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Stem cells: cross-talk and developmental programs

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The thesis advanced in this essay is that stem cells—particularly those in the nervous system—are components in a series of inborn 'programs' that not only ensure normal development, but persist throughout life so as to maintain homeostasis in the face of perturbations—both small and great. These programs encode what has come to be called 'plasticity'. The stem cell is one of the repositories of this plasticity. This review examines the evidence that interaction between the neural stem cell (as a prototypical somatic stem cell) and the developing or injured brain is a dynamic, complex, ongoing reciprocal set of interactions where both entities are constantly in flux. We suggest that this interaction can be viewed almost from a 'systems biology' vantage point. We further advance the notion that clones of exogenous stem cells in transplantation paradigms may not only be viewed for their therapeutic potential, but also as biological tools for 'interrogating' the normal or abnormal central nervous system environment, indicating what salient cues (among the many present) are actually guiding the expression of these 'programs'; in other words, using the stem cell as a 'reporter cell'. Based on this type of analysis, we suggest some of the relevant molecular pathways responsible for this 'cross-talk' which, in turn, lead to proliferation, migration, cell genesis, trophic support, protection, guidance, detoxification, rescue, etc. This type of developmental insight, we propose, is required for the development of therapeutic strategies for neurodegenerative disease and other nervous system afflictions in humans. Understanding the relevant molecular pathways of stem cell repair phenotype should be a priority, in our view, for the entire stem cell field.

Keywords: degeneration; neural stem cells; regeneration; tissue engineering; inflammation; transplantation

1. NSCs: THE PROTOTYPICAL SOMATIC STEM CELL

In thinking about the practical application of stem cell biology to clinical situations—particularly for the CNS—it is instructive to remember that study of the NSC—a prototype for somatic stem cells in general—emerged as the unanticipated by-product of investigations by developmental neurobiologists into fundamental aspects of neural determination, commitment and plasticity. Stem cell behaviour is ultimately an expression of developmental principles, an alluring vestige from the more plastic and generative stages of organogenesis. In attempting to apply stem cell biology therapeutically, it is instructive always to bear in mind what role the stem cell plays in development and to what cues it was 'designed' to respond in trying to understand the 'logic' behind its behaviour (both what investigators or clinicians want to see and what they do not want to see). Furthermore, in transplantation paradigms, the interaction between engrafted NSCs and recipient host is a dynamic, complex, ongoing reciprocal interaction where both entities are constantly in flux.

Almost two decades ago, a handful of investigators interested in fundamental neural development began to identify, within cultures obtained from the developing and mature CNS, cells with surprising plasticity, multipotency and a propensity for dynamically shifting their fates (Ryder *et al.* 1990; Reynolds & Weiss 1992; Snyder *et al.* 1992). The existence of such cells—if indeed they represented a population normally resident in the brain—challenged the prevailing dogma that the nervous system was rigidly and immutably constructed. 'NSCs', as these plastic cells came to be termed, began to garner the interest of not just the developmental community but also that of the neural repair, gene therapy and transplant communities when it was recognized that they could be expanded in culture and reimplanted into the mammalian brain where they would

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reintegrate appropriately and stably express foreign genes (Snyder *et al.* 1992). Their abundance, multipotency, ease of manipulation and engraftability made this strategy an attractive alternative for CNS gene therapy and repair. Compared with extant techniques, NSCs presented certain advantages: they were a homogeneous and relatively well-defined neural cell population that could be easily stored and expanded on demand, and, if necessary, genetically manipulated *ex vivo* to express a wide variety of foreign transgenes. These transduced genes, as well as their inherent genetic repertoire, could be effectively imported into the CNS 'Trojan Horse'-style after transplantation almost anywhere into the developing and mature host brain. Furthermore, NSCs and their progeny possessed a capacity to integrate not only locally at their site of implantation, competing with and interdigitating seamlessly with endogenous cells (Snyder *et al.* 1992, 1997; Park *et al.* 1999; Rosario *et al.* 1997; Auerbach *et al.* 2000; Zlomanczuk *et al.* 2002), but also more broadly (Snyder *et al.* 1995; Ourednik *et al.* 1999; Yandava *et al.* 1999; Park *et al.* 2002). They were quite migratory—particularly if implanted into germinal zones—allowing cell and gene therapy to be contemplated for disseminated, even global, CNS disease processes. In that sense, NSCs had a distinct advantage over transplanted foetal tissue and non-neural cells for cell replacement and over most viral vectors and protein infusion devices for gene delivery. Even such alternative cellular vectors as haematopoietic cells, when used for protein delivery in bone marrow transplantation paradigms, could not efficiently circumvent the restrictions of the blood–brain barrier and integrate throughout the CNS as effectively as NSCs. A single *bona fide* NSC clone could take up residence in, and accommodate to, any nervous system region, allowing an economy of resources. In addition, NSCs were attracted by degenerating neural tissue (Flax *et al.* 1998; Park *et al.* 1999; Aboody *et al.* 2000), effectively replacing dead or dysfunctional cells in those regions. In these pathological niches, these multipotent cells, in response to signals still poorly understood (though probably linked to inflammatory cytokines as well as to neurotrophic factors), would shift their progeny's fate towards that of those neural lineages most in need of repletion; even if beyond the classical developmental window for genesis of that cell type. Indeed, these observations gave birth to the hypothesis that certain neurodegenerative environments recapitulate developmental cues because NSCs responded to neurogenic signals not only during their normal embryological expression, but also when recreated by particular types of cell death. NSCs, in other words, were sufficiently sensitive to 'sense' niches of neurogenesis and/or small niduses of pathology in the brain (Rosario *et al.* 1997; Snyder *et al.* 1997; Flax *et al.* 1998; Yandava *et al.* 1999).

In this essay, we will attempt to offer a 'logic' for various stem cell behaviours that are rooted in developmental processes. In introducing such concepts, we hope to provide a framework for viewing, in a cohesive manner, findings (by us and by others) that might otherwise seem disparate.

2. STEM CELLS AS COMPONENTS IN A SERIES OF DEVELOPMENTAL 'PROGRAMS'

Central to the views espoused in this essay is the belief that we may more intelligently promote neural stem/progenitor cell compensation in adulthood by first examining perturbations to the developing, less mature nervous system so as to discern the mechanisms by which the CNS shifts developmental patterns to maintain *homeostasis*. Those mechanisms are probably the key ones to be 'recapitulated' in adulthood. In fact, there is likely to be a continuum between the response of the injured CNS in adulthood and that in the paediatric age group. For sceptics of this view, it is important to recognize that, once beyond foetal life, the temporal 'windows' and spatial regions for neurogenesis are not significantly different in the paediatric brain than in the adult brain, but the *response* to perturbations (e.g. injury) appears to be more robust. We can learn from understanding what constitutive, homeostasis-preserving *developmental 'programs'* are *inherently* in place to deal with perturbations. Indeed, such an understanding may even explain the degree of spontaneous recovery that is often, yet inexplicably, seen in adults.

There exist, we believe, intrinsic 'developmental programs' that constitutively unfold throughout life that are 'designed' to respond to the exigencies of survival, and that integrate and coordinate with non-neural as well as other neural systems. The NSC, we believe, is a mediator of, and a repository for, this inherent plasticity.

We have somewhat arbitrarily delineated *three categories of program*. The first classification we call '*Macro Programs*'. In such programs, NSCs that emanate from primary germinal zones participate in organogenesis. However, in our view, organogenesis entails not only 'putting the nervous system together', i.e. creating the structures, regions and cytoarchitectonics of the brain, but also establishing 'reservoirs' for maintaining homeostasis throughout life. These reservoirs are typically secondary germinal zones that persist throughout life.

The second category of programs we call '*System Programs*', i.e. programs that constitutively unfold and allow the structure (or indeed the entire organism) to respond to perturbations. Such perturbations may be those that occur during the remainder of the complex yet precise process of development, or the 'minor' perturbations that occur 'day-to-day' throughout the lifespan of an organism in the 'wild', or the 'extreme' perturbations that often are encountered (e.g. ischaemia, trauma, toxins). Compensation for the last perturbations is often insufficient to reattain baseline, leaving the organism with a persistent handicap. Failure of these programs to respond to the more routine perturbations of daily life may also be the essence of some slowly progressive degenerative neuropathological processes. The 'system' invoked by some programs may entail the mobilization of stem cells from secondary germinal zones and the redistribution (in time and/or space) of the expression of molecular cues.

The third class of programs we term '*Micro Programs*'. These are programs that direct the process by which cell type and functional diversity spontaneously emerge within a given region of the CNS. Some of these programs appear to allow the multiple progeny of a single stem cell to self-assemble in an autonomous manner, spontaneously allocating various neural cell type identities to these progeny such that they interweave with each other to form the 'fabric' of a given region of nervous system.

We suggest that a better understanding of these fundamental developmental 'programs' will ultimately yield not

only a better understanding of development but also how best to exploit stem cell biology for therapy. For example, it might lend insight into the previously unclear teleology of 'secondary' germinal zones and 'vestigial' stem cells in the 'post-developmental' or adult CNS. It may also pinpoint and elucidate those developmentally programmed compensatory mechanisms that need to be re-invoked and/or exploited for purposes of repair.

Interestingly, the exploration of some of these fundamental processes may actually be abetted by the use of exogenous NSCs—not simply as therapeutic vehicles—though their utility in that regard will become obvious—but as biological 'tools'. In other words, we will introduce the notion of the NSC as a '*reporter cell*'—by way of analogy, at the cellular level, to a reporter gene as used in molecular biology—to reflect (or signal) when a particular process has occurred. Such exogenous stem cells can 'interrogate' the environment and track (i.e. 'report on') the behaviour of endogenous CNS progenitor/stem cells that are otherwise 'invisible' to such monitoring, too few to track reliably, and whose clonal relationships and degree of homogeneity may be less certain.

3. 'MACRO' PROGRAMS

Recently we observed that, early in embryogenesis, even in primates, a developmental neurogenic program constitutively allocates the progeny of a single NSC to either participate in organogenesis (pool no. 1) or to constitute secondary germinal zones, the type that persist into adulthood (pool no. 2) (Ourednik *et al.* 2001; figure 1). We determined this by exploring the process by which the progeny of a single clone of hNSCs became segregated during cerebrogenesis. A traceable clone of hNSCs was implanted into the ventricular zone (a primary germinal zone) of foetal Old World monkeys. Presumably reflecting prevalent morphogenetic programs, the NSCs self-distributed into two subpopulations: one contributed to corticogenesis by migrating along radial glia towards the cortical plate, terminating at temporally appropriate layers, and differentiating into lamina-appropriate neurons or glia; the other subpopulation remained undifferentiated and contributed to a secondary germinal zone (the subventricular zone) with occasional members interspersed throughout brain parenchyma. These findings suggested the existence of a unitary embryonic neurogenetic program allocating the progeny of NSCs either for immediate use in organogenesis or to quiescent pools for later use in the 'post-developmental' (including adult) brain for maintaining homeostasis and/or subserving self-repair. If true, then the prediction would be that, in the face of a perturbation, without specific instruction, induction or non-physiological manipulations, this developmental pattern (and the secondary stem/progenitor cell pool that constitutes it) should shift constitutively in the face of a disequilibrating force towards an attempted re-establishment of equipoise. Although this remains to be proven, we have preliminary evidence that suggests the presence of such 'System Programs', as described in § 4.

4. 'SYSTEM' PROGRAMS

To test the hypothesis posed in § 3, in pilot studies we tracked the response of a seemingly hard-wired mammalian program to extreme perturbations in the mammalian forebrain. The program chosen for study was the well-characterized prototypical developmental program known as the SVZa–RMS–OB axis. In this program, in the intact brain, endogenous neural progenitors within the SVZa seem to be invariantly fated throughout life to become interneurons in the OB. Just as such programs are established to replenish regions of neuronal turnover (such as the OB), we proposed that predictable shifts in these patterns (to compensate for perturbations to other regions of the brain) themselves constitute inborn programs; programs by which a system self-adjusts. We call these 'system' programs.

This SVZa–RMS–OB developmental pattern was examined after confrontation with a pathological process that is common and devastating to the paediatric population: *HI brain injury*, a major cause of cerebral palsy. Experimentally, unilateral HI injury is produced by ligation of one of the carotid arteries followed by hypoxia. To trace the brain's intrinsic response to this focal injury, experimental mice, at the instant of asphyxiation, were treated in two ways. First, they were pulsed with BrdU to label proliferative progenitors. Second, their lateral cerebral ventricles were injected with a retroviral vector transducing the *lacZ* reporter gene that would selectively mark SVZa cells transiting through the S phase. In these preliminary studies, we indeed observed that this ostensibly stereotypical, rigid program shifted to maintain homeostasis within the brain. SVZa progenitors were redirected from supplying the OB to instead attempting to reconstitute regions that had lost cells to asphyxia. The cell types lost after HI are of all neural lineages, although neurons and oligodendrocytes are particularly vulnerable. Although the mammalian cortex is classically considered a region to which neurons can no longer be added (i.e. a 'non-neurogenic' region), new neurons were indeed supplied by the spontaneous redirection of this developmental program; so, too, were new oligodendrocytes, astrocytes and even immature, undifferentiated progenitors. The new neurons were integrated as interneurons—receiving synaptic input, becoming appropriately activated. Intriguingly, although this type of injury is traditionally viewed as 'focal', it appeared to change the terrain of the entire cerebrum: SVZa progenitors on the *opposite* 'intact' side of the brain were also directed away from their respective OB and instead migrated across the midline to the injured side. In other words, developmental programs even on the *contralateral* side were similarly altered. The transiently diffuse upregulation of nestin expression *throughout* the cerebrum in response to this focal insult further supported the view that an injured brain changes in a global manner.

Briefly, programs established during the earliest stage of development and persisting into the 'post-developmental epochs' are spontaneously redirected to re-establish equipoise, this change itself constituting a 'program'. Molecules employed in the establishment and maintenance of such patterns in embryogenesis are involved in these switches. For example, *slit-2* is a chemorepulsive molecule that is believed to play a role in establishing and maintaining the SVZa–RMS–OB axis by propelling progenitors from the SVZa towards the OB. After HI injury, however, the typical pattern of *slit* expression, preliminary data suggest, dissipates and is redistributed so that it no

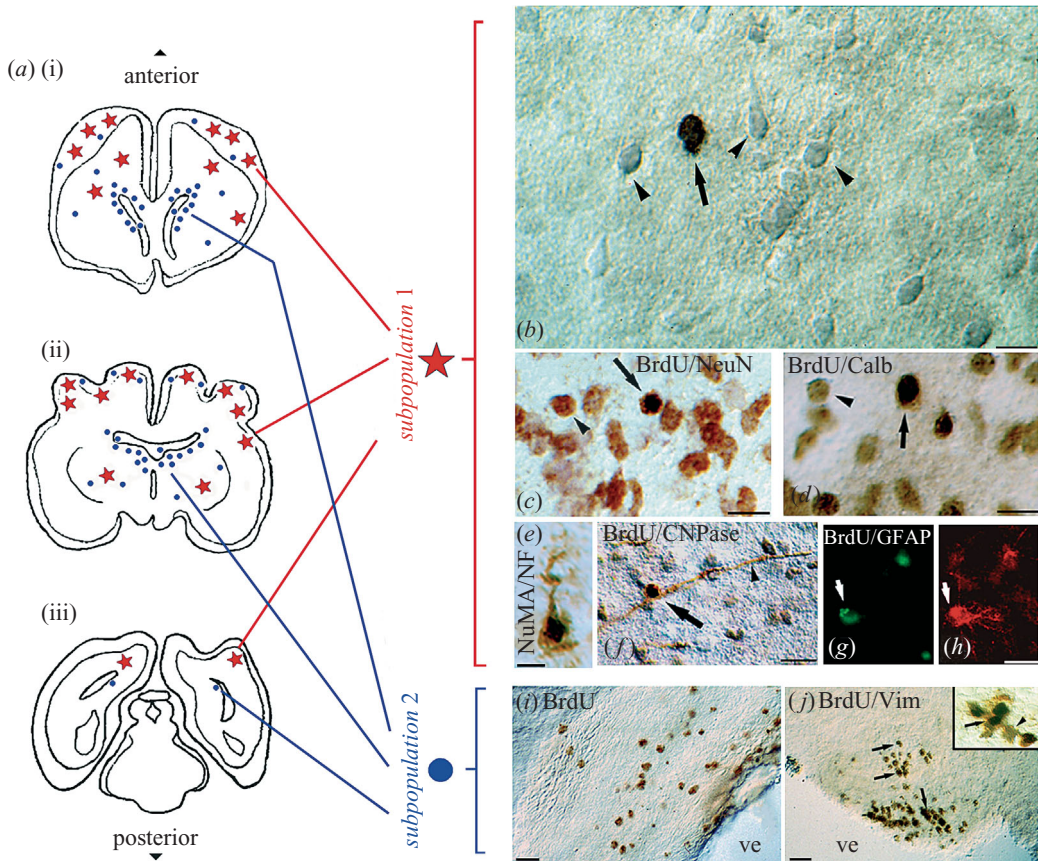

Figure 1. Segregation of the fates of hNSCs and their progeny into two subpopulations after implantation into the ventricular zones (VZ) of developing Old World monkey brains (modified from Ourednik *et al.* 2001). Schematics (left) and photomicrographs (right) illustrating the cells. Coronal sections from representative levels throughout the telencephelon are illustrated on the left. hNSCs (pre-labelled with BrdU and implanted) dispersed throughout the ventricular system and integrated into the VZ. From there, clonally related hNSC-derived cells pursued one of two fates, as shown by immunocytochemical analysis (*b–j*). Those donor cells that migrated outwards from the VZ along radial glial fibres into the developing neocortex constituted one pool or subpopulation. The differentiated phenotype of cells in this subpopulation 1 (red stars in the schematic) (particularly in layers (II/III) are pictured in (*b–h*). (*b*) A human NSC-derived (black nucleus, arrow), probably according to its size, morphology, large nucleus and location, is visualized (under Nomarski optics) intermingled with the monkey's own similar neurons (arrowheads) in neocortical layers II/III. The neuronal identity of such donor-derived cells is confirmed in (*c–e*). (*c*,*d*,*f–h*) High-power photomicrographs of human donor-derived cells integrated into the monkey cortex double-stained with antibodies against BrdU and cell-type-specific markers: (*c*) NeuN and (*d*) calbindin for neurons (arrows: donor-derived cells; arrowheads: host-derived cells); (*f*) CNPase for oligodendroglia (arrow: BrdU+ black nucleus in CNPase+ brown cell; arrowhead indicates long process emanating from the soma); (*g*,*h*) GFAP for astroglia (anti-BrdU+ via fluorescein in (*g*); anti-GFAP via Texas Red in (*h*)). The human-origin of the cortical neurons is further independently confirmed in (*e*) where the human-specific nuclear marker NuMA (black nucleus) is co-localized in the same cell with neurofilament (NF) immunoreactivity (brown). Progeny from this same hNSC clone were also allocated to a second cellular pool—subpopulation 2; blue dots in the schematic and pictured in ((*i*,*j*) arrows)—that remained mainly confined to the SVZ and stained only for an immature neural marker (vimentin (brown) co-localized with BrdU (black nucleus) in inset, arrows; arrowhead indicates host vimentin+ cell). Some members of subpopulation 2 were identified within the developing neocortex intermixed with differentiated cells. Panels (*g*,*h*) employ immunofluorescence; the other immunostains use a DAB-based colour reaction. The photomicrographs were taken from different animals as representative of all animals. Abbreviations: 've', lateral cerebral ventricle; arrow, BrdU+ donor-derived cell; arrowhead, BrdU-negative, host-derived cell. Scale bars: 30 μm (*b–d*); 20 μm (*e–j*).

longer directs progenitors to the OB but rather 'forces' them instead towards the injured cortex. Similarly, in other pilot studies, another developmentally regulated molecule, *SDF1-α*, known as an inflammatory chemokine but recently determined to be an attractant that helps establish migratory pathways during cerebrogenesis, is upregulated within the injured region, drawing SVZ progenitors (which bear receptors to this attractant) to the region. Activated microglia, the omnipresent first wave of 'invaders' in most neurodegenerative processes, also appear to produce factors that beckon NSCs (surprisingly, NSCs bear receptors to such 'non-neural' ligands). NSCs also bear receptors to vascular endothelium. When the blood–brain barrier is breached by insults, endothelium is typically exposed. NSCs are thus presented with another 'beacon' to guide their homing response.

These observations suggesting the presence of intrinsic systems for maintaining homeostasis may explain the degree of spontaneous recovery long witnessed in infants and children who have suffered a stroke. It may also

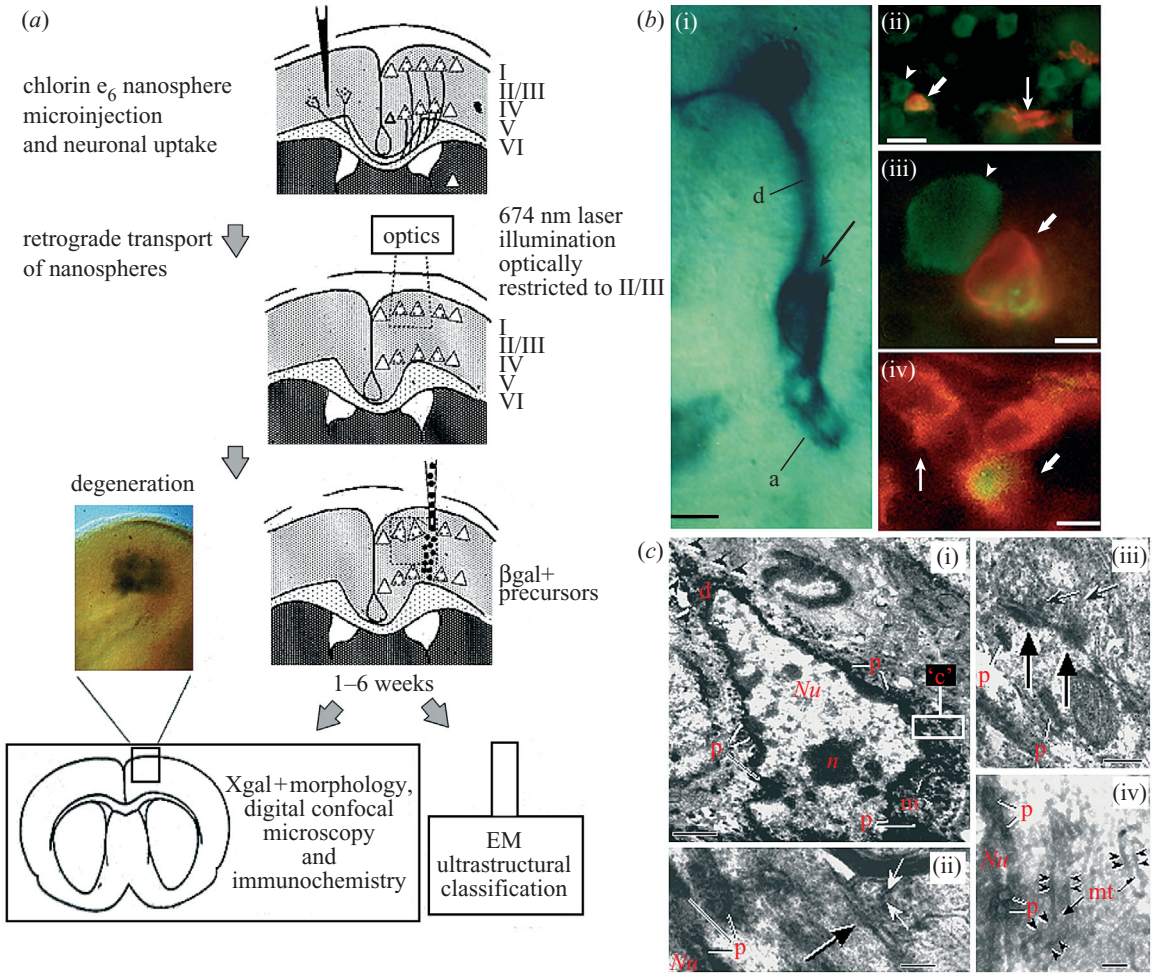

Figure 2. Multipotent NSCs acquire a neuronal phenotype in regions of adult neocortex subjected to targeted apoptotic neuronal degeneration (modified from Snyder *et al.* (1997)). (*a*) Schematic of procedure by which pyramidal neurons in layer II of the adult mouse neocortex are induced to die an apoptotic death by injecting nanospheres into the target region of their transcallosal projections, allowing them to be retrogradely transported back to their somata, and then energizing them via a laser illumination such that they promote degeneration of those neurons. NSCs were then implanted into the degenerated region. (*b*) NSCs differentiate appropriately into only glia (or remain undifferentiated) in intact adult cortex, where neurogenesis has normally ceased but gliogenesis persists. However, 15% of engrafted cells (identified by their *lacZ* expression) in regions of apoptotic neurodegeneration developed neuronal morphology, resembling pyramidal neurons (PNs) within layer II/III six weeks after transplantation, at 12 weeks of age (i) and immunocytochemical properties consistent with a neuronal phenotype (ii–iv). (i) Donor-derived cell (which stains blue after Xgal histochemistry to detect *lacZ* expression) with pyramidal neuronal morphology and size (large arrow) under bright field microscopy: apical dendrites ('d'); with descending axons ('a'). These features are readily confirmed by ultrastructural criteria under EM (*c*), where donor-derived cells are noted to receive both axo-somatic and axo-dendritic synaptic input. Donor-derived cells in control intact adult cortices had only morphologic, ultrastructural and immunocytochemical features of glia (astrocytes and oligodendrocytes; not shown here but pictured in Snyder *et al.* (1997)). (ii–iv) Immunocytochemical analysis. Donor-derived *lacZ*-expressing cells (identified by an anti-βgal antibody conjugated to Texas Red) are immunoreactive for NeuN (fluorescein, green), a marker for mature neurons. In (ii), and at higher magnification in (iii), a βgal+ cell (large arrow) double-labels for NeuN. Other small βgal+ cells with non-neuronal morphology are NeuN− (small arrow). Remaining host neurons (NeuN+) are βgal− (arrowhead). (iv) Donor-derived neuron (βgal+, NeuN+; large arrow) adjacent to two NeuN− donor-derived cells (small arrow). Scale bars: (i,ii) 25 μm; (iii,iv) 10 μm. (*c*) Ultrastructure of donor-derived neurons in regions of adult cortex subjected to targeted apoptotic neuronal degeneration. Donor-derived neurons were restricted to the cortex within the degenerated region of layers II/III. In all panels, Xgal precipitate ('p') is visible in the nuclear membrane, cytoplasmic organelles and processes. (i) EM characteristic suggestive of layer II/III pyramidal neurons are present six weeks after transplantation at 12 weeks of age: large somata (20–30 μm); large nuclei ('*Nu*'), prominent nucleoli ('n'), abundant endoplasmic reticulum and mitochondria ('m') and apical dendrites ('d'). An afferent synapse is indicated on the donor-derived neuron in (i) (box 'c', expanded in (ii)). (ii) Higher magnification of the axosomatic synapse boxed in (i): presynaptic vesicles (white arrows) and postsynaptic specialization (large arrow); the postsynaptic region is in continuity with the donor cell nucleus via uninterrupted cytoplasm. Both the cytoplasm and the nuclear membrane contain precipitate ('p'). (iii) Axodendritic synapse on the dendrite of a donor-derived neuron: crystalline, linear Xgal precipitate ('p') in the postsynaptic region of the dendrite confirms its donor origin; postsynaptic specialization with a hazy, nonlinear, non-crystalline appearance immediately under the membrane (large arrows); presynaptic vesicles clustered near the synaptic densities (small arrows). (iv) Microtubules (arrows, 'mt') of 20–26 nm diameter (outlined by arrowheads) in a donor-derived neuron near precipitate ('p') in the nuclear membrane. Scale bars: (i) 1 μm; (ii) 200 nm; (iii) 40 nm; (iv) 500 nm.

provide a handle for exploiting that response beyond childhood. Although, in the most devastating cases, this self-repair mechanism is typically insufficient, the presence of this altered niche suggests that these responses might be augmented. To achieve such augmentation, as well as to better understand this process, in another series of pilot studies we implanted exogenous 'reporter' NSCs into animals subjected to HI. The insights derived from these experiments are described in the following sections.

5. CONCEPT OF A 'REPORTER CELL' TO 'INTERROGATE' THE ENVIRONMENT

Although the cortex is classically deemed to be a 'non-neurogenic' region, the findings above, as well as our past studies (Snyder *et al.* 1997; Park *et al.* 2002) and those of others (Magavi *et al.* 2000; Arvidsson *et al.* 2002), suggest that conclusions derived from the intact cortex may not accurately reflect the milieu of the *abnormal*, *injured* or *degenerating* cortex; that neurodegeneration might convert the 'non-neurogenic' cortex into a 'neurogenic' niche from the 'viewpoint' of the NSC in certain areas, and at certain times, through the recrudescence of signals not normally seen (or the elimination of restrictive factors/barriers that normally emerge). Indeed, it was the observation that exogenous multipotent NSCs could respond to the prevailing cues of normal and abnormal microenvironments that first suggested the existence of spontaneous compensatory mechanisms for genetic (Snyder *et al.* 1995; Rosario *et al.* 1997; Flax *et al.* 1998; Park *et al.* 2002) or acquired deficiencies (Snyder *et al.* 1997; Park *et al.* 1999) including neurogenesis beyond its normal confines under certain circumstances.

Several years ago, we and our collaborators first observed that such a phenomenon might take place in the adult neocortex (figure 2). When a clone of murine NSCs was implanted into an adult mouse neocortex in which pyramidal neurons of a circumscribed region were induced experimentally to undergo apoptosis, 15% of the transplanted NSCs 'altered' the differentiation path that they would have otherwise taken. Instead, they differentiated specifically into pyramidal neurons despite the fact that the 'normal' developmental window for cortical neurogenesis had long since passed. In intact neocortices, these same NSCs yielded exclusively glia. Such results suggested that the *'probe' that can best 'interrogate' the CNS and reflect prevailing developmental processes is likely to be the NSC itself*. NSCs can mirror subtle changes quite sensitively. Indeed, in the experiment just described, NSCs could detect the presence of a small niche—only 3600 μm in diameter—of neurogenesis by yielding neurons there, and only there. In cortical regions beyond the perimeter of that circumscribed area of apoptosis, NSCs, although robustly engrafted, yielded only non-neurons. Furthermore, the same clone placed in a *given* region of the intact brain at *different developmental stages* could similarly detect the shift of developmental programs; for example, in the cortex from a neurogenic region when implanted *prenatally* to a *non*-neurogenic region when implanted *postnatally* and, in the cerebellum, from yielding Purkinje cells *prenatally* to only small interneurons *postnatally* (Snyder *et al.* 1992; Rosario *et al.* 1997). Subtleties in neurogenesis can be assayed so sensitively using NSC clones that we have come to think of them as 'reporter cells', integrating and screening a panoply of cues to yield a relevant read-out (e.g. differentiation towards a particular phenotype). This technique has been useful to other investigators as well, for example, in hippocampus and OB (Suhonen *et al.* 1996), reinforcing the notion that the same NSC clone that can give rise to neurons as well as glia in culture will yield neurons *in vivo* only when placed into neurogenic niches; never when placed into environments unsupportive of neurogenesis.

We used exogenous NSCs to test the hypothesis that injury and degeneration can change the cortex into a region that does support neurogenesis. The signals modifying exogenous and endogenous NSC behaviour are doubtless a complex of various mitogens and chemokines, trophic and tropic agents, adhesion and extracellular matrix molecules, chemotactic and angiogenic factors, some probably elaborated by reactive astrocytes, activated microglia, inflammatory cells, invading macrophages and damaged neurons and glia; some with positive, others with negative actions on NSCs. If the brain's 'attempt' to repair itself with its own NSCs falls short solely because their supply is insufficient in number or insufficiently mobilized, then augmenting that population with strategically placed exogenous NSCs and/or exogenous trophic factors (perhaps delivered by NSCs) seems feasible. In pilot studies, such an approach proved useful for NSC-mediated neuron replacement in the asphyxiated cerebrum (Park *et al.* 1999), as detailed below.

6. ENHANCING NEURONAL DIFFERENTIATION

In preliminary studies, when exogenous NSCs are transplanted into brains of young mice subjected (as described above) to unilateral HI injury (optimally within 3–7 days), donor-derived cells migrate preferentially to, and integrate extensively within, the large ischaemic areas that typically span the injured ipsilateral hemisphere. Even donor cells implanted in more distant locations (including the contralateral hemisphere) migrate towards the regions of HI injury (emulating what endogenous progenitors appear to do). (Waiting five weeks post-HI yields virtually no engraftment, suggesting a 'window' for this phenomenon.) A subpopulation of donor NSCs, particularly in the penumbra, 'shift' their differentiation fate towards neurons (5%) and oligodendrocytes, the neural cell types typically damaged after asphyxia/stroke although no neurons and few oligodendrocytes are derived from NSCs in intact postnatal neocortex. Clearly, as in the targeted apoptosis model described above, novel signals appear to be transiently elaborated to which NSCs respond. Because engrafted NSCs continue to express their *lacZ* reporter transgene, it appeared feasible that desired differentiation of both host and donor-derived cells might be enhanced if donor NSCs were genetically manipulated *ex vivo* to (over)express certain bioactive transgenes, for example, the neuron-inducing factor NT-3, a neurotrophic factor it expresses at baseline low amounts. In pilot studies, a subclone of the NSCs was transduced with a retroviral vector encoding NT-3. The engineered NSCs produced large amounts of NT-3 *in vitro* (Liu *et al.* 1999; Himes *et al.* 2001). We determined, in pilot studies, that both the parent clone and its NT-3-producing subclone expressed trkC receptors, that these

Figure 3. The injured brain interacts reciprocally with NSCs to reconstitute lost tissue—evidence from HI injury (modified from Park *et al.* 2002). (*a*) Characterization of NSCs *in vitro* when seeded on a PGA scaffold. Cells, seen with scanning electron microscopy at 5 days after seeding, were able to attach to, impregnate and migrate throughout a highly porous PGA matrix (arrow). The NSCs differentiated primarily into neurons (greater than 90%) that sent out long complex processes that adhered to, enwrapped and interconnected the PGA fibres. Scale bar: 20 μm. (*b*) Implantation of NSC/PGA complexes into a region of cavity formation after extensive HI brain injury and necrosis. Supporting the NSCs on the scaffold not only provided a template and support, but also allowed protracted interaction between the cells and the degenerating environment. (i) Brain of an untransplanted ('non-Tx') mouse subjected to right HI injury with extensive infarction and cavitation of the ipsilateral right cortex, striatum, thalamus and hippocampus (arrow). Contrast with (ii), the brain of a similarly injured mouse implanted with an NSC/PGA complex ('PGA+NSCs') (generated *in vitro* as per (*a*) into the infarction cavity 7 days after the induction of HI (arrow) ($n = 60$). At maturity (age-matched to the animal pictured in (i)), the NSC/scaffold complex appears, in this whole-mount, to have filled the cavity (arrow) and become incorporated into the infarcted cerebrum. Representative coronal sections through that region are seen at higher magnification in (iii) and (iv) in which parenchyma appears to have filled in spaces between the dissolving black polymer fibres (white arrow in (iii)) and, as seen in (iv), even to support neovascularization by host tissues (blood vessel indicated by closed black arrow in (iv); open arrow in (iv) points to degrading black polymer fibre). Scale bars: (iii, iv) 100 μm. (*c*) Characterization *in vivo* of the neural composition of NSC/PGA complexes within the HI-injured brain. At two weeks after transplantation of the NSC/PGA complex into the infarction cavity, donor-derived cells showed robust engraftment within the injured region. An intricate network of multiple long, branching NF+ (green) processes were present within the NSC/PGA complex and its parenchyma enwrapping the PGA fibres (orange autofluorescent tube-like structures under a Texas Red filter), adherent to and running along the length of the fibres (arrows), often interconnecting and bridging the fibres (arrowheads). Those NF+ processes were of both host and donor derivation. In other words, not only were donor-derived neural cells present, but also host-derived cells seemed to have entered the NSC/PGA complex, migrating along and becoming adherent to the PGA matrix. In a reciprocal manner, donor-derived (*lacZ*+) neurons (NF+ cells) within the complex appeared to send processes along the PGA fibres out of the matrix into host parenchyma as seen in (*d*). Scale bar: 100 μm. (*d*) Long-distance neuronal connections extend from the transplanted NSC/PGA complexes in the HI-injured brain towards presumptive target regions in the intact contralateral hemisphere. By six weeks after engraftment, donor-derived *lacZ*+ cells appeared to extend many exceedingly long complex NF+ processes along the length of the disappearing matrix apparently extending into host parenchyma. To confirm the suggestion that long-distance processes projected from the injured cortex into host parenchyma, a series of tract tracing studies were performed. (i–vi) BDA-FITC was injected (iii) into the contralateral intact cortex and external capsule (green arrow) at eight weeks after implantation of the NSC/PGA complex into the infarction cavity ('NSC/PGA-Tx'). Axonal projections (labelled green with fluorescein under an FITC filter) are visualized (via the retrograde transport of BDA) leading back to (across the interhemispheric fissure ('IHF') via the corpus callosum ('cc')) and emanating from cells in the NSC/PGA complex within the damaged contralateral cortex and penumbra (seen at progressively higher magnification in (ii) (region indicated by arrow to (iii)) and (i) (region indicated by arrow and asterisk (∗) in (iii)). In (i), the retrogradely BDA-FITC-labelled perikaryon of a representative neuron adherent to a dissolving PGA fibre is well visualized. That such cells are neurons of donor derivation is supported by their triple-labelling (iv–vi) for *lacZ* (βgal)(iv), BDA-FITC (v), and the neuronal marker NF (vi); arrow in (iv–vi), indicates the same cell in all three panels. Such neuronal clusters were never seen under control conditions—i.e. in untransplanted cases or when vehicle or even an NSC suspension unsupported by scaffolds was injected into the infarction cavity. Scale bars: (i) 500 μm; (iii) 20 μm; (iv–vi) 30 μm (scale bar in (vi) also applies to (iv) and (v)). (*e*) Adverse secondary events that typically follow injury (e.g. monocyte infiltration and astroglial scar formation) are minimized by, and within, the NSC/PGA complex. (i–iv) Photomicrographs of H&E-stained sections prepared to visualize the degree of monocyte infiltration in relation to the NSC/PGA complex and the injured cortex three weeks after implantation into the infarction cavity. Monocytes are classically recognized under H&E as very small cells with small round nuclei and scanty cytoplasm (e.g. inset in (iv), arrowhead). Although some very localized monocyte infiltration was present immediately surrounding a blood vessel ('BV' in (iii), arrow) that grew into the NSC/PGA complex from the host parenchyma, there was little or no monocyte infiltration either in the centre of the NSC/PGA complex (ii) or at the interface between the NSC/PGA complex and host cortical penumbra (i)—in stark contrast to the excessive monocyte infiltration seen in an untransplanted infarct of equal duration, age and extent (iv), the typical histopathologic picture otherwise seen after HI brain injury (see inset, a higher magnification of the region indicated by asterisk in (iv); a typical monocyte is indicated by the arrowhead). Whereas neural cells (nuclei of which are seen in (i–iii)) adhere exuberantly to the many polymer fibres ('P' in (i–iii)), monocyte infiltration was minimal compared with that in (iv). (v,vi) Astroglial scarring (another pathological condition confounding recovery from ischaemic CNS injury) is also much constrained and diminished after implantation of the NSC/PGA complex. Although GFAP+ cells (astrocytes) were among the cell types into which NSCs differentiated when in contact with the PGA fibres, away from the fibres (∗) there was minimal astroglial presence either of donor or host origin. (v) GFAP immunostaining recognized by a fluorescein-conjugated secondary antibody (green) is observed. Note little scarring in the regions indicated by an asterisk. Under a Texas Red filter (vi) (merged with the fluorescein filter image), the tube-like PGA fibres (arrowhead in both panels) become evident (as an autofluorescent orange) and most of the donor-derived astrocytes (arrows) (yellow because of their dual *lacZ* and GFAP immunoreactivity) are seen to be associated with these fibres, again leaving most regions of the infarct (∗) astroglial scar-free (arrows in (v) and (vi) point to the same cells). Far from creating a barrier to the migration of host- or donor-origin cells or to the ingrowth/outgrowth of axons of host- or donor-origin neurons (as per (*c*) and (*d*)), NSC-derived astrocytes may actually have helped provide a facilitating bridge. Scale bars: (i) 10 μm; (iii–vii) 20 μm.

receptors were appropriately tyrosine-phosphorylated in response to exogenous NT-3, that this phosphorylation could be blocked by K252a, and that the signal was appropriately transduced via MAP kinase. Therefore, it appeared that this engineered NSC subclone could not only secrete excess NT-3, but could also respond to NT-3 in an autocrine/paracrine fashion. In culture, NT-3-over-expressing NSCs, like the parent clone, still differentiate

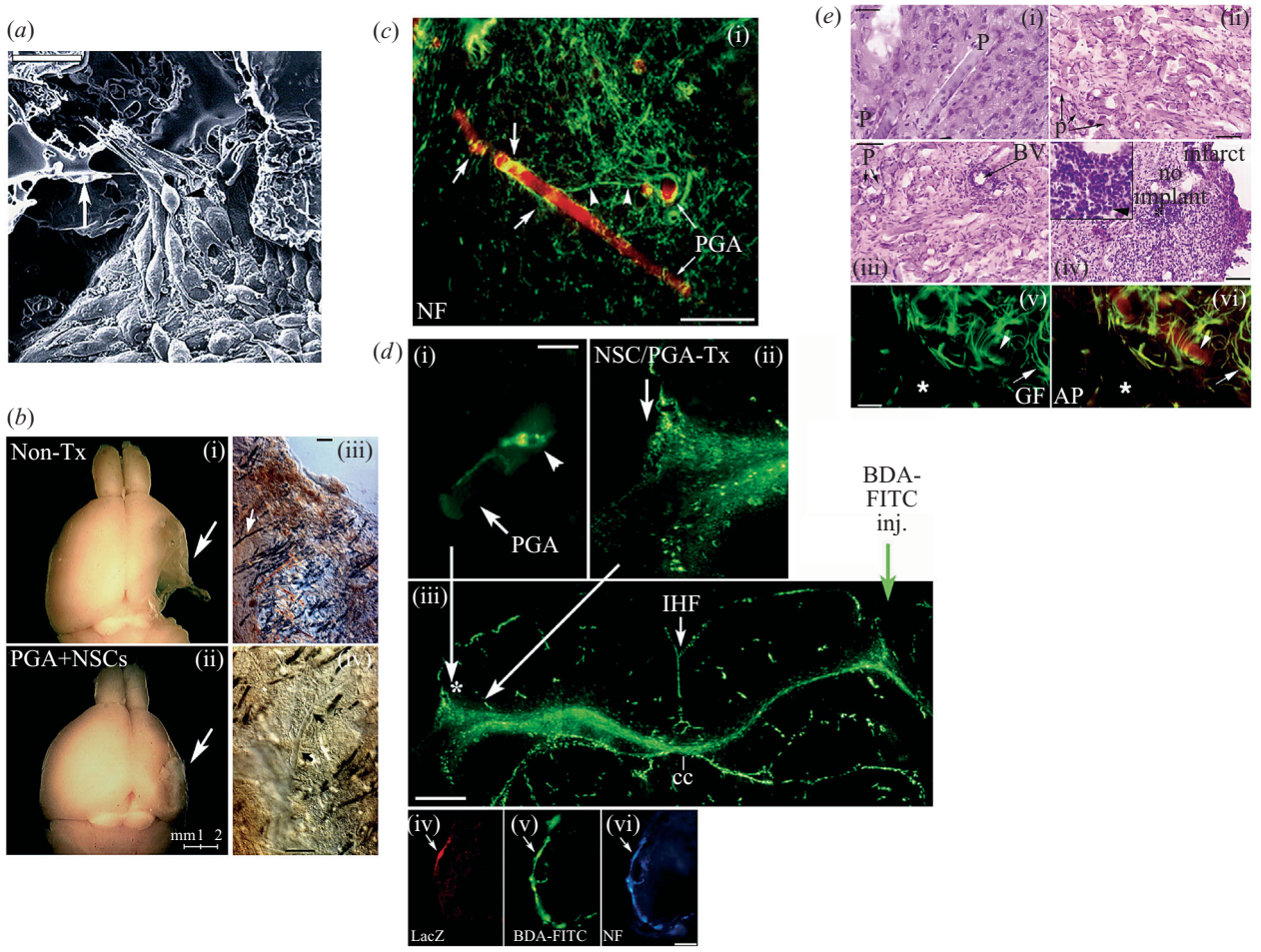

Figure 3. (*Caption p. 829.*)

into neurons, astrocytes and oligodendrocytes. However, unlike the parent clone whose percentage of neurons falls as new cells are born, the percentage of this NT-3-overexpressing subclone that remained neuronal *in vitro* was *ca.* 95%. Therefore, pilot studies were performed in which NT-3-overexpressing NSCs were implanted into the infarct. NT-3 expression remained robust *in vivo*. The percentage of donor-derived neurons was increased from 5% (in the above-described pilot experiments) to 20% in the infarct and to more than 80% in the penumbra. Many of the neurons became cholinergic, glutamatergic or GABAergic. NT-3 was also probably acting on host as well as donor cells in a paracrine fashion to enhance their neuronal differentiation. It is quite plausible that the original yield of 5% new neurons is actually the correct ratio and that 80% neurons is actually a prescription for dysfunction. Indeed, we have come to adopt the aphorism that even the 'dumbest stem cell is smarter than the smartest neurobiologist'. However, the NT-3 experiment serves as a proof-of-concept: the observations suggest that, when a molecular mechanism underlying a naturally occurring NSC-based process in a degenerative environment is known, it can be augmented via genetic engineering. It also enunciated the potential use of migratory NSCs for simultaneous gene therapy *and* cell replacement during the same procedure in the same recipient using the same cells, an intriguing NSC ability. This work constitutes a paradigm for using NSCs to express other trophic factors in other instances, such as spinal cord injury. Indeed, we have evidence that the same parent NSC clone can be variously engineered to express a range of gene products serving a variety of therapeutic ends, including NT-4/5, GDNF, BDNF, NGF, L1, *sonic hedgehog*, *wnt-1*, *wnt-3a* as well as a variety of biosynthetic and metabolic enzymes. Hence, implantation of genetically engineered NSCs expressing bioactive transgenes, when used in a thoughtful manner, might enhance neuronal differentiation, neurite outgrowth and proper connectivity.

7. THE INJURED CNS INTERACTS RECIPROCALLY WITH NSCs TO RECONSTITUTE LOST TISSUE

Once again, using exogenous NSCs to 'interrogate' the environment and using HI brain injury as a prototype for insults to the CNS (or other organs) characterized by extensive tissue loss, we confirmed that a reciprocal dynamic indeed occurs between NSCs and degenerating neural tissue. Seeding NSCs onto a biosynthetic scaffold, which is subsequently implanted into the large infarction cavities of mouse brains injured by HI, not only provided a template for bridging extensive cystic lesions and guiding restructuring, but served to 'fix' NSCs in space as well as to 'fix' and prolong the effects of: (i) molecules emanating from the injured brain; and (ii) molecules emanating from the NSCs (figure 3). In so doing, we were allowed to observe, in a manner previously unavailable, the multiple robust reciprocal interactions that spontaneously ensue

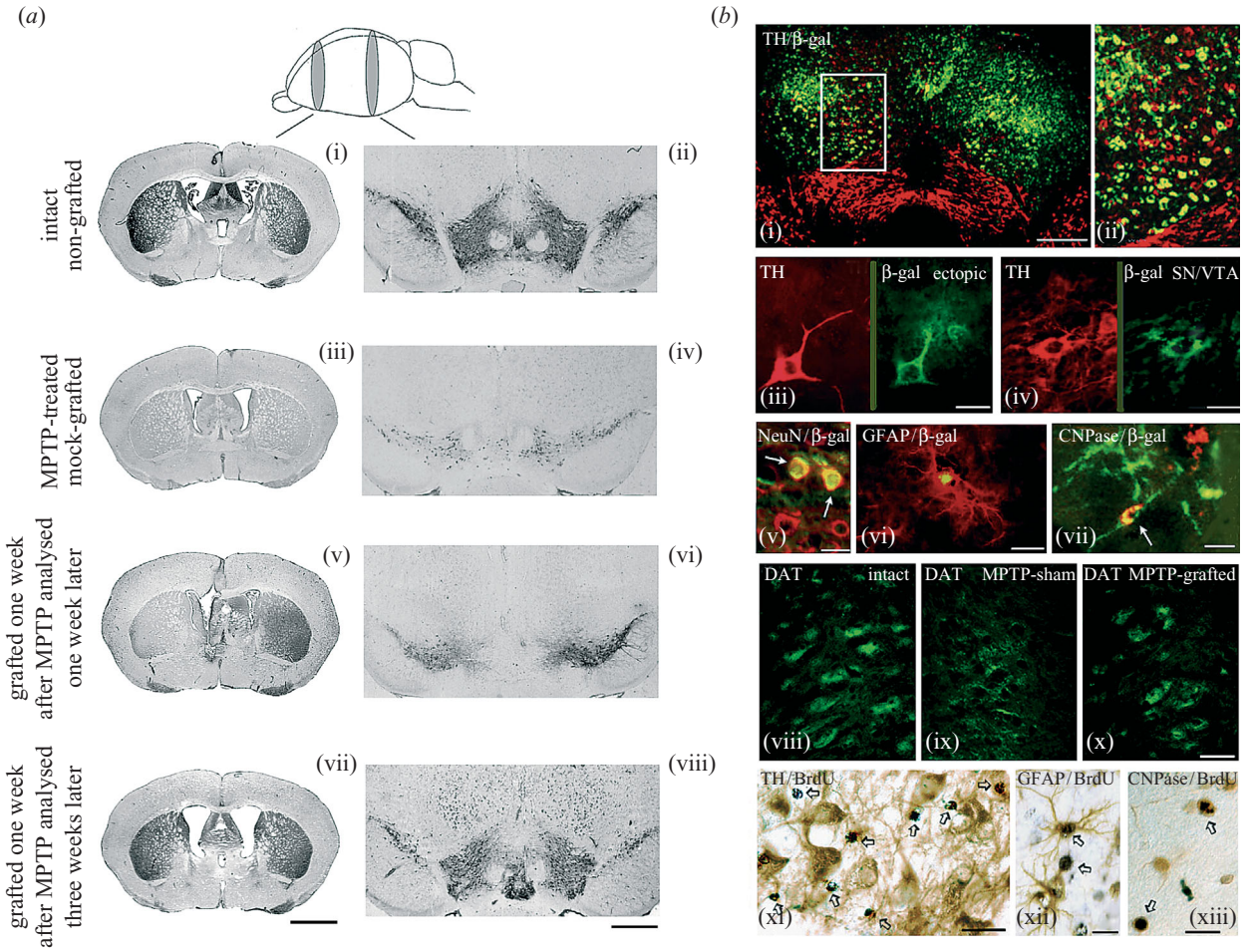

Figure 4. (*Caption p. 832.*)

between NSCs and the extensively damaged brain. NSCs grew exuberantly into a lattice of neurons and glia, parenchymal loss was dramatically reduced, an intricate meshwork of many highly arborized neurites of both host- and donor-derived neurons emerged, and some anatomical connections were reconstituted. The exogenous NSCs, nestled within the necrotic host parenchyma, altered the trajectory and complexity of *host* cortical neurites promoting their entrance into the matrix. In a reciprocal manner, tract tracing demonstrated donor-derived neurons extending processes into host parenchyma as far as the opposite hemisphere. Of interest was the degree to which these neurons were capable of seemingly directed, target-appropriate neurite outgrowth *without* specific external instructive guidance cues, induction or genetic manipulation of host brain or donor cells. These NSC/scaffold complexes, these 'biobridges', appeared to unveil and/or augment a constitutive reparative response by facilitating a series of reciprocal interactions between NSC and host CNS tissue (both injured and intact), including promoting neuronal differentiation, enhancing the ingrowth/outgrowth of neural processes, fostering the reformation of cortical tissue and promoting connectivity after brain injury. Inflammation and scarring were also reduced, facilitating reconstitution.

That certain reciprocal interactions spontaneously unfold between transplanted NSCs and an injured host, as if 'pre-programmed', suggests that NSCs might influence the fate of host cells as profoundly as the fate of the NSC itself is changed. The power and implications of this under-recognized axis of influence is discussed in § 8.

8. NSCs DISPLAY AN INHERENT MECHANISM FOR RESCUING DYSFUNCTIONAL NEURONS AND AXONS

The notion that stem cells, as exemplified by NSCs, may actually possess an intrinsic capacity to 'rescue' dysfunctional host neurons and their connections was first confirmed in two very different situations: the brains of aged mice (Ourednik *et al.* 2002; figure 4) and the injured spinal cords of adult rats (Teng *et al.* 2002; figure 5).

One of these studies focused on a neuronal cell type with stereotypical projections that is commonly compromised in the aged brain—the DA neuron (Ourednik *et al.* 2002; figure 4). The DA-selective neurotoxin (and complex I inhibitor) MPTP (via its metabolite MPP^{+}) was administered systemically to intensify and accelerate permanent impairment of these neurons bilaterally (an experimental lesion that emulates Parkinson's disease). Unilateral implantation of murine NSCs into the midbrains of these aged Parkinsonian mice promoted reconstitution of the mesostriatal system, an impressive outcome supported by both histological and functional assays. The recovery of DA activity mirrored the spatio-temporal distribution of donor-derived cells. Although the spontaneous conversion of some donor NSCs into replacement DA neurons contributed to nigral reconstitution in

Figure 4. NSCs possess an inherent mechanism for rescuing dysfunctional neurons: evidence from the effects of NSCs in the restoration of mesencephalic DA function (modified from Ourednik *et al.* 2002). (*a*) TH expression in mesencephalon and striatum of aged mice after MPTP lesioning and unilateral NSC engraftment into the SN/VTA. A model that emulates the slow dysfunction of ageing DA neurons in SN was generated by giving aged mice repeated high doses of MPTP. Schematic on top indicates the levels of the analysed transverse sections along the rostrocaudal axis of the mouse brain. Representative coronal sections through the striatum are presented in (i), (iii), (v) and (vii) and through the SN/VTA area in (ii), (iv), (vi) and (viii). (i,ii) Immunodetection of TH (black cells) shows the normal distribution of DA-producing TH+ neurons in coronal sections in the intact SN/VTA (ii) and their projections to the striatum (i). (iii,iv) Within one week, MPTP-treatment caused extensive and permanent bilateral loss of TH immunoreactivity in both the mesostriatal nuclei (iii) and the striatum (iv), which lasted throughout life. Shown in this example, and matching the time-point in (vii,viii), is the situation in a mock-grafted animal four weeks after MPTP-treatment. (v,vi) Unilateral (right side) stereotactic injection of NSCs into the nigra is associated, within one week after grafting, with substantial recovery of TH synthesis within the ipsilateral DA nuclei (vi) and their ipsilateral striatal projections (v). By three weeks post-transplant, however (vii,viii), the asymmetric distribution of TH expression disappeared, giving rise to TH immunoreactivity in the midbrain (viii) and striatum (vii) of both hemispheres that approached that of intact controls (i,ii) and gave the appearance of mesostriatal restoration. Similar observations were made when NSCs were injected four weeks after MPTP-treatment (not shown). Scale bars: (i), (iii), (v) and (vii) 2 mm; (ii), (iv), (vi) and (viii) 1 mm. Note the ectopically placed TH+ cells in (viii). These are analysed in greater detail, together with the entire SN, in (*b*). (*b*) Immunohistochemical analyses of TH, DAT, and BrdU-positive cells in MPTP-treated and grafted mouse brains. The presumption was initially that the NSCs had replaced the dysfunctional TH neurons. However, examination of the reconstituted SN with dual βgal (green) and TH (red) ICC showed that (i) 90% of the TH+ cells in the SN were host-derived cells that had been rescued and only 10% donor-derived (iv). Most NSC-derived TH+ cells were actually just above the SN ectopically (blocked area in (i), enlarged in (ii)). These photomicrographs were taken from immunostained brain sections from aged mice exposed to MPTP, transplanted one week later with NSCs, and sacrificed after three weeks. The following combinations of markers were evaluated: TH (red) with βgal (green) (i–iv); NeuN (red) with βgal (green) (v); GFAP (red) with βgal (green) (vi); CNPase (green) with βgal (red) (vii); as well as TH (brown) and BrdU (black) (xi); GFAP (brown) with BrdU (black) (xii); and CNPase (brown) with BrdU (black) (xiii). Anti-DAT-stained areas are revealed in green in the SN of intact (viii), mock-grafted (ix), and NSC-grafted (x) brains. Three different fluorescence filters specific for Alexa Fluor 488 (green), Texas Red (red) and a double-filter for both types of fluorochromes (yellow) were used to visualize specific antibody binding; (iii,iv), and (viii–x) are single-filter exposures; (i,ii) and (v–vii) are double-filter exposures. (i) Shows a low-power overview of the SN+VTA of both hemispheres, similar to the image in figure 4*a*(viii). Most TH+ cells (red cells in (i)) within the nigra are actually of host origin (*ca*. 90%) with a much smaller proportion being of donor derivation (green cells) (*ca*. 10%) (representative close-up of such a donor-derived TH+ cell in (iv)). Although a significant proportion of NSCs did differentiate into TH+ neurons, many of these actually resided ectopically, dorsal to the SN—(boxed area in (i), enlarged in (ii); high power view of donor-derived (green) cell that was also TH+ (red) in (iii))—where the ratio of donor-to-host cells was inverted: *ca*. 90% donor-derived compared with *ca*. 10% host-derived. Note the almost complete absence of a green βgal-specific signal in the SN+VTA whereas, ectopically, many of the TH+ cells were double-labelled and thus NSC-derived (appearing yellow-orange in higher power under a red/green double-filter in panel (ii)). (iii–vii) NSC-derived non-TH neurons (NeuN+) ((v), arrow), astrocytes (GFAP+) (vi) and oligodendrocytes (CNPase+) ((vii), arrow) were also seen, both within the mesencephalic nuclei and dorsal to them. (viii–x) The green DAT-specific signal in (x) suggests that the reconstituted mesencephalic nuclei in the NSC-grafted mice (as in (*a*(viii)) were functional DA neurons comparable to those seen in intact nuclei (viii) but not in MPTP-lesioned sham-engrafted controls (ix). This further suggests that the TH+ mesostriatal DA neurons affected by MPTP are, indeed, functionally impaired. (Note that sham-grafted animals (ix), contain only punctate residual DAT staining within their dysfunctional fibres, whereas DAT staining in normal (viii) and, similarly, in engrafted (x) animals was normally and robustly distributed both within processes and throughout their cell bodies.) (xi–xiii) Any proliferative BrdU+ cells after MPTP insult and/or grafting were confined to glial cells whereas the TH+ neurons (xi) were BrdU−. This finding suggested that the reappearance of TH+ host cells was not the result of neurogenesis but rather the recovery of extant host TH+ neurons. Scale bars: (i) 90 μm; (ii–v) 20 μm; (vi) 30 μm; (vii) 10 μm; (viii–x) 20 μm; (xi) 25 μm; (xii) 10 μm; and (xiii) 20 μm.

DA-depleted areas, the *majority* of DA neurons in the mesostriatal system were actually 'rescued' host cells. Pools of undifferentiated donor NSCs in, and adjacent to, the mesostriatal nuclei appeared to have mediated this 'rescue'. That these pools spontaneously expressed such neuroprotective substances as GDNF provided, in part, a plausible molecular basis for this phenomenon. This unexpected and novel observation suggested that host structures might benefit not only from NSC-derived replacement of lost neurons but also from the 'chaperone' effect of other NSC-derived progeny. This process, dominant in this Parkinson's disease model, probably represents a mechanism of NSC action for a range of neurodegenerative disorders (we have already begun to witness a similar effect in mouse models of amyotrophic lateral sclerosis) and provides insight into the broader use of stem cells from and for other organ systems.

A similar mechanism plays a pivotal and equally unanticipated role in a model of acquired neural impairment, spinal cord injury (figure 5). Murine NSCs were implanted into the extensive injury site that results when the spinal cords of adult rats are subjected to a hemi-resection between thoracic levels 9 and 10 (T9–10) (Teng *et al.* 2002). (As described for the extensive cerebral ischaemic lesions detailed above (Park *et al.* 2002), these NSCs, too, were supported by a biodegradable synthetic scaffold to fix them in space.) Hindlimb deficits were evaluated weekly for up to a year. By 2–3 months post-implant, engrafted rats exhibited coordinated weight-bearing stepping whereas the lesion-control group failed to ambulate even months after injury (see Movie 1 www.pnas.org). Improvement persisted for at least a year (when the study was terminated). Histology, immunocytochemistry (including for expression of GAP-43, a marker of

regeneration) and tract-tracing all suggested that the long-term reduction in functional deficits resulted not from replacement of neural fibres by the NSCs, but rather from their role in providing trophic support, reducing scar formation, increasing the amount of preserved host tissue (including neuronal) by mitigating secondary cell death, promoting host fibre regrowth through the lesion epicentre, and catalysing regeneration of damaged host tissue. Indeed, the NSCs themselves remained largely immature nestin-expressing cells; *not* differentiating into neurons but also *not* becoming astrocytes that might contribute to the glial scar (which, in fact, was significantly diminished). As noted above for neurodegenerative diseases, it appeared that NSCs in their *non-neuronal* state, within the traumatized CNS, also produced growth factors, anti-inflammatory factors, angiogenic factors and differentiation factors, to a degree not witnessed if they had become neuronally committed. Indeed, NSCs in this state may be superior tools for promoting repair in some acquired conditions (e.g. trauma, ischaemia, toxins)—via their promotion of *host* axonal regeneration and/or preservation.

Suspecting that NSCs may constitutively express these factors as part of their fundamental biology, we began to explore the possibility that their natural production of neurotrophic agents (particularly those known to promote sensory and motor axon growth) might be by used intentionally to promote regeneration. To examine, *in vivo*, the effects of NSCs on *host* axonal regeneration, adult rats underwent lesions of the cervical (C3) spinal cord with a microwire knife (Lu *et al.* 2003). This cervical lesion transects the dorsal columns bilaterally, thereby disrupting both descending motor cortico-spinal projections and ascending dorsal column proprioceptive pathways. Murine NSCs were injected into the lesion cavity immediately post-lesion. NSCs survived well *in vivo* after grafting, filling the lesion site, becoming well vascularized. Their extension stopped at the borders of the injury; there was no deformation of the spinal cord or tumour formation. In this model, the NSCs remained undifferentiated, labelling for nestin but not for neuronal, astroglial or oligodendroglial markers. Despite the absence of NSC differentiation, or possibly because of it, axons were observed penetrating the grafts directly from the host stem cell (to a significantly greater extent than control fibroblasts grafts). Furthermore, specific classes of host axons grew extensively within the grafts: motor axons labelled with choline acetyl transferase (ChAT), and sensory axons labelled with calcitonin gene-related peptide (CGRP) or p75. These findings further suggested that NSCs can *inherently* provide permissive substrates and factors to promote growth of host axons *in vivo*. Indeed, attempting to intervene in the natural expression of the various neurotrophic factors in their various proportions through genetic manipulation actually appeared to throw the system into somewhat of an *imbalance* (Lu *et al.* 2003). For example, in the above-described experiments, enhancing expression of NT-3 in a given clone of NSCs actually extinguished its expression of GDNF, obliterating its promotion of *motor* axonal ingrowth, which instead became supplanted by the enhanced ingrowth of *sensory* axons. In other words, manipulating one aspect of a delicately balanced natural system may yield desirable effects if the consequences are understood, but may also yield unanticipated and undesirable effects if the system and its 'logic' from the 'viewpoint' of the NSC are not.

The molecular mechanism underlying all of these above-described observations, we believe, constitutes the normal constitutive expression of yet another developmental program—one we term a '*Micro Program*'—and is discussed in § 9.

9. RECIPROCAL SIGNALLING: EXPRESSION OF DEVELOPMENTAL 'MICRO' PROGRAMS

In preliminary studies, it was determined via enzyme-linked immunosorbent assay (ELISA), Western blots and immunocytochemistry that murine and human NSCs constitutively produce a broad range of peptide neurotrophic and neurite outgrowth-promoting factors that function appropriately in appropriate bioassays (e.g. the promotion of motor neuron outgrowth from organotypic spinal explants). Conditioned medium from NSCs contained significant quantities of NGF (7.5 ± 2.5 pg per 10^6 cells per day), BDNF (7.1 ± 0.1 pg per 10^6 cells per day), GDNF (70 ± 1 pg per 10^6 cells per day) (Lu *et al.* 2003), and others. Fibroblasts expressed no detectable levels. Out of the various factors, GDNF was of particular interest because of its known neuroprotective and outgrowth-promoting effect on such ventralized neural cell types as nigral DA neurons and spinal anterior horn motor neurons—cell types we had established to be impacted *in vivo* by NSCs (Ourednik *et al.* 2002; Teng *et al.* 2002; Lu *et al.* 2003). We elected, in preliminary studies, to use GDNF as an index neurotrophic agent and explore the NSC's regulation of its intrinsic GDNF expression to help reveal what we believed was a little recognized but pervasive NSC developmental mechanism with powerful therapeutic possibilities. In pilot studies, using motor neuron axon outgrowth from spinal explants as a quantifiable bioassay, it was determined that NSCs could mimic the effect of exogenously administered GDNF peptide; GDNF antisense or a soluble 'scavenger' GDNF receptor was sufficient to blunt NSC-induced neurite outgrowth whereas stem cell explants obtained from the ret (GDNF-receptor)-null mouse, when used in this bioassay, elaborated dramatically fewer axons towards the NSCs. Of interest was the observation that, when the progeny of a given NSC clone existed in a *non*-neuronal (undifferentiated or glial) state, intrinsic GDNF expression was robust. Curiously, however, the cell in that state could not respond to the GDNF it had just produced because it did bear a GDNF (ret) receptor. By contrast, when the same clonal progeny was induced to differentiate into *neurons*, GDNF production diminished virtually to nil, but gave way to expression of a functional *ret*-receptor, one that could be appropriately phosphorylated by GDNF. This dynamic suggested a developmental program—a 'micro' program, if you will—by which a single 'mother' NSC gives rise to progeny that, in a symbiotic fashion, provide reciprocal support for each other; serving as '*chaperones*'.

Using subclones of NSCs that contained various neural cell-type-specific promoters driving green fluorescent protein expression to signal when various fate decisions were made by various equipotent members of a given clone, we began to amass preliminary evidence that pointed to an 'autonomous CNS self-assembly program' pursued constitutively by

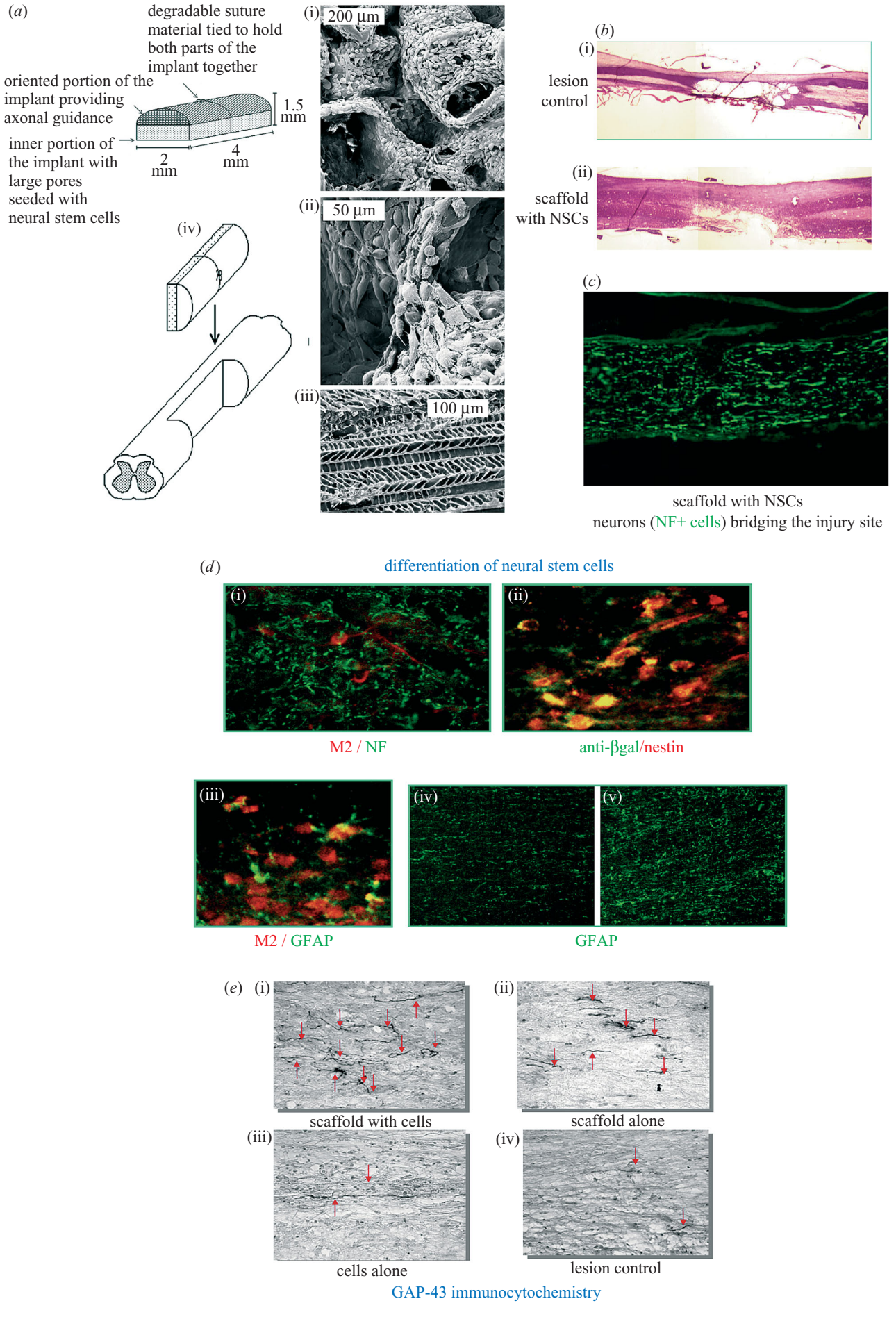

Figure 5. (*Caption p. 835.*)

Figure 5. Functional recovery after traumatic spinal cord injury mediated by a unique polymer scaffold seeded with NSCs (modified from Teng *et al.* 2002). (*a*) Schematics of scaffold design showing inner and outer portions. (i,ii) Inner scaffold seeded with NSCs. Outer scaffold created to have long, axially oriented pores for axonal guidance and radial pores to allow fluid transport and inhibit ingrowth of cells. (iv) Schematic of surgical insertion of implant into SC. (*b*) Based on BBB open-field walking scores, the 'scaffold+NSCs' group showed significant improvement in open-field locomotion compared with 'lesion-control' groups ($p < 0.007$). Histology (H&E) of longitudinal sections from (i) lesioned untreated group and from (ii) 'scaffold+cells' groups was revealing. Note greater integrity of parenchyma in the latter. (*c*) Examination of composition of the tissue at the lesion site demonstrated numerous NF+ cells and process. However, as illustrated in (*d*), the neurons were host and not donor NSC-derived. (*d*) The neurons were host and not donor NSC-derived. The murine NSCs were identified with M2, a mouse-specific marker that works reliably in the rat host stem cell. The mNSCs were neither NF+ (i) nor even GFAP+, the latter finding suggesting that they did not contribute to the glial scar. In fact, glial scarring was diminished in 'NSC+scaffold' stem cells (iv) compared with 'lesion control' stem cells (v) based on GFAP immunoreactivity. Most mNSCs remained undifferentiated nestin+ cells (ii). (*e*) 'Scaffold+NSC' implantation significantly increased the presence of GAP-43+ fibres relative to other controls, a marker for regenerating neurites. After administration of BDA for antegrade tracing, BDA+ axons (not shown, but see Teng *et al.* (2002)) were coursed through the lesion epicentre (as in (*c*)) to reach areas caudal to the lesion in the scaffold-containing groups. This suggests an anatomical substrate for the functional improvement seen in those animals (a mean of 14 on the 21-point BBB scale).

NSCs. Key to that model was the preliminary observation that NSCs that have become neuronally committed (seemingly their default differentiation pathway), appear to actively promote the *non*-neuronal differentiation of their equipotent sister cells via a membrane-associated mechanism. Briefly, within a clone of equipotent sister NSCs, when the first cell exits the cell cycle and commits to a neuronal phenotype (either stochastically, by default, or by instruction) it then actively inhibits the neuronal differentiation of its equipotent sister cells that subsequently exit the cell cycle (even under conditions that would ordinarily propel them towards a neuronal lineage). It effects this inhibition, we believe, via membrane-associated factors that exert their influence by direct cell–cell contact. Interestingly, these factors appear to be independent of *notch-delta* or bone morphogenic protein signalling. Membranes from sister cells that differ only in that they have not made such a neuronal commitment do not have this effect.

Such an autonomous self-assembly developmental scheme establishes a network independent of external instruction wherein a neuron is always flanked by non-neurons (often astroglia). One can envision the primitive fabric of the brain being spontaneously woven based on this scheme starting with a few multipotent NSCs. A possible biological 'rationale' for the existence of such a developmental 'program' is provided by taking into account our recent observations (described previously) of GDNF peptide and receptor expression by various differentiation states of the same NSC. The non-neurons are forced to be 'chaperones' for, and by, their sibling neuron, providing the trophic support needed by the neuron. Such a developmental scheme for NSC-mediated CNS self-assembly, cell-type determination, and 'division of labour', based on intercellular communication between members of a single NSC clone ensures that each neuron is surrounded by cells that can vouchsafe its survival. Although GDNF may be 'intended' by non-neuronal 'chaperones' for support of their juxtaposed *neuronal* clonal members, this factor probably has a broader sphere of influence, including support and/or neuroprotection of 'bystander' host neurons. In other words, when one transplants NSCs into a diseased recipient, host cells, as 'bystanders' of this developmental program, become the indirect beneficiaries of this trophic factor production.

It is also significant to realize that the GDNF is not produced by the NSCs tonically, but seems to be released in a sporadic pulsatile manner. In attempting to dissect the signal transduction pathway mediating GDNF expression and the type of production observed, inhibitors of either the MAP kinase kinase pathway were employed versus inhibitors of the PI3 kinase pathway; the former pathway broadly subserving more permanent changes in neural progenitors (e.g. differentiation, apoptosis, etc.), the latter pathway subserving responses that are more transient adjustments to environmental influences (e.g. proliferation, stress, etc.). Such preliminary experiments suggested that it was the PI3 kinase pathway that mediated the GDNF response. Nitric oxide donor molecules, simulating environmental stress, increased GDNF expression.

Such evidence that GDNF (presumably representative of other neurotrophic agents) is expressed in a regulated, stimulus-appropriate, region- and cell-type-specific manner supports a view that NSCs may serve as better gene delivery vehicles than non-neural cells or non-cellular vectors because the production of neural-relevant gene products by NSCs is part of their fundamental biology. This point was reinforced in a recent experiment (Himes *et al.* 2001) in which the ability of NT-3 to rescue neurons in Clarke's nucleus after axotomy was explored. Rescue was found to be greater when NT-3 was delivered from engrafted NT-3-expressing NSCs than by administering the peptide alone. The suggestion was that the NSCs might provide additional factors or provide more physiological regulated concentrations of the factors, or act as a target or bridge that supports regenerating axons. This observation gets to the heart of some of the advantages of NSCs—whether unmanipulated or genetically engineered—in CNS dysfunction. We believe that trophic support is best supplied by cellular vehicles of neural origin, specifically NSCs, because, as suggested above, these molecules can be released in a regulated fashion, targeted in a site-specific manner from members of the parenchyma, with less concern for transgene downregulation (given their intrinsic basal expression by the NSC) while simultaneously providing the possibility of cellular replacement.

Whether one chooses to use transplanted NSCs or attempts to manipulate endogenous NSCs, an understanding of the reciprocal interactions between genes and NSC biology, between differentiation state and gene expression, and between injured host and NSC, will be critical. Their use must be dictated by a greater knowledge of NSC biology

and of how various neurotrophic agents interact within the NSC and with the degenerating host environment. Isolated, well-characterized, homogenous clones of NSCs may make such study more easily observed and controlled.

10. A 'PROGRAM' MAY CALL FOR NSCs TO MEDIATE THE INTEGRATION AND COORDINATION OF NEURAL WITH NON-NEURAL SYSTEMS

Although we, as scientists, need to focus upon one cell type or one organ system to design interpretable experiments, it should never escape our notice that a given structure in the body is actually not composed simply of one system isolated from the others. For an appendage or an organ to grow, multiple developmental processes, neurogenesis, osteogenesis, angiogenesis, myogenesis, chondrogenesis, etc., must all be intimately coordinated, presumably by a network of constant reciprocal cross-talk between progenitors of the various systems. Furthermore, from the vantage point of a neurobiologist, it is instructive to realize that ultimately every organ is innervated (often by the autonomic, enteric and peripheral nervous systems): every blood vessel, every sweat gland, every sphincter, every bone, and could probably not function without that neural component. An interesting series of pilot experiments has hinted at the pivotal role the nervous system, and the NSC as its effector, may play in initiating, catalysing, directing and synchronizing the assembly of some of these organs, exemplified by the neuromusculo-skeletal-vascular unit. Another 'Macro-Program' may be suggested.

In the first pilot experiments, rat myoblasts and murine NSCs were mixed in a collagen solution and injected subcutaneously in a nude mouse. Although each cell type injected alone failed to flourish and died, in combination, the cells began to self-organize into a mass that resembled muscle tissue. Moreover, this subcutaneous 'muscle' began to attract innervation from the intercostal nerves and vascularization from the intercostal arteries of the adjoining thoracic segment. Ultimately, a mature capillary network permeated the new muscle. The presence of *bona fide* muscle tissue was confirmed by immunostaining for myosin heavy chain. The mature myofibrils, it is crucial to emphasize, derived from the implanted rat myoblasts; they did *not* result from transdifferentiation of the murine NSCs. (The species differences between the two progenitor populations ensured that proper lineage could be assigned.) Organized muscle bundles were, however, surrounded by undifferentiated NSCs.

In the next pilot experiments, this paradigm was extended to a more physiologically relevant context to observe whether NSCs could, indeed, promote skeletal muscle regeneration by catalysing the creation of a functional muscle *in situ* and guide its local innervation and vascularization. The tibialis anterior muscle was entirely removed from a nude mouse. A combination of murine NSCs and rat myoblasts (supported by biological matrix, bladder submucosa) was implanted into the empty muscle bed. After three weeks, once again, the implanted cells began to self-assemble into a coherent mass. Mature muscular bundles coursed throughout the new tissue and NSCs had distributed themselves evenly throughout. New vessels penetrated the mass. Importantly, a branch of the sciatic nerve could be seen coursing towards and entering the new muscle. Again, the new myofibrils were derived from the myoblasts, not from the NSCs; there was no transdifferentiation. Interestingly, the NSCs also did not differentiate into neurons. Rather they interacted with the regenerating sciatic nerve endings, guiding them to the acetylcholine receptors on the new muscle. When the sciatic nerve was electrically stimulated, the new mass contracted appropriately, a contraction that could be measured electrically and recorded. The creation of a contractile organ reactive to electrical stimulation emerged only when both NSCs and myoblasts were intermixed; samples containing no cells, or bladder submucosa alone, or solely NSCs or solely myoblasts produced no contractile structure.

In summary we, and others, have spent a good deal of effort demonstrating that NSCs interact with their neural environment. For the first time, however, we have begun to observe an additional set of programs that make immense sense for a more global view of organogenesis: that NSCs interact with and help promote the development of other systems with which they are in intimate contact; for example, muscle, vasculature, skin, etc. In these preliminary experiments, NSCs acted on local non-neural progenitors to create an innervated, functional contractile structure assimilated appropriately *in vivo*. The NSC may prove to be a useful tool for dissecting the developmental processes by which a functional organ emerges from the interactions between seemingly disparate components. In acquiring a better understanding of this process, we may also gain an insight into how to use NSCs for promoting the repair and regeneration of even non-neural systems.

11. CLOSING THOUGHTS

In summary, NSCs are not only able to mediate cell replacement and gene therapy, but also to perform other intriguing functions as a result of their fundamental biology and the role they play in some complex developmental programs. For example, transplanted NSCs trigger and/or augment constitutive reparative responses by facilitating a series of reciprocal interactions between themselves and host CNS tissue (both injured and intact) including:

(i) promoting neuronal differentiation;
(ii) enhancing ingrowth/outgrowth of neural processes;
(iii) fostering reformation of neural tissue;
(iv) promoting connectivity after injury;
(v) promoting preservation of parenchyma;
(vi) reducing secondary injury processes;
(vii) reducing inflammation/scarring (facilitating reconstitution);
(viii) coordinating interaction among other somatic systems.

The study of NSCs by neurobiologists over almost the past two decades has helped stimulate investigators of other organ systems to search for 'stem-like' cells even within tissues generally held to be more regenerative, more forgiving and/or more redundant than the CNS. The results have been rewarding indeed. Hence, the NSC—in effect the first 'solid organ stem cell' isolated and exploited—served, and continues to serve, as a model for other somatic stem cells. Importantly, despite the spotlight of therapeutic promise the NSC has thrown upon itself

(and other stem cells), it is critical to remember that its existence was unveiled in the course of understanding *development* and that, in the end, the stem cell is simply one player in a broad and exceedingly complex, interdependent, finely tuned developmental system; one that requires fundamental developmental understanding. In this endeavour, the CNS continues to serve as an instructive model for the stem cell field in general.

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GLOSSARY

BBB: Basso–Bresnahan–Beatie
BDA: biotinylated dextran amine
BDNF: brain-derived neurotrophic factor
BrdU: bromodeoxyuridine
CNPase: 2′3′-cyclic nucleotide-3′-phosphodiesterase
CNS: central nervous system
DA: dopaminergic
DAT: dopamine transporter
GDNF: glial-cell-line-derived neurotrophic factor
H&E: haematoxylin and eosin
HI: hypoxic–ischaemic
hNSC: human neural stem cell
MPTP: methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF: neurofilament
NGF: nerve growth factor
NSC: neural stem cell
NT-3: neurotrophin-3
OB: olfactory bulb
PGA: polyglycolic acid
RMS: rostral migratory stream
SN: substantia nigra
SVZa: anterior subventricular zone
TH: tyrosine hydroxylase
VTA: ventral tegmental area