Immature human NT2 cells grafted into mouse brain differentiate into neuronal and glial cell types

Alessandra Ferrari[a], Elisabeth Ehler[b], Roger M. Nitsch[a], Jürgen Götz[a,*]

[a] *Division of Psychiatry Research, University of Zürich, August Forel Str. 1, 8008 Zurich, Switzerland*
[b] *Institute of Cell Biology, ETH, Zurich, Switzerland*

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Abstract NT2 cells are a transfectable human embryonal carcinoma cell line, that can be differentiated into postmitotic neuron-like cells (NT2N cells), and transplanted into rodent brains. Differentiation requires a 5-week-long treatment with retinoic acid prior to transplantation. Here, we show that this step can be omitted, and that undifferentiated NT2 cells migrate over long distances and differentiate into both neuron- and oligodendrocyte-like cell types upon grafting into brains of immunocompetent newborn mice. Grafted cells can be traced by fluorogold, with no evidence for tumor formation. Our approach provides an experimental model system which allows the immunohistological and biochemical study of neuronal and glial differentiation of human cells in vivo, and which may be suitable as an in vivo model for pharmacological studies.

Key words: Transplantation; Differentiation; NT2 cell; Neuron; Oligodendrocyte

1. Introduction

A common neuropathological feature of neurodegenerative diseases is neuronal cell loss which is restricted to a particular cell type in some diseases [1,2]. In Parkinson's disease (PD), for example, only the tyrosine hydroxylase neurons of the substantia nigra are degenerating, whereas in other diseases including Alzheimer's disease (AD), several distinct neuronal cell types in different brain areas are affected. For the treatment of these diseases, several approaches can be envisaged: Whereas pharmacological treatment of PD with either L-3,4-dihydroxyphenylalanine or dopamine agonists has been effective to some extent, similar pharmacotherapies, such as the use of cholinesterase inhibitors or muscarinic agonists, were not very promising in AD. An alternative approach is cell replacement therapy. For PD, clinical trials have already been initiated by transplanting human fetal brain tissue in order to substitute for the loss of dopaminergic neurons in PD [3,4]. However, several aborted fetuses are required for the therapy of a single patient. For practical and ethical reasons, this approach is not very useful [5]. A third approach is the use of engineered murine embryonal stem (ES) cells [6,7] or human cell lines. One of the best established human cell lines is the embryonal carcinoma cell line NTera-2 (in short NT2), which is transfectable, capable of differentiating into postmitotic neuron-like cells (NT2N cells) following treatment with retinoic acid, and transplantable into brain or spinal cord of immunocompetent and immunodeficient rodents [8–10]. Intracerebral grafting of NT2N cells has been successfully used to promote functional recovery of ischemic rats [11]. However, differentiation of NT2 cells is an elaborate procedure [8]. Prior to grafting, NT2 cells have to be incubated with retinoic acid for at least 5 weeks, followed by three consecutive replatings, in the presence of mitotic inhibitors, onto poly-D-lysin- and laminin-coated dishes. As most of the NT2 cells differentiate into neurons under these conditions, they have been used as replacement therapy for PD [12]. Neuronally differentiated NT2 cells (NT2N cells), grafted into athymic or immunosuppressed mice, survived for at least a year in the host brain [13,14]. Undifferentiated NT2 cells, grafted into immunodeficient mice, formed tumors [15] unless they were grafted into the caudoputamen where the grafted cells remained and survived for up to a year [13]. They differentiated into postmitotic neuron-like, but not glial-like, cell types [13].

In the present study, we show that fluorogold-labeled, undifferentiated NT2 cells can be grafted into immunocompetent newborn mice (P0), without tumor formation. In addition, many NT2 cells migrated over distances of several mm and differentiated not only into neuron-, but also glial-like cell types.

2. Materials and methods

2.1. Cell culture

NT2 cells were obtained from Dr. Roland Brandt (Heidelberg) and cultured as previously described [16]. Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–F12 medium (Gibco), supplemented with 10% fetal calf serum (Seromed), 5% horse serum (Seromed), and 1% penicillin/streptomycin (Gibco). Twice a week, NT2 cells were split 1:5 onto uncoated tissue culture dishes. Two days before grafting, the tracer substance fluorogold (Fluorochrome) was diluted 1:10 000 in DMEM/F12 medium, incubated overnight at 37°C, and added to NT2 cells for another day. Cells were washed five times in phosphate-buffered saline (PBS), trypsinized for 4 min at 37°C, centrifuged at $900 \times g$ for 5 min, taken up in PBS with a final concentration of 50 000 cells/μl, and kept on ice until grafting, for less than 1 h. Primary embryonic day 18 (E18) neuronal cultures were established from the cerebral cortices of C57BL/6×DBA/2 F1 (B6D2F1) mice following standard procedures and cultured on poly-L-lysin-(Gibco) and fibronectin (Gibco)-coated coverslips in MEM medium (Gibco) containing 4 g/l glucose (Sigma), 100 μg/ml transferrin (Sigma), 5 μg/ml insulin (Sigma), 20 nM progesterone (Sigma), 100 μM putrescine (Sigma), 30 nM selendioxide (Sigma), 1 mM sodium pyruvate (Sigma), 0.1% ovalbumin (Sigma) and 5 μM AraC (Sigma), for 8 days prior to staining.

*Corresponding author. Fax: (41)-1-634 8874.
E-mail: goetz@bli.unizh.ch

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2.2. Transplantation

Immunocompetent C57BL/6×DBA/2 F1 (B6D2F1) females were obtained from Biological Research Laboratories, and kept in a conventional animal facility. They were hormonally stimulated with 5 U PMS gonadotropin (Folligon, Intervet) at day −3, 5 U HCG gonadotropin (Chorulon, Intervet) at day −1, and mated with B6D2F1 males as described [17]. Immediately after birth, the newborn mice (P0) were placed on a pre-warmed glass plate, and injected intracerebrally into or close to the ventricle with approximately 1 μl of NT2 cells in PBS, using a glass capillary. This capillary had an outer diameter of 50–100 μm and was plugged into a tube, with a mouth piece inserted at the other end of the tube. In order to monitor the grafting procedure, some of the injected NT2 aliquots were mixed with bromophenol blue or trypan blue. Immunohistochemistry was done with at least six grafted animals per experiment, and stainings were done in duplicate for every grafted mouse brain. The transplanted cells were monitored at days P1, P7, P14 and P21 after grafting. Approximately 100 sections were obtained from each grafted brain, of which, at days P14 and P21, between 20 and 30 contained substantial numbers of grafted cells. Immunohistochemical stainings were done with three sections each per antibody.

2.3. Immunohistochemistry

Antibody MAB353 (Chemicon, used at 1:200 dilutions) was used to detect nestin; anti-glial fibrillary acidic protein (GFAP) antibody AM-2185-11 (InnoGenex, used at 1:1000 dilutions) was used to detect astrocytes, anti-CNPase antibody AM-2033-11 (InnoGenex, used at 1:500 dilutions) was used to detect oligodendrocytes, anti-MAP-2 antibody (Roche, # 1284959, used at 1:500 dilutions) was used to detect neurons, anti-EndoA antibody Troma-1 [18] was used to detect cytokeratins, and anti-FG antibody (Fluorochrome, 1:5000) was used to detect the fluorogold-labeled human NT2 cells. As secondary antibodies, fluorescein isothiocyanate (FITC)- and Cy3-labeled anti-mouse and anti-rabbit IgG antibodies (Molecular Probes) were used at 1:1000 dilutions.

Cultures of NT2 cells and frontal vibratome sections (30–50 μm thick) of mouse brain were incubated with antibodies directed against fluorogold and differentiation markers, and analyzed by confocal or conventional fluorescence microscopy. For confocal analyses, sections were washed and mounted in 0.1 M Tris–HCl (pH 9.5)/glycerol (3:7) including 50 mg/ml *n*-propyl gallate as anti-fading reagent [19]. Sections were viewed by using a Leica TCS confocal microscope, and images were processed with the IMARIS software package (Bitplane). To estimate the survival of grafted cells, vibratome sections were viewed in the UV channel of a conventional fluorescence microscope. NT2 and primary cell cultures were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and stained as described previously [20].

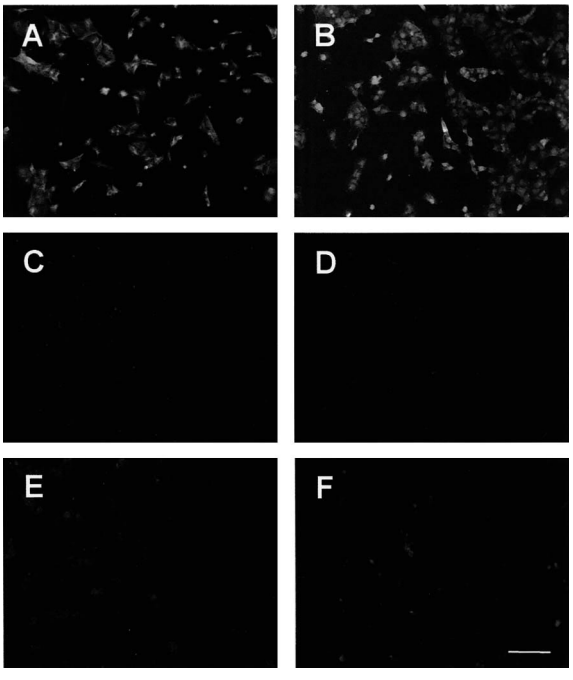

Fig. 1. Cultured NT2 cells express epithelial cell-specific cytokeratins (A). The intermediate filament protein nestin is expressed by approximately 20–30% of the NT2 cells (B). Differentiation markers, such as MAP-2 for neurons, CNPase for oligodendrocytes, and GFAP for astrocytes, are not expressed (C–E). A negative control is included where the primary antibody has been omitted (F). Scale bar: 100 μm.

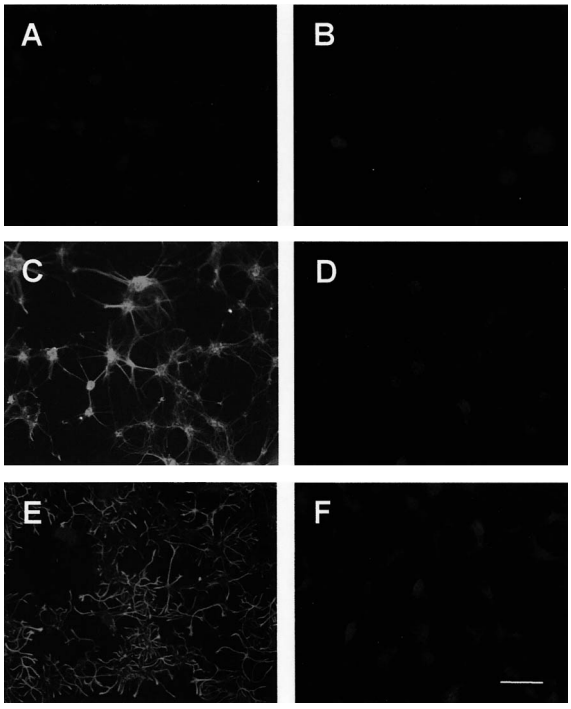

Fig. 2. Cortical primary neurons obtained from mouse brain are not stained by the endothelial cell-specific antibody Troma-1 or an antibody directed against the neural progenitor marker nestin (A, B). Cultures express the neuronal marker MAP-2 (C), but not the oligodendrocytic marker CNPase (D). Astrocytes were also detected, as indicated by GFAP staining (E). A negative control is included where the primary antibody has been omitted (F). Scale bar: 100 μm.

3. Results

3.1. Characterization of undifferentiated NT2 cells in culture

To determine the differentiation capacity of NT2 cells after grafting into mouse brain, we analyzed undifferentiated NT2 cells in culture, before grafting, by immunohistochemistry (Fig. 1). We found that approximately 60–70% of the NT2 cells expressed epithelial cell-specific cytokeratin as revealed with antibody Troma1 [18] (Fig. 1A), whereas typical neuronal and glial differentiation markers, such as MAP-2 for neurons, CNPase for oligodendrocytes, and GFAP for astrocytes, were negative at this stage of culture (Fig. 1C–E). The intermediate filament protein nestin, which is a marker for neuroglial progenitor cells committed to neuronal cell fate [21], was expressed in approximately 20–30% of the NT2 cells, demonstrating heterogeneity of the NT2 cell culture (Fig. 1B). As a negative control, the primary antibody was omitted from the reaction (Fig. 1F).

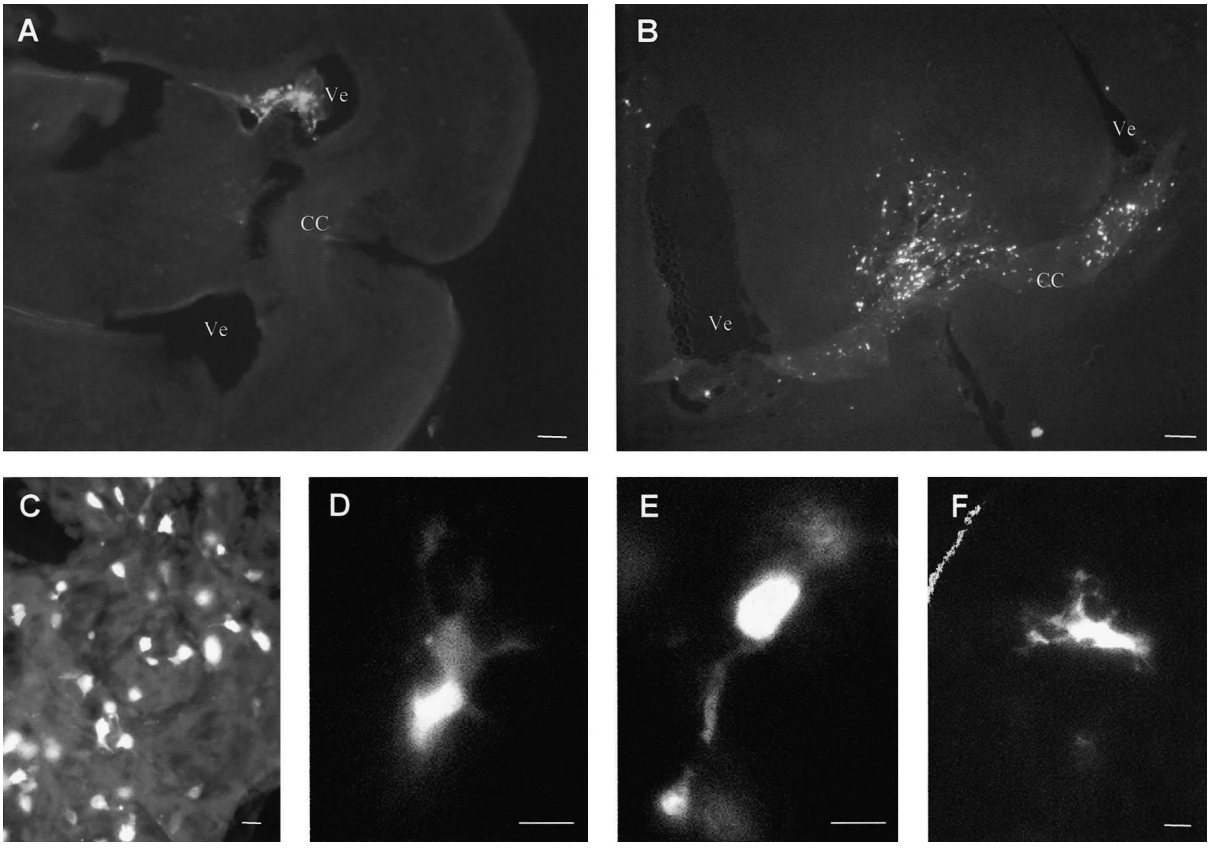

Fig. 3. Upon intraventricular grafting into P0 mouse brain, undifferentiated fluorogold-labeled NT2 cells migrate and undergo morphological changes. Between days P1 and P7 after grafting, the fluorogold-labeled NT2 cells form a mass of cells clustered around the injection site (A). Subsequently, cells start to migrate and are found, at day P21 after grafting, several mm distant from the injection site, along the corpus callosum (CC) and the lining of both ventricles (Ve) (B). Between P14 and P21 after grafting, NT2 cells lose their round shape (C) and acquire different types of morphologies: flattened cells with relatively few processes, bipolar cells and cells with a rich arborization (D–F). Scale bar: A, 250 μm; B, 100 μm; C, 25 μm; D–F, 10 μm.

3.2. Characterization of primary neuronal cultures

In order to compare the differentiation potential of grafted NT2 cells to primary neuronal cultures, cortical primary neurons obtained from mouse brain were analyzed immunohistochemically after 8 days in culture (Fig. 2). Primary neuronal cultures did not express cytokeratins as shown with epithelial cell-specific antibody Troma-1 (Fig. 2A). However, they expressed the neuronal marker MAP-2 (Fig. 2C), but not the neural progenitor marker nestin or the oligodendrocytic marker CNPase (Fig. 2B,D). Cultures were stained by an antibody directed against GFAP reflecting the presence of astrocytes (Fig. 2E). As a negative control, the primary antibody was omitted from the reaction (Fig. 2F).

3.3. Survival and morphology of undifferentiated NT2 cells grafted into mouse brain

Undifferentiated NT2 cells were preincubated with fluorogold for 1 day, washed and grafted intracerebrally into newborn (P0) mice. Mice were sacrificed and brains analyzed at postnatal days P1, P7, P14 and P21, respectively. Between days P1 and P7 after grafting, the fluorogold-labeled NT2 cells clustered around the injection site. After day P7, the majority of surviving NT2 cells started to migrate from the injection site along the surface of the lateral ventricle, following myelinated fibers, in particular of the corpus callosum, to the contralateral hemisphere (Fig. 3A,B). Until day P21, some of the grafted cells even migrated over distances of several mm. At that time, at least 1% of the injected cells had survived, and the remaining cells showed cell shrinkage and nuclear condensation most likely reflecting apoptotic mechanisms. The histological analysis did not provide any evidence for tumor formation or pathological abnormalities.

3.4. In situ differentiation of undifferentiated NT2 cells grafted into mouse brain

Between P14 and P21 after grafting, fluorogold-positive cells changed their morphology and lost their round shape (Fig. 3C). At least three different types of morphologies were found: flattened cells with relatively few processes, bipolar cells and cells with a rich arborization (Fig. 3C–F).

Vibratome sections of grafted brains were costained with antibodies directed against fluorogold and differentiation markers, and analyzed by confocal microscopy (Fig. 4). At days P7 until P14 after grafting, we identified fluorogold-positive NT2 cells which had retained nestin expression (Fig. 4K–M). At days P18 until P21, some of the NT2 cells expressed neuronal markers as indicated by MAP-2 staining (Fig. 4A–F). The morphology was similar to a neuronal arborization (Fig. 4D–F) or a bipolar morphology (Fig. 4A–C). In addition, another subset of grafted NT2 cells were identified which

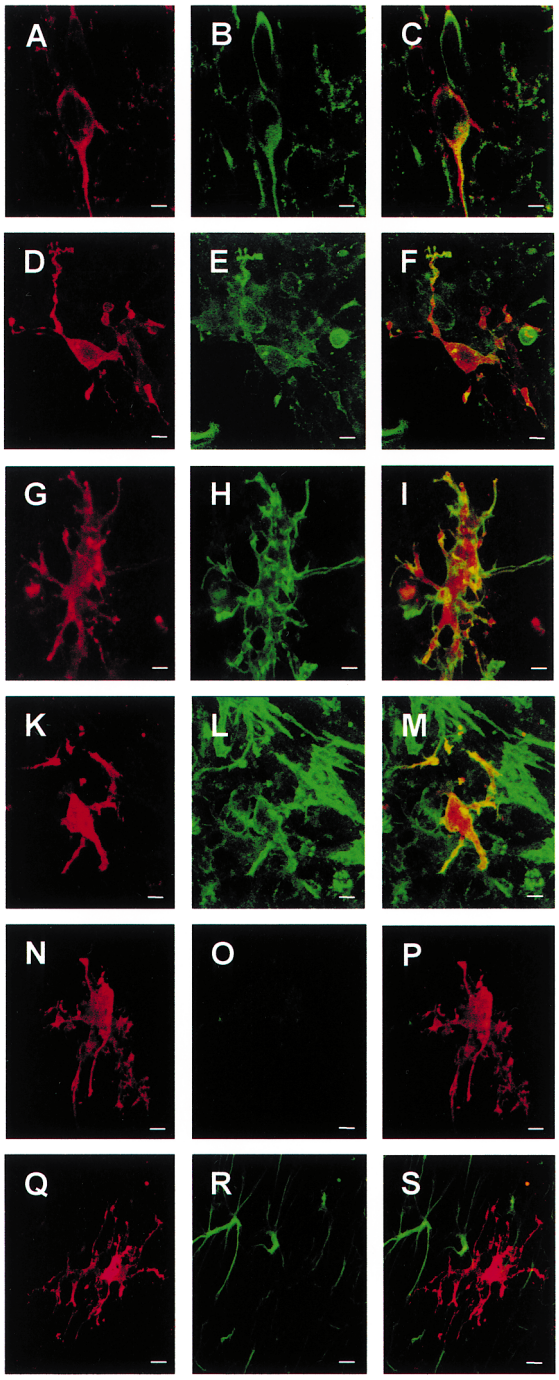

Fig. 4. Confocal microscopy reveals that immature NT2 cells grafted into mouse brain are capable to differentiate into neuronal and glial cell types. Vibratome sections of grafted brains are co-stained with antibodies directed against fluorogold, to identify NT2 cells (first column, Cy3 channel), and differentiation markers (second column, FITC channel). Stainings were done with antibodies directed against MAP-2 (A–F), CNPase (G–I), nestin (K–M), cytokeratin (N–P) and GFAP (Q–S). The overlay (third column) reveals MAP-2-, CNPase- and nestin-positive NT2 cells, respectively. Scale bar: 10 μm.

belonged to the oligodendrocytic cell lineage as shown by expression of the CNPase marker (Fig. 4G–I). We could not identify any GFAP-positive NT2 cell in the grafted brain indicating that NT2 cells did not differentiate into cells of the astrocytic cell lineage (Fig. 4Q–S). Also, we did not identify NT2 cells that had differentiated into endothelial-like cells, as shown by the absence of Troma-1 reactivity (Fig. 4N–P).

4. Discussion

Our study shows that undifferentiated human NT2 embryonal carcinoma cells migrate over distances of up to 5 mm and differentiate into neuron- and glial-like cell types upon grafting into brains of newborn (P0) immunocompetent mice, with no evidence for tumor formation.

4.1. Differentiation potential of grafted NT2 cells

It has previously been shown that undifferentiated NT2 cells in culture expressed cytokeratins as predominant intermediate filament protein, and that only a fraction of cells expressed vimentin, and none expressed GFAP or neurofilament chains [16]. Immature NT2 cells never went on to establish polarized neurites with the characteristics of axons or dendrites as evidenced by either the distribution of cytoskeletal markers (such as MAP-2) or by functional assays [16,22,23]. We confirmed these published characteristics for the batch of NT2 cells which was used for our grafting experiments, and included CNPase as marker for oligodendrocytes (Figs. 1 and 2). Undifferentiated NT2 cells in culture expressed nestin and cytokeratins, but no neuronal, astrocytic or oligodendrocytic markers. Grafting into newborn (P0) immunocompetent mice was not accompanied by tumor formation. It has been shown previously that the caudoputamen (CP) of immunodeficient nude mice specifically inhibits proliferation of NT2 cells grafted into the CP [13], although the NT2 cells formed lethal tumors following grafting into peripheral tissues and into many regions of the nude mouse central nervous system [15]. This suggests that endogenous factors modulate cell proliferation and differentiation, and that the P0 immunocompetent brain used in our studies is similar to the CP of adult nude mice, with respect to tumor formation. Grafting into P0 immunocompetent mice induced differentiation not only into neuron-, but also oligodendrocyte-like cells. Some NT2 cells retained nestin expression, but did not express cytokeratins, which indicates that these cells had initiated differentiation without final commitment to either the neuronal or glial cell lineage (Fig. 4). In contrast, grafting of undifferentiated NT2 cells into the caudoputamen of immunodeficient adult mice only induced differentiation into neuron-like cells [13]. Intrauterine transplantation of murine ES cells into brain generated neural chimeras composed of ES cell-derived neurons, oligodendrocytes and astrocytes [7,24]. This suggests that the P0 brain produces the cues for neuronal and oligodendrocytic differentiation of NT2 cells, and that immature NT2 cells have the potential to differentiate into neuron- and oligodendrocyte-like cells. It remains to be established whether intrauterine injections into embryonic mouse brain of immature NT2 cells induces astrocytic differentiation, as in the case of murine ES cells.

A major advantage of the present grafting approach is that grafted NT2 cells can be discriminated from murine brain cells by fluorogold labeling, and that the state of differentiation can be monitored by expression of differentiation antigens. Therefore, this approach is suited also to biochemical characterization of NT2 differentiation in situ. Grafted cells either can be isolated by fluorescence-activated cell sorting or

by UV-laser dissection methods of single cells from endogenous tissue [25] at various time points after grafting, sorted according to their differentiation state, and subjected to differential screenings to identify mRNAs whose expression levels are altered during differentiation. Alternatively, gene expression can be monitored as a function of time and differentiation using microarray techniques [25].

4.2. Migration and survival of grafted NT2 cells

We showed in our study that NT2 cells migrated from the ventricle along the corpus callosum and ventricular lining to the contralateral hemisphere, for as far as 5 mm (Fig. 3B). Migration of neuronally differentiated NT2 (NT2N) cells grafted into adult brain, in contrast, depended on the site of integration: NT2N cells grafts introduced into the septum were dispersed over a relatively wide area (several mm) within the septum, while grafts which were introduced into the neocortex were completely confined within the neocortex [14]. Murine ES cells, grafted intrauterinely, migrated over distances of several mm. This suggests that migration of grafted cells (NT2 or ES cells) is mainly determined by the age and maturity of the host brain.

4.3. Reconstructive approaches in the nervous system: deterministic versus non-cell-autonomous concepts

Transplantation is one of the most effective strategies for restoring cell loss and tissue function. Widely used in a variety of organ systems, transplantation approaches in the nervous system are still in their infancy. The complexity of the tissue and our limited knowledge about the mechanisms required for neuronal integration have restricted therapeutic efforts to reconstitute local, well characterized neurotransmitter deficits, such as restoring the nigrostriatal dopaminergic system in PD patients. Such an approach puts a lot of emphasis on the donor cell population, and a considerable effort in the transplantation field is still directed at establishing and improving dopaminergic donor cells. Although this deterministic strategy has been used successfully for PD patients, such a strategy is not useful for the treatment of more widespread neurodegenerative diseases, and non-cell-autonomous concepts provide a more promising perspective. These concepts emphasize the plasticity of individual precursor cells and regard region-specific differentiation as a response of pluripotent precursor cells to local environmental cues [3].

The finding, that even undifferentiated NT2 cells, which can be grown in high quantities, do differentiate into neuron- and oligodendrocyte-like cells, emphasizes the usefulness of NT2 cells in therapeutic approaches. NT2 cells are particularly valuable as they may be used as vehicles for the release of neurotrophic factors into the host brain environment. In addition, our approach provides an experimental model system which allows the study of neuronal and glial differentiation of human cells in vivo, and which may be suitable as an in vivo model for pharmacological studies. Moreover, a comparative analysis of the caudoputamen of adult nude mice and the immature brain of newborn immunocompetent mice may provide insights into inductive mechanisms of the host brain that make possible both the inhibition of proliferation and the induction of NT2 cell differentiation into postmitotic neuron- and glial-like cells in situ.

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