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Time associated decline in neurotrophic properties of neural stem cell grafts render them dependent on brain region-specific environmental support

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ABSTRACT

Fetal neural stem/precursor cells (NPCs) possess powerful neurotrophic properties by which they can facilitate self repair processes in the adult central nervous system. The therapeutic value of NPC therapy in neurodegenerative diseases is critically dependent on their long term survival and enduring functional properties. An important aspect of NPC neurotrophic properties is their ability to support their own survival independent of any exogenous growth factor. Here, we examined whether NPCs survive and maintain their properties for extended periods of time, or become dependent on environmental support. Two months following transplantation to naïve brains, large grafts were detected in the ventricles and hippocampus, but only little survival was evident in the striatum. To point at possible regional characteristics which underlie the differential survival of NPC grafts we performed several manipulations of the brain environment. Acute neurotoxic injury with 6-hydroxydopamine induced a 3-fold increase in striatal graft survival, associated with induction of nestin, CD31, β1-integrin, GFAP and cycling cells. Disruption of the extracellular matrix structure of this reactive niche by continuous blockage of host striatum β1-integrin caused 73% reduction in graft survival. In the hippocampus, NPC graft survival did not correspond to β1-integrin and CD31 expression. This suggests that hippocampal environmental support to graft survival rely on different mechanisms than in the reactive striatum. In correlation with in vivo findings, long term cultured neural precursors exhibited an increase in apoptotic cells and dramatic decline in neurotrophic effects, as indicated by two in vitro functional assays. We conclude that long-term changes in transplanted NPC properties render them dependent on region specific environmental support. The major extracellular matrix protein β1-integrin is essential for providing tissue support for graft survival in the striatum. The neurotrophic properties of transplanted neural stem cells are limited in time, representing a shortcoming which should be taken into consideration when developing clinical transplantation protocols for chronic neurological disorders.

Introduction

A major problem in the field of regenerative medicine is the progressive decline in the self repair capacity of the adult central nervous system (CNS). A therapeutic alternative to endogenous repair is neural stem/precursor cell (NPC) transplantation. Recent studies have highlighted also the powerful immune-regulatory and neurotrophic properties of NPCs. For example, transplanted NPCs enhance axonal regeneration after spinal cord (Teng et al., 2002) and optic nerve injury (Charalambous et al., 2008; Hill et al., 2009; Zhang et al., 2007) by production of neurotrophic growth factors (Lu et al., 2003) and induction of tissue remodeling (Zhang et al., 2007) and angiogenesis (Rauch et al., 2009). Transplanted NPCs were shown to promote endogenous myelin repair following primary chronic toxic-induced demyelination (Einstein et al., 2009) and secondary demyelination in spinal cord injury models (Keirstead et al., 2005). Also, transplanted NPCs can enhance endogenous neurogenesis and memory functions in the physiologically aging brain (Hattiangady et al., 2007), as well as in pathological conditions, such as prenatal exposure to opioids causing impaired learning associated with reduced neurogenesis (Ben-Shaanan et al., 2008). While the multileveled beneficial therapeutic effects of NPCs have been well characterized in the acutely-injured brain, it is not clear whether transplanted NPCs can survive in the host brain and maintain their trophic properties for extended periods of time. Clearly, the therapeutic value and clinical translation of NPC therapy are critically dependent on their long term survival and functional properties. While dissociated NPCs rapidly die if not supplemented with mitogenic growth factors, in the form of spheres they support their own survival independent of any exogenous growth factor (Einstein et al., 2006). This self-supportive effect of NPCs indicates an important trophic property, and therefore one would expect that transplanted NPC spheres

Abbreviations: NPCs, neural precursor cells; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; FGF2, fibroblast growth factor 2; 6-OH DA, 6-hydroxydopamine; BrdU, bromodeoxyuridine; GalC, galactocerebroside; PSA-NCAM, Polysialylated-neural cell adhesion molecule; NeuN, neuronal specific nuclear protein; PDGF, Platelet derived growth factor.

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should survive for long period of time in the host brain independently of environmental support. Here, we challenged this notion and found that transplanted NPC sphere survival is highly environment dependent. Specifically, there was marked difference between transplanted NPC-survival in various brain regions. Grafts introduced to the naïve striatum exhibited poor survival, whereas transplantation into the lateral ventricle or dentate gyrus of the hippocampus (which are close to the sub-ventricular and sub-granular zones) resulted in extensive graft survival. Manipulation of the host brain environment by various means caused marked changes in the brain's ability to support graft survival. In accordance, in vitro experiments on long term cultured NPC spheres showed loss of their neurotrophic properties, rendering them dependent on environmental support.

Methods

Animals

C57BL/6 female mice were supplied by Harlan and were grown under specific pathogen-free conditions. Animal experimentation was approved by the institutional Ethics committee.

Isolation and growth of mouse NPCs

Multipotential NPCs were isolated from the forebrain of transgenic C57BL/6 mouse (expressing green fluorescent protein (GFP); obtained from Japan SLC) embryos on 13.5 day of pregnancy and grown as free-floating neurospheres. The tissue was dissociated using EBSS containing 0.25 mg/ml trypsin and 10 μg/ml DNase I (5 min at 37 °C). Tissue was further mechanically dissociated by aspiration and expulsion with a 5 ml Falcon pipette. Dissociated cells were transferred to T-75 flasks (5×10^6 cells/flask) and grown as suspended neurospheres ("fetal spheres") in a serum-free DMEM/F-12 medium containing B27 supplement. Basic fibroblast growth factor (FGF2, 10 ng/ml, R&D systems), was added to cultures daily. In some experiments, FGF2 was removed from the culture medium of formed NPC spheres after 5 days of expansion. These spheres were maintained in FGF2-deprived DMEM/F-12 medium containing B27 supplement for one month ("long term spheres").

6-OH DA lesion induction and bromo-deoxyuridine injection

Mice were anesthetized using a combination of ketamine (80 mg/kg; i.p.) and xylazine (20 mg/kg; i.p.) prepared in normal saline. 6-hydroxydopamine (6-OH DA) toxin (55 mg/mice) was injected ICV (coordinates: A = 0, L = 1 mm, H = 2.2 mm from bregma) and tyrosine hydroxylase staining was used to determine the extent of the injury. Following 6-OH DA injection the mice exhibited a slight ataxic gait. In some mice, 10 days after lesion induction cell transplantation to the striatum was performed (see below). In order to assess lesion influence on the cell proliferative state in the brain; naïve or 6-OH DA lesioned mice were injected intraperitoneally with bromodeoxyuridine (BrdU, Sigma-Aldrich, 50 μg/1 g body weight) for 7 consecutive days, for identifying proliferating cells.

NPCs transplantation and mini pump insertion

Mice were anesthetized using ketamine and xylazine (see above). Quantities of 2×10^3, 4×10^3 or 5×10^3 GFP + NPC spheres in a volume of 2 μl, 4 μl or 5 μl of F12/DMEM were injected into the hippocampus (A = −2.2 mm, L = 2 mm, H = 2.7 mm), naïve/lesioned striatum (A = 0.5 mm, L = 2 mm, H = 4.5 mm) and lateral ventricles respectively. Since the GFP + NPCs were of the same C57BL/6 background, no immunosuppression was used. Two days and two months post transplantation, animals were sacrificed for histopathological analysis (see below). In some experiments, Alzet mini pumps were implanted in the striatum (using the same coordinates as above) to provide continuous delivery of 100 ng/day neutralizing anti-β1 integrin antibodies (or isotype control) for 28 days, according to manufacturer instructions.

Brain irradiation

Mice were put into a pipe like plastic devise, followed by a selective 10 Gy irradiation of mice head. There was some hair loss evident at 1 month post irradiation but the mice continued to gain weight similar to their normal counterparts, and no neurological deficits were observed. Transplantation of GFP + NPC spheres was performed 3 weeks post irradiation.

Histopathology

Animals were anesthetized with a lethal dose of pentobarbital and brains were perfused via the ascending aorta with ice-cold PBS followed by cold 4% paraformaldehyde. Tissues were deep frozen in liquid nitrogen, serial 10 μM coronal sections were prepared and imunofluorescent stainings were performed as previously described (Einstein et al., 2003). The following antibodies were used: mouse anti-PSA-NCAM (Chemicon), mouse anti-Nestin (Chemicon), rabbit anti NG2 (Chemicon), rabbit anti galactocerebroside (GalC, Chemicon), rabbit anti-glial fibrillary acidic protein (GFAP, Dako), mouse anti neuronal nuclei (NeuN, Chemicon), rat ant-BrdU (Serotec), rat anti-CD31 (Pharmingen) and rat anti-β1 integrin (Millipore). Goat anti mouse Alexa-fluor555, goat anti rabbit Alexa-fluor555 and goat anti rat Alexa-fluor555 were used as secondary antibodies where appropriate. For in vitro stainings, floating spheres were adhered to 35-mm tissue culture dishes coated with 10 μg/ml poly-D-lysine and 5 μg/ml fibronectin (Sigma). Stainings for PSA-NCAM, nestin, anti-Sox2 (Abcam), BrdU, GFAP, mouse anti-βIII-tubulin (Sigma) and GalC were performed as previously described (Einstein et al., 2006). Cell death was assessed using TUNEL TMR Red kit (Roche) according to manufacturer instructions.

Determination of graft survival

Images spanning the entire graft were obtained from every third section. The entire GFP + graft area per section was measured in pixels, using computerized software. In order to compare graft survival at 2 months as fraction of 2 days old grafts, we took into account the dissemination and change in size of transplanted cells in the host brain with time. First, confocal microscopy was used to determine the percent of GFP positive cells within the graft area. To that end, seven random fields were chosen in each mouse. The average percent was calculated independently for each time point (2 days and 2 months), in order to account for cell dissemination. Second, to account for changes in cell size, average cell size (in pixels) was measured independently for each time point in 50 cells per mouse, using computerized software. The fraction of surviving graft was calculated by using the following equation:

$$\frac{\textit{Total graft area in pixels} \times \textit{Percent of GFP(+)cells}}{\textit{Cell size in pixels}}.$$

PC12 cultures and NPC spheres

PC12 cells (a generous gift from Prof. P. Lazarovici, The Hebrew University) were plated in 24-well dishes (Costar, Corning), at a density of 10,000 cells per well in 0.5 ml B27 medium. To induce neurite outgrowth, 100 ng/ml of human NGF or 0.5 ml of fetal or long term NPC sphere supernatants were added. After 48 h, cells were visualized