Transfected neural precursor cultures exhibit dopaminergic properties when coated with porn/laminin

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Introduction: Dopaminergic (DA) neurons are important regulators of motor control, behavior, emotion, cognition and motivation. In Parkinson's disease (PD), progressive loss of these neurons in the substantia nigra leads to severe motor dysfunction. Exogenous cell replacement, which is achieved by grafting fetal DA neurons into the striatum represents a possible therapy for late stages of PD. However, this approach is limited by a lack of donor tissue, poor survival of grafted cells and ethical problems. Furthermore, the identification of transplanted cells *in situ* remains difficult. To overcome these limitations, stem cells and neural precursors provide attractive prospects for transplantation in neurodegenerative diseases. Studer et al. expanded ventral mesencephalic precursor cells in response to basic fibroblast growth factor and subsequent differentiated them upon mitogen withdrawal into DA neurons *in vitro* (Studer et al., 1998). However, the *in vivo* survival and functional effects were inferior to embryonic cells from later stages used so far (Brundin and Bjorklund, 1998). Therefore, aims of the present study were (i) to establish an optimized protocol for attached cultures and single-cell transplantation, (ii) to characterize the cells during differentiation using different techniques, (iii) to investigate how long one has to pre-differentiate the cells before grafting them, and (iv) what coating substrates fulfill these requirements best. In addition, the cells were transfected with both, expression reporters like eGFP and DsRed and with growth factors like FGF-2.

Methods: Ventral mesencephali of embryonic rats were dissected and dissociated to a single cell suspension. The highest proliferation rates were achieved by the treatment with a medium containing 3%FCS for the first day and the use of a Porn / Laminin coated culture surface. After 24 hours, the medium was removed, the cells were cultivated in serum-free proliferation medium in the presence of 20 ng/ml FGF-2 as mitogen for 6 additional days and afterwards differentiated for 2-7 days in the absence of the mitogen. The differentiation medium contained DMEM/F12, 0.25%BSA, B27, 1%FCS, 100µM ascorbic acid and 2mM glutamine. The cells were plated at a density of 40,000 cells / 24 well plate and were expanded one week and subsequent differentiated. We used different techniques for transfection: lipofection with Lipofectamine 2000 reagent (Invitrogen), electroporation with EasyJecT Optima electroporator (EquiBio Ltd), and nucleofection with Nucleofector device (Amaxa GmbH). Immunocytochemistry was performed with different antibodies (e.g. anti-tyrosine hydroxylase (TH), nestin), and cell ELISA was used for quantification. In addition, mRNA for different markers was analyzed by RT-PCR. AMPA receptors were characterized with calcium imaging. For *in vivo* testing of the generated cells, the precursors were transplanted into 6-hydroxydopamine lesioned rats and behavioral tests were done.

Results / Discussion: We report that CNS precursors can be expanded *in vitro* up to 40-fold and afterwards be efficiently differentiated into DA neurons. Both, the vitality and attachment on the substrate surface after seeding was important with respect to the proliferation capacity of the progenitors. The highest proliferation rates were achieved by treatment with 3%FCS for up to 24 hours of culturing and the use of Porn/laminin substrate. Other substrates, like poly-ornithine/fibronectin or PLL, were less effective. Another proliferation-promoting aspect was to leave some very small clusters within the single cell suspension, because they acted as “starting points“ for expansion. The number of cells expanded from 30,000 seeded cells to about 750,000 ± 173,000 cells within one week. After 4-5 days under differentiation conditions, more than 70% of the neurons were TH+, equivalent to 30% of the total cell population. The highest transfection rate of up to 47% was achieved using nucleofection. Calcium imaging revealed the presence of calcium permeable AMPA receptors in the differentiated precursors which are capable to contribute to many developmental processes. In addition, neuronal progenitor cells change their expression pattern after differentiation into DA neurons. RT-PCR analysis showed that significantly higher RNA levels after differentiation were found for dopamine receptor 2 (D2R) and TH (the relative increase after differentiation was for D2R: +70.8 ± 31.6%, and for TH: +112.1 ± 12.7). The *in vitro* differentiation period prior to transplantation greatly influences the overall outcome *in vivo*. The survival rate, degree of reinnervation and the behavioral performance after transplantation of 4-days *in vitro* differentiated cells were significantly better (survival: 928 ± 69 TH+ neurons) compared to longer (survival after 6 days: 217 ± 43) and shorter (2 days: 573 ± 65) differentiation periods, respectivley. Already 3 weeks post implantation, the rats showed significant improvements in behavioral scores.

Conclusions: These results indicate that neuronal progenitors can be cultured on adherent surfaces and afterwards be grafted using micro-transplantation. However, there is only a short time-window during *in-vitro*-differentiation, in which enough cells are already differentiated towards DA phenotypes and simultaneously not too mature for implantation. Taken together, these modifications of earlier protocols are beneficial in promoting the technique of culturing neural precursors in terms of both, expansion rate and ratio of TH+ cells. This could help to establish an alternative cell recourse. This study shows, that neural progenitors provide attractive perspectives in neurodegenerative diseases and could be even more promising when transfected with (a) neurotrophic factor(s).

References: Brundin P, et al. Nat Neurosci. 1998;1:537.
Studer L, et al. Nat Neurosci. 1998;1:290-295.