Neural Stem Cell Systems: Diversities and Properties after Transplantation in Animal Models of Diseases

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Currently available effective treatments of the diseased or damaged central nervous system (CNS) are restricted to a limited pharmacological relief of symptoms or those given to avoid further damage. Therefore the search is on for treatments that can restore function in the CNS. During recent years replacement of damaged neurons by cell transplantation is being enthusiastically explored as a potential treatment for many neurodegenerative diseases, stroke and traumatic brain injury. Several references in both scientific journals and popular newspapers concerning different types of cultured stem cells, potentially exploitable to treat pathological conditions of the brain, raise important questions pertinent to the fundamental and realistic differences between grafts of primary neural cells and the transplantation of *in vitro* expanded neural stem cells (NSCs). Our aim is to review the available information on the grafting of different NSC types into the adult rodent brain, focusing on critical aspects for the development of clinical therapies to replace damaged neurons.

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INTRODUCTION

In the last two decades, cell replacement approaches have offered a basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of brain diseases (12). These therapeutic approaches are based on the idea of transplanting cells into the central nervous system (CNS) that are able to substitute for lost elements and rewire interrupted circuitries, thus reestablishing regular brain function. An alternative and possibly more realistic goal in cell transplantation approaches considers the possibility that the transplanted cells may act in a paracrine manner to influence the survival and activity of endogenous cells (76).

As mature neurons do not readily survive the necessary dissection and grafting procedures, immature or proliferating cells constitute the best source of donor cells for transplantation to the CNS. Transplantation experiments in animal models of brain lesions or neurodegeneration have revealed that fetal cells (ie, freshly dissociated primary cells) are capable of integrating into the host brain and ameliorate functional defects. Based on these results, clinical trials have been carried out in patients affected by Parkinson's Disease (PD), Huntington's Disease (HD) and vascular and traumatic brain damage (12, 83). While these trials have provided results on safety and, in individual cases, on short- or long-term efficacy of this approach, there are well-known technical and ethical difficulties in relying exclusively on cells that have to be extracted from fetal tissues recovered from elective abortions. For example, the composition and status of the donor cells cannot be standardized because they have to be extracted *ex novo* prior to every single surgical session. In addition, the yield of extraction is low and the fetal material scarce. While spontaneously aborted fetuses are exempted from ethical problems, they are unlikely to represent a reliable source for future clinical trials because of the possibility of intrinsic genomic alterations, serious difficulties in identifying the tissue or region of interest, and the lack of experimental evidence of the properties and efficacy of the donor cells in animal disease models (91). The demonstration of the existence of cells in the developing and adult nervous system that can be isolated and grown *in vitro*, where they behave as *bona fide* neural stem cells (NSCs), has provided new strategies to the field (64). Current approaches are therefore aimed at exploiting the intrinsic regenerative potential of such cells through their *ex vivo* or *in vivo* experimental manipulation (3, 76, 77).

NSCs are defined by two functional traits: (i) their ability to self-renew and to generate identical progeny and (ii) their multipotentiality, that is, the ability to generate cells capable of producing neurons, astrocytes, and oligodendrocytes (31). These cells are often confused with neural precursors, which are expandable cells lacking the capacity for self-renewal and exhibiting a more restricted potential for differentiation (70, 106).

A remarkable number of papers have been published in the last 10 years in which protocols to isolate and grow, in an unlimited fashion, putative NSCs in a laboratory setting have been described. These cells have been transplanted in many models of neurodegenerative diseases. However, in most cases, the beneficial effects observed did not seem to derive from cell replacement or rewiring of the donor cells into neuronal circuitries, but rather from their ability to release molecules capable of stimulating or inhibiting the activity of endogenous cell elements. Thus, when considering the transplantation of tissue-derived NSCs, a great deal of basic research is still needed, because their *in vitro* properties remain poorly understood, and other strategies to obtain cells from alternative sources than the CNS, such as embryonic stem cells (ESCs), are under active consideration.

SOURCES AND METHODS TO GENERATE AND EXPAND MAMMALIAN NSCS

The ideal cell for brain transplantation approaches should be extremely plastic in its nature. Stem cells match this condition given their intrinsic capability to respond to different environmental cues. Both ESCs and tissue-derived NSCs might represent the perfect source for cell replacement approaches in the CNS. Even in the absence of a fully exhaustive molecular characterization, it is evident that NSCs possess features that are extraordinarily promising for the goals of restoring normal CNS functionality. They are undifferentiated, endowed with proliferative capacity, often highly mobile cells, relatively resistant to hypoxia and other injuries and able to produce mature neurons and glia (106). ESCs also have extraordinary capacities for self-renewal and differentiation (99). For years they have been the only cell type that could be propagated in homogenous cultures. These and other properties have made ESCs extremely powerful tools to uncover molecules and pathways involved in self-renewal and lineage specification. Their ability to differentiate into multiple cell types has made them extremely useful for biological studies, drug screening and experimental transplantation (14, 36).

Embryonic stem cells. Embryonic stem cells (ESCs), derived from the inner cell mass (ICM) of the pre-implantation embryo, have the widest developmental potential. ESCs can be propagated indefinitely under defined *in vitro* conditions while retaining a normal pluripotency and karyotype (99). Classically, mouse ESCs are maintained in a serum-supplemented medium on a feeder layer or grown on a gelatin substrate, in feeder-free conditions, in the presence of serum and leukemia inhibitory factor (LIF) (99). LIF and BMP4 have been shown to be capable of sustaining the serum-free feeder-free expansion of ESCs (116). ESC lines can be established from virtually all mammals although relevant differences are observed among different species (82). Human ESCs, for example, are known to have characteristics distinct from mouse ESCs, including slower doubling time, different *in vitro* needs for self-renewal, diverse morphological features and different cell surface antigen phenotype.

The power of ESCs lies in their largely homogeneous propagation and in the possibility of enriching for undifferentiated stem cell-like or progenitor populations as well as differentiated cell types (99). Neural cell types can be efficiently generated from mouse and human ESCs. Originally, the generation of neurons of various subtypes, as well as of astrocytes and oligodendrocytes, was accomplished by using embryoid body (EB) differentiation in the presence of retinoic acid (5, 78). Subsequently, EB differentiation combined with genetic lineage selection have enabled the initial enrichment of neural progenitor cells (60). More recently, methods have been described that enable the precise conversion of an ESC into a neural fate in adherent monolayer cultures without feeders, serum or addition of exogenous growth factors (107, 118). These protocols have provided the foundation for studies aiming at identifying the molecular mechanisms of neural induction, although the scientific debate about inductive or default models is still ongoing (100).

Although ESCs have remarkable capacities for self-renewal and phenotypic plasticity, their tumorigenicity after *in vivo* transplantation represents a major obstacle to their clinical use (13). To partially overcome such a limitation, protocols have been developed to obtain *in vitro* high numbers of cell type-specific neural precursors (eg, motor neurons, dopaminergic neurons, oligodendroglial cells) from ESCs (6, 15, 48, 111). To these efficient inductive protocols, lineage selection-based strategies have been added in order to eliminate residual ESCs in the cultures prior to grafting (109). Nevertheless, the development of homogeneous ESC-derived long-term expandable tissue-restricted stem cell lines would represent the safest solution. The generation of dopaminergic neurons from mouse and human ESCs has been extensively investigated by various researchers in the last decade (6, 13, 48, 51, 58, 119). In the developing CNS, the induction of midbrain dopamine-producing neurons is dependent on the soluble signaling molecules fibroblast growth factor (FGF-8) and sonic hedgehog (Shh). Treatment with these two factors during the generation of neuronal precursor cells resulted in a considerable amount of mouse ESCs differentiating into tyrosine hydroxylase (TH)-expressing neurons *in vitro* (58).

Genetic manipulation has also been exploited for the generation of dopaminergic neurons from ESCs. ESC-derived dopaminergic neurons have been obtained by generating stable nuclear receptor-related (Nurr)1 ESCs. Nurr1 is a transcription factor that has been shown to play a role in the differentiation of midbrain precursors into dopamine neurons and its overexpression in mouse ESCs promotes an increase in the proportion of TH^+ neurons from 5% to 45% *in vitro*. This proportion was further increased by combining Nurr1 overexpression with FGF-8 and Shh stimulation, resulting in nearly 80% of Nurr1–mouse ESCs differentiating into TH-expressing neurons *in vitro* (22, 51). Similarly, differentiation of spinal motoneurons has been achieved by exposing ESCs to a specific sequence of environmental stimuli, known to regulate motoneuron generation *in situ*, such as retinoic acid and Shh (111). Other strategies have employed a protein named stromal cell-derived inducing activity (SDIA) in order to induce and support neural differentiation of mouse ESCs into dopaminergic neurons *in vitro* (48, 53, 73).

Although extremely significant technical progress has been reported in the last few years, the future development of therapeutic approaches based on human ESCs is at risk because of ethical constraints related to the use of human blastocysts to generate new ESC lines, a controversial issue that has been pushed forward by part of society. While clear rules and legislation are present in countries like the United Kingdom, where this research area has been strongly supported, allowing the use of public funds for obtaining new ESC lines from the surplus of frozen blastocysts derived from *in vitro* fertilization procedures or from embryos produced by nuclear transfer or parthenogenetic egg activation for research investigation, other countries are still waiting for clearer legislation in this area and have to rely on the use of preexisting cell lines.

Brain-derived NSCs. The study of mammalian NSCs made its first progress toward the end of the 1990s, when it was demonstrated that proliferating cells isolated from the forebrain can differentiate

into neurons *in vitro* (18, 88, 89, 105). These results and subsequent technical progress, including the improvement of molecular probes and immunocytochemical tools, allowed for more reliable and accurate identification of the phenotype of the various neural cells, thus prompting strong interest and development in the NSC field.

Different groups have shown that cells with properties of NSCs could be isolated from the fetal telencephalic germinal zone [or adult subventricular zone (SVZ)] and propagated as neurospheres, a floating clump of cells that would form promptly upon plating onto uncoated dishes. In the absence of efficient and validated procedures for their prospective isolation, the ability to grow these cells *in vitro* as neurospheres has been used as an operative definition for NSCs. Neurospheres have been prepared from many regions of the fetal CNS (56, 66, 87, 115). Noteworthy, many studies focused on early passaged neurospheres assuming that the results obtained at this stage could be extended and generalized to mid-term and long-term expanded neurosphere cultures. However, interpretation of results from studies performed at these early passages may be difficult given the heterogeneous composition of the cultures (90). On the other hand, at subsequent passages, when enrichment of NSCs is expected, the neurospheres seem to change in composition and become more gliogenic (103, 112). According to available data, neurospheres may contain a variable fraction of NSCs (ranging from 0.4% to 5%, depending on the developmental stage, the area and the different growth procedures employed by the various groups) at early passages but become progressively depleted during *in vitro* growth. In addition, transformation events may take place over time *in vitro* (75).

Given the mixed nature of the neurospheres, results from transplantation or gene expression profiling are also difficult to trace back to the activity of the stem cells present in the culture (104). Nevertheless, the neurospheres have proved invaluable as a system for estimating neural stem/progenitor number and their ability to release factors has generated beneficial results in experimental transplantation paradigms. As a consequence of the limits described above, neurospheres have been less useful in the molecular dissection of events that control self-renewal, or in the identification of the nature of neurosphere-forming cell.

Commonly, tissue samples used for the isolation of mouse NSCs are harvested from embryonic or fetal brain by standard micro-dissection procedures. Most studies employ fetal tissue taken from rodent embryos ranging from embryonic day 14 (E10.5) to E18. The tissue is typically triturated by gentle mechanical dissociation to a nearly single-cell suspension (87). Some laboratories use proteases (generally, collagenase or trypsin) together with DNAse treatment in order to allow complete dissociation of the tissue. Precursor cells removed from the brain are cultured in growth factor-supplemented serum-free medium. For the expansion of NSCs, cells are plated in low-attachment culture flasks in serum-free media containing supplements, such as N2 or B27 and, in order to promote replication, the medium is usually supplemented with epithelial growth factor (EGF; 10–20 ng/mL) and/or FGF-2 (10–20 ng/mL) (46). There have been also reported alternative protocols in which EGF is employed alone as a mitogen. In some cases of human NSCs cultures, LIF has also been added to stimulate proliferation (7, 17). The details of this process, including the particular regions from which the cells are derived, the media in which they are grown, and the substrates on which they are plated, can have significant effects on the fate of the precursors (49, 121).

Generally, the differentiation protocols for NSCs rely on the removal of growth factors or mitogens in serum-free media on standard culture growth surfaces, such as poly-L-lysine or laminin. It is also known that the differentiation program can be influenced by exposure to various factors, such as growth factors or cytokines (46). In the CNS, the relevant patterning along the anterior–posterior (A-P) and dorsal–ventral (D-V) axes takes place early on, along with neural induction (95). Accordingly, it has been suggested that stem cells and restricted precursors display regionalization. Spinal cord stem cells generate spinal cord progeny, whereas stem cells from the forebrain generate more γ-aminobutyric-acid-containing neurons than dorsal stem cells cultures under identical conditions. Consistently, only NSCs isolated from the ventral midbrain differentiate into functional mature dopaminergic neurons. There are several reports in the literature indicating that neurospheres generated from different CNS regions express region-specific markers and generate region-specific progeny, although most of these studies have been conducted on shortly expanded neurosphere cultures (reviewed by 90).

The presence of stem cells has also been extensively investigated in two regions of the adult mammalian brain, the SVZ and the hippocampus (27, 81). The NSCs derived from these regions can self-renew and differentiate into neurons, astroglia, and oligodendroglia *in vitro*.

Although the identity of the adult NSCs in the SVZ is still debated (74), a number of experiments have been carried out to influence their fate and study their potential, both *in vitro* and *in vivo*. *In vitro*, SVZ NSCs have been exposed to several soluble and adhesion factors in order to test their behavior. Generally, EGF and FGF-2 both induce the proliferation of SVZ NSCs and can influence their differentiation (38, 39). EGF tends to direct cells to a glial fate, FGF-2 is more neurogenic (110) while bone morphogenetic proteins promote differentiation of SVZ NSCs into an astroglial fate (35). By contrast, brain-derived neurotrophic factor (BDNF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-1) promote differentiation of SVZ NSCs into neurons and support their survival (8, 98).

Hippocampal precursor cells are studied *in vitro* much like SVZ NSCs: they are removed from the brain, dissociated, and typically cultured in EGF and/or FGF-2. To induce differentiation, the mitogens are removed, and the cells exposed to the growth factors or supplements of interest. Hippocampal precursors proliferate in response to FGF-2 and can differentiate into astroglia, oligodendroglia, and neurons *in vitro*. BDNF increases both, neuronal survival and differentiation, while NT-3, NT-4/5, and ciliary neurotrophic factor (CNTF) might have more limited effects (44, 55, 80, 81, 101). Further demonstrating the existence of precursors in the adult human, multipotent precursors derived from the adult human brain can be cultured *in vitro* (92).

Novel NSC systems: NS cells. Homogenous propagation of somatic stem cells *ex vivo* has always been a major challenge for biologists, based on the hypothesis that specialized niches or microenvironments would be necessary in order to avoid stem cells differentiating (1, 26, 30, 67, 102, 108). With respect to NSCs, their growth as neurospheres finds support from the assumption that the heterogeneous composition of the aggregate would reconstitute a niche-like environment (1, 16, 33). This idea has been challenged by the recent demonstration that NSCs grown in monolayer and in serum-free media could be propagated in homogeneous cultures (24). Prior to this study, the ability to expand long-term stem cells in a precisely controlled and homogeneous manner was restricted to ESC cultures (19, 99). Thanks to these well-characterized experimental conditions, ESC technology has revealed its potential by capturing undifferentiated stem cell-like or progenitor populations as well as fully differentiated cell types. Protocols and strategies have been developed in order to efficiently generate neural cell types from mouse ESCs (6, 9, 65, 94, 109, 117).

A recent research effort performed in the context of EuroStemCell (http://www.eurostemcell.org) has allowed for the first time the identification of a novel and powerful strategy for NSC derivation and stable propagation. Starting from ESCs, it was demonstrated that ES-derived neural precursor cells, normally fated to rapidly differentiate to neurons and glia, can be efficiently expanded as adherent clonal stem cell lines by means of EGF and FGF-2 stimulation (24). Importantly, cells in these conditions undergo symmetrical divisions without accompanying differentiation and while retaining the ability to generate neurons and astrocytes *in vitro* and upon transplantation after long-term expansion. Molecular characterization of these cells revealed a close correspondence to forebrain radial glia, recently indicated as the fetal source of both neurons and glia during CNS development (37, 42, 54, 68). In a previous study, cells with similar properties were only transiently present in culture (9). Instead, our results indicate that pluripotent ESCs can be efficiently converted to distinct homogeneous somatic stem cell lines, which have been named NS cells (from Neural Stem), that can be expanded *in vitro* in adherent conditions in the long term. Additionally, this study indicates that expansion of these EGF-dependent NS cells can occur in the absence of a complicated cellular niche. Thus, adherent NS culture appears to suppress spontaneous differentiation and allow propagation of homogeneous radial glia-like stem cells. Importantly, we have demonstrated that NS cells are not an unusual property of ESC differentiation. In fact, similar lines can also be obtained from fetal or adult CNS and from established long-term neurosphere cultures. It is therefore possible that NS cells represent the resident stem cell population within neurospheres. Further characterization of different mouse NS cell lines has demonstrated a close similarity in self-renewal, neuronal differentiation potential and molecular markers, independently from their origin. NS cells are expanded in defined serum and feeder-free basal media and divide through symmetrical self-renewal with very little spontaneous cell death or differentiation. This homogeneity and unlimited expansion are particularly well suited for approaches aimed at investigating stem cell self-renewal and differentiation. NS cells are a versatile model system for studying basic questions in stem cell biology and may represent the ideal system to create cellular models of neurodegenerative diseases, given their ability to differentiate into electrophysiologically active neurons.

The attributes of NS cells render them particularly suited for high-throughput chemical screening to identify small molecules that control stem cell behavior. Because of their purity and stability upon extensive *in vitro* expansion and absence of tumorigenic potential upon transplantation, NS cells may also represent a valuable source of cells for transplantation approaches in models of neurodegenerative diseases. Nevertheless, further studies have to be performed in order to test their ability to generate various neuronal lineages *in vitro* and *in vivo* and to assess their long-term stability and functional *in vivo* reconstitution.

CONCEPTS AND CRITERIA TO BE FULFILLED FOR STEM CELL THERAPY IN BRAIN DISEASES

The ability of NSCs to divide and adopt specific phenotypic traits *in vitro* does not guarantee that, once transplanted, they will actually be incorporated into the recipient tissue leading to functional repair. In fact, to produce benefits (i) donor cells may need to achieve a certain degree of morphological and functional integration into the host tissue. In addition; and (ii) inductive signals present in the adult damaged brain may more promptly direct the donor cells into a gliogenic rather than neuronal differentiation program.

In the case of neuronal degeneration, the success of cell replacement is also likely to depend on the complexity and precision of the pattern of connectivity that needs to be restored. In PD, the affected dopaminergic neurons in the substantia nigra (SN) exert a modulator action on the target circuits (the striatum) mostly through the release of diffusible molecules (dopamine) (10, 64). This system is defined as "paracrine" and, in such conditions, even a partial pattern repair may lead to a significant functional recovery. The donor cells are transplanted directly into the target region to circumvent the problem of long-distance neuritic growth in the adult CNS. Despite the ectopic location, grafted fetal mesencephalic cells produce dopaminergic neurons and restore a regulated and functionally efficient release of dopamine in both experimental animals and in a number of patients. Nevertheless, these results are obtained after transplantation of freshly dissociated fetal mesencephalic cells or tissue pieces, which are limited in amount and contain immature progenitors already committed to generate dopaminergic neurons. When using *in vitro* expanded adult neurospheres that contain a number (albeit low) of neural stem-like cells, the situation becomes even more complicated, by virtue of the heterogeneity of the cultures and, more importantly, by the fact that cells are not specified to a given phenotype prior to transplantation and will have to rely on the *in vivo* environment for subsequent specification. Additionally, in most of the cases, implantation of cells from adult neurospheres into the brain leads to the generation of glial cells and in few other cases, cells remain fully undifferentiated (86). Diseases in which the need for pattern repair may be even more demanding include HD, amyotrophic lateral sclerosis (ALS) and diseases characterized by global degeneration such as trauma, stroke, and Alzheimer's Disease (AD) (91).

Although pattern repair is critical to obtain permanent efficacy in the brain, it should be emphasized that stem cells transplanted into the brain may also be beneficial via the release of molecules that may either stimulate the regenerative potential of local cells (where present) or increase the survival (and decrease disease progression) of the remaining host elements.

Differently from the abovementioned situations, a good target of cell replacement with limited requirements of pattern repair is represented by diseases characterized by glial degeneration, such as multiple sclerosis (MS) (85). Attempts in transplanting putative adult NSCs (in the form of neurosphere-forming cells) have produced encouraging results in an animal model of the disease. Although the nature of the cells and the mechanism by which recovery is obtained appears to be independent from cell replacement events, this result shows that transplanted adult stem-like cells can be beneficial, at least in the short term (84, 86).

NSCs grafting in models of PD. The potential of cell transplantation for neurodegenerative diseases was first challenged in clinical trials for PD (11). So far, transplantation of primary fetal tissue has been completed in more than 300 patients with PD (28, 63). Prior to the beginning of the clinical trials, the characteristics and properties of the primary cells that were to be transplanted had been extremely well described in the literature. On the contrary, the use of expanded NSCs is less developed and understood. The potential of ESC-derived cells for PD has been analyzed in several experimental paradigms. However, since the early studies, the therapeutic use of ESCs was hindered by the intrinsic capability of ESC to generate teratomas and to give rise to extraneural cell types when grafted to the brain at high density.

In an attempt to avoid these undesired effects, Bjorklund and coworkers, grafted low density poorly differentiated mouse ESCs into the striatum of 6-OHDA-lesioned adult rats (13). In this study, 56% of the animals exhibited differentiation of the grafted ESCs into mature dopaminergic neurons, with significant improvements in motor asymmetry occurring at 7 and 9 weeks post transplantation. Restoration of hemodynamic response to an amphetamine challenge in the grafted striatum, also measurable on functional magnetic resonance imaging (MRI), further indicated that donor cells integrated into the host striatum as functional dopaminergic neurons. The remaining 44% of grafted animals did not exhibit any dopaminergic differentiation and, among them, six recipients did not show surviving grafts. Furthermore, five animals that did not survive were found to have teratoma-like masses at the transplant site and in three additional animals mesodermal or ectodermal markers were found in the grafts.

Using a different inductive protocol, Kawasaki et al generated neurons expressing TH from cynomolgus monkey ESCs by using a 12-day coculture with PA6 stromal cells, followed by mitomycin C treatment in order to eliminate mitotic cells (47). The cultures were transplanted into the striatum of 6-OHDA-lesioned parkinsonian mice. Fifteen days after grafting, the survival rate of TH-expressing neurons was 22%, notably greater than the 5%–10% survival rate in primary human fetal dopaminergic neurons. Under these conditions, no teratoma formation was observed in any grafted animal. These results are quite noteworthy because they present for the first time the implementation of two strategies that could advance the practical use of ESC grafts: pretransplant induction of a desired phenotype and elimination of tumorigenic cells.

A different method, involving direct genetic manipulation of ESCs, was employed by Kim and coworkers (51). These authors have transduced mouse ESCs with the nuclear cell receptor Nurr1, a transcription factor directly activating transcription activity of the TH promoter. Grafting of the Nurr1-transduced cells into the striatum of 6-OHDA-lesioned rats led to considerable behavioral improvement. Noteworthy, treatment of Nurr1–mouse ESCs with FGF-8 and Shh enhanced the differentiation into TH-expressing neurons *in vitro*. When these cells were transplanted into the striatum of immunosuppressed 6-OHDA-lesioned rats, a significant number of them exhibited both immunohistochemical and electrophysiological properties of midbrain dopaminergic neurons. These neurons innervated the striatum by extending processes up to 2 mm from the graft site and forming functional synapses. Behavioral studies also showed a significant reduction in motor asymmetry in the animals that had undergone transplantation. Once more, the induction of differentiation prior to transplantation resulted in the absence of teratoma formation, even 8 weeks post transplantation and grafts were also shown to be negative for the expression of the proliferation marker Ki-67. Although a long-term follow-up of the behavior of the transplanted cells is not available, this study indicates the potential of the combination of genetic and epigenetic manipulation in order to commit ESCs to a non-tumorigenic, well-differentiated state.

More recently, Barberi and colleagues described a more efficient method of generating multiple subtypes of neurons from both fertilization and nuclear transfer-derived ESCs (6). In this study, human ESCs were originally cultured on stromal cell feeder layers, regional fate specification was guided *in vitro* by manipulating growth environment in a step-by-step manner, similar to what occurs during development *in vivo*. The authors have transplanted their "committed" human ESCs and could demonstrate that cells surviving the grafting procedure lead to a behavioral recovery in 6-OHDA-lesioned mice. A couple of months after transplantation, grafted animals demonstrated more than 70% functional improvement in rotational asymmetry tests. In addition, the dopamine neuron survival rate *in vivo* was higher for nuclear transfer-derived ESC grafts, demonstrating the potential of therapeutic cloning at least in this disease model.

Less promising results have been achieved with transplantation of brain-derived expanded neurospheres. Ostenfeld and colleagues have assessed the ability of long-term expanded human fetal NSCs (in the form of neurospheres) to survive and mature in the striatum of 6-OHDA-lesioned adult rats (79). Cortical precursor cells derived from an 11-week-old human fetus were expanded 100-fold prior to transplantation. Around 20 weeks post transplantation, human neurofilament-positive neurites were observed throughout the rat striatum, with less than 1% of grafted cells still dividing. Few transiently TH-expressing cells were observed at 6-week survival, indicating the need for more

precise characterization and modification of the cells prior to transplantation in order to obtain effective differentiation.

Armstrong et al have investigated the ability of expanded porcine fetal NSCs to reconstruct the nigrostriatal circuitry in 6-OHDA-lesioned rats (2). Most of the research in rodents has demonstrated that allografts to the lesioned SN do not result in fiber projections that reinnervate the striatum, a circumstance necessitating the ectopic placement of primary fetal grafts in the striatum in humans. In contrast, grafted porcine forebrain-derived fetal NSCs differentiated into neurons capable of projecting extensively along white matter tracts and of forming synapses in targets appropriate to their implantation site. Nine weeks after transplantation, intramesencephalic grafts projected to the striatal complex, and intrastriatal grafts projected abundantly to the pallidum, entopeduncular nucleus, and the SN. Less than 1% of graft-derived neurons expressed TH, however, with no evident functional recovery. The failure of most grafted cells to adopt a dopaminergic fate also occurred with porcine fetal NSCs expanded from the ventral mesencephalon.

Sanchez-Pernaute and coworkers were to some extent successful in facilitating survival of TH-expressing donor cells in parkinsonian rats by inducing the differentiation of a subgroup of short-term cultured human ventral short-term expanded mesencephalic fetal NSCs with dibutyryl cyclic adenosine monophosphate and ascorbic acid treatment for 2 days prior to transplantation (93). Six weeks after intrastriatal transplantation, grafts confirmed robust TH expression, and a significant reduction in amphetamine-induced circling behavior. Nevertheless, it remains to be determined whether these cells retain an identical skill for TH instruction after expansion in culture for longer periods of time.

In agreement with the idea of neuronal progenitor turnover in non-neurogenic areas, multipotent progenitors from non-neurogenic regions have been successfully isolated from the adult rat SN by using a buoyancy separation method. Lie and colleagues used the buoyancy partition method to separate neuronal progenitors *in vitro* from the adult rat SN (62). When these isolated SN progenitors were expanded *in vitro* in the presence of FGF-2 or FGF-8, the exposure to retinoic acid induced neuronal differentiation but, once transplanted into a healthy adult SN, these SN-derived progenitor cells were exclusively capable of producing a glial phenotype.

Recently, Dziewczapolski and colleagues reported the first results on the transplantation of adult rat hippocampal progenitor cells (AHPs) into the striatum of a rat model of PD (29). In an effort to increase the capacity of neuronal differentiation, rat AHPs were primed with retinoic acid 1 day prior to transplantation, and retinoic acid was administered daily following transplantation. Although 60% of grafted rat AHPs survived, exhibited widespread distribution in the striatum, and integrated morphologically with host tissue, the expression of the neuronal marker β3-tubulin was exceedingly rare (0.1%).

NSC grafting in models of HD. Neuro-transplantation has been proposed over the last years as a potential treatment for HD. To modify disease progression, fetal tissue transplantation into the striatum has been tested in humans, and has been found to show some transient favorable effects. As for PD, the first clinical trials employed freshly isolated cells taken from the ganglionic eminence of aborted fetal material. In the year 2000, Bachoud-Levi and colleagues (4) explored in an open-label trial whether grafts of human fetal striatal tissue could survive and have detectable effects in five patients with mild to moderate HD. This clinical condition seems to be a requirement for fetal transplantation. In fact, patients with advanced HD are at risk for subdural hematoma (SDH) after transplantation surgery (43). After 2 years of preoperative assessment, patients were grafted with human fetal neuroblasts into the right striatum followed by transplantation into the left striatum after 1 year. The first results were assessed 1 year later on the basis of neurological, neuropsychological, neurophysiological, and psychiatric tests. The results obtained were compared with those of a cohort of 22 untreated patients at similar stages of the disease who were followed up in parallel. The positron emission tomography (PET)-scan assessment showed increased metabolic activity in various subnuclei of the striatum in three of five patients. These results contrast with the progressive decline recorded in the two other patients present in the trial, which was comparable to the decline seen in patients with untreated HD. Small areas of even higher metabolic activity, coregistered with spherical hypo signals on MRI were also present in the same three patients, suggesting that grafts were functional. Accordingly, motor and cognitive functions were improved or maintained within the normal range, and functional benefits were seen in daily-life activities in these three patients, but not in the other two. The improved clinical changes in these three patients were associated with a reduction of the striatal and cortical hypometabolism, demonstrating that grafts were able to restore the function of striato–cortical loops (34). A long-term follow-up study (ie, 6 years after surgery) indicated that transplantation of primary neuronal cells in HD provides a period of several years of improvement and stability, but not a permanent cure for the disease. It is therefore possible that even in the presence of ideal stem cell lines, future therapeutic strategy against the disease will have to rely on a combination of principles, including neuroprotection and neuronal replacement (91).

Recently, it has been found that intrastriatal transplants of human fetal cortical cells expanded *in vitro* as neurospheres elicit behavioral and anatomical recovery after transplantation into the striatum of quinolinic acid (QA)-lesioned rats (69). Twenty-four rats received unilateral QA injections into the striatum and 1 week later, rats were transplanted with cells derived from human fetal cortex (12 weeks post conception) that were pretreated in culture media with the differentiating cytokine CNTF. Each rat was injected with a total of 200 000 cells and a third group of rats was given a sham injection of vehicle. It was found that rats transplanted with the *in vitro* expanded cells performed significantly better over the 8 weeks of testing on the cylinder test compared with those treated with vehicle. Moreover, treating neurospheres with CNTF led to increased striatal volume. Grafted cells were also observed in the globus pallidus, entopeduncular nucleus, and SN pars reticulata, areas of the basal ganglia receiving striatal projections. Some of the human

nuclei-positive cells coexpressed glial fibrillary acidic protein (GFAP) and NeuN, suggesting that they had differentiated into neurons and astrocytes. Taken together, these data indicate behavioral and anatomical recovery in a rodent model of HD after implantation of *in vitro* expanded cortical cells. Given the limited differentiation of the transplanted cells, it is also possible that release of molecules from the transplanted cells account for most of the beneficial effects observed.

A different strategy has tested the efficacy of NSCs transplantation at a remote site and with less invasive methods (20, 21, 45). Specifically, Lee and colleagues have investigated whether intravenously (i.v.) injected human NSCs can migrate and be engrafted into the striatum in a rodent HD model. The results showed that i.v. transplanted human NSC reduce excitatory asymmetry and progressive atrophy in the rat brain (59).

In 2004 Kim and coworkers have investigated the effectiveness of transplantation of human NSC (generated from the human fetal telencephalon) in adult rat striatum prior to striatal damage induced by the mitochondrial toxin 3-nitropropionic acid (3-NP) (52). Systemic 3-NP administration caused widespread neuropathological deficits similar to those found in HD including impairment in motor function and extensive degeneration of cresyl violet positive neurons, glutamic acid decarboxylase-positive neurons and calbindin-positive striatal neurons. Animals receiving intrastriatal implantation of human NSC 1 week prior to 3-NP treatments exhibited significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections. In contrast, transplantation of human NSC at 12 h following 3-NP administration did not show any protective effects against 3-NP-induced behavioral impairment and striatal neuronal damage. The neuroprotection sustained by the proactive transplantation of human NSC in the rat model of HD appears to be mediated by BDNF secreted by the transplanted human NSC. Active production of BDNF by human NSC *in vivo* and *in vitro* was firmly established by studies using RT-PCR, immunocytochemistry, dot-blot and ELISA.

These findings suggest that transplanted human NSCs can integrate well in the striatum and support the survival of host striatal neurons against neuronal injury induced by 3-NP toxicity. As the genetic screening of HD gene and neuroimaging can provide an indication of signs and symptoms progression in potential HD patients, the results of this study indicate that early intervention using brain transplantation with human NSC overexpressing neurotrophic factors might be effective in blocking the progression of clinical pathology in HD.

NSCs grafting in models of MS. Neurological function is impaired in MS because of damage to both myelin and the myelin-producing cells known as oligodendrocytes, resulting in the disruption of electrical signaling. MS is characterized by permanent demyelination and axonal loss and clinically by neurological impairment and disability. Although some spontaneous remyelination is known to occur in MS, it is an inadequate and unsustained process.

The supplementation of endogenous remyelination by the delivery of cells capable of providing trophic support or myelinating axons is therefore an attractive therapeutic strategy (40). MS is by definition multifocal, and injecting cells into each and every lesion is unlikely to be practical. Targeted implantation at the site of symptomatic spinal cord or brainstem plaques might circumvent this problem, but there is increasing evidence that axonal loss is a major substrate of chronic disability. As remyelination of clinically silent plaques may protect axonal tracts from chronic damage, a more global treatment may therefore be of benefit in the long term. This will necessitate a more complex strategy for cell delivery unless the cells themselves retain tropism for areas of pathology and can migrate to sites of damage following intraventricular or intrathecal delivery. Intravenous delivery of stem cells may also be a realistic alternative in MS and other neuroinflammatory conditions because of the breakdown of the blood–brain barrier that occurs in areas of active inflammation. Oligodendrocyte progenitors are a logical candidate for use in MS transplantation therapy, as these cells are responsible for most spontaneous remyelination (96, 113). Unfortunately, while oligodendrocyte progenitors can be isolated from the adult human brain (97), and can undergo limited proliferation *in vitro*, the number of these cells is limited and their migration through normal brain is considerably low. Furthermore, much of the knowledge about these cells comes from work on rodents, and significant species differences require this work to be repeated in humans. Stem cells, with their significant replicative potential, provide a possible solution for this problem.

When transplanted in rodent models of either genetically determined or chemically induced demyelination (both within the brain and the spinal cord), ESCs have been able to differentiate into glial cells and reensheath demyelinated axons *in vivo*. The first demonstration of this is from the work of Brüstle et al (15), in which ESC-derived precursors were shown to interact with host neurons and efficiently myelinate axons in brain and spinal cord. In this study, to initiate differentiation, ESCs were aggregated into embryoid bodies and plated in a defined medium that favors the survival of ESC-derived neural precursors. To investigate whether ESC-derived oligodendrocytes can form myelin *in vivo*, cells grown in the presence of FGF2 and PDGF were transplanted into the spinal cord of 1-week-old myelin-deficient rats, an animal model for the hereditary human myelin disorder Pelizaeus-Merzbacher disease (PMD).

These data show that cell type-specific somatic precursors can be generated from ESCs and used for nervous system repair. As ESCs can be maintained and expanded in an undifferentiated state, it is possible to generate a virtually unlimited number of cells for transplantation. Previous transplant studies involving ES-derived neural cells generated without growth factors or with retinoic acid treatment were complicated by the formation of heterogeneous tissues and teratomas.

In addition, Pluchino and colleagues have investigated the ability of these neural precursors to reach multiple demyelinating areas of the CNS and to ameliorate disease-related, clinical, neurophysiological, and pathological signs when injected both intraventricularly or systemically in an experimental model of MS—(Relapsing-remitting Experimental Autoimmune Encephalomyelitis) R-EAE (84). Through-

out this study, the authors have used early passage neurosphere cultures that were established from the periventricular region of the forebrain ventricles of adult C57BL/6 mice, expanded *in vitro*, and labeled with a lentiviral vector through which the expression of the *Escherichia coli* β-galactosidase (β-gal) gene (*lacZ*) was directed to the nuclear compartment. To investigate the migratory capacities of neural precursor *in vivo*, cells were injected into the cisterna magna intracerebroventricular (i.c.) or into the bloodstream (i.v.) of syngenic, untreated C57BL/6 mice as well as in mice that had been pretreated with lipopolysaccharide (LPS; i.v.) or with tumor necrosis factor-α (TNF-α) and interleukin (IL)-1beta (i.c.) in order to force the opening of the blood–brain barrier, thus mimicking a CNS-restricted inflammatory-like condition. Thirty days after injection, many of the donor cells were localized deeply within the brain parenchyma and displayed a marked distribution pattern: most of them were confined within areas of demyelination and axonal loss, and only very few were found in regions with preserved myelin architecture. A portion of the neurosphere-derived cells was capable of differentiating into oligodendrocytes. In addition, reduction of reactive astrogliosis occurring along with demyelinating was observed in mice injected i.c. or i.v with the neural cells.

In a follow-up paper, the authors have confirmed the persistence of the neurosphere-derived cells in the inflamed perivascular areas of the CNS for up to 3 months after transplantation in R-EAE mice (86). It was found that neurosphere-derived cells enter the CNS via constitutively activated integrin and chemokine receptors and accumulate within perivascular areas, where they induce apoptosis of CNS-infiltrating encephalitogenic T cells. The authors also propose that when neurodegeneration prevails, transplanted cells acquire a mature functional phenotype and replace damaged neural cells; on the other hand, when neuroinflammation predominates, transplanted cells survive recurrent inflammatory episodes by retaining both an undifferentiated phenotype and the ability to proliferate. In this condition, cells release molecules that contribute to reduce local inflammation at least for the time examined.

NSCs grafting in models of ALS. In its common form, ALS is characterized by progressive dysfunction and degeneration of both upper and lower motor neurons. To replace lost motoneurons, transplanted cells should be guided to differentiate into cholinergic motoneurons in the ventral horn of the spinal cord. In addition, the new motoneurons should send axons through appropriate nerves to form synapses on muscle cells causing improved motor function. Successful attempts to generate cholinergic neurons from mouse ESCs have been achieved through exposure of the cells to inductive factors such as retinoic acid and Shh. However, ESCs or tissue-derived NSCs do not form typical motoneurons in adult spinal cord, presumably because of lack of sufficient environmental cues. Kerr and colleagues (50) investigated the potential of human pluripotent cells to restore function in rats paralyzed with a virus-induced motor neuronopathy. Cells derived from embryonic germ cells, termed embryoid body-derived (EBD) cells, introduced into the cerebro–spinal fluid (CSF) were distributed extensively over the rostrocaudal length of the spinal cord and migrated into the spinal cord parenchyma in paralyzed, but not uninjured, animals. Some of the transplanted human cells expressed the neuroglial progenitor marker nestin, whereas others expressed immunohistochemical markers characteristic of astrocytes or mature neurons. Few transplanted cells developed immunoreactivity to choline acetyltransferase (ChAT) and sent axons into the sciatic nerve as detected by retrograde labeling. Paralyzed animals transplanted with EBD cells partially recovered motor function 12 and 24 weeks after transplantation, whereas control animals remained paralyzed. They concluded that most human embryonic germ cells, when grafted into rats with diffuse motor neuron injury, remain undifferentiated and only a few become cholinergic, and these neither become motor neurons nor connect to muscle. To try to obtain a higher proportion of stem cell-derived cholinergic neurons, Wu and coworkers developed an *in vitro* priming technology that resulted in human fetal NSCs (propagated as neurospheres) differentiating into cholinergic neurons *in vitro*, with many acquiring a cholinergic phenotype when grafted into intact adult spinal cord (114). Therefore, this study demonstrates for the first time that motoneurons generated from primed human fetal NSC emit axons that innervate peripheral muscle targets, a finding that correlates with partial improvement of motor function and indicates that appropriate environmental cues for forming new connections may still be present in axotomized adult rats with chronic motoneuron degeneration. Moreover, advances presented recently show that mouse ESC-derived motoneurons can extend axons into ventral roots only after dibutyryl cAMP treatment (41). However, even with this treatment, those axons were confined to proximal ventral roots, and no neuromuscular junctions were detected. All these findings represent a conceptual advance and may shed light on the development of stem cell replacement techniques for neural disorders characterized by motoneuron loss.

CAN EXTRANEURAL STEM CELLS BE EMPLOYED TO TREAT BRAIN DISEASES?

According to the literature, bone marrow stromal cells (BMSCs) appear to have the capacity to generate a multitude of tissues, including bone, cartilage, hepatocytes, muscle and adipose cells (57, 71, 72). When exposed to selected growth factors, human BMSCs appear to be also able to differentiate into cells expressing markers of neural progenitors. There are several reports of BMSCs-derived cells having reached the normal and damaged brain through the circulation and having differentiated to neural-resembling cells within the parenchyma (25). The debated potential of BMSCs to differentiate into neural cells has attracted special attention and criticisms, caused by overinterpretation of the results and by the lack of control experiments, often missed or not adequate to conclusively exclude incorrect interpretation.

Overall, the available evidence indicates that the number of BMSCs able to transdifferentiate, if any, is extremely low and irrelevant when thinking of their potential clinical exploitation. In addition, such low and often not consistent numbers (between different groups) have been interpreted by some authors as the result of technical artifacts or the consequence of cell fusion (assimilation of transplanted

cells or their progeny into existing neurons and formation of heterokaryons). Nevertheless, various laboratories have continued to suggest that these cells may be able to contribute to the generation of new neurons in the adult brain through transdifferentiation or transdetermination, that is, a direct switch of grafted stem cells into a stem cell of a diverse embryonic origin. The hypothesis that BMSCs may be developmentally plastic has encouraged many attempts to use BMSCs for brain repair, also caused by the potential of adult human BMSCs to provide an unlimited source of autologous transplantable cells, especially based on the relatively easy way in which they can be harvested and expanded.

BMSCs have recently been injected into animals affected by experimental demyelination, ischemic stroke and ALS. Several groups have reported that BMSCs can improve recovery after focal cerebral ischemia in rats. Zhao and colleagues reported partial and limited neural differentiation of adult human BMSCs grafted into the adult rodent brain (120). Adult BMSCs from the bone marrow of volunteers were enriched and expanded for approximately 26 passages prior to transplantation in an experimental stroke model. Recipients included immunosuppressed adult rats in which cortical brain ischemia had been produced by permanent right middle cerebral artery ligation. Six weeks after transplantation into the ischemic cortex, grafted cells expressed neuronal, astrocytic, and oligodendrocytic markers and had migrated along the corpus callosum, although the cells were morphologically immature. Among the 2 million BMSCs injected into the carotid artery, only 0.02% stained for neural markers in the ischemic hemisphere. Both, nestin and human BMSCs markers were also found in the grafts, indicating that a subset of transplanted cells maintained a stem cell phenotype, as has also been observed in the transplantation of fetal neurospheres.

Adult mouse BMSCs have also been grafted into adult parkinsonian mice lesioned with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, leading to a significant improvement in rotational asymmetry after 35 days in the absence of differentiation, although few scattered grafted cells expressed TH (61). This latter point has been subject to criticism by many other groups because of the lack of adequate controls for the unequivocal identification of grafted cells. In rats with focal chemical demyelination of the spinal cord, i.v. or direct microinjection of mouse BMSCs into the demyelinated spinal cord have been reported to induce an increase of new myelin forming cells, with subsequent improvement in axonal conduction velocity (57). While these results are of interest, more studies are necessary to identify both the nature of the cells that evoke beneficial effects *in vivo* and the underlying mechanisms, and finally whether these effects persist for a long term.

Human umbilical cord blood (hUCB) has been proposed as a second extra-neural source of multipotential stem cells for brain repair. Garbuzova-Davis and colleagues investigated the long-term effects of i.v. delivery of mononuclear hUCB cells into the presymptomatic G93A mice, a mouse model of ALS (32). In this study, the authors examined the distribution of transplanted cells inside and outside the CNS, as well as the migration of transplanted cells to degenerating areas in the brain and spinal cord, and their immunophenotype. It was found that donor cells survived 10–12 weeks after infusion and colonized regions of motor neuron degeneration in the brain and spinal cord. There, the cells migrated into the parenchyma of the brain and spinal cord and a minor fraction of them expressed an immature neural marker (Nestin) and early post-mitotic (βIII-tubulin) neuronal and glial (GFAP) markers. Furthermore, the authors reported a limited but significant 2–3-week delay in disease progression and (limited) increase in lifespan of diseased mice.

Based on the fact that a low number of donor cells expressed neural antigens, one can conclude from this study that cell-based treatment of ALS may provide protection of motor neurons rather than neuronal cell replacements. In this context, a number of questions have been raised as to the safety of bone marrow-derived cells in the brain. What ultimately occurs to these cells in the brain? Do they survive long term in the lesioned region? If the cells do survive, do they differentiate into different mature cell types or become extraneous tissue and potentially tumorigenic? To answer all these issues, long-term follow-up studies are needed. A second issue is that only a percentage of transplanted cells administered i.v. migrate to the ischemic area, the remaining spread to other areas of the brain and to other organs, where they could potentially change organ physiology and promote the formation of ectopic tissue masses. Homing of bone marrow stem cells to sites of organ injury, including the ischemic and neoplastic brain tissues, is believed to occur through chemotactic factors, which are likely responsible for attracting transplanted cells to ischemic regions of the brain. It might be that other routes of administration, such as an intracarotid approach, may be necessary to minimize spread of donor cells to other tissues.

CONCLUSION

Earlier trials in PD and HD patients have already identified a number of critical clinical issues that need to be addressed in order to develop effective cell replacement therapies for brain diseases. An alert to consider more global therapeutic interventions for neurodegenerative brain diseases comes from the recent conclusion of a long-term, 6-year, follow-up study in HD patients in which neuronal transplantation is described as providing a period of improvement but not a permanent cure for the disease (91).

Stem cells may contribute to prolong the efficacy of cell therapies by virtue of their biological flexibility and potential ability to homogeneously replace lost cell elements. In this context, the recent demonstration that ESCs can be converted into a homogeneous and stable population of NSCs has provided a unique tool to investigate the potential of experimental transplantation of pure stem cells.

With respect to tissue-derived putative stem cells, most of the animal trials performed so far indicate that a great fraction of the observed efficacy may derive from the ability of the cells to release factors capable of activating endogenous pathways and cell elements, or that preserve them from dying. It remains unclear whether these factors represent a specific repertoire of the NSCs or whether other, more accessible, cells produce a similar profile. The search for NSCs that fully accomplish the requisites for cell therapy in models of human disease is still an open field.

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