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REVIEW

Global gene and cell replacement strategies via stem cells

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The inherent biology of neural stem cells (NSCs) endows them with capabilities that not only circumvent many of the limitations of other gene transfer vehicles, but that enable a variety of novel therapeutic strategies heretofore regarded as beyond the purview of neural transplantation. Most neurodegenerative diseases are characterized not by discrete, focal abnormalities but rather by extensive, multifocal, or even global neuropathology. Such widely disseminated lesions have not conventionally been regarded as amenable to neural transplantation. However, the ability of NSCs to engraft diffusely and become integral members of structures throughout the host CNS, while also expressing therapeutic molecules, may permit these cells to address that challenge. Intriguingly, while NSCs can be readily engineered to express specified foreign genes, other intrinsic factors appear to emanate spontaneously from NSCs and, in the context of reciprocal donor–host signaling, seem to be capable of neuroprotective and/or neuroregenerative functions. Stem cells additionally have the appealing ability to 'home in' on pathology, even over great distances. Such observations help to advance the idea that NSCs – as a prototype for stem cells from other solid organs – might aid in reconstructing the molecular and cellular milieu of maldeveloped or damaged organs.
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Introduction

Cell-based therapies such as neural transplantation have, until recently, been reserved for focal or regionally restricted neurologic diseases. These are best exemplified by Parkinson's disease,[1–4] where the goal has been the engraftment and enhanced survival of dopamine-producing cells within the striatum, or by forestalling degeneration of dopaminergic neurons within the substantia nigra (SN). However, the pathologic lesions of most neurogenetic diseases – indeed, most neurologic disorders – are usually widely disseminated in the brain and spinal cord and have not typically been regarded as within the purview of neural transplantation. Such diseases include not only the inherited neurodegenerative diseases of the pediatric age group (eg lysosomal storage diseases, leukodystrophies, inborn errors of metabolism, hypoxic-ischemic encephalopathy), but also such adult maladies as Alzheimer's disease (AD), Huntington's disease (HD), multi-infarct dementia, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and brain tumors (especially glioblastomas). Therapeutic approaches for such 'global' problems have typically depended on pharmacologic or genetic interventions. They have been regarded as beyond the purview of cellular-mediated approaches. Cell replacement therapies have largely been limited to transplantation of somatic cells derived from the hematopoietic system administered via bone marrow transplantation (BMT). In the majority of these disorders, such strategies have been unsatisfactory for treating the CNS component of the disease.

The recognition that neural progenitor or stem cells, or cells that model their behavior, might integrate appropriately throughout the mammalian CNS following transplantation (for example, Refs 5–9) has unveiled a new role for neural transplantation and gene therapy and a possible strategy for addressing the CNS manifestations of diseases that heretofore had been refractory to intervention. Multipotent neural stem cells (NSCs) are operationally defined by their ability to self-renew, to differentiate into cells of all glial and neuronal lineages throughout the neuraxis, and to populate developing or degenerating CNS regions (reviewed in Refs 10–15). Thus their use as graft material can be considered analogous to hematopoietic stem cell-mediated reconstitution and gene transfer.

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Limitations of various gene transfer and cellular repair strategies for the CNS

A number of inherited metabolic diseases, in which a single gene product is deficient, can be partially treated by BMT or enzyme replacement. In many cases, such interventions have been successful in addressing peripheral manifestations but have been disappointing in reversing or forestalling damage to CNS because the blood–brain barrier (BBB) restricts entry of therapeutic molecules from the vascular compartment. BMT also usually involves conditioning regimens such as irradiation, that are deleterious to the developing CNS. Pharmacologic agents for CNS disease administered systemically often have erratic effects, transient efficacy, and undesirable side-effects.

The delivery of gene products directly to the CNS might circumvent these problems. Gene transfer may be achieved by the direct delivery of genetic material to the host's own neural tissue. The vectors presently available, however, are sometimes difficult to target *in situ* to the specific neural cell types and regions most in need of correction. For example, retroviral vectors infect only mitotic cells, which are less abundant in the post-developmental CNS and often not the cells needing therapy. Although encouraging progress is being made in the use of lenti- and adeno-associated virus (AAV)-based vectors for post-mitotic neural tissue, they do not often target the widespread lesions and multiple cell types characteristic of many neurogenetic diseases. Only cells in a relatively spatially restricted area are typically corrected. AAV, although possessing a wider distribution, may have a selectivity for neurons to the exclusion of oligodendrocytes and astrocytes. The safety and efficacy *in vivo* of many of these vectors remains to be established.

Alternatively, gene products may be imported into the host CNS by the implantation of synthetic 'pumps', or genetically modified donor cells that can reside within the CNS to deliver exogenous factors to host cells. Genetically engineered non-neural cells (eg fibroblasts) can be used for localized delivery of discrete molecules to the CNS and can be implanted autologously.[16,17] However, this approach is limited to correction of disease only in the vicinity of the graft and they lack the ability to incorporate widely into host cytoarchitecture in a functional manner following implantation. Thus, essential circuits may not be reformed and the regulated release of important factors through feedback loops may be missing. For some substances and impairments, the unregulated, inappropriate, excessive, or ectopic release may be harmful to the host (for example, NGF[16] and dopamine[17]). The choice of vehicle for a particular disease process may, therefore, need to be determined on an individual basis by a better understanding of the pathobiology and replacement needs underlying a particular defect. Of note, down-regulation of neural gene expression in engineered non-neural cells may leave them 'incapacitated'. However, donor tissue originating from the brain may sustain expression of neural genes longer. Low levels of normal neural cell products expressed intrinsically by donor neural-derived cells may enhance the therapeutic effects of such engineered cells.

Many neurogenetic diseases are characterized by the degeneration of specific neural cell types or circuits. These losses may be due to the presence of certain toxins in the milieu, an insufficiency of various trophins in the microenvironment, or to pathologic processes intrinsic to the metabolic deficiency of the diseased cell. Therefore, ideal grafts would not only provide exogenous therapeutic gene products, but would also effect repair of damaged host brain by becoming integral components of the host cytoarchitecture and circuitry. Indeed, one of the major deficiencies for most extant gene therapy techniques for neurodegenerative diseases is that they involve inserting new genetic information on old neural substrates that may have already become dysfunctional or degenerated. The challenge is to create new substrates on which these therapeutic genes can operate. Tissue-derived from the CNS may also provide as yet unrecognized endogenous neural-specific substances that are beneficial to the host. It has already been recognized that NSCs intrinsically produce a broad range of peptide neurotrophic factors (eg GDNF, BDNF, NGF, NT-3, NT-4/5).[19]

Mature or even young neurons derived from the CNS would seem to be the ideal graft material. However, there are restrictions on the types and ages of neurons that successfully survive implantation in a functionally meaningful way for prolonged periods. Primary neurons also have limited usefulness as vehicles for stable gene transfer since their limited mitotic capacity restricts their ability to be expanded into adequate numbers for grafting, as well as their ability to be transduced *ex vivo* by retroviral vectors. Primary fetal neuronal tissue has historically been the most successful donor tissue for CNS grafting and has shown promise for the amelioration of certain neurologic conditions (reviewed in Ref. 19). However, the use of fetal tissue involves significant concerns including: the ready availability of sufficient amounts of suitable disease-free material; ensuring survival of desired cells in tissue which is typically heterogeneous and contains non-neural cells; augmenting the expression of biological molecules by donor fetal tissue; and limited or very focal integration of the fetal graft into the host brain.

Properties of NSCs useful for therapeutics

The recognition that NSCs propagated in culture could be reimplanted into mammalian brain, where they could reintegrate appropriately and stably express foreign genes[20,21] made this strategy an attractive alternative for CNS gene therapy and repair. Numerous subsequent studies over the past decade (reviewed in Ref. 15) reaffirmed that neural progenitors from many regions and developmental stages could be maintained, perpetuated, and passaged *in vitro* by a number of epigenetic and genetic methods. Examples include the transduction of genes interacting with cell cycle proteins (eg v*myc*) and by mitogen stimulation (eg EGF and/or bFGF).[20–26] Some of these methods may operate through common cellular mechanisms. This speculation is supported by the observation that many progenitor cell lines behave similarly in their ability to reintegrate into the CNS despite the fact that they were generated by different methods, obtained from various locations, and reimplanted into various CNS regions. Some of these NSC lines appear sufficiently plastic to participate in normal CNS development from germinal zones of multiple regions along the neuraxis and at multiple stages of development from embryo to

old age.[5,6,9,21,27–30] They appear, as well, to model the *in vitro* and *in vivo* behavior of some primary fetal and adult neural cells,[31–36] suggesting that insights gleaned from these NSC lines may legitimately reflect the potential of CNS progenitor or stem cells.

Some of the inherent biologic properties of NSCs may circumvent some of the limitations of other techniques for treating metabolic, degenerative, or other widespread lesions in the brain. They are easy to administer (often directly into the cerebral ventricles), are readily engraftable, and circumvent the BBB. A preconditioning regime is not required before administration (eg total body irradiation) as is required for BMT. One important property of NSCs is their apparent ability to develop into integral cytoarchitectural components[37]of many regions throughout the host brain as neurons, astrocytes, oligodendrocytes, and even incompletely differentiated but quiescent progenitors. Therefore, they may be able to replace a range of missing or dysfunctional neural cell types. A given NSC clone can give rise to multiple cell types within the same region. This is important in the likely situation where return of function may require the reconstitution of the whole milieu of a given region – eg not just the neurons but also the glia and support cells required to nurture, detoxify and/or myelinate the neurons. They appear to respond *in vivo* to neurogenic signals not only when they occur appropriately during development, but even when induced at later stages by certain neurodegenerative processes, eg during apoptosis.[21,38] NSCs may be attracted to regions of neurodegeneration in the young as well as in the aged.[39–41]

NSCs also appear to accommodate to the region of engraftment, perhaps obviating the necessity for obtaining donor cells from many specific CNS regions or the imperative for precise targeting during reimplantation. The cells might express certain genes of interest intrinsically (for example, many neurotrophic factors), or they can be engineered *ex vivo* to do so since they are readily transduced by gene transfer vectors. These gene products can be delivered to the host CNS in a direct, immediate, and stable manner.[5,8,40,42]While NSCs can migrate and integrate widely throughout the brain particularly well when implanted into germinal zones, allowing reconstitution of enzyme or cellular deficiencies in a global manner,[5,8,42] this extensive migratory ability is present even in the parenchyma of the diseased adult[5,40] and aged[43] brain. Despite their extensive plasticity, NSCs never give rise to cell types inappropriate to the brain (eg muscle, bone, teeth) or yield neoplasms.

Testing the therapeutic potential of NSCs

These attributes of NSCs may provide multiple strategies for treating a range of CNS dysfunction. As proof-of-principle, they were first tested experimentally in mouse models of genetically based neurodegeneration. Their ability to mediate gene therapy was affirmed in a model of the neurogenetic lysosomal storage disease (LSD), mucopolysaccharidosis type VII (MPS VII).[5] Mice homozygous for a frameshift mutation in the β-glucuronidase gene are devoid of the secreted enzyme β-glucuronidase (GUSB). The enzymatic deficiency results in lysosomal accumulation of undegraded glycosaminoglycans in the brain and other tissues, causing a fatal progressive degenerative disorder. Treatments for MPS VII and most other LSDs are designed to provide a source of normal enzyme for uptake by diseased cells, a process termed 'cross-correction'.[44] The goal of *ex vivo* gene therapy is to engineer donor cells to express the normal GUSB protein for export to other host cells. The engraftment and integration of GUSB overexpressing NSCs throughout the newborn MPS VII mutant brain succeeded in providing a sustained, lifelong, widespread source of cross-correcting enzyme in a manner not previously achieved.[5]

A rapid intraventricular injection technique was devised for the diffuse engraftment of the NSCs. Injecting the progenitors into the cerebral ventricles presumably allowed them to gain access to most of the subventricular germinal zone (SVZ), as well as to networks of cerebral vasculature, along the surface of which they would also migrate. This approach worked equally well in the fetus where donor NSCs gained access to the ventricular germinal zone (VZ),[42] migrating into the parenchyma within 24–48 h. This engraftment technique, exploiting many of the inherent properties of NSCs, permitted missing gene products to be delivered without disturbing other neurobiological processes and suggested a strategy for gene therapy of a class of neurogenetic diseases that, heretofore, had not been adequately treated (Figure 1a). While MPS VII may be regarded as 'uncommon', the broad category of diseases that it models (neurogenetic conditions) afflicts as many as 1 in 1500 children and serves as a model for many adult neurodegenerative processes of genetic origin (AD, for example, could broadly fall into this category). Therapy instituted early in life might arrest disease progression and prevent irreversible CNS alterations. Even in the adult brain, there are routes of relatively extensive migration followed by both endogenous and transplanted NSCs.[45,46] If injected into the cerebral ventricles of normal adult mice, NSCs (including those expressing transgenes) will integrate into the SVZ and migrate long distances, eg to the olfactory bulb, where they differentiate into interneurons, and occasionally into subcortical parenchyma where they become glia.[6,27,28,47,48] Although these migratory paths are still relatively restricted and stereotyped compared with that seen in the fetal or newborn brain, in the degenerating, abnormal, or injured adult brain (as discussed below), migration by foreign gene-expressing NSCs can be extensive and directed specifically to regions of pathology, a phenomenon observed to date with stroke, head injury, dopaminergic dysfunction, brain tumors and amyloid plaques.

The therapeutic paradigm described above can be extended to other untreatable neurodegenerative diseases characterized by an absence of gene products and/or the accumulation of toxic metabolites. In almost all cases, NSCs, because they are normal cells, constitutively express normal amounts of the particular enzyme in question. The extent to which this amount needs to be augmented may vary from model to model and enzyme to enzyme. Reassuringly, in most inherited metabolic disease, the amount of enzyme required to restore normal metabolism and forestall CNS disease may be quite small. It is significant to note that, while the histograms in Figure 1b illustrate the widespread distribution of a lysosomal enzyme, they could similarly reflect the NSC-mediated distribution of other diffusible (eg synthetic enzymes, neurotrophins, viral vectors)[49,50] and non-diffusible (eg myelin, extracellular matrix) factors, as well as

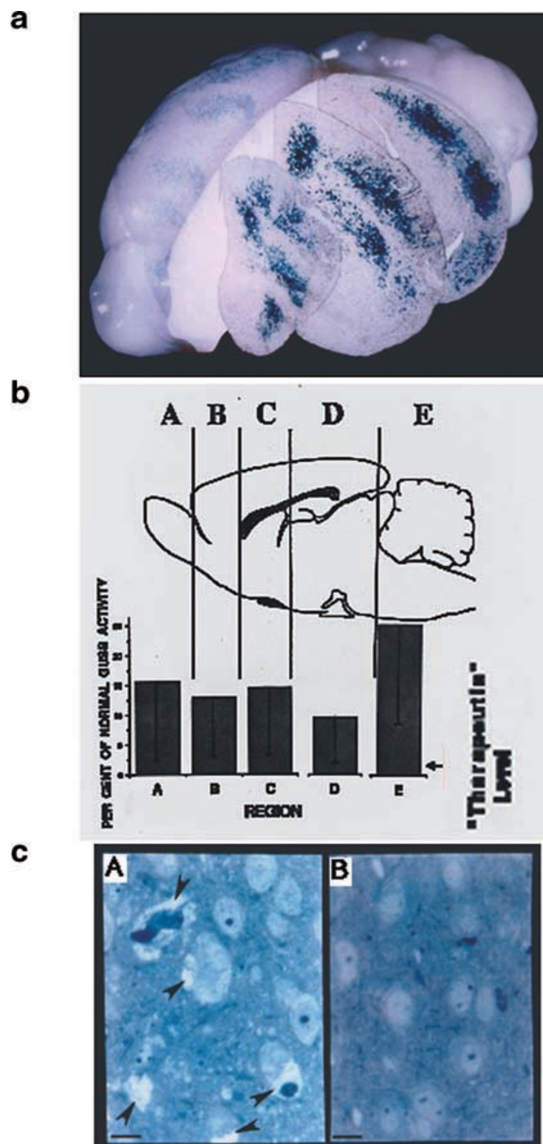


Figure 1 Widespread engraftment of NSCs expressing GUSB throughout the brain of the MPS VII mouse (adapted from Ref. 5). (a) Brain of a mature MPS VII mouse after receiving a neonatal intraventricular transplant of murine NSCs expressing GUSB. Donor NSC-derived cells, identified by their X-gal histochemical reaction (blue precipitate) for expression of the LacZ marker gene, have engrafted throughout the recipient mutant brain. Representative coronal sections – placed at their appropriate level by computer – show these cells to span the rostral-caudal expanse of the brain. (b) Distribution of GUSB enzymatic activity throughout brains of MPS VII NSC transplant recipients. Serial sections were collected from throughout the brains of transplant recipients and assayed for GUSB activity. Sections were pooled to reflect the activity present within the regions demarcated in the schematic. The regions were defined by anatomical landmarks in the anterior-to-posterior plane to permit comparison among animals. The mean levels of GUSB activity for each region ($n = 17$) are presented as the percentage of average normal levels for each region. Untreated MPS VII mice show no GUSB activity biochemically or histochemically. Enzyme activity of 2% of normal is corrective based on data from liver and spleen. (c) Decreased lysosomal storage in a treated MPS VII mouse brain at 8 months of age. (A) Extensive vacuolation representing distended lysosomes (arrowheads) in both neurons and glia in the neocortex of an 8-month-old, untransplanted control MPS VII mouse. (B) Decrease in lysosomal storage in the cortex of an MPS VII mouse treated at birth from a region analogous to the untreated control section in (A). The other regions of this animal's brain showed a similar decrease in storage compared with untreated, age-matched mutants in regions where GUSB was expressed. Scale bars, 21 μm.

the distribution of 'replacement' neural cells (see section below). For example, neural progenitors and stem cells have been used for the local expression of NT-3 within the rat spinal cord,[51,52] NGF and BDNF within the septum,[53,54] and tyrosine hydroxylase[55,56] and Bcl-2[57] and GDNF[58] to the striatum. These earlier studies helped to advance the idea that NSCs – as a prototype for stem cells from any solid organ – might aid in reconstructing both the molecules as well as the cells of a maldeveloped or damaged organ. A further complexity, however, is the recognition that the same NSC may not be able to be engineered to express certain neurotrophic agents simultaneously because they may be processed antagonistically within the cell and/or within the environment. Therefore a greater knowledge of the NSC processing of certain molecules is a prerequisite.[18]

Replacing more than genes: cells, myelin, extracellular matrix

Many neurologic diseases, even those that can be ameliorated by replacement of a gene product, are characterized by the degeneration of cells or circuits. These cytoarchitectural components may need to be replaced in a functional manner and be resistant to residual toxic processes. One reassuring insight from the classic fetal transplant literature is that even modest anatomical reconstruction may sometimes have an unexpectedly beneficial functional effect.[18] Early experiments with NSC clones in various rodent mutants and injury models have provided evidence that NSCs may be able to replace some degenerated or dysfunctional neural cells. In the meander tail (*mea*) mutant mouse, which is characterized by a deficiency of cerebellar granule cell (GC) neurons, NSCs, implanted at birth, were capable of 'repopulating' large portions of the GC-poor internal granular layer (IGL) with neurons[59] (a phenomenon that was subsequently duplicated with human NSCs.[6] A pivotal observation to emerge from this work, with implications for fundamental stem cell biology, was that cells with the potential for multiple fates will 'shift' their differentiation to compensate for a deficiency in a particular cell type. As compared with their fate in normal cerebella, a majority of these donor NSCs in GC-deficient regions pursued a GC phenotype in preference to other potential phenotypes, suggesting a 'push' on undifferentiated, multipotent NSCs towards repletion of the 'unmet' quota. This work presented a possible developmental mechanism with therapeutic value. Indeed, in the typically 'non-neurogenic' milieu of the adult neocortex, it was learned that NSCs would 'choose' to differentiate into neurons under conditions where host neurons were experimentally eliminated by a targeted apoptotic process.[21]The neuronal phenotype assumed by these NSCs suggested that this circumscribed region of degeneration created a microenvironment permissive or instructive for a neuronal fate choice, likely through reactivation of signals ordinarily available only during embryonic corticogenesis.

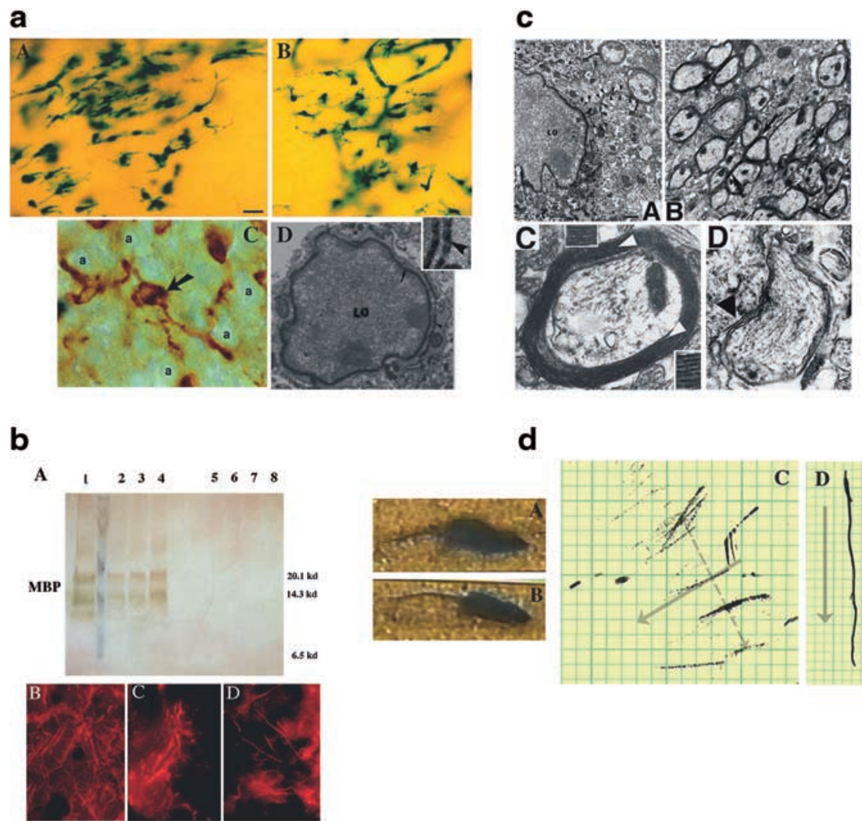
Preliminary work in another mutant, the reeler (*rl*) mouse, has suggested that NSCs may not only replace developmentally impaired or degenerating cells, but may

also help correct certain aspects of abnormal cytoarchitecture, particularly those characterized by deficiencies in extracellular matrix (ECM). The laminar assignment of neurons in *rl* brain is abnormal due to a mutation in a gene encoding the secreted ECM molecule, Reelin. NSCs, implanted at birth into the defective developing *rl* CB, appeared not only to replace missing GCs, but also to promote a more wild-type laminated appearance in engrafted regions by 'rescuing' aspects of the abnormal migration, positioning, and survival of host neurons most likely by providing molecules (including Reelin) at the cell surface to guide proper histogenesis. These findings suggested a possible NSC-based strategy for the gene therapy of CNS diseases characterized by abnormal cellular migration, lamination and cytoarchitectural arrangement.

White matter disease as a model system

It was hypothesized that the techniques described for the widespread engraftment of NSCs for gene replacement could be extended to the remediation of other types of diffuse neuropathologies requiring other kinds of interventions. Mutants characterized by CNS-wide white matter disease provided ideal models for testing whether NSCs might be useful for widespread cell replacement.[8] The oligodendroglia of the dysmyelinated shiverer *(shi)* mouse are dysfunctional because they lack myelin basic protein (MBP) which is essential for proper myelination. Therapeutic intervention, therefore, requires widespread replacement with oligodendrocytes expressing MBP. NSCs transplanted at birth – employing the same intracerebroventricular implantation technique devised for diffuse engraftment of enzyme-expressing NSCs to treat global metabolic lesions – resulted in widespread engraftment throughout the *shi* brain (Figure 2a) with repletion of significant amounts of MBP (Figure 2b). Accordingly, of the many donor-derived oligodendroglia (NSCs indeed 'shifted' to yield a higher proportion of such cells), a subgroup myelinated up to 52% of host neuronal processes with better compacted myelin (Figure 2c). Some animals experienced a decrease in their symptomatic tremor (Figure 2d).[8] Therefore, 'global' cell replacement seems feasible for some pathologies if cells with stem-like features are employed. More specifically, the ability of NSCs to generate myelinating cells is important because disordered myelination plays a critical role in many genetic and acquired (injury, infectious) neurodegenerative processes. Oligodendroglial pathology is prominent in stroke, spinal cord injury, head trauma, ischemia and may account for a significant proportion of the neurologic handicap seen in asphyxiated and premature newborns. More broadly, complementation studies in mutants such as those described above, help support an NSC-based approach – whether with exogenous cells or with mobilized endogenous ones – for compensating for neurodevelopmental problems of many etiologies.

Accordingly, the approach employed in *shi* has been extended to other demyelinated and oligodendrocyte-deficient mutants. Loss of galactocerebrosidase-α (GALC) activity results in the accumulation in oligodendrocytes of psychosine, a toxic glycolipid. The resultant disease in humans is Krabbe's or globoid cell leukodystrophy (GLD). Children afflicted with GLD exhibit inexorable psychomotor deterioration and early death, presumably as a result of dysfunctional and ultimately degenerated oligodendrocytes with loss of myelin. The twitcher (*twi*) mouse is an authentic model of GLD. In preliminary studies, murine NSCs have been transplanted into the brains of both neonatal and symptomatic juvenile *twi* mice. The NSCs engrafted exuberantly throughout the brains, particularly in newborns (as per Figures 1 and 2), and differentiated extensively into healthy oligodendrocytes that elaborated normal-appearing myelin. Each NSC-derived oligodendrocyte appeared to remyelinate up to 30–50 host axons. Interestingly, unlike in *shi*, cell replacement even to this extent and magnitude was, nevertheless, unable to remediate symptoms or prolong the life of the *twi* mouse. This observation became instructive, however, as to how one might need to treat some complex global neurogenetic diseases. Although, engraftment of these unengineered NSCs did not appear to result in symptomatic improvement, the fact remained that exogenous NSCs could survive and differentiate into myelinating oligodendrocytes within the *twi* environment. There is a prevailing belief that GLD pathophysiology is related to the toxic psychosine-filled environment, one that kills not only host cells, but theoretically any new cells placed into that milieu, hence dooming any attempts at cell replacement. The *twi* brain, however, was not inherently non-permissive. One could hypothesize that the absence of GalC not only permits the toxic build-up of psychosine, but predisposes Krabbe neural cells (especially oligodendrocytes) to be more vulnerable to that toxicity in a way that wild-type NSCs may not. Pilot experiments were, therefore, performed in which the NSCs that had differentiated into oligodendrocytes in the *twi* brain, were exposed in culture to increasing concentrations of psychosine, particularly concentrations that were inimical to Krabbe cells. The NSCs proved to be resistant to those toxic effects. As the concentrations were further increased, the NSCs did begin to show some toxicity. However, interestingly, when populations of those same NSCs were engineered to overexpress GalC (enough to cross-correct fibroblasts from Krabbe patients in culture) they were now no longer affected by that and even higher psychosine concentrations *in vitro*. Although preliminary, these results suggest a number of important points regarding the use of cellular therapies against complex degenerative diseases, such as GLD. A component of treatment will likely not only be cell replacement, but also cross-correction of host cells to overexpress GalC and hence be more resistant. While oligodendrocyte replacement alone is not a sufficient treatment for GLD (even when extensive), the replacement of both cells and molecules – eg with NSCs that can both become oligodendrocytes and be pumps for GalC remains a promising basis for a multidisciplinary strategy. Additionally, it is not unexpected that the present experimental design did not result in prolonged lives in animals with system-wide disease (ie cerebellar, spinal and peripheral, as well as intracerebral; extracranial as well as intracranial). Interventions may call for implantation of NSCs at multiple locations, as well as at multiple time points in the evolution of the disease, beginning, if possible, presymptomatically and continuing after disease is established. Interventions will likely need to combine NSC implantation in the brain with strategies to address extracranial manifestations (eg BMT) that together may prolong life. Most neurological disease is complex in this way and will



likely require multifaceted approaches, perhaps with NSCs serving as the 'glue'. Importantly, we learn, however, that NSCs may be useful even in cell non-autonomous diseases if the NSCs are intrinsically resistant to or neutralizing of the toxic milieu – or can be engineered to be so.

'Homing in' on pathology from even long distances

It is tempting to speculate that the ability of NSCs to pursue alternative differentiation paths in response to certain types of neurodegeneration[21,59] and their ability to migrate and express foreign genes can be targeted selectively to the regions most in need. Evidence (some published, some preliminary) in various models suggests that this may be feasible: during phases of active neurodegeneration, as yet unidentified factors seem to be transiently elaborated to which NSCs may 'home'.

Hypoxic-ischemic brain injury

In pilot studies, when NSCs are transplanted into brains of young mice subjected to unilateral hypoxic-ischemic (HI) brain injury (a model for cerebral palsy), donor-derived cells migrated preferentially to and integrated extensively within the large ischemic areas that typically spanned the injured hemisphere. A subpopulation of donor NSCs, particularly in the penumbra of the infarct, 'shifted' their differentiation fate towards neurons and oligodendrocytes, the neural cell types typically damaged following asphyxia/stroke. Furthermore, there appeared to be an optimal window of time following injury (3–7 days) during which signals were elaborated within the degenerating region and to which NSCs responded with migration and reconstitution of lost neural cells. (Similar observations have been noted by Hodges *et al*[60]).

Because engrafted donor-derived cells continue to express their *LacZ* reporter gene, it appeared feasible that desired differentiation, neurite outgrowth, and connectivity of both host and donor-derived cells might be enhanced if donor NSCs were genetically manipulated *ex vivo* to express certain trophins, cytokines, or other factors. When, in pilot studies, a subclone of the same murine NSCs were engineered via retroviral transduction to overexpress neurotrophin-3 (NT-3) (known to play a role in inducing neuronal differentiation) and then implanted into asphyxiated mouse brains, the percentage of donor-derived neurons increased from 5% (in the above described experiments) to 20% in the infarction cavity and up to >80% in the penumbra. It seemed likely that the NSCs (which bear a functional trkC receptor) were producing a factor that worked in an autocrine/paracrine fashion. While it remains far from clear that one would even desire so many neurons, this observation suggests that a naturally occurring NSC-based process in a degenerative environment can be augmented via genetic engineering. Furthermore, migratory NSCs may be capable of simultaneous gene therapy and cell replacement during the same transplantation procedure in the same recipient.

Brain tumors

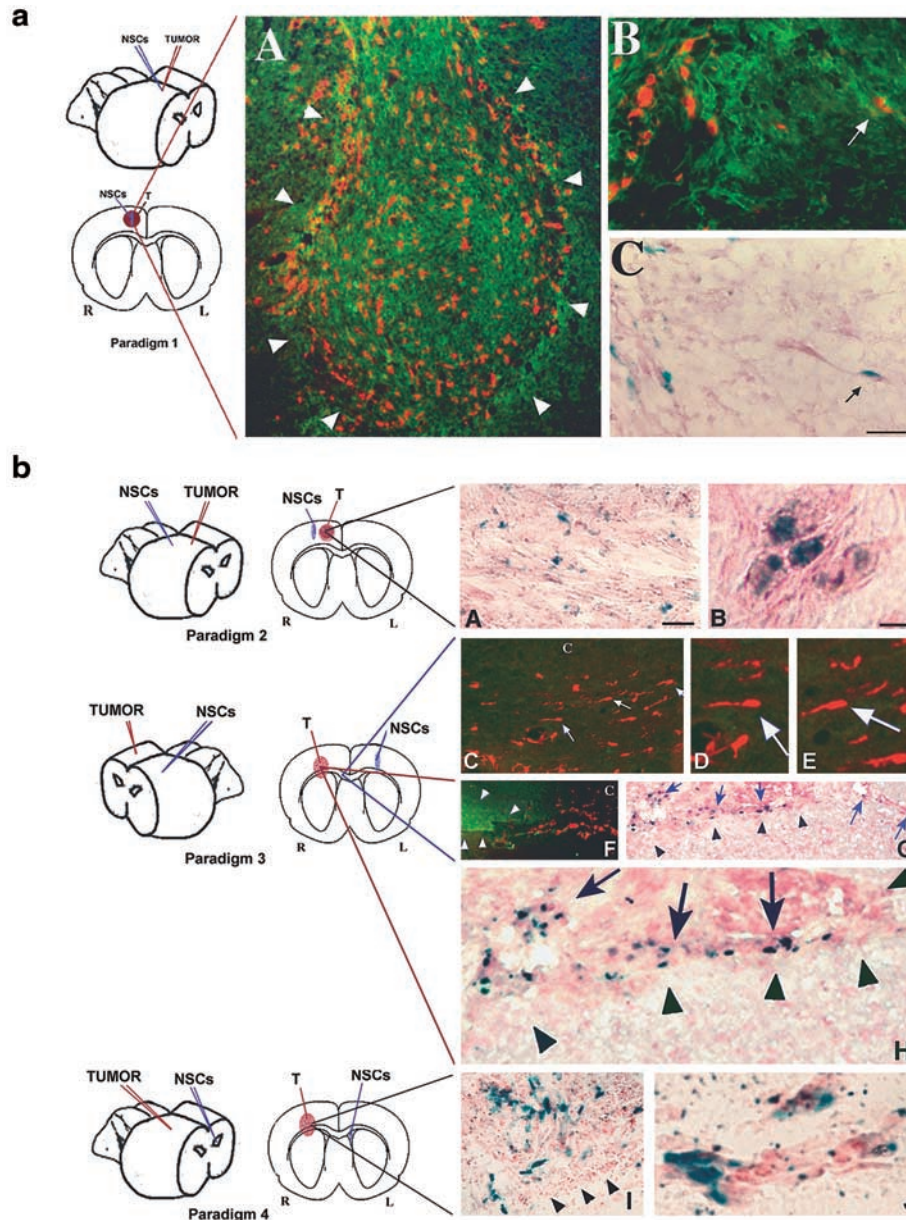
Another dramatic example of using NSCs as gene delivery vehicles that home in on pathology is illustrated by

Figure 2 'Global' cell replacement is feasible via NSC transplantation: evidence from the dysmyelinated shiverer (shi) mouse brain (modified from Ref. 8). (a) NSCs engraft extensively throughout the shi dysmyelinated brain, including within white tracts, and differentiate into oligodendrocytes. LacZ-expressing, β-galactosidase (βgal)-producing NSCs were transplanted into the cerebral ventricles of newborn shi mutants and analyzed systematically at intervals between 2and 8 weeks following engraftment. Coronal sections through the shi brain at adulthood demonstrated widely disseminated integration of blue Xgal+ donor-derived cells throughout the neuraxis, similar to the pattern seen in Figure 1a in the MPS VII mutant mouse. Donor-derived cells in the shi mouse brain are shown at higher magnification and greater detail in (A–D). (A, B) Donor-derived Xgal+ cells in representative sections through the corpus callosum possessed characteristic oligodendroglial features (small, round or polygonal cell bodies with multiple fine processes oriented in the direction of the neural fiber tracts). (C) Close-up of a representative donor-derived anti-βgal immunoreactive oligodendrocyte (arrow) extending multiple processes towards and beginning to enwrap large adjacent axonal bundles ('a') viewed on end in a section through the corpus callosum. That cells such as those in panels A–C (and in b(B–D) were oligodendroglia was confirmed by the representative electron micrograph in panel D (and in c) demonstrating their defining ultrastructural features (see Ref. 8). A donor-derived Xgal+ oligodendrocyte ('LO') can be distinguished by the electron dense Xgal precipitate that is typically localized to the nuclear membrane (arrow), ER (arrowhead) and other cytoplasmic organelles. The area indicated by the arrowhead is magnified in the inset to demonstrate the unique crystalline nature of individual precipitate particles. (b) MBP expression in mature transplanted and control brains. (A) Western analysis for MBP in whole brain lysates. The brains of three representative transplanted shi mutants (lanes 2–4) expressed MBP at levels close to that of an age-matched unaffected mouse (lane 1, positive control), and significantly greater than the amounts seen in untransplanted (lanes 7, 8, negative control) or unengrafted (lanes 5, 6, negative control) age-matched shi mutants. (Identical total protein amounts were loaded in each lane.) (B–D) Immunocytochemical analysis for MBP. (B) The brain of a mature unaffected mouse was immunoreactive to an antibody to MBP (revealed with a Texas Red-conjugated secondary antibody). (C, D) Age-matched engrafted brains from shi mice similarly showed immunoreactivity. Because untransplanted shi brains lack MBP. MBP immunoreactivity has also classically been a marker for normal donor-derived oligodendrocytes in transplant paradigms. (c) NSC-derived 'replacement' oligodendrocytes are capable of myelination of shi axons. In regions of MBP-expressing NSC engraftment, shi neuronal processes became enwrapped by thick, better compacted myelin. (A) At 2 weeks post-transplant, a representative donor-derived, labeled oligodendrocyte ('LO') (recognized by extensive Xgal precipitate ('p') in the nuclear membrane, cytoplasmic organelles, and processes) was extending processes (a representative one is delineated by arrowheads) to host neurites, and was beginning to ensheathe them with myelin ('m'). (B) If engrafted shi regions, such as that in (A), were followed over time (eg to 4 weeks of age as pictured here), the myelin began to appear healthier, thicker and better compacted (examples indicated by arrows) than that in age-matched untransplanted control mutants. (C) By 6 weeks post-transplant, these matured into even thicker wraps; ~40% of host axons were ensheathed by myelin (a higher power view of a representative axon is illustrated in panel C) that was dramatically thicker and better compacted than that of shi myelin (an example of which is shown in panel D (black arrowhead) from an unengrafted region of an otherwise successfully engrafted shi brain). In panel C, white arrowheads indicate representative regions of myelin that are magnified in the adjacent insets; MDLs are evident. (d) Functional and behavioral assessment of transplanted shi mutants and controls. The shi mutation is characterized by the onset of tremor and a 'shivering gait' by the 2nd–3rd postnatal week. The degree of motor dysfunction in animals was gauged in two ways: (1) by blindly scoring periods of standardized videotaped cage behavior of experimental and control animals; and (2) by measuring the amplitude of tail displacement from the body's rostral-caudal axis (an objective, quantifiable index of tremor). Video freeze-frames of representative unengrafted and successfully engrafted shi mice are seen in panels A and B, respectively. The whole body tremor and ataxic movement observed in the unengrafted symptomatic animal (A) causes the frame to blur, a contrast with the well-focused frame of the asymptomatic transplanted shi mouse (B). Sixty percent of transplanted mutants showed nearly normal-appearing behavior as in panel B and attained scores that were not significantly different from normal controls (see Ref. 8 for details). Panels C and D depict the manner in which whole body tremor was mirrored by the amplitude of tail displacement (hatched gray arrow in panel C) measured perpendicularly from a line drawn in the direction of the animal's movement (solid gray arrow, represents the body's long axis). Measurements were made by permitting a mouse, whose tail had been dipped in India ink, to move freely in a straight line on a sheet of graph paper as shown. Large degrees of tremor cause the tail to make widely divergent ink marks away from the midline, representing the body's axis (C). Absence of tremor allows the tail to make long, straight, uninterrupted ink lines on the paper congruent with the body's axis (D). The distance between points of maximal tail displacement from the axis was measured and averaged for transplanted and untransplanted shi mutants and for unaffected controls (hatched gray arrow). Panel C shows data from a poorly engrafted mutant that did not improve with respect to tremor, while panel D reveals lack of tail displacement in a successfully engrafted asymptomatic mutant. Overall, 64% of transplanted shi mice examined displayed at least a 50% decrement in the degree of tremor or 'shiver'. Several showed 0 displacement (see Ref. 8 for details).

their use against brain tumors. One of the impediments to the treatment of such tumors as gliomas has been the degree to which they expand, infiltrate surrounding tissue, and migrate widely into normal brain, usually rendering them 'elusive' to effective resection, irradiation, chemotherapy, or gene therapy. Aboody *et al*[40] (Figure 3) demonstrated that migratory murine and human NSCs, when implanted into intracranial gliomas in adult rodents, distributed quickly and extensively throughout the tumor bed (Figure 3aA) and migrated uniquely in juxtaposition to aggressively advancing tumor cells, while continuing to stably express a foreign gene (Figure 3aB,C). The NSCs 'surrounded' the invading tumor border while 'chasing down' migrating infiltrating tumor cells. Furthermore, when implanted intracranially at distant sites from the tumor bed in adult brain (eg into normal tissue (Figure 3bA,B), into the contralateral hemisphere (Figure 3bC–H), or into the cerebral ventricles (Figure 3bI,J), the donor NSCs crossed from one hemisphere to the other, migrating through normal tissue to the tumor on the opposite side, as if 'drawn' to and targeting tumor cells. The NSCs appeared to be attracted by either factors elaborated by tumors or by the tissue destruction they rendered. NSCs could deliver a therapeutically relevant molecule – the oncolysis-promoting enzyme cytosine deaminase – such that *in vitro* (Figure 4a) and *in vivo* (Figure 4b) a dramatic reduction in tumor cell burden resulted. These data suggested the adjunctive use of inherently migratory NSCs as a delivery vehicle for more effectively targeting a wide variety of therapeutic genes and vectors to refractory, migratory, invasive brain tumor cells. More broadly, they suggested that NSC migration could be extensive, even in the adult brain and along non-stereotypical routes, if pathology (as modeled here by tumor) is present.

Amyloid plaques

More recently this notion has been extended in preliminary studies by Tate *et al* in our group,[61] to address lesions present in an adult animal model of AD-like pathology. The widespread amyloid deposits characteristic of AD make it difficult to address with conventional grafting approaches (for the potential replacement of dead or dying neurons) or with traditional gene delivery vectors (for molecular therapy). While the signals that stimulate migration of NSCs are not yet identified, inflammatory molecules are among the likely candidates. Tate pre-



viously demonstrated that chronically infused human amyloid will cause an inflammatory response in the rat brain. In preliminary studies, Tate *et al*[61] observed that murine NSCs placed in the opposite lateral ventricle will migrate to and surround areas of amyloid infusion. Pilot studies in transgenic mouse models of AD (harboring a similarly inflamed brain) suggest a similar tropism of foreign gene-expressing NSCs for amyloid depositions. NSCs may, therefore, play a role in the delivery of therapeutic molecules in adult global degenerative diseases like AD. (It is not yet known whether they can similarly replace neural cells that die in such diseases.)

Reciprocal NSC–host interactions and augmenting this dynamic

While most work to date in stem cell biology has focused on the concept that the host CNS environment – as it changes over the course of development and aging or as it is altered by injury or degeneration – influences or instructs the stem cell, Ourednik *et al*[41]have accumulated preliminary data suggesting that communication also occurs in a reciprocal fashion: the NSC inherently expresses genes that signal and instruct the host CNS. This speculation derives in part from an interesting observation following the implantation of murine NSCs unilaterally into the SN of aged mice that, 1 month previously, had received repetitive systemic administrations of high-dose MPTP, a neurotoxin that produces a persistent impairment (though not death) of mesencephalic dopaminergic (DA) neurons and their striatal projections (similar to a dysfunction seen in the aging brain).[41] Unilaterally implanted NSCs not only migrated and integrated extensively within both hemispheres but were associated with dramatic reconstitution of tyrosine hydroxylase (TH) and dopamine transporter expression

Figure 3 NSCs display extensive tropism for pathology in the adult brain: evidence from intracranial gliomas (modified from Ref. 32) (a) NSCs migrate extensively throughout a brain tumor mass in vivo and 'trail' advancing tumor cells. Paradigm 1, in which NSCs are implanted directly into an established experimental intracranial glioblastoma, is illustrated schematically. (A) The virulent and aggressively invasive CNS-1 glioblastoma cell line, used to create the tumor, has been labeled ex vivo by transduction (via a retroviral vector) with green fluorescent protein (GFP) cDNA, allowing those cells to fluoresce green (enhanced when revealed by an anti-GFP antibody). The NSCs stably express LacZ and produce β-galactosidase (βgal), allowing them to be visualized as red (by anti-βgal immunocytochemistry under fluorescence microscopy) or as blue (by Xgal histochemistry under light microscopy). This panel, processed for double immunofluorescence using an anti-ßgal antibody (NSCs, red) and an anti-GFP antibody (glioblastoma cells, green), shows a section of brain (under low power) from an adult nude mouse killed 10 days after NSC injection into the CNS-1 glioblastoma; arrowheads demarcate approximate edges of the tumor mass where it interfaces with normal tissue. Donor red βgal+ NSCs can be seen extensively distributed throughout the mass, interspersed among the green tumor cells. This degree of interspersion by NSCs occurs within 48 h following injection. Interestingly, while NSCs have extensively migrated and distributed themselves within the mass, they largely stop at the junction between tumor and normal tissue except where a tumor cell is infiltrating normal tissue; then NSCs appear to 'follow' the invading tumor cell into surrounding tissue. This 'trailing' of individual glioblastoma cells migrating away from the main tumor bed is examined in greater detail in panels B and C. (B) High power view, under fluorescence microscopy, of single migrating infiltrating GFP+ tumor cells (green) in apposition to βgal+ NSCs (red) (white arrow). In a similar and perhaps even more impressive demonstration, the section in panel C is co-stained with Xgal (allowing the LacZ-expressing NSCs to stain blue, arrow) and with neutral red (allowing the elongated glioblastoma cells to stain dark red. The blue NSC is in direct juxtaposition to a single migrating, invading neutral red+, spindle-shaped tumor cell (arrow), the NSC 'riding' the glioma cell in 'piggy-back' fashion. Scale bars: [C] 60 μm. (b) NSCs implanted at various intracranial sites distant from main tumor bed migrate through normal adult tissue towards glioblastoma cells. (A, B) Same hemisphere (paradigm 2): Shown here is a section through the tumor from an adult nude mouse 6 days following NSC implantation caudal to tumor. In panel A (as per the schematic) a coned down view of a tumor populated as pictured under low power in a(A), note Xgal+ blue NSCs interspersed among dark neutral red+ tumor cells. (B) High power view of NSCs in juxtaposition to islands of tumor cells. (C–H) Contralateral hemisphere (paradigm 3): (C–E) As indicated on the schematic, these panels are views through the corpus callosum ('c') where ßgal+ immunopositive NSCs (red cells, arrows) are seen migrating from their site of implantation on one side of the brain towards tumor on the other. Two representative NSCs indicated by arrows in panel C are viewed at higher magnification in panels D and E, respectively, to visualize the classic elongated morphology and leading process of a migrating neural progenitor oriented towards its target. In panel F, ßgal+ NSCs (red) are 'homing in' on the GFP+ tumor (green) having migrated from the other hemisphere. In panel G, and magnified further in panel H, the Xgal+ blue NSCs (arrows) have now actually entered the neutral red+ tumor (arrowheads) from the opposite hemisphere. (I, J) Intraventricular (paradigm 4): Shown here is a section through the brain tumor of an adult nude mouse 6 days following NSC injection into the contralateral cerebral ventricle. In panel I, as per the schematic, blue Xgal+ NSCs are distributed within the neutral red+ main tumor bed (edge delineated by arrowheads). At higher power in panel J, the NSCs are in juxtaposition to migrating islands of red glioblastoma cells. Fibroblast control cells never migrated from their injection site in any paradigm. All Xgal-positivity was corroborated by anti-βgal immunoreactivity. Scale bar: (A): 20 μm, and applies to panel C; (B): 8 μm, and is 14 μm in panels D and E, 30 μm in panels F and G, 15 μm in panel H, 20 μm in panel I, and 15 μm in panel J.

bilaterally throughout the aged mesostriatal system. While there was spontaneous conversion of NSCs to TH+ cells in DA-depleted areas, and while TH+ cells of donor-origin contributed to nigral reconstitution, the majority (~80–90%) of TH+ cells in the 'reconstituted SN' were actually host cells 'rescued' presumably by constitutively produced NSC-derived factors.

While the mechanism underlying this inherent NSC-mediated protection or activation of a host regenerative capacity remains uncertain, one mode is likely to be the production by NSCs of trophic and tropic agents. NSCs constitutively produce a broad range of peptide neurotrophic factors (including NT-3, NT-4/5, NGF, BDNF, GDNF), adhesion molecules (eg L1), ECMs (eg reelin), and lysosomal enzymes. In the example above, it was interesting to note that, intermixed among NSCs that had differentiated into TH+ neurons, was a larger subpopulation of clonally related undifferentiated or glial-differentiated donor-derived cells that intrinsically expressed GDNF (among other peptides), a molecule known to be neuroprotective of DA neurons. The broader implications for CNS repair are that host structures may benefit not only from NSC-derived replacement of missing neurons but also from the 'chaperone' effect of undifferentiated/glial-differentiated NSCs equally necessary for promoting optimal reconstitution. While NSCs have been touted for cell and gene therapy, these findings suggest a third mechanism by which therapeutic outcomes might be achieved: an inherent capacity of NSCs to create host environments sufficiently rich in trophic and/or neuroprotective support to promote the recovery of damaged endogenous cells. If insufficient on their own, these effects can likely be augmented.

Human NSCs

The identification of human NSCs (hNSCs)[6,48,62–65] seemed to vouchsafe the conservation of certain neurodevelopmental principles – initially gleaned from rodents – to the human CNS. Stem cell-based strategies, therefore, seemed a reasonable consideration for human neurodegenerative conditions.

Lines of engraftable hNSCs have been isolated from normal human fetuses (ideally from the ventricular zone) that, in many ways emulate their rodent counterparts (reviewed in Refs 11 and 13) (albeit with a three to four times longer cell cycle). Insights into how therapeutic goals might be achieved with such cells have derived from observations of rodent NSCs, which, by and large, the hNSCs have mimicked. These shared behaviors likely reflect a fundamental biology that cuts across species. For example, hNSCs can participate in CNS development, including migration from germinal zones along migratory streams, to widely disseminated CNS regions.[6,28,48,66] They retain a responsiveness to regional and temporal developmental cues to become multiple cell types in these regions. Genetically manipulatable *ex vivo*, hNSCs in these widely disseminated locations, can express a retrovirally transduced transgene, offering promise for future gene therapy applications. Secretory products from hNSCs can cross-correct genetic metabolic defects.[6] hNSCs can differentiate into neuronal subtypes that are deficient in mouse mutants,[6] suggesting their potential for cell replacement. hNSCs are drawn to pathology (as modeled by brain tumor) from as far as the opposite side of the adult rodent brain.[40] In the contused adult rat spinal cord, hNSCs (in pilot studies) can yield

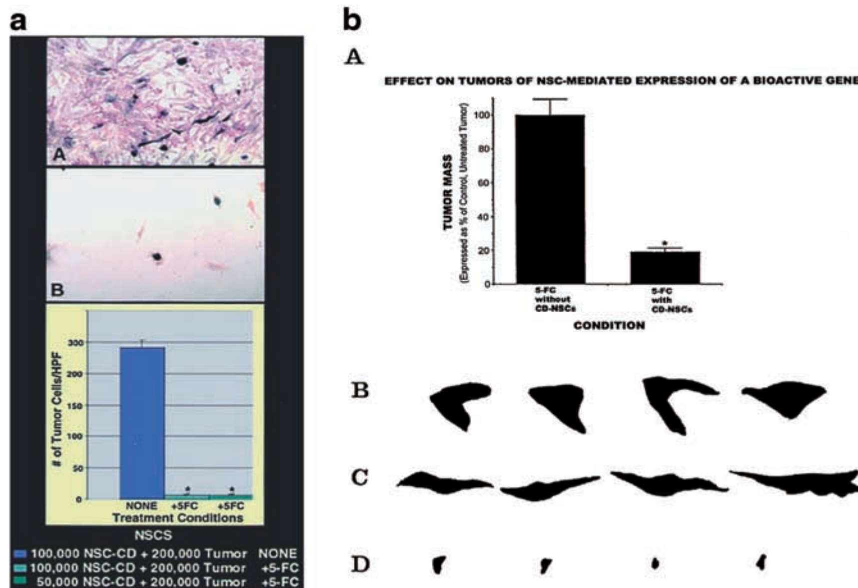


Figure 4 NSCs can express functional genes within a pathological situation (modified from Ref. 40). (a) Bioactive transgene (cytosine deaminase (CD)) remains functional (as assayed by in vitro oncolysis) when expressed within NSCs. CNS-1 glioblastoma cells (red) were co-cultured with CD-transduced murine NSCs (CD-NSCs) (A, B) (blue). Co-cultures unexposed to 5-fluorocytosine (5-FC) grew healthily and confluent (A), whereas plates exposed to 5-FC showed dramatic loss of tumor cells (B) represented quantitatively by the histograms ($* = P < 0.001$).The oncolytic effect was identical whether 1×105 CD-NSCs or half that number were co-cultured with a constant number of tumor cells. (In this paradigm, subconfluent NSCs were still mitotic at the time of 5-FC exposure and thus also subject to self-elimination by the generated 5-FU and its toxic metabolites.) (b) Expression of a bioactive transgene (CD) delivered by NSCs in vivo as assayed by reduction in tumor mass. The size of an intracranial glioblastoma populated with CD-NSCs in an adult nude mouse treated with 5-FC was compared with that of tumor treated with 5-FC, but lacking CD-NSCs. These data, standardized against and expressed as a percentage of a control tumor populated with CD-NSCs receiving no treatment, are presented in the histograms in panel A. These measurements were derived from measuring the surface area of tumors (like those in Figure 3), representative camera lucidas of which are presented in panels B–D. Note the large areas of a control non-5-FC-treated tumor containing CD-NSCs (B) and a control 5-FC-treated tumor lacking CD-NSCs (C) as compared with the dramatically smaller tumor areas of the 5-FC-treated animal who also received CD-NSCs (D) (~80% reduction as per the histogram in panel A, $* = P < 0.001$), suggesting both activity and specificity of the transgene. The lack of effect of 5-FC on tumor mass when no CD-bearing NSCs were within the tumor (C) was identical to the effect of CD-NSCs in the tumor without the gene being employed (B).

neurons (including motor neuron-like cells) that can make long distance connections both rostral (to higher centers) and caudal to the lesion, have the ability to conduct corticospinal signals, and result in apparent functional improvement.

If insights from rodents were applicable to humans, then experiments with monkeys would provide a necessary intermediate step for ensuring translation of those insights. hNSCs transplanted into normal fetal Old World monkeys via intracerebroventricular injections that allowed the cells access to the VZ, integrated throughout the developing brain, yielding neurons and glia appropriate to given cortical laminae, as well as contributing to such secondary germinal zones as the SVZ (that serve homeostatic and self-repair functions into adulthood).[9] It is worth noting that this intervention could be viewed as somewhat of a 'dress rehearsal' for procedures that are actually feasible for *in utero* therapy of human neurogenetic disorders. Such prenatal treatments could be directed not only at congenital disorders but also, theoretically, at neurodegenerative diseases that are not expressed until adulthood or middle age, but whose antenatal genetic diagnosis is possible (eg HD).

Pilot experiments have begun involving hNSCs in lesioned non-human primates – often the primate equivalent of some of the rodent models described above. Such experiments not only help determine the cells' safety and efficacy, but also provide a more direct understanding of 'human-like' development and the response of human stem cells to 'human-like' neurodegenerative environments. They also permit formulation of the logistics of cell administration to an anatomy more relevant to humans. For example, analysis of the fate and impact of hNSCs in the MPTP-induced model of DA depletion and Parkinsonism in Old World St Kitts African Green Monkeys has begun. In encouraging pilot studies, hNSCs appeared to survive in the SN and some spontaneously converted to TH+ cells. Improvement in DA activity in some recipient pilot monkeys (as assessed by SPECT) might either be the result of DA cell replacement by NSCs or reflect the provision by NSCs of factors promoting the survival of host DA neurons (as described above for the mouse), or a function of both. Either mechanism, if corroborated, will likely be therapeutically important.

The field of NSC biology is at a very early stage of development. Many of our suggestions are highly speculative, and much needs to be learned about the properties of such cells. While work is ongoing on the isolation, propagation, and transplantation of hNSCs, many important questions need to be addressed experimentally before using such cells in clinical applications. For instance, what factors optimize the expansion, stability, engraftment, migration and differentiation of transplanted NSCs? What variables dictate the efficiency of foreign gene expression by engrafted NSCs? What are the fundamental pathophysiological needs in a given disease for reversing progression and/or restoring function? When is the proper time to administer cells? What are

the limits of reconstitution in the brain? Do donor-derived cells function normally?

Broader implications

With the recent attention paid to embryonic stem cells (ESCs) and their ability to give rise to neural precursors,[29,30,67] with the discovery of stem cells in other solid organ systems, and with the possibility that some of these tissue-resident stem cells from other organ systems may enter the CNS (possibly giving rise to neural elements), the question arises as to whether these other varieties of stem cells can also address the global demands of neurological diseases or will this still be the unique niche for NSCs. Only future research will determine whether such other stem cells can meet the gold standard of safety, efficiency, simplicity and efficacy established by NSCs. Regardless of how that question is resolved by future experiments, it seems likely that NSCs have unveiled a novel therapeutic paradigm that may now be emulated for other organs. The existence of stem cells in other organs or the ability of ESCs to give rise to such precursors suggests that stem cell-mediated widespread repair of those tissues (eg heart, liver, muscle, pancreas, etc) may similarly be possible in much the same way as NSCs seem to do for the CNS. The demand for organ donation may be forestalled, for example. While approaches to disease have heretofore focused on stopping pathology, stem cells now allow for the complementary approach of 'starting over' – replacing defective components with more normal ones. The possibility of doing autografts with stem cells derived from an adult remains controversial, particularly for genetic diseases. The cells derived from an individual with a genetic impairment likely already harbor that genetic defect or predisposition and may not be useful or effective.

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