

Cross-Talk Between Stem Cells and the Dysfunctional Brain is Facilitated by Manipulating the Niche: Evidence from an Adhesion Molecule

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ABSTRACT

In the injured brain, the behavior of neural stem/progenitor cells (NSCs) is regulated by multiple converging factors encountered in the niche, which is composed of several neural and non-neural cell types. Signals emanating from the host influence the migration, survival, distribution, and fate of transplanted NSCs, which in turn can create host microenvironments that favor a return to homeostasis. We tested the hypothesis that overexpression of key facilitatory molecules that define the injury niche might enhance this bidirectional stem cell–host interaction to therapeutic advantage. As proof of concept, we investigated whether conditioning the niche with the neural cell adhesion molecule L1 might enhance recovery in a prototypical neurodegenerative milieu—the MPTP-induced model of Parkinson's disease in aged mice—where cross-talk between NSCs and imperiled host dopaminergic neurons is known to be pivotal in rescuing the function and connectivity of the latter. In lesioned mice (and in unlesioned controls), we overexpressed L1 in the NSCs to be transplanted into the ventral mesencephalon. Several pairwise experimental combinations were tested based on variations of engraft-

ing L1 overexpressing versus nonoverexpressing NSCs into wild-type (WT) versus L1-overexpressing transgenic mice (specifically L1 transcribed from the GFAP promoter and, hence, overexpressed in host astrocytes). Enrichment for L1—particularly when expressed simultaneously in both donor NSCs and host brain—led to rapid and extensive distribution of exogenous NSCs, which in turn rescued (with an efficacy greater than in nonengineered controls) dysfunctional host dopaminergic nigral neurons, even when grafting was delayed by a month. L1 overexpression by NSCs also enhanced their own differentiation into tyrosine hydroxylase-expressing neurons in both WT and transgenic hosts. Graft–host interactions were thus favored by progressively increasing levels of L1. More broadly, this study supports the view that manipulating components of the niche (such as an adhesion molecule) that facilitate cross-talk between stem cells and the dysfunctional brain may offer new strategies for more efficacious neurotransplantation, particularly when treatment is delayed as in chronic lesions or advanced stages of a neurodegenerative disease. *STEM CELLS* 2009;27:2846–2856

Disclosure of potential conflicts of interest is found at the end of this article.

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INTRODUCTION

A growing appreciation that dysfunctional organs and transplanted stem cells engage in an elaborate cross-talk has prompted investigations into the molecular basis of this dynamic. One manifestation of this cross-talk has been the exertion, by exogenous stem cells, of a rescue or protective effect on degenerating host cells [1–5], a phenomenon dubbed the “chaperone effect.” This stem cell–mediated action can be particularly well-characterized in the central nervous system (CNS) because clearly-defined neuronal cell types become dysfunctional under well-established, selective lesioning conditions.

It was previously observed that the neural stem/progenitor cell (NSC) possesses an intrinsic capacity to rescue dysfunctional dopaminergic neurons in the brains of aged mice subjected to systemic, repetitive exposure to the dopamine transporter (DAT) selective neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [1], creating a well-characterized model of Parkinson’s disease (PD). Unilateral implantation of NSCs was associated with reconstitution of the mesostriatal system bilaterally; locomotor recovery paralleled this reconstitution of tyrosine hydroxylase (TH) and DAT function. Although spontaneous differentiation of a small proportion of grafted NSCs to TH⁺ cells may have contributed to this nigral reconstitution, the majority of dopaminergic neurons in the mesostriatal system were actually rescued host cells that seemed to have benefited from trophic and neuroprotective support from NSC-derived cells. That NSC–host cross-talk underlay this effect was reinforced by the observation that rescue of host DA neurons precisely mirrored the spatiotemporal distribution of migrating donor NSC-derived cells: rescue of imperiled DA neurons could be linked to their juxtaposition with exogenous NSCs. The greater the migration of the NSCs, the greater the rescue of host cells appeared to be. While initially described in this rodent model of PD, identical phenomena have now been reported in primate models that authentically mimic true human PD [67, 68]. Indeed, the “chaperone effect” has now been found to account for a large proportion of the stem cell–mediated therapeutic actions reported in a range of organ systems by a range of stem cell types (neural and non-neural), including spinal cord injury, stroke, neurodegenerative conditions, heart disease, etc.

Such observations have suggested that one dimension critical to the intercellular communication so fundamental to the chaperone effect is the injury/degenerative niche itself. NSC behavior in vivo is an integrated response to converging signals emanating from a variety of neighboring neural and non-neural cell types (e.g., glia and endothelium, respectively), as well as molecules that mediate their cross-talk [16–18, 22–24]. Furthermore, this microenvironment is constantly in flux. We hypothesized that manipulating the niche such that molecular dialogues are enhanced might, in turn, positively influence these stem cell–host dynamics and yield greater therapeutic benefit by optimizing the chaperone action of NSCs [25].

Among molecules in the niche that are most pivotal to bidirectional stem cell–host cross-talk are adhesion molecules. Therefore, as proof of concept for this approach, we focused on this component of the niche. As a prototypical neural adhesion molecule, we chose the well-characterized glycoprotein L1. A member of the immunoglobulin superfamily, L1 is associated with several functions that make it an appealing target, including cell migration, neurite outgrowth and fasciculation, myelination, synaptic plasticity, and regeneration [7]. L1 is involved in homophilic (i.e., self-binding) and het-

erophilic signal transduction events leading to changes in intracellular messengers [8, 9]. The importance of L1 in development is underscored by the morphological and functional abnormalities associated with L1 loss-of-function mutations [9, 10]. In using L1 as a prototypical adhesion molecule, we hoped to gain insight into the potential contributions of other candidate adhesion molecules (e.g., other immunoglobulin superfamily molecules, integrins, cadherins, ephrins, and constituents of the extracellular matrix (ECM)).

In this study, we studied the interactions and impact of NSCs engineered to overexpress L1 (compared to nonoverexpressing NSCs) implanted into the nigra of aged Parkinsonian host mice, which themselves either did or did not constitutively overexpress L1 (specifically in astrocytes, the astrocyte having been identified as pivotal in neuron–glia interactions and for defining the stem cell niche). Several pairwise experimental combinations were tested. Host dopaminergic neuron survival and activity, in addition to NSC fate (e.g., migration, distribution, or differentiated phenotype) served as reliable quantifiable functional readouts of the subsequent cross-talk between stem cell and host. Indeed, we found that L1 served to enhance NSC–host interactions, which suggests that, in the array of molecular interactions between stem cells and the adult CNS (which likely include signaling mediated by multiple trophic factors and chemokines [11, 12]), cell surface-expressed adhesion molecules play an important role in allowing stem cells to help restore a homeostatic milieu. Furthermore, manipulating the niche (e.g., by overexpression of adhesion molecules that play a facilitatory role in stem cell–host cross-talk) might be one strategy for optimizing the therapeutic efficacy of neurotransplantation.

MATERIALS AND METHODS

Animals

We used aged (20-month-old) mice from wild-type (WT) strain C57BL/6J and from a C57BL/6J-derived transgenic line in which L1 cDNA is constitutively transcribed from the glial fibrillary acidic protein (GFAP) promoter, allowing it to be overexpressed in astrocytes (L1-mice) [15]. The L1-mice expressed ~1.5 times more L1 than the age-matched control WT mice as estimated with Western blot analysis of total brain homogenates of young (2-month-old) nonlesioned mice. The transgenic mice were genotyped with the use of polymerase chain reaction. Numbers of animals and the experimental design duplicated those reported in our previous study [1] and are thus comparable, yet none of the data from the previous study were used for the present one; all animals in the two studies were different.

Neural Stem/Progenitor Cells (NSCs)

We used a well characterized, widely used clone of NSCs in which the stemness gene *myc* [55–58] has been enhanced to preserve self-renewal and multipotency in vitro (by being placed under control of a retroviral long terminal repeat (LTR)), but is nevertheless constitutively downregulated upon cell contact, engraftment, or differentiation [59]. In addition to expressing a broad range of stemness genes and fulfilling the operational definition of a stem cell [1–3, 13, 21, 25–27, 55–60], this C57BL/6J-BalbC-derived murine NSC clone (designated C17.2) has been successfully and safely used for transplantation, integration, and therapeutic benefit in a wide range of animal models in the hands of many investigators [1–3, 21, 25–27]. Because they are clonal, are readily expandable into large uniform cell cycle- and differentiation state-synchronized clonal populations, and constitutively express *lacZ* under transcriptional control of a retroviral LTR

(hence producing the readily detectable marker *Escherichia coli* β -galactosidase [β -gal]), these NSCs have been widely regarded as a useful tool for revealing and studying fundamental prototypical stem cell behaviors. Cells from this clone were grown under standard conditions in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and 5% horse serum. When used in this manner without further manipulation, the cells are designated wt-NSCs. For preparation of L1-overexpressing NSCs (L1-NSCs), the above clones of NSCs were lipotransfected with a bicistronic expression vector containing an internal ribosome entry site where the transcription of full-length mouse L1 and green fluorescent protein (GFP) was driven from the same promoter, a rat tyrosine hydroxylase (TH) promoter [60]. This construct drives GFP with L1 expression, allowing GFP to serve as a reliable dynamic marker for transgenic L1 expression in a neurally L1-expressing host environment (i.e., effectively distinguishing transgenic L1 from any endogenous L1). For positive clone selection based on drug-resistance, cells were cotransfected with a plasmid encoding puromycin N-acetyl transferase driven by the phosphoglycerate kinase promoter [61]. Prior to transplantation, undifferentiated cells were harvested at 70% confluence by minimal trypsinization (48 hours after passaging and plating), washed twice in Hank's balanced salt solution (HBSS), and resuspended at a concentration of 10^5 cells/ μ l HBSS.

In our initial *in vitro* tests with L1-NSCs (Figure 1B, 1C), the TH promoter, although theoretically active only in cells producing TH, showed an unexpected constitutive activity in proliferating and undifferentiated NSCs. While the reasons for this remain unknown and may have to do with some particular regulation of this promoter activity in NSCs, its broad activity in the subsequent *in vivo* studies did not change their utility in these paradigms. In fact, although the initial aim was to study the effects of L1 expression only in TH⁺ cells, the constitutive expression of L1 in all grafted NSCs may have helped reveal the influence of the transgene on NSC plasticity and host cell rescue.

MPTP Lesioning

Aged (20 months old, experimental) and young (2 months old, control) C57BL/6J adult WT female mice ($n = 104$ animals) were subcutaneously injected twice with 30 mg/kg MPTP (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) at 0 days and 5 days and transplanted after the last day of injection 1 week or 4 weeks later (early versus delayed intervention, respectively). One group of mice was sacrificed for analysis 1 week after transplantation and another group 3 weeks after transplantation (to assess early NSC behavior versus later NSC behavior, respectively).

Transplantation of NSCs

Mice were anesthetized (sodium pentobarbital, 75 mg/kg), the skull was disinfected, and a parasagittal incision of skin and subcutaneous tissue (1 cm long) was performed above the right tegmental area. Using a stereotactic frame through a hole in the skull (0.9 mm diameter; 2.6 mm anterior to lambda; 1.5 mm lateral to the sagittal suture), 2 μ l ($4\text{--}5 \times 10^4$ cells/ μ l) of NSCs in HBSS was injected (over 2 min) unilaterally into the right substantia nigra/ventral tegmental area (SN/VTA) (depth: 4 mm). Mock-grafted controls received the same volume of HBSS. The skin was reapproximated with cyanoacrylate glue.

Histology and Immunohistochemistry

Mice were perfused with 4% paraformaldehyde in saline and brains were embedded in polyester wax as described [14]; 20- μ m serial coronal sections were collected. Dewaxed and rehydrated sections were blocked, and β -gal was detected with a mouse monoclonal antibody (1:300; Promega, Madison, WI, <http://www.promega.com>) or a rabbit polyclonal antibody (1:100; ICN Biomedicals, Irvine, CA; <http://www.icnbiomed.com>); TH was

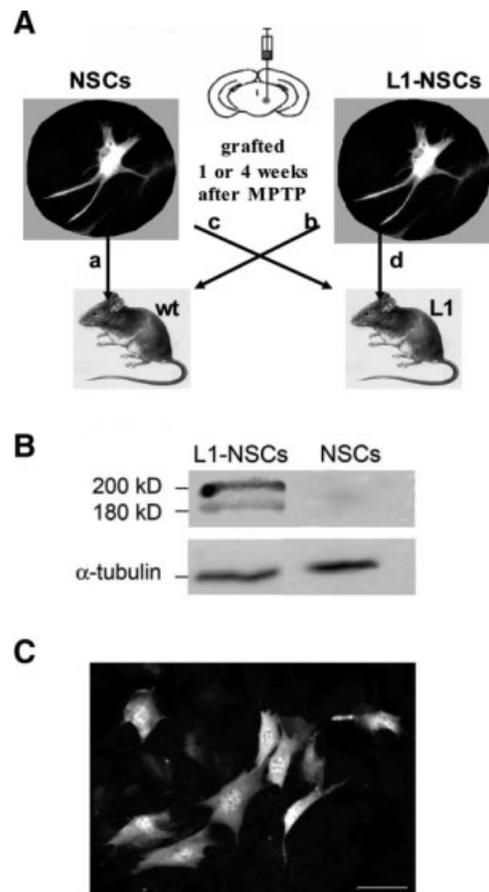


Figure 1. Grafting paradigm and characterization of L1 overexpression in NSCs. (A) Scheme illustrating the various combinations of grafted NSCs and host animals with respect to their overexpression of L1 in astrocytes and time of grafting after MPTP lesioning. All grafts consisted of a single stereotactic deposit of 10^5 NSCs above the right substantia nigra/ventral tegmental area (SN/VTA) area. (B) Western blot analysis of murine NSCs transfected with the L1 construct (subclone: L1-NSC) compared with the control parental NSCs without transfection (NSCs). To confirm expression of the L1 transgene, total cell lysates were subjected to SDS-PAGE and Western blot analysis using L1-555 monoclonal antibody, which yields the typical two bands at \sim 200 kD and 180 kD. (C) L1-NSCs were engineered to express the marker green fluorescent protein (GFP) from the same promoter as their full-length mouse L1 molecule. The photomicrograph shows L1-NSCs in culture for detection of GFP prior to grafting. (See text as well as legend to Fig. 4 for more detailed description and discussion.) The same cell culture was used for the Western analysis shown in (B). Abbreviation: NSC, neural stem/progenitor cell.

detected with a mouse monoclonal antibody (1:5,000; Sigma). Dilutions were made in blocking solution [13], and incubations were carried out overnight at 4°C. Primary antibodies were detected with biotinylated antibodies and 3,3'-diaminobenzidine tetrahydrochloride according to the Vectastain kit protocol (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>). For simultaneous detection of β -gal and TH, double immunostaining using epifluorescence was carried out with secondary antibodies coupled to fluorochromes Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes Inc., Eugene, OR, <http://probes.invitrogen.com>) and Texas Red (Vector Laboratories) (used according to the manufacturer's protocol).

Quantitative Analysis

Cell counts were obtained from three sections per animal taken from the rostral (level 335), medial (level 343), and caudal (level

360) regions of the area serially cut between levels 330 and 361 [40] containing the major portions of SN and VTA, the borders of which were delineated with TH immunostaining of intact brains. From these, camera lucida overlays were prepared and superimposed on sections from corresponding brain regions and oriented according to structures such as hippocampus and ventricles.

Cell counts in SN/VTA were evaluated on blind-coded slides using an inverted Nikon Eclipse microscope (Nikon USA, Melville, NY, <http://www.nikonusa.com>), with a Hamamatsu C4742-95 digital camera (Graftek Imaging Inc., Austin, TX, <http://www.graftek.com>) controlled with Prairie Technologies software (Middleton, WI, <http://www.prairie-technologies.com>), with an image relay to a desktop computer and a motorized X-Y stage. Sampling occurred under a 40 \times objective by moving the counting frame systematically through the SN/VTA region using the motorized X-Y stage. The Metaphorb offline software (Universal Imaging, West Chester, PA, <http://www.microscopy.info>) was used to automatically count and average cell numbers. The combined numbers of labeled cells from the three SN/VTA levels obtained from the left and right hemispheres were then compared within an animal, or between the experimental groups, and expressed as mean percentage values \pm SEM in comparison with intact controls using a reticule eyepiece (magnification \times 400) and expressed either in cells/mm² or cells/section totaling donor (β -gal⁺) and TH⁺ cells. Cell counts per unit area were corrected for split cell error [71]. ANOVA with post hoc Newman-Keuls test was used to analyze differences in cell numbers between groups. A paired *t* test was used to compare interhemispheric differences in dopaminergic neuron counts ($p < .001$ and $p < .01$). As a confirmation of the sensitivity of the system described above for assessing even subtle changes in cell numbers, the following preliminary calculations were performed: in an intact SN/VTA, there are on average 480 NeuN⁺ neurons/mm², 48% of which (230 cells) are TH⁺. A reduction of TH positivity by 61% corresponds to \sim 140 dopaminergic neurons/mm². Neuronal cell loss of NeuN⁺ cells, would be reflected as a 29% reduction, i.e., from 480 to 340 cells/mm², readily detectable by this technique. Such total neuron losses were not detected, although losses in TH immunoreactivity within the same cell population could sensitively be assayed. Results are presented as means \pm SEM.

For this study, a parameter not used in our previous study [1] was added: the migration ratio (MR). This is the ratio of the number of donor-derived cells in the contralateral to the number of donor-derived cells in the ipsilateral hemisphere. The numbers used were donor cells per section as assayed using the constitutively expressed donor NSC marker β -gal, which remains stably and reliably expressed in $>90\%$ of cells in vitro and $>80\%$ in vivo [72] during the time period monitored in this study.

RESULTS

To investigate the role that manipulation of an adhesion molecule plays in mediating NSC–host interactions in a disease niche, the transgenic expression of L1 in NSCs and/or in host astrocytes in Parkinsonian mice was investigated with particular attention to how the various permutations of L1 expression by host and donor elements affected the migratory patterns of exogenous NSCs, and how these patterns, in turn, impacted the ability of NSCs to rescue imperiled host dopaminergic neurons.

Cells from a subclone of murine NSCs engineered to overexpress L1 and GFP (L1-NSCs) or cells from the non-overexpressing parental murine NSC clone (wt-NSCs) were implanted unilaterally above the right SN/VTA of 20-month-old (aged) mice that had been exposed systemically to repetitive high doses of the dopaminergic neurotoxin MPTP. The

biochemical and neuropathological effects of MPTP intoxication (particularly related to oxidative stress) closely mimic those observed in idiopathic PD [14] as well as the progressive dopaminergic dysfunction of aging. Two strains of mice were lesioned (i.e., wt-mice or mice transgenic for L1 transcribed from the mouse GFAP promoter (L1-mice)) and hence characterized by ectopic L1 expression in astrocytes [15, 16]. The astrocyte was chosen for expression of L1 because it is one of the cells pivotal in creating the stem cell niche and effecting neurogenesis [17–20], including the survival [1] and identity [21] of NSC-derived neurons. The NSCs were implanted 1 week or 4 weeks after MPTP exposure (Fig. 1A). Controls included age-matched intact and sham-injected littermate mice. Sham animals received vehicle (HBSS) but no cells (HBSS-injected MPTP-lesioned mice). Because the parental NSC clone constitutively and stably expressed *lacZ*, all donor-derived cells could be identified by immunohistochemical detection of β -gal, as well as by GFP fluorescence in the case of L1 overexpressing NSCs. After verification of substantial (70%–80%) overlap of L1 and GFP expression by L1-NSCs in vitro (Fig. 1B, 1C), GFP was used as a marker to monitor L1-transgene expression in grafted L1-NSCs to avoid detection errors that might have resulted from baseline endogenous L1 expression in recipient brains. The number and distribution of donor-derived cells in the mesencephalon, as well as recovery of TH expression in the host SN/VTA region, were evaluated 1 week or 3 weeks after grafting in the right (ipsilateral) and left (contralateral) hemispheres for each experimental group (Figs. 2 and 3). These time points for interval analysis and sampling of the movements of the donor cells (assessing early versus later NSC behavior) were chosen on the basis of our extensive previous experience on the speed of migration of murine NSCs in adult mouse brains [1].

As we previously observed [1], β -gal⁺ cells were present in all grafted brains, their distribution and density varying based on the time of transplantation after MPTP lesioning. In the present study, however, the behavior of the transplanted NSCs was significantly influenced by L1 overexpression and whether L1 was expressed in the NSCs, in the host, or both. As presented below, it significantly changed the subsequent graft–host interactions and patterns of TH expression in the mesostriatal pathway.

Migration

Transplantation of NSCs into MPTP-lesioned mice resulted in different degrees of donor cell migration depending upon whether the NSCs were wt-NSCs or L1-NSCs (Fig. 2A, a and b; $p < .001$). The MR was significantly greater in L1-NSCs (red lines) compared with wt-NSCs (blue lines) regardless of whether grafting occurred shortly after MPTP lesioning or was delayed. Three weeks after even delayed grafting (Fig. 2A, b), L1-NSCs were able to reach an MR of nearly 1.0, indicating that the distribution of unilaterally implanted L1-NSCs essentially came to be equal between ipsilateral and contralateral hemispheres. In comparison, wt-NSCs achieved an MR of only \sim 0.3 (indicating that, 21 days after grafting, their numbers were still more than twice as high ipsilaterally as contralaterally, suggesting significantly less migration). While L1-NSCs continued to migrate efficiently even when grafting was delayed, wt-NSCs began to show a significant drop in their MR.

With regard to the speed of migration, L1-NSCs injected into a wt host brain were detected in the contralateral mesencephalon after a shorter period of time than wt-NSCs

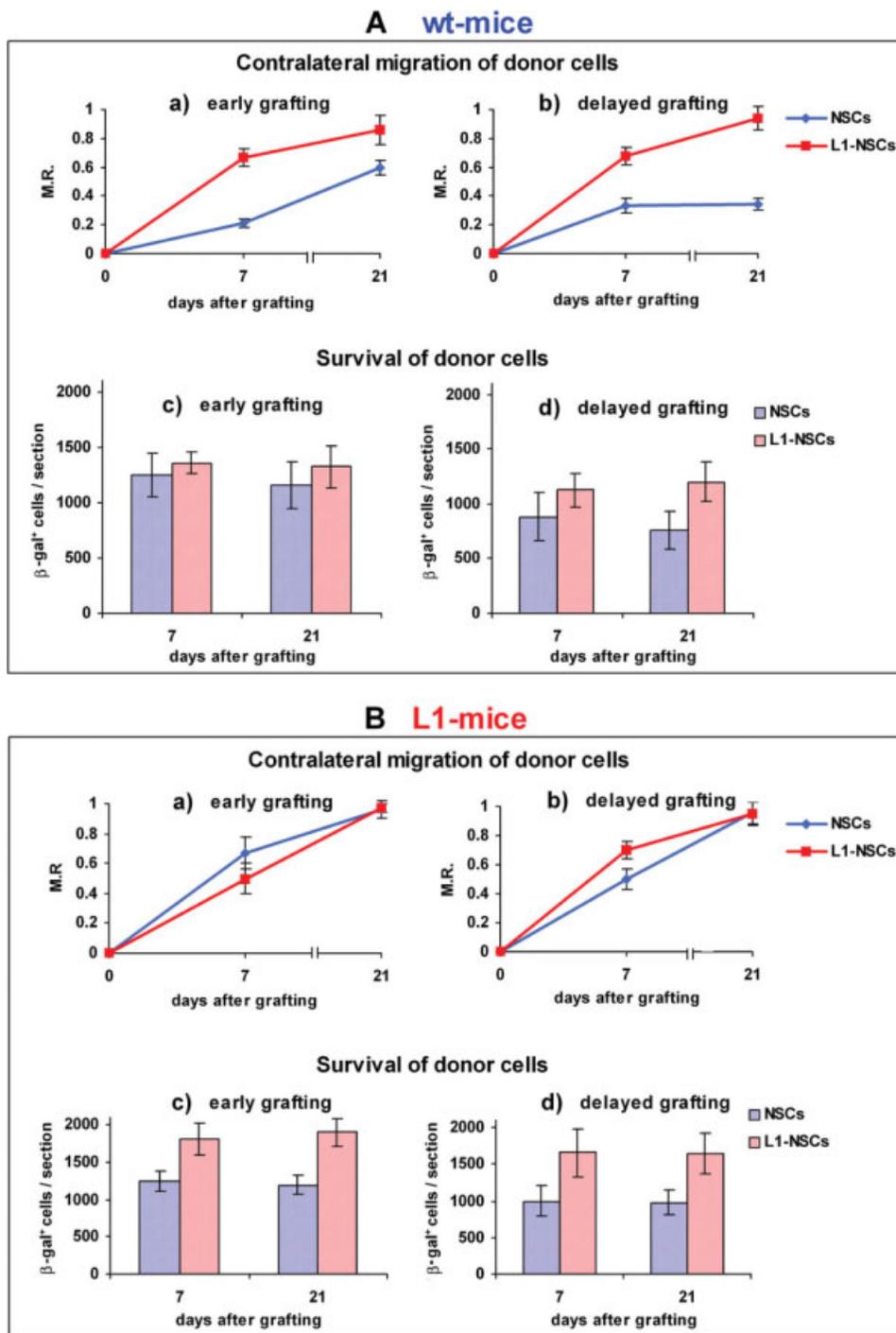


Figure 2. Effects of transgenic L1 expression on distribution and survival of grafted NSCs. Aged wt-mice (A) or L1-mice (B) were treated systemically with repeated high doses of MPTP. A single deposit of 10^5 L1-NSCs (red line) or the parental wild-type non-L1-overexpressing NSCs (blue line) were administered to the hosts unilaterally above the right substantia nigra/ventral tegmental area (SN/VTA) area 1 week (early grafting [a, c]) or 4 weeks (delayed grafting [b, d]) after MPTP treatment. After 7 days and 21 days, grafted NSCs were analyzed for their distribution and migration into the contralateral hemisphere (expressed by the M.R., i.e., the ratio between the number of donor cell in the contralateral-to-the-ipsilateral hemisphere [line graphs a and b in (A) and (B)]). The absolute number of β -gal⁺ (donor-derived) cells sampled, as described in Materials and Methods, provided an index of cell survival at those time points [bar charts c and d in (A) and (B)]. All values are represented as means \pm SEM (see text for more detailed description and discussion.) Abbreviations: L1-mice, transgenic mice overexpressing L1 in normally L1-negative astrocytes; L1-NSC, L1-overexpressing NSC; M.R., migration ratio; NSC, neural stem/progenitor cell; wt-mice, wild-type mice.

similarly injected, suggesting a more rapid migration rate (Fig. 2A, a and b).

Intriguingly, many of the differences between wt-NSCs and L1-NSCs almost vanished when cells were grafted into host mice that already overexpressed L1 (GFAP-L1 mice) (Fig. 2B, a and b) ($p < .001$). Specifically, wt-NSCs in L1-mice achieved MRs almost three times those seen in wt mouse brains at 1 week after transplantation after early grafting and reached almost 1.0 by 2 weeks later. This degree of heightened migration was noted even when grafting was delayed by 3 weeks after lesioning, with only a moderate reduction of the MR of wt-NSCs (from 0.6 to 0.4) after the

first week after transplantation (compare blue lines in 2B, a and b). The migratory behavior of L1-NSCs, on the other hand, which was already efficient in wt hosts, showed only minor improvement in the L1-mouse brain when engrafted early. In the delayed grafting paradigm, however, there was a small but significant difference between wt- and L1-NSCs 1 week after transplantation, with a higher MR for the L1-NSCs in this environment (compare blue and red lines in 2B, b). However, this difference disappeared by 2 weeks later when the MR for both approached 1.0.

It appeared that L1 overexpression in either donor NSCs or host astrocytes (i.e., in either the migratory cell or in the

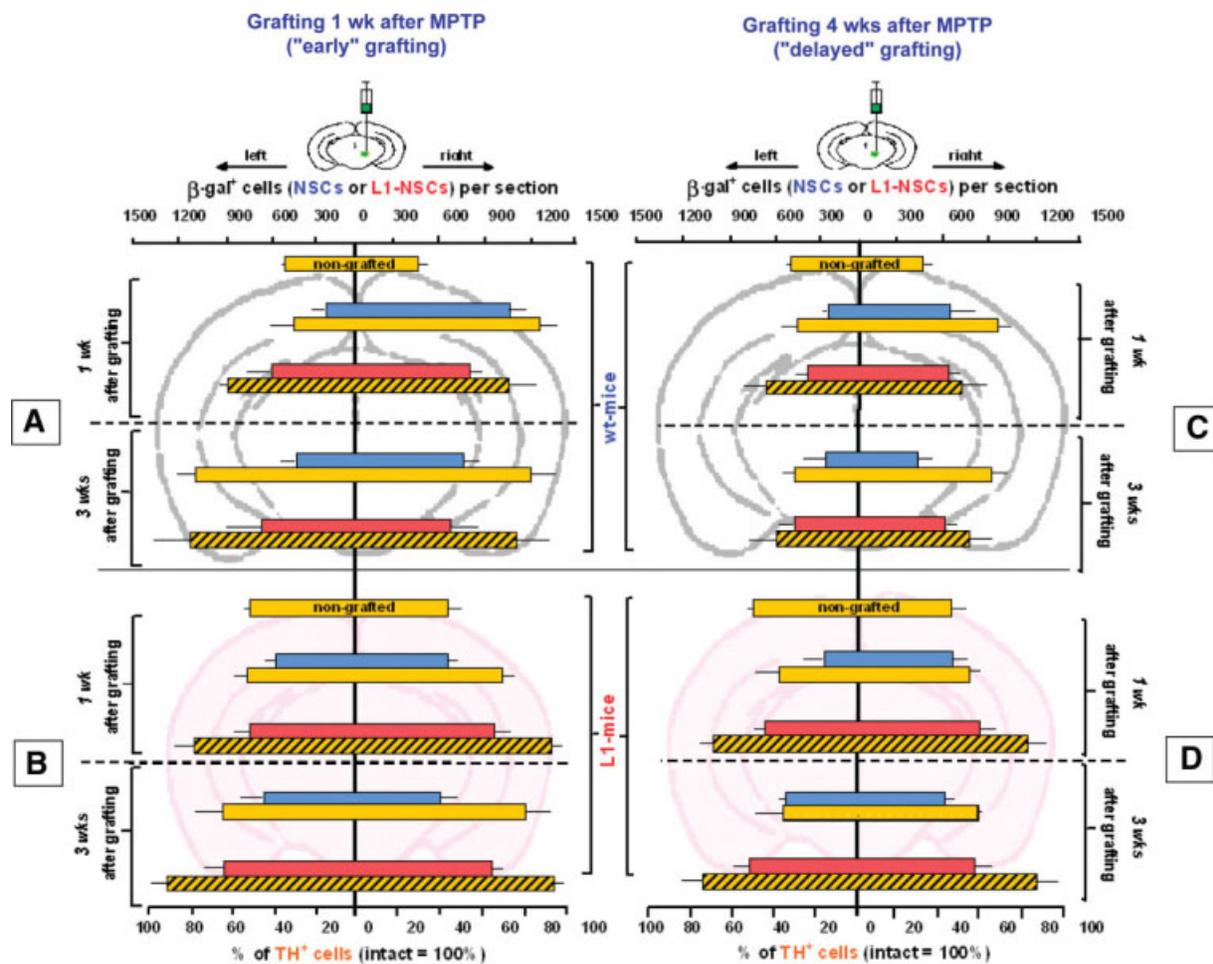


Figure 3. Bilateral reactivation of TH production in the substantia nigra/ventral tegmental area (SN/VTA) region of MPTP-lesioned mice by grafted NSCs. Wild-type mice (wt-mice) (A, C) and transgenic mice overexpressing L1 in normally L1-negative astrocytes (L1-mice) (B, D) treated with repeated systemic high doses of MPTP were left for 1 week (early grafting) (A, B) or 4 weeks (delayed grafting) (C, D) before receiving a single unilateral injection of 10^5 NSCs above the right SN/VTA area. Controls were injected with vehicle (Hank's balanced salt solution) instead of cells (nongrafted). One week (top half of each graph) or 3 weeks (bottom half of each graph) later, coronal sections through SN/VTA were processed for anti- β -gal immunohistochemistry to detect each type of NSC (blue bars indicate wt-NSCs; red bars indicate L1-NSCs) as well as for anti-TH immunohistochemistry (yellow bars when assessing the impact of wt-NSC distribution (blue bars) and yellow-hatched bars when assessing the impact of L1-NSC distribution (red bars)). The numbers of β -gal⁺ cells and TH⁺ neurons were quantified. In each graph, the midline between both mesencephalic hemispheres is represented by a vertical line. The horizontal bars across this line represent the quantification of interhemispheric distribution of the two transplanted NSC types (red or blue) as well as the host TH⁺ cells (yellow or yellow-hatched). The data for β -gal⁺ donor cells (top horizontal bar of each pairing) are expressed as absolute cell numbers ranging from 0–1,500 cells on each side of the midline; see the β -gal scale at the top of the graphs. The data for rescued host TH⁺ neurons (bottom horizontal bar of each pairing) are expressed as percentage of TH⁺ host neurons present in intact non-MPTP-lesioned age-matched controls; see the "TH" scale at the bottom of the graphs. Consistent pairing of blue horizontal top bars with yellow horizontal bottom bars and red horizontal top bars with yellow-hatched horizontal bottom bars was done to facilitate comparison between TH⁺ cell rescue and the distribution of the respective type of transplanted NSC. All values are means \pm SEM per section (sampled as per Materials and Methods), with the respective zero values positioned at the midline ($p < .001$). The yellow horizontal bars labeled as nongrafted represent the surviving dopaminergic (TH⁺) neurons in MPTP-lesioned, mock-grafted, age-matched, and genotype-matched control mice. Please see text for more detailed description and discussion of results. Abbreviations: L1-NSC, L1-overexpressing NSC; NSC, neural stem/progenitor cell; TH, tyrosine hydroxylase.

microenvironment) was equally effective in promoting donor NSC migration. The migratory advantage conferred by L1 expression to NSCs was less obvious in the context of a host mouse brain already overexpressing L1 (i.e., L1-mice) (Fig. 2B, a and b), where wt-NSCs and L1-NSCs had similar MRs. Simultaneous overexpression of L1 in both graft and host did result in a slightly greater rate for achieving such widespread distribution, but it ultimately did not result in a more extensive end-distribution of the cells, the cells likely having already achieved the maximal possible degree of dispersion for integrated NSCs within a nigrostriatal system (a notion rein-

forced in Fig. 3). These data also imply a role for host astrocytes in the migration or guidance of grafted NSCs.

Survival

We also assayed the impact of L1 overexpression on NSC survival in the MPTP lesion paradigm. In animals grafted 1 week after MPTP lesioning, wt-NSCs were found in large numbers 3 weeks after transplantation (Fig. 2A, c). When grafting was delayed until 4 weeks after MPTP exposure, NSC survival dropped from \sim 1,200 cells/section (after early

grafting) to 750 cells/section (Fig. 2A, c and d (blue bars)), while L1-NSC survival rates between early and delayed grafting remained essentially unchanged (compare red bars in Fig. 2A, c and d). In other words, there was a tendency for better survival of L1-NSCs compared to wt-NSCs, an improvement most noticeable 3 weeks after delayed transplantation into wt hosts (Fig. 2, d). L1-Enhanced NSC survival became even more robust when L1-NSCs were transplanted into L1-mice, apparent at all grafting and analysis time points (Fig. 2B, c and d, compare blue and red bars) ($p < .001$). Thus, with regard to NSC survival, there were minor differences between L1-NSCs and wt-NSCs in wt mice; a small decrease in the number of wt-NSCs was detected 21 days after delayed grafting (Fig. 2A, c and d) ($p < .05$). However, in L1-mice, prominent differences appeared (Fig. 2B, c and d) ($p < .001$) where the number of surviving L1-NSCs was higher than that of wt-NSCs, suggesting that exogenous NSCs were somehow being shielded from noxious microenvironmental influences.

Rescue of Host Dopaminergic Neurons

MPTP impairs TH function in dopaminergic neurons but does not kill them until very late in the course [1]. Hence, it is possible to rescue host dopaminergic neuronal function under the proper circumstances. We previously showed that NSCs have that capacity [1, 67]. Having determined that various permutations of L1 expression by host and donor cells in a diseased niche affect the migratory and survival patterns of transplanted NSCs, we next assessed how these patterns, in turn, impacted the consequent graft–host interactions and patterns of TH expression in the mesostriatal pathway. Brain sections at corresponding coronal levels were double-immunostained for β -gal and TH to quantify the donor and host components of the recovering mesencephalic neurons both ipsilateral and contralateral to the graft site (Fig. 3). Reappearance of TH production in SN/VTA, presumably reflecting reactivation/rescue of imperiled host dopaminergic neurons by exogenous NSCs, was obvious by 1 week after grafting (Fig. 3, yellow bars) and mirrored the distribution and migration patterns of β -gal⁺ donor NSCs (Fig. 3, blue & red bars). In wt mice, early grafting of either wt-NSCs or L1-NSCs led to a robust recovery of TH expression in the MPTP-challenged hosts. Nevertheless, while both NSC clones were effective, the improved migration of L1-NSCs (Fig. 2A, a) was reflected in a significantly more rapid reactivation of contralateral host dopaminergic neurons one week after grafting (Fig. 3A, upper half, hatched yellow bar), with equivalence of the two NSCs clones being achieved only after 2 weeks (compare yellow with yellow-hatched bars). Delayed grafting into wt mice was marked by two observations: fewer overall rescued host dopaminergic neurons and persistent lateralization of this rescue when wt-NSCs were used (Fig. 3C, yellow bars). This observation was consistent with the fact that wt-NSCs migrated less efficiently in the wt hosts than did L1-NSCs after delayed grafting (Fig. 2A, b). Hence, the lower number of wt-NSCs contralaterally and the longer duration of MPTP-induced host dopaminergic neuron impairment without grafting prevented the wt-NSCs from achieving a bilateral reconstitution of mesencephalic TH production similar to that observed after earlier transplantation. On the other hand, the better survival, migration rate, and wider distribution of L1-NSCs provided an advantage in the delayed grafting paradigm: although lower than with earlier grafting, the numbers of rescued dopaminergic neurons were evenly distributed across both mesencephalic hemispheres by 3 weeks after transplantation (Fig. 3C, yellow-hatched bars).

The switch in recipient mouse from a wt host (Fig. 3A, 3C) to an L1 overexpressing host (Fig. 3B, 3D) had a significant additive effect on the degree and distribution of NSC-mediated reactivation of host dopaminergic neurons. As rapidly as 1 week after early grafting of wt-NSCs into L1-mice, cells achieved better contralateral reactivation of host TH expression than they did in wt-mice, presumably due to their improved migration and subsequent engagement with host cells (Fig. 3A, 3B; compare blue bars in upper half of 3A with upper half of 3B). The impact of L1-NSCs on the same process was improved as well because of their higher survival and extent of migration (Fig. 3B, red bars) leading, in turn, to an overall higher percentage of rescued host dopaminergic neurons (Fig. 3B, compare yellow bars with yellow-hatched bars). Finally, a similar result was found even after delayed transplantation: the robust and bilateral reactivation of TH production in long-term MPTP-challenged host dopaminergic neurons in L1-mice by grafted L1-NSCs stood in contrast to the more lateralized, less broad, and lower number of host dopaminergic neurons after grafting with wt-NSCs (Fig. 3D, compare yellow bars with yellow-hatched bars). It warrants noting that, although the number of TH⁺ neurons in L1-mice was slightly less diminished by MPTP exposure than in wt-mice (Fig. 3, compare the nongrafted yellow horizontal bar in the lower two graphs to the same bar in the upper two graphs)—possibly by an L1-mediated protective effect—there was no spontaneous recovery of TH⁺ cells in the L1-mice in the absence of NSC engraftment, as illustrated by the nongrafted MPTP-lesioned control mice in the delayed grafting experimental groups (Fig. 3C, 3D).

To analyze the possible contribution (if any) of donor-derived cells to the pool of TH⁺ mesostriatal neurons after transplantation, sections were incubated with antibodies against TH and β -gal (or GFP as an alternative donor cell marker). The wt-NSCs or L1-NSCs that coexpressed TH were counted. As we previously reported [1], the number of β -gal⁺/TH⁺ or GFP⁺/TH⁺ double-positive cells in or adjacent to the mesostriatal nuclei was very low, under both early and delayed NSC transplantation conditions (Fig. 4). Nevertheless, the frequency of double-positive cells was slightly higher when both host astrocytes and donor NSCs overexpressed L1. More specifically, when wt hosts were engrafted with wt-NSCs, only low numbers of the TH⁺ cells in SN/VTA were β -gal⁺ (hence, donor-derived) (Fig. 4A, arrow): ~5% donor cell contribution after early grafting and ~2% after delayed grafting. When L1 was overexpressed on either graft or host cells (Fig. 4A), the proportion of donor-derived cells that were also TH⁺ was slightly increased (but not to a statistically significant extent). However, when L1 was overexpressed simultaneously in both graft and host (Fig. 4B), the proportion rose to ~10% (after early transplantation) and ~8% (after delayed transplantation)—a statistically significant change, although still a minor component of the total TH⁺ population.

In summary, the rapid distribution of grafted L1-NSCs in both host types and that of wt-NSCs in L1-mice was accompanied by bilateral rescue and reactivation of host dopaminergic neurons in a pattern that mirrored the location of these donor-derived cells. These beneficial actions became evident as quickly as 1 week after early grafting in both wt- and L1-mice where improved migration of both donor NSC types led to better TH expression than in the case of wt-NSCs grafted into wt-mice. The increased survival (and, hence, numbers) of L1-NSCs in L1-mice resulted in a more abundant reactivation of host dopaminergic neurons, reaching nearly the values in intact animals—including, importantly, after delayed grafting (Fig. 3B, 3D, yellow-hatched bars). The delay in grafting into

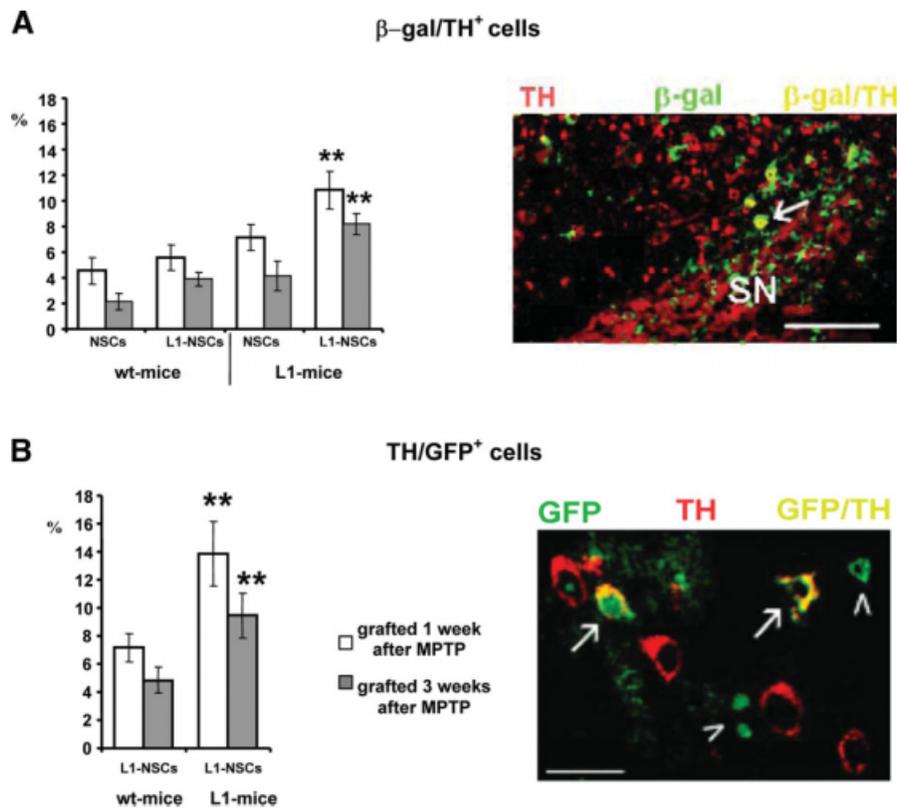


Figure 4. Quantification of donor-derived TH-expressing cells in relation to graft/host combinations and post-MPTP grafting times. (A) The graph depicts the increasing numbers of TH-expressing donor-derived cells as a function of the increasing amount of L1. A maximum of donor-derived TH expression was reached when both graft and host overexpressed the L1 transgene; this correlated with the donor cells' higher survival rates. High-power micrograph of the right substantia nigra (SN) and the surrounding midbrain parenchyma in a right-side representative grafted animal. Sections were immunostained for the NSC marker β -gal (green) and TH (red); yellow fluorescent cells indicate dual immunoreactivity to both markers (arrow). These dual-expressing cells were quantified. Note that, despite the presence of donor-derived TH⁺ cells, the SN is composed mainly of reactivated host (β -gal-negative) dopaminergic neurons surrounded by mostly undifferentiated donor NSC-derived β -gal⁺ (green only) cells. Microbar: 110 μ m. (B) Donor-derived TH expression (red) correlated also with GFP-positivity (green) in L1-NSCs. The high-power image of cells in the SN/ventral tegmental area of an MPTP-lesioned L1-mouse grafted 1 week later with L1-NSCs reveals GFP⁺ (green only, arrowheads) and GFP⁺/TH⁺ (yellow, arrows) progeny of the grafted NSCs, as well as TH⁺ host cells (red only). The GFP marker in the grafted L1-NSCs was driven by the same promoter as the L1 transgene and was used to monitor its expression *in vivo* (distinguishing it from any endogenous L1). The presence of GFP⁺/TH⁻ cells (green only, arrowheads), however, confirmed that, in the donor stem cell progeny, the L1 transgene was expressed also in other than just TH⁺ cell types, as should be expected. Bar: 40 μ m. Abbreviations: GFP, green fluorescent protein; L1-NSC, L1-overexpressing NSC; NSC, neural stem/progenitor cell; TH, tyrosine hydroxylase.

MPTP-damaged brains was more of an impediment for wt-NSCs, always resulting in lower numbers of rescued host dopaminergic neurons (even taking into account their improved migration in L1-hosts). The high numbers of broadly distributed engrafted L1-NSCs, especially in L1-mice, led to remarkable and unmatched reactivation of the subchronically damaged mesostriatal pathway. Given these findings, it is reasonable to speculate that enhanced migration and survival of donor NSCs in the niche-manipulated environments had positive consequences for the reactivation of TH production in the imperiled host dopaminergic neurons, discussed more fully in the next section.

DISCUSSION

In the present study, transplantation into the aged MPTP-lesioned substantia nigra—a well-characterized model that focuses on a clearly-identifiable neural population (dopaminergic neurons) in a well-defined region (the mesostriatal pathway)—provided an ideal opportunity to test the hypothe-

sis that manipulation of the disease niche could itself enhance the therapeutic actions of stem cells. Specifically, as proof of concept, we evaluated the beneficial features of the adhesion molecule L1 in this prototypical neurodegenerative disease model.

Previously we observed that, while wt-NSCs grafted into a mouse model of PD could, to a minor degree, assume a compensatory dopaminergic phenotype, their most impressive action was their rescue of aged host dopaminergic neurons that were otherwise rendered permanently dysfunctional and moribund by MPTP [1]. Since that time, we [e.g., 2, 3, 25–27, 67–70] and others [e.g., 28–31] have observed that exogenous stem cells possess an inherent ability to create host microenvironments that favor recovery or preservation of damaged or imperiled cells, mobilization of host progenitors, remodeling of an impaired milieu, and triggering or augmenting latent intrinsic regenerative and protective host mechanisms. We hypothesized that this action reflected cross-talk in the system. The present study supports the view that enhancing a component of the niche—as exemplified by the adhesion molecule L1, which favors this cross-talk—will, in turn, enhance the benefits derived from this interaction.

L1 overexpression was achieved in two ways: enhancing expression within the grafted NSC and within a key component of the host's niche, the astrocyte. The metric assayed was augmentation of NSC-mediated restoration of TH expression in host dopaminergic neurons. While the data support the view that overexpression of L1 improved migration and survival of donor NSCs, the magnitude of the effects varied according to the combinations of L1 expression in graft and in host. L1-NSCs injected into a wt host brain were detected in the contralateral mesencephalon after a shorter period of time than wt-NSCs similarly injected, suggesting a more rapid rate of migration (Fig. 2A, a, b). Compared with wt-NSC, L1-NSCs achieved an almost equal distribution in both hemispheres by 21 days after unilateral implantation. Even after delayed grafting (Fig. 2A, b), L1-NSCs continued to migrate efficiently. The migratory advantage L1 conferred to NSCs was less obvious in the context of an already L1 overexpressing host brain (i.e., L1-mice) (Fig. 2B, a and b), where wt-NSCs and L1-NSCs had comparable MRs. While L1-NSCs had a better MR than wt-NSCs in the first week, this difference disappeared within 2 weeks as the migration ratio for both approached 1.0. Nevertheless, the speed of donor cell migration did have an impact on efficacy of rescue of injured host dopaminergic neurons.

With regard to NSC survival, there were no significant differences between L1-NSCs and wt-NSCs in wt brains after early grafting (Fig. 2A, c) and slightly more pronounced, though still minor, differences after delayed grafting in that host (Fig. 2A, d). However, prominent differences appeared in L1-enriched host brains where the number of surviving engrafted L1-NSCs remained significantly higher than wt-NSCs, suggesting that exogenous NSCs might be somehow shielded from noxious microenvironmental influences. This increased number of L1-NSCs in L1-mice, in turn, had positive consequences for the rescue of host dopaminergic neurons, which now achieved nearly the values of an intact non-lesioned animal. This effect became evident not only after early but, importantly, after delayed grafting. For wt-NSCs, a delay in grafting had negative consequences for their efficacy, resulting in lower numbers of engrafted cells and a less symmetric distribution of rescued dopaminergic neurons. Not even the improved migration of wt-NSCs in L1-hosts could mitigate this. On the other hand, the higher numbers of surviving engrafted L1-NSCs, especially in L1-mice, reversed this effect and led to reactivation of the subchronically damaged mesostriatal pathway.

Although in this study, as in our previous report [1], TH⁺ cell replacement by NSC differentiation contributed negligibly to nigrostriatal restitution, there did appear to be slightly increased numbers of TH⁺ donor-derived cells as a function of increased levels of transgenic L1. Maximum donor-derived TH expression was reached when both graft and host over-expressed L1; this correlated with the donor cells' higher survival.

In summary, the enhanced distribution of grafted L1-NSCs in both host types and that of wt-NSCs in L1-hosts had a beneficial effect on bilateral reactivation of TH production in the imperiled host dopaminergic neurons (compared to wt-NSCs in wt hosts). In addition, simultaneous expression of L1 in donor NSCs and host astrocytes favored cell interactions the most.

How might an adhesion molecule promote these functions? For example, how might L1 overexpression promote better migration and survival of donor cells and, hence, host cell well-being? L1 binds to a diverse set of molecules on neural cell surfaces and in the extracellular matrix (ECM). Its extracellular domain helps cluster multiple binding partners into a multipotent complex, while its cytoplasmic do-

main organizes intracellular cytoskeletal and signaling proteins [7, 8, 35, 36]. The resulting cellular activities include neuron-glia adhesion, migration, sprouting, and cell survival [6, 34, 37, 38, 65, 66]. Hence L1 contributes to developmental and regenerative plasticity [34, 35, 65, 66]. These activities diminish in the adult and aged CNS, likely limiting the endogenous constitutive repair mechanisms that might normally be in play during early development or in younger brains. Re-expression of L1 may have helped enhance a therapeutic response by restoring aspects of the niche that, at earlier developmental stages, normally mediates intercellular cross-talk.

Although, the neuron has historically received the most focus when examining the cause and treatment of neurodegenerative diseases, the role of the astrocyte has recently received increasing attention, not only as one of the cells pivotal in creating the stem cell niche and in affecting the genesis [19, 20, 23], identity [21], and survival [1, 67–70] of stem cell-derived neurons, but as also ameliorating the severity of CNS injury [3, 25–27, 70] and degeneration [39]. Astrocytes play crucial roles in maintaining normal brain physiology in response to injury and disease [19, 40–42]. For example, under oxidative stress astrocytes protect neurons by producing anti-oxidants, such as glutathione or ascorbic acid, and anti-oxidant enzymes, such as superoxide dismutase, catalase, or glutathione reductase [43–46]. They contain glutamate transporters that protect neurons from excitotoxicity [47] and produce factors that are trophic for neurons [1, 67]. Given that astrocytes are the stem cell progeny thought to play a chaperone-like role for both NSC-derived and host neurons, it seems plausible that molecules that enhance the juxtaposition of astrocytes with imperiled neurons would also enhance these beneficial effects. Increased L1 expression may enhance homophilic or heterophilic L1-mediated contacts between donor NSCs and host astrocytes and hence expose the former to more optimal interaction with astrocyte-derived survival factors. The present study suggests that L1-expressing astroglia are a better migratory substrate for donor NSCs *in vivo* (even when transplantation is delayed), thereby at least partially abrogating the impediment to cell migration and axonal outgrowth an astroglial environment often presents [40]. This more favorable substrate became especially apparent when wt-NSCs were grafted into L1-mice, resulting in a considerable increase in their migration.

The beneficial effects of NSC grafts in the injured or diseased CNS have usually been described in relation to cell and gene replacement [48, 49]. However, by their capacity to provide trophic and neuroprotective support, engrafted NSCs also evoke regenerative processes in the abnormal host brain by protecting and rescuing imperiled host cells before death occurs (the chaperone effect) [1–5]. The effectiveness of neural transplantation, however, depends in part on survival of grafted cells and their ability to infiltrate damaged regions. Unfortunately, although NSCs may be resistant to certain types of stress [32, 63], their migration and survival are still drastically reduced in the adult or aged CNS and in the presence of chronic insults [50–52]. The reasons for this impairment include depletion of neurotrophic factors, presence of myelin-associated and glia-derived inhibitory molecules, and age- or lesion-dependent accumulation of ECM molecules (e.g., chondroitin sulfate proteoglycans and some tenascins) that interfere with the conducive signaling between NSCs cells and their environment [53, 54]. The findings in this study suggest that the opportunity exists to manipulate the stem cell niche to enable more effective cross-talk between NSCs and the diseased brain in order to help restore a homeostatic milieu, e.g., NSC migration to sites of damage and enhancement of NSC-mediated recovery of dysfunctional

neurons. Overexpression of beneficial adhesion molecules like L1 offers new options for more efficacious transplantation when grafting is delayed or must occur in the context of chronic lesions or advanced stages of a neurodegenerative disease in the aged CNS. The techniques employed here can be used to investigate other factors mediating stem cell–host cross-talk and offer practical strategies for optimizing the therapeutic efficacy of neurotransplantation.

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DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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