.

**Materials and Methods**

**Transfection of ESCs With a β-Actin Promoter and GFP**

*Vectors.* The pCX-(β-act)-EGFP expression vector—which contained the enhanced version of the GFP coding sequence under the chicken β-actin promoter and modified as follows: Sal I Xba I restriction fragment containing the Neomycin (G418) resistance gene (NeoR) from pTTL2NeoR—to insert blunt-end ligation into the Sal I site of pCX-EGFP.

*Electroporation and Selection Procedure.* The pCX-(β-act)-NeoR was linearized by Sca I restrictase and electroporated.

*Electroporation Conditions.* A 0.4-cm electroporation cuvette and a 240-V electroporation apparatus (500 μF) were used to obtain concentrations of 7 × 10^6 cells/ml, which were suspended with 20 to 40 μg of DNA in 0.8 ml of PBS. After electroporation, the cell suspension was placed on ice for 20 minutes and transferred to a 10-cm tissue-quality petri dish with a G418-resistant feeder layer in 10 ml of DMEM with 15% FCS. Two days later, 300 μg/ml of G418 was added for selection of G418-resistant cells. Medium supplemented with G418 (300 g/ml) was changed every other day. After 8 to 10 days of selection, emerging drug-resistant colonies were tested for GFP expression by using fluorescence microscopy. After the medium was replaced with PBS, the colonies were picked up, trypsinized, and plated separately on 48-well plates containing 418 feeder layers. After 2 to 4 days of growth, cells from each well were propagated on 24-well and 60-mm culture dishes.

*Preparation of ESCs*

Mouse ESCs of the D3/clone 7 line were cultivated independent of feeder with supplementation by 100 nM of LIF in 15% FCS containing DMEM, as described in previous studies. The cells were differentiated into embryo-like aggregates (so-called EBs) in hanging-drop preparations. The hanging drops were composed of initial populations of 400 cells/20 μl and maintained in culture for 2 days in DMEM supplemented with 10% FCS, 2 mM glutamine, nonessential amino acids (stock solution 1:100), 100 U/100 μg/ml penicillin–streptomycin, and 50 μM β-mercaptoethanol. Hanging drops in which EBs had formed were rinsed off after 2 days and subsequently cultivated in suspension (DMEM, 10% FCS) for another day. Finally, on Day 3, EBs were transferred to tissue culture dishes (DMEM, 10% FCS) and adhered within 12 hours.

**Selection of Neural Precursor Cells**

One day after plating the EBs, the culture medium was switched to an astrocyte-conditioned serum-free medium (DMEM/F12) supplemented with insulin, transferrin, selenium, and fibronectin as differentiation factors. Astrocyte preconditioning was attained by incubation of the medium with 5-azacytidine–immortalized astrocytes for 1 day to allow adoption of precursor cells to a rat environment.

As analyzed using phase-contrast microscopy and confirmed on immunocytochemical studies, the first neural precursor cells differentiated 2 days after cultivation in the serum-free medium, and approximately 7 days later a very pure population of nestin-positive cells was observed and were used for the various assays.

**Monitoring in Vitro Capacity of Selected Neural Precursor Cells**

To assess the differentiation capacity of neural precursor cells used for transplantation, an aliquot of the cells was replated on laminin-coated culture dishes and incubated for 7 days with astrocyte-conditioned DMEM/F12 medium supplemented with B27. Immunocytochemical analysis of neuronal and glial differentiation was performed by detection of GFAP/Thy-1-positive cells.

**Transplantation Procedure**

Han Wistar rats weighing 300 to 350 g were used as intracerebral transplant recipients. An average of 300,000 viable neural precursor cells was inserted into the right striatum of the rat hosts. For all surgical procedures, the animals were anesthetized with intraperitoneal injections of Avertin (1 ml/100 g) and secured in a Kopf stereotactic frame. Neural precursor cells were implanted stereotactically (distance from bregma: anterior +1.5 mm, lateral −2 mm, ventral −4.5 mm). A 10-μl Hamilton syringe attached to a 22-gauge needle (inner/outer diameter 0.41:0.71 mm) was used to deliver 5 μl of cells suspended in DMEM/F12 without supplements (rate: 1 μl/min, allowing an additional 2 minutes for the final injection pressure to equilibrate before slowly withdrawing the needle).

**Histological Procedures**

At defined times following the operation (1–4 weeks), the animals were killed with an overdose of Avertin (1 ml/100 g body weight), then perfused intracardially with 100 ml heparin saline (0.1% heparin in 0.9% saline), followed by fixation with 400 ml paraformaldehyde (4% in PBS). The rat brains were removed and postfixed for 8 hours in the same 4% paraformaldehyde solution, after which they were equilibrated in sucrose (18% in PBS), cut into 16-μm sections on a freezing microtome, and collected on poly-L-lysine–coated glass slides.

After detection of GFP autofluorescence in the sections from the transplantation area, the slices were processed for guinea pig polyclonal GFAP (1:200 dilution) and mouse monoclonal Thy-1, as well as OX-42 immunohistochemistry. Sections were incubated in 1.2% normal goat serum in PBS for 60 minutes before primary antibody incubation. After the incubation period, the primary antibody was rinsed out with PBS. The sections were subsequently incubated in Cy3-labeled secondary antibody (goat-antirabbit or goat–antimouse, depending on the primary antibody species), and then rinsed three times in PBS before microscopic analysis was performed.

Immunocytochemical investigation of the in vitro cultures was performed again by using Cy3-labeled secondary antibody for fluorescent visualization of nestin. Horseradish peroxidase visualization with the three-step avidin–biotin horseradish peroxidase method was used for the following antigens: cardiac α-actin, PECAM, MAP2, calretinin, Thy-1, and GFAP. Visualization of the enzymatic reaction was achieved by incubating the sections for 5 to 30 minutes in 0.04% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine in Tris-buffered saline. Control assays were performed on selected sections prepared with the omission of the primary antibody to verify the specificity of staining. After immunostaining, slides were prepared with coverslips and analyzed with brightfield microscopy.

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Morphometric Analysis

Morphometric analysis of the area covered by GFP-positive cells was performed using commercially available software. Distribution of GFP-positive cells was assessed by measuring the distance of most peripheral cells from the border of the injected cell mass.

Sources of Supplies and Equipment

The electroporation cuvette and apparatus (Gen Pulser) were obtained from BioRad, Hercules, CA. The multwell culture dishes were acquired from Falcon, Heidelberg, Germany. The EGFP was provided by Clontech Laboratories, Palo Alto, CA, and the FCS, LIF, DMEM, DMEM/F12, and glutamine were purchased from Life Technologies, Heidelberg, Germany, as was the geneticin (G418). The stock solution of nonessential amino acids was obtained from Biochrom, Berlin, Germany. The guinea pig polyclonal GFAP was purchased from Progen, Heidelberg, Germany. The mouse monoclonal Thy-1 (clone G7) was acquired from the American Type Culture Collection, Manassas, VA, and the Cy3-labeled monoclonal antibody for nestin (Rat 401) was obtained from the Developmental Hybridoma Bank, Iowa City, IA.

Antibodies were purchased from the following companies: cardiac α-actin, Alamon Labs, Jerusalem, Israel; PECAM, Pharmingen, San Diego, CA; MAP2, Sigma, Deisenhufen, Germany; and calretinin, SWANT, Bellinza, Switzerland. The 3,3′-diaminobenzidine was also purchased from Sigma. The Cy3-labeled secondary antibodies were from Rockland, Biotrends, Cologne, Germany. The staining kit for the avidin–biotin method (Vectastain ABC ELITE kit) was acquired from Vector Labs, Burlingame, CA. The software for morphometric analysis was obtained from Optimas Corp., Bothell, WA.

The pCX-(β-act)-EGFP expression vector and the pTL2NeoR were kindly supplied by Dr. Okabe22 (University of Osaka, Japan), and Dr. Tarakhovsky (Institute for Genetics, Cologne, Germany), respectively.

Results

Differentiation Capacity of D3/Clone 7 in Vitro

To determine whether the β-actin GFP–transfected D3 cell clone (clone 7) used for the transplantation experiments had differentiation properties similar to those described for the wild-type D3 clone, morphological and immunohistochemical analyses of the differentiation capacity of clone 7 were performed using endothelial, cardiomyocyte, and neuronal differentiation properties.

Endothelial Differentiation. According to established differentiation protocols for the D3 cell line, this subclone shows development of dense vascular networks at 3 to 5 days postplating, as revealed by staining with an antibody against PECAM, which is specific for endothelial cells (Fig. 1a).

Cardiomyocyte Differentiation. From Day 3 postplating, several independently beating areas could be detected in plated EBs, the number of which is comparable with other normal D3 clones and indicates cardiomyocyte differentiation. The suggested differentiation of early heart muscle is confirmed by immunohistochemical studies for the α-actin antibody, which gives rise to a fibrillate pattern in immunoreactive cells (Fig. 1b).

Neuronal Differentiation. According to the standard differentiation protocol, the induction of neuronal differentiation was observed, with the typical features of neuronal
Transplantation of fluorescence-labeled embryonic stem cells

Fig. 3. Photomicrographs showing in vitro cell differentiation of selected neural precursor cells. a: A large number of neuronal cells can be detected via Thy-1, an antibody that is specific for mouse neurons. b: A subpopulation of these cells showing mainly bi- and multipolar morphological characteristics can be traced using calretinin immunocytochemistry. c: Astrocyte differentiation shown by GFAP immunostaining. Scale bar = 100 μm (a and b); 20 μm (c).

development that had been identified in similar investigations in control wild-type D3 cells. Neuronal differentiation could already be detected at 2 to 3 days after plating, with increasing formation of processes as well as an increasing formation of neuronal networks. Differentiation of neurons and glial cells was assessed using antibodies to cell type–specific antibodies such as MAP2 for neurons and GFAP for astroglia (Fig. 1c). Synaptophysin, as well as different neurotransmitters (γ-aminobutyric acid, glutamate, glycine) were also detected (data not shown). With the double labeling of cells by GFP expression and by cell type–specific antibodies, we could evaluate the number of neurally differentiated cells of the GFP-labeled cell population.

Generally, all cells independent of commitment were GFP-positive in vitro. However, there was a variation in brightness, depending on the cell type and localization within the cells (for example, cell processes show a weaker green fluorescence than cell somata).

Selection of Neural Precursor Cells

During the first 48 hours of neural precursor selection in synthetic astrocyte-conditioned medium, a large number of cells died, whereas the surviving cells acquired an elongated phenotype similar to neuroepithelial precursor cells. After 7 days of selection, most of the remaining GFP-expressing cells (> 95%) expressed nestin, an intermediate filament common to the nondifferentiated cells derived from the neuroectoderm. Efficiency was evaluated by nuclear counterstaining in which Hoechst dye was used (Fig. 2). These cells were harvested by trypsinization and portioned for two different sets of experiments.

In Vitro Differentiation of Neural Population After Second Trypsinization

In one set of experiments parallel to the transplantation studies, neural precursor cells were seeded on laminin-coated tissue culture dishes to assess further differentiation in vitro. After 4 days in B27-supplemented astrocyte-conditioned medium, cultured cells started to differentiate into neurons. Six days after reseeding, approximately 50% of the population stained positively for MAP2 (data not shown) or for Thy-1 (Fig. 3a), a marker for mouse neuronal cells. A subpopulation expressed calretinin, a calcium-binding protein known to be expressed early during neuronal differentiation (Fig. 3b).

Neuronal and Glial Differentiation After Intrastriatal Transplantation

Selected neural precursor cells were implanted into the striatum of adult rats. Cryostat sections of striata containing the transplants were studied 1, 2, 3, and 4 weeks postoperatively. In brain sections obtained in animals 1 week after transplantation, the tissue defect induced by the injection cannula was clearly visible. However, 2 weeks after transplantation there was almost no detectable tissue injury, indicating the fast regenerative capacity of rodent brain tissue (Fig. 4a). No immunological reactions, which were checked by the microglial markers 5C6 (data not shown) and Ox42, or obvious indications of tumor formation were detected. Grafts were clearly identifiable by the GFP fluorescence in the brain sections.

The histological appearance and size of the grafts varied greatly, with the mean size ranging from 4 to 25 mm³ depending on the length of time after transplantation. Whereas almost no spreading of grafted cells into the surrounding tissue was visible 1 week posttransplantation (mean size range 5–10 mm³), continuous spreading was noticed from 2 weeks onward. After 4 weeks, grafted cells were found in the surrounding host tissue as far away as 300 to 500 μm from the center of the graft (size range 15–25 mm³; Fig. 4b–d).

From 2 weeks posttransplantation onward, green fluorescent cells observed in the outermost region of the graft exhibited typical neuronal morphological characteristics. Counterstaining with Thy-1, a mouse- and neuron-specific antibody used in other grafting experiments, confirm...
Fig. 5. Photomicrographs showing identification of differentiated cells by cell type–specific markers for neurons (a–c) and astrocytes (d–f) 4 weeks posttransplantation. a: Neuronal morphological characteristics (arrows) visualized after GFP fluorescence. b and c: Identification of neurons by the mouse- and neuron-specific marker Thy-1. d: Green fluorescent protein–labeled cells in the striatum. Selected cells are marked by arrows. e: Same site as in d after immunostaining with GFAP. Note the GFAP staining in the same cells (arrows) as marked in d. f: Double fluorescence image in which GFP labeling and GFAP-immunoreactive cells are visualized (arrows). Differentiation of GFP-labeled cells to GFAP-positive astroglia is evident. Scale bar = 15 μm (a); 5 μm (b and c); and 30 μm (d and f).
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firmed the differentiation of grafted cells into neurons. Cells exhibited bipolar and multipolar morphologies (Fig. 5a–d). Although the number of these Thy-1–positive neuronal cells was restricted, there was a much higher number of glial cells differentiating from the transplanted precursor cells, as demonstrated by the glia-specific marker GFAP (Fig. 5e–g).

Discussion

With the data presented here we demonstrate for the first time that ESC-derived neural precursor cells selected on a synthetic medium are able to differentiate into Thy-1–positive neurons and GFAP-positive astrocytes in the recipient adult rat brain. Using an ESC clone with general GFP expression under the control of a β-actin promoter, it is possible easily to identify the whole population of transplanted cells in the host striatum at different times after transplantation, independent of cell fate during differentiation and integration. Using the same construct, expression of GFP was previously shown in transgenic animals in all tissues including the brain, with the exception of erythrocytes and hair.21 Furthermore, the initial state, pluripotency, and normal development and functional capacities of this subclone are similar to those identified in other ESC lines. When cultured without LIF, they can differentiate into a variety of types such as hematopoietic, muscle, and cardiomyocyte as well as neuronal cells.2,3,5,6,11,12,15,24,26,28

As already described by Okabe, et al.,23 and Brüstle, et al.,9 by applying appropriate protocols ESCs can generate proliferating neuronal precursor cells, which can then be expanded, showing that lineage restrictions observed in the developing embryo can be replicated in the in vitro culture system in a precisely determined manner. In our investigation, we have used rat astrocyte-conditioned medium to allow adaptation of mouse neural precursor cells to a rat environment such as that found in the target tissue. Moreover, during further cultivation and differentiation in serum-free medium supplemented with B27 astrocyte-conditioned medium was again applied. This was done for better comparability with the in vivo situation. By this procedure cells give rise to a large number of neuronal cells similar to a brain primary culture.

This pretreatment consequently means that neuronal precursor cells are able to differentiate into mature neuronal phenotypes after transplantation. Because these cells, including their processes, express the mouse- and neuron-specific antigen Thy-1,20 it can be concluded that this subpopulation of the transplanted ESC-derived neuronal precursor cells actually differentiates into neurons within the host tissue. Furthermore, it can be demonstrated that integration of donor cells is not restricted to the neuronal lineage, because large numbers of GFP-positive cells were consistently detected to express GFAP. However, it remains to be determined, using electrophysiological studies, whether these cells behave similarly to neighboring host cells and whether they are functionally integrated into the host tissue. Earlier studies, in which the α-actin promoter was used when investigating cardiomyogenes, demonstrate that GFP cell labeling does not alter the physiological behavior of the marked cells.16

By applying GFP cell labeling, it is possible to investigate further the suitability of ESCs as a source of future cell replacement therapies in neurodegenerative diseases. This has already been suggested by both Dinsmore, et al.,11 and Deacon, et al.,10 who demonstrated the potential of ESCs to differentiate into either γ-aminobutyric acidergic or dopaminergic/serotoninergic neurons, respectively. However, the RA treatment used in their experiments did not prevent differentiation of nonneuronal cells as shown by Wobus, et al.21

The advantage of using ESC-derived neural precursor cells is that they do not exhibit nonneuronal differentiation in vitro. Moreover, ESC-derived precursor cells induced by differentiation protocols, supplemented by specific growth and differentiation factors,20,22 have been shown to contribute to embryonic rat brain development after transplantation,9 including myelination in a myelin-deficient rat model.8

Conclusions

Our investigation demonstrates a reliable system for a highly efficient differentiation of neural precursor cells into Thy-1–positive neurons and GFAP-positive glia. This opens up several opportunities for the application of these cells in transplantation strategies when treating neurodegenerative diseases such as PD. Because PD is caused by a loss of dopaminergic neurons, the transplanted cells must have the potential to differentiate efficiently into tyrosine hydroxylase– or dopamine-positive neurons. In contrast with transplanted neural precursor cells isolated directly from embryonic striatum or hippocampus,18 ESC-derived precursor cells lack a temporal or spatial commitment. The challenge is to establish appropriate protocols in vitro to promote lineage-restricted differentiation (for example into dopaminergic neurons) after transplantation in vivo. Mesencephalic progenitor cells have already been shown to differentiate with different cytokines along with either glial cell–derived neurotrophic factor or brain-derived neurotrophic factor.17

References


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