CHAPTER

5

Survival, differentiation, and connectivity of ventral mesencephalic dopamine neurons following transplantation

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Abstract

The reconstruction of midbrain dopamine (DA) circuitry through intracerebral transplantation of new DA neurons contained in embryonic ventral mesencephalon (VM) is a promising therapeutic approach for Parkinson's disease (PD). Although some of the early open-label trials have provided proof-of-principal that VM grafts can provide sustained improvement of motor function in some patients, subsequent trials showed that the functional response can be highly variable. This chapter reviews an extensive body of basic and clinical research on the survival, differentiation, and connectivity of DA neurons in VM grafts, and also looks at how these parameters are affected by certain host- and donor-specific variables. We also review how technical advances in the tools available to study the integration of grafted DA neurons, such as transgenic reporter mice, have made significant contributions to our understanding of the capacity of different DA neuronal subtypes for target-directed growth and innervation of appropriate host brain structures. Our established and on-going understanding of the capacity of grafted DA neurons to structurally and functionally integrate following transplantation forms an important basis for the refinement and optimization of VM grafting procedures, and also the development of new procedures based on the use of stem cells.

Keywords

Parkinson's disease, midbrain, regeneration, transplantation, cell therapy

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1 INTRODUCTION

Parkinson's disease (PD) is an irreversible neurodegenerative condition involving the progressive loss of midbrain dopamine (DA) neurons as the primary pathological feature (German et al., 1989; Hornykiewicz, 1975). The mDA neurons reside in the ventral part of the mammalian brain and send long-distance axonal projections to various forebrain targets, including the putamen and caudate nucleus (Björklund and Dunnett, 2007; Fallon and Moore, 1978). When the loss of DA neurons reaches around 50%, resulting in a substantial reduction in striatal DA, the first signs of motor dysfunction become apparent, including tremor at rest and difficulties in initiating and executing movements (Fearnley and Lees, 1991; Hornykiewicz, 1975). Most of the current therapies for PD are aimed at restoring dopaminergic signaling in order to reinstate a normal pattern of information flow through the basal ganglia, thereby improving motor function. The most widely used and successful approach to date has been through the systemic delivery of DA agonists or the DA precursor L-DOPA. Although these pharmacotherapies can provide excellent results in the early phase of the disease, prolonged treatment invariably leads to complications, including a substantial waning of the therapeutic effect and the development of unwanted side effects such as dyskinesias. Thus, there is an on-going need for better therapies for PD, either through the refinement of currently available treatments or the development of new ones.

Cell therapy is an experimental approach with significant potential as a restorative treatment for the motor deficit in PD. The concept was originally developed through experiments showing that DA progenitors in fetal ventral mesencephalic (VM) tissue could survive, differentiate, and functionally integrate into a host brain after intracerebral transplantation in order to restore motor function in a rodent model of PD (Björklund and Stenevi, 1979; Perlow et al., 1979; see Fig. 1). This led to the first, open-label clinical trials in patients with advanced PD, which showed that a number of patients can experience long-term symptomatic relief of motor dysfunction after VM grafting, with substantially fewer side effects compared to long-term drug treatment (Dunnett et al., 2001; Lindvall and Björklund, 2004; Lindvall and Hagell, 2000). Since these early experiences, more than 30 years of basic and clinical research in this field has led to a considerable body of work describing the survival, differentiation, growth, and connectivity of VM grafts following intracerebral transplantation. This chapter reviews some of the key studies in this area, with an emphasis on the role of donor- and host-specific variables that impact on the survival and integration of VM grafts.

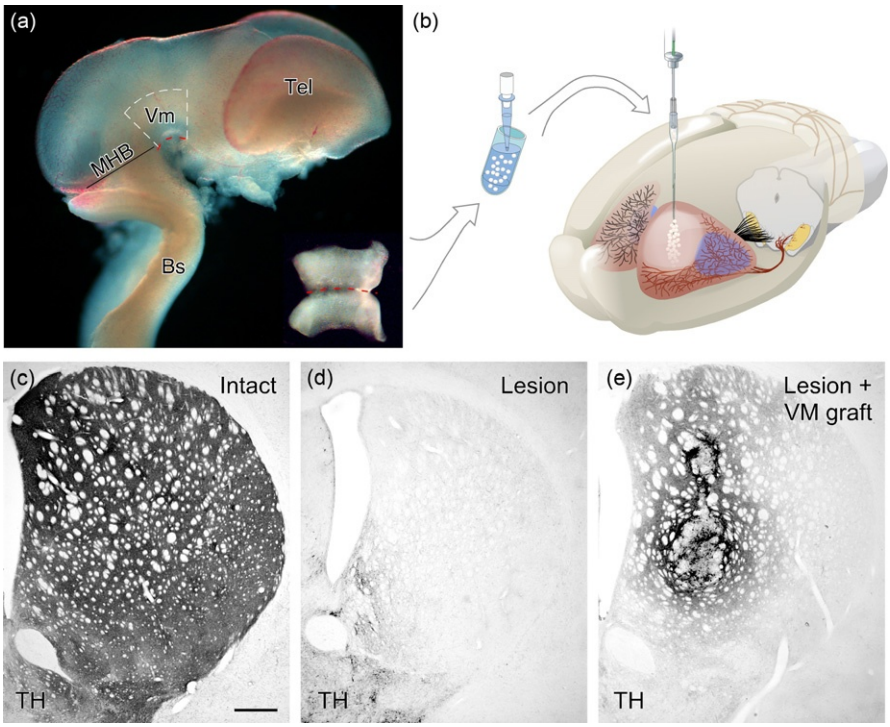

FIGURE 1

Cell therapy for Parkinson's disease. (A) The developing mouse brain at embryonic day 12.5. The dashed lines indicate the approximate region of ventral mesencephalon dissected in order to generate cell preparations for grafting. The inset shows a representative piece of dissected VM. The numbers indicated are for orientation relative to the intact brain. The red dashed line marks the midline. (B) A schematic overview of a typical transplantation procedure, whereby the dissected VM is prepared as a single cell suspension (through trypsin digestion and mechanical dissociation), before the cells are microinjected into the host brain. The example used here shows placement into the striatum. (C–E) Tyrosine hydroxylase (TH) immunohistochemistry in coronal sections through the adult rat brain. The dark staining of the striatum in the intact animal (C) represents the dense terminal network of TH+ fibers originating from midbrain DA neuronal projections. Lesioning of the midbrain DA neurons through injection of 6-hydroxydopamine (6-OHDA) removes this TH+ afferent innervation of the striatum (D). Panel (E) illustrates a 6-OHDA-lesioned animal 6 weeks after grafting of 1.0×10^5 E12.5 mouse VM cells into the striatum. The graft itself can be seen as a discrete teardrop-shaped deposit of darkly stained TH+ cells, while the dark gray area surrounding the graft represents the new TH+ innervation of the host striatum provided by the grafted midbrain DA neurons. Scale bar: (C), 500 μm. Abbreviations: Bs, brainstem; MHB, mid-hindbrain border; Tel, telencephalon; Vm, ventral mesencephalon.

(*This figure is a modified reproduction from Thompson and Björklund, 2009.*)

2 SURVIVAL OF DA NEURONS IN VM GRAFTS

Restoration of motor function following grafting of primary VM tissue requires the survival and integration of DA neurons so that a new terminal network is established in the host striatum that can functionally compensate for the degeneration of the intrinsic system. In PD patients, where striatal uptake of [^{18}F]-fluorodopa (FD) is typically only 30–35% of normal values, meaningful clinical outcomes following grafting require restoration to 50–60% of normal (see Hagell and Brundin, 2001, for review, and Chapter 10, for a discussion of imaging studies of graft integration in patients). The magnitude of the change in FD uptake is very likely related to the degree of reinnervation of the host striatum by the grafted DA neurons. Studies in rats indicate that both the amount of graft-derived striatal innervation (Nakao et al., 1995; Schierle et al., 1999a) and the extent of behavioral recovery in motor function (Brundin et al., 1994) are related to the number of grafted DA neurons. Thus, although a range of donor- and host-specific factors are known to contribute to the overall functional outcome following VM grafting, the final number of DA neurons (notably, of the right kind, see below) remains a fundamentally important parameter.

The relatively poor survival of DA neurons per VM tissue piece has meant that patients with the best clinical outcomes have generally required multiple (2–7) fetal donors per grafted hemisphere (for reviews, see Hagell and Brundin, 2001; Lindvall and Björklund, 2004; Winkler et al., 2005). Given the obvious scarcity of human fetal tissue as a donor source, this has stimulated an intense research interest into improving survival of DA neurons following VM grafting with the goal of reducing the number of VM tissue required for each patient (Barker et al., 1995; Brundin et al., 2000; Sortwell, 2003). The yield of DA neurons in VM grafts is often reported as a percentage of the total number of cells grafted and is typically in the order of 0.5–3%. While this provides a useful index of the *efficiency* of dopaminergic yield per volume of tissue, which will depend on the boundaries used for the VM dissection, it does not necessarily reflect the *survival* rate *per se*, which is related to the total amount of VM tissue used per graft. Attempts to calculate the survival rate of DA neurons following transplantation have been based on representing the final numbers of grafted DA neurons as a fraction of either: (a) the expected total amount of DA neurons in the mature midbrain from the same species or (b) the number of DA neurons contained in the VM piece at the time of grafting.

Both methodologies present caveats when calculating absolute survival rates. For example, comparison against published figures for total number of DA neurons in the mature midbrain is complicated by a lack of consensus on the accurate figure (e.g., see German et al., 1983; Pakkenberg et al., 1991), will not necessarily take into account sex or strain differences, and does not allow for the possibility of higher initial numbers of DA neurons in the embryo because of programmed cell death known to occur during the early postnatal period (Janec and Burke, 1993). On the other hand, using the number of DA neurons in VM tissue at the time of grafting, estimated at 8–10% in rodents based on TH+ cell numbers (Nakao et al., 1995; Sauer et al., 1992; Schierle et al., 1999b), does not account for the presence of DA progenitors not yet

expressing TH as a possible source of the DA neurons in mature grafts. In fact, recent cell-sorting studies suggest that the majority of grafted DA neurons from cell suspension preparations are derived from early progenitors prior to the onset of TH expression (Jonsson et al., 2009; Thompson et al., 2006).

Nonetheless, the use of these VM-specific common denominators provides a valuable means to standardize comparisons of survival across studies. Experiments in rodents have reported survival rates of DA neurons under baseline conditions that vary between 1% and 20% (for reviews, see Brundin and Björklund, 1987; Brundin et al., 2000). Similar results of 5–10% survival have been observed in xenografting studies of human VM into immunosuppressed rats (Brundin et al., 1988; Frodl et al., 1994) and also following *postmortem* analysis of PD patients with human VM grafts (Freed et al., 2001; Kordower et al., 1996, 1998; Mendez et al., 2005).

As part of efforts to improve the survival of grafted DA neurons, experiments in animals have identified important variables that affect the yield of DA neurons per VM donor after transplantation. Important donor-specific aspects include the age of the dissected VM tissue and parameters related to the preparation and handling of the tissue—such as the incubation medium, whether the tissue is prepared as solid pieces or as a cell suspension, and hibernation periods between dissection and grafting. Key host-specific aspects include the site of implantation, the age of the host, and to some degree, the status of the intrinsic midbrain DA system. Excellent reviews on this topic have been published elsewhere (Barker et al., 1995; Brundin et al., 2000). This chapter looks at renewed interest in the impact of donor age on DA neuron yield and also provides a brief overview of other important donor- and host-related variables affecting numbers of DA neurons in primary VM grafts.

2.1 Donor age of VM preparations

Donor age is a particularly important parameter in VM grafting procedures. The VM must be obtained during a specific time window spanning the neurogenic period for DA neurons. There must be sufficient maturity to allow commitment to a DA neuron phenotype but with limited differentiation in order for the DA progenitors to survive the transplantation procedure. Experiments using rat tissue have established the upper limits of age at around embryonic day (E) 17–19 when using solid VM pieces (Simonds and Freed, 1990; Stenevi et al., 1976) or around E15–16 when the tissue is prepared as a cell suspension (Brundin et al., 1985a). Following these early experiments, it has become more or less conventional to use a donor age of E14 for rat VM or E12 for mouse VM, corresponding to a time that overlaps with the peak phase of DA neurogenesis in both species.

The influence of donor age has since received detailed attention in studies by Torres et al. (2007) and Bye et al. (2012). The study by Torres et al. compared the yield of DA neurons in a rat model of PD following grating of cell suspensions prepared from rat VM tissue dissected at E11 (crown-rump length of 4 mm), E12 (6 mm), E13 (9 mm), or E14 (10.5 mm). Interestingly, when grafting the equivalent number of cells to a single VM piece at each age, the E12 donor preparation resulted

in the greatest number of DA neurons (11.248 ± 1226), in fact, fivefold more compared to the E14 donor preparation (2221 ± 201). The E13 group gave similar results to E14 donors, while very few DA neurons were observed in animals grafted with E11 donors. Given there are substantially fewer cells in the younger VM pieces, the differences in the yield of DA neurons represented as a fraction of total cells grafted is even more dramatic—approximately 2.5% of the 441,000 cells grafted at E12 and 0.26% of the 844,000 grafted at E14. A calculation of survival rate based on 35,000 DA neurons in the adult rat midbrain (Brundin et al., 2000) shows figures at each donor age of around 32% for E12 compared to 6% for E14 donor VM. To put this into clinical perspective, improvement on this scale in the yield of DA neurons per human VM piece would potentially allow for meaningful functional outcomes using single VM donors, in place of the 3–4 that are currently required.

The average volume of grafts derived from the E12 donor group (around 4 mm^3) was also significantly larger than the older E13 (1 mm^3) and E14 (0.4 mm^3) groups. This may reflect that greater survival at this age is not DA neuron-specific but is a more general feature of the grafted tissue. It is also notable that, unlike the E13 and E14 donor groups, cell preparations derived from E12 VM included meningeal tissue, which is difficult to remove at earlier ages. Consequently, the presence of a mesenchymal cell fraction capable of growth after transplantation may contribute to the graft size, although the very small grafts arising from the earlier E11 donor group (also including meningeal layers) suggests this is unlikely to be a significant element. An interesting hypothesis put forward by the authors of this study is that the ventral meningeal layers closely apposed to the DA germinal zone during normal development secrete trophic factors that support the growth and survival of DA neurons.

The use of growth and neuroprotective factors as a strategy for improving survival of grafted DA neurons has been explored extensively and reviewed in detail elsewhere (Brundin et al., 2000; Sortwell, 2003). Cytoprotective agents, such as caspase inhibitors (Schierle et al., 1999a) and lazaroids (Nakao et al., 1994), have been shown to significantly increase the survival rate of grafted DA neurons when added directly to the cell preparation medium. A number of neurotrophic factors have also been utilized, including GDNF and members of the fibroblast growth factor and neurotrophin families. While these can also be used as additives to the cell preparation (Sullivan et al., 1998; Zeng et al., 1996), they have more commonly been applied to the host brain at or near the site of implantation, for example, using osmotic minipumps (Yurek, 1998), viral vectors (Thompson et al., 2009), or overexpressing cell lines (Sautter et al., 1998; Zeng et al., 1996).The most robust and reproducible results have been obtained using GDNF, which is now conventionally used as a growth supplement for the *in vitro* growth and differentiation of DA neurons in both primary VM and stem cell cultures. Overexpression of GDNF in the host target site prior to or at the time of transplantation of primary VM cells significantly enhances the survival of grafted DA neurons (Rosenblad et al., 1996; Sinclair et al., 1996; Thompson et al., 2009; Torres et al., 2005).

Interestingly, this phenomenon may be dependent on the age of the donor VM used. A study by Torres et al. (2005) found that survival of DA neurons from grafts

of E14 or E15 rat VM was significantly enhanced by overexpression of GDNF at the implantation site, while there was no effect on grafts of younger (E12 and E13) donor tissue. Notably, however, the use of sonic hedgehog in place of GDNF significantly improved the yield of DA tissue from E12 but not the older E13, 14, or 15 VM grafts. The context-specific action of different growth factors will clearly be important to consider as part of strategies for augmenting DA neuron survival through selection of optimal VM donor age combined with trophic support.

The continued growth of rodent VM grafts derived from earlier donors through cell division may also contribute to the larger volumes and DA neuron numbers. An important study by Sinclair et al. (1999b) showed that the vast majority of DA neurons in grafts of E14 rat VM are postmitotic at the time of grafting. This was later corroborated by cell-sorting studies using mouse VM from a similar developmental age (E12), which showed that the transplantable DA neurons were largely derived from a newly postmitotic cell fraction (Jonsson et al., 2009; Thompson et al., 2006). Interestingly, however, the study by Jonsson et al. (2009) suggests that, at earlier ages, grafted DA neurons are derived predominately from an actively dividing population. At E10, for example, the germinal zone for DA neurons is composed mainly of dividing, ventricular zone (VZ) progenitors (Fig. 2A). When isolated by fluorescence-activated cell sorting using antibodies targeted to the transmembrane protein "Corin," which is expressed by VZ cells (Fig. 2C), these cells give rise to DA neurons after transplantation (Jonsson et al., 2009). This finding is further supported by a report from Bye et al. (2012) showing that up to 3% of DA progenitors continue to divide *after* intrastriatal transplantation of mouse VM dissected at E10 compared to less than 1.5% when using E12 VM (Fig. 2D). Thus, continued cell division is likely to contribute to the growth of VM grafts, including the DA neuron component, when using younger donor tissue.

2.2 Tissue handling

Gradual improvements on various aspects related to tissue handling prior to implantation have also greatly enhanced the yield of DA neurons from VM pieces after grafting. While optimization of parameters such as dissection technique and choice of media have clearly been important (Barker et al., 1995; Brundin et al., 1985b, 2000), one of the most fundamental changes to the preparation of VM tissue for transplantation has been the development of the cell suspension technique, whereby the solid VM piece is dissociated into a roughly single cell suspension through incubation with trypsin (and DNase) followed by mechanical dissociation (Björklund et al., 1983a,b). The approach has important practical advantages over solid VM piece grafts, including the possibility to consistently deliver deposits of predefined cell numbers across multiple graft sites and animals. It is also thought to aid in the elimination of particularly immunogenic tissue components, such as vascular elements, that may remain intact in solid tissue grafts and facilitate a greater host immune response (Baker-Cairns et al., 1996; Broadwell et al., 1990). The preparation of cell suspensions from solid VM pieces does not in itself appear to improve

cell survival. A study by Redmond et al. (2008) found no difference in the survival rate of DA neurons following allografting of either solid VM pieces or cell suspensions in a primate model of PD. Notably, however, there was a significantly greater host astroglial response in animals grafted with solid pieces. The overhandling of VM tissue is, in fact, more likely to be detrimental, and excessive mechanical trituration can have unfavorable results on cell survival (Barker et al., 1995). Cell-sorting experiments suggest that it is the more mature DA neurons, rather than the progenitors, that are particularly vulnerable in cell suspension preparations (Jonsson et al., 2009). In these experiments, the overall survival rate was quite low, around 0.5% of all cells grafted, compared to similar studies by the same

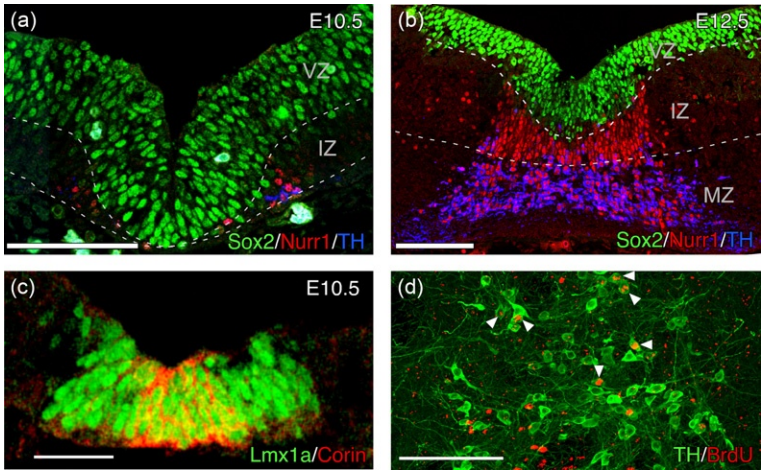

FIGURE 2

Different progenitor states of transplantable midbrain DA neurons at different embryonic ages. (A) Immunohistochemistry in coronal sections of embryonic mouse shows at E10.5, the DA germinal zone around the ventral midline is largely composed of actively dividing ventricular zone (VZ), many of which express Sox2 (green). As the cells exit the cell cycle, the newly postmitotic DA progenitors in the intermediate zone (IZ) express Nurr1 (red) and then also tyrosine hydroxylase (TH) as they continue to differentiate. (B) By E12.5, the structure has expanded substantially to include large populations of Nurr1+ DA progenitors in the IZ and TH+ neurons in the MZ. Cell-sorting experiments have shown that, at this age the vast majority of transplantable DA neurons are derived from the newly postmitotic IZ progenitors. (C) The earlier DA progenitors in the VZ at E10.5 (defined here by the transcription factor Lmx1a, green) can also be isolated in cell-sorting experiments using antibodies against the transmembrane protein "Corin" (red). (D) Many of these earlier DA progenitors will continue to divide after transplantation, as shown by incorporation of BrdU (red) in TH+ (green) neurons when host animals receive a single intraperitoneal injection of BrdU (150 mg/kg) 2 h after transplantation. Scale bar: (A), (B), and (D), 100 μm; (C), 50 μm.

(*Some of the images shown here are modified reproductions from Jonsson et al., 2009.*)

investigators without a cell-sorting step (3–4%; Grealish et al., 2010; Thompson et al., 2005), further highlighting that extra tissue handling can be detrimental for survival of DA neurons.

Nonetheless, an advantage of carefully prepared cell suspension preparations is that it facilitates "microtransplantation" of small deposits of cells using a fine glass cannula with minimal damage to the host brain. This approach was originally described in a series of studies by Nikkhah et al. (1993, 1994b, 2000) with results from allografting studies in rodents showing improved survival of grafted DA neurons from around 0.5% to 5–6%, and a greater degree of striatal reinnervation and improvement in motor function. The improved survival may well relate to reduced trauma and inflammation in the host brain around the implantation site. In support of this are studies showing reduced survival of DA neurons when using cannulae with larger outer diameters (Brundin et al., 1990) and also experiments showing that the time between injection of the cell preparation and trauma at the host target site prior to implantation can influence graft survival (Sinclair et al., 1999a). Thus, while the preparation and handling of VM tissue can impact markedly on the survival of grafted DA neurons, parameters related to the host environment are also important to consider.

2.3 Host-specific variables affect VM graft survival

A multicenter double-blind study designed to assess the efficacy of intrastriatal VM grafting relative to a placebo control group failed to reach the primary end point as measured by change in the motor component of the Unified Parkinson's Disease Rating Scale (Freed et al., 2001; Olanow et al., 2003). Notably, however, comparison of the placebo group with either a younger subset of grafted patients (Freed et al., 2001) or those with less severe disease progression (Olanow et al., 2003) showed a significant treatment effect. These results highlight the significance of host-related variables as determinants of clinical outcome. The limited availability of *postmortem* tissue from grafted patients makes it difficult to assess the impact of these variables on survival-grafted DA neurons, but experimental work in rodents suggest they are relevant to consider in this context.

At least, two studies have looked directly at the impact of host age following transplantation and found that DA neuron survival is significantly diminished in aged compared to young adult rats—17% survival in 12-week-old compared to 4% survival in 2-year-old hosts (Collier et al., 1999; Sortwell et al., 2001). This finding may well reflect a diminished neurotrophic tone in the striatum with advancing age. There is an extensive body of work demonstrating age-related decline in the expression of neurotrophic factors in the brain including those known to support DA neuronal survival, such as GDNF (Kornblum et al., 1997; Schaar et al., 1993; Seroogy et al., 1993; Strömberg et al., 1993; Widenfalk et al., 1997). It is also worth considering that the pathological environment in the PD-affected brain can impact on survival of grafted DA neurons. In addition to the substantial loss of DA neuron numbers as part of the removal and preparation of VM donor material, there is an acute phase of cell death that occurs within the first week after grafting (Barker et al., 1996; Duan et al., 1995; Emgard et al., 1999) and it is likely that this may be exacerbated by an inflammatory environment, for

example, associated with PD pathology (for review, see Brundin et al., 2000). Thus, the combination of low neurotrophic tone in the aged brain and a high degree of inflammation and oxidative stress associated with advanced pathology may constitute a particularly unfavorable environment for survival of grafted DA neurons. Incidentally, Chapter 11 discusses histological studies of long-term surviving grafts in patients that show that the grafted DA neurons can develop Lewy body pathology. However, this is likely a phenomenon that develops slowly over time rather than affecting the acute phase of DA survival after transplantation.

The target location is also known to play a role in DA neuron survival in VM grafts. Early work in this area established that implantation into well-vascularized sites, including previously aspirated cortical cavities (Stenevi et al., 1985) or the anterior eye chamber (Olson et al., 1983), was favorable for survival of solid tissue pieces. As discussed above, subsequent development of the cell suspension technique allowed for implantation of cell deposits into deep sites within the host neuropil with minimal tissue damage. The striatum and the substantia nigra are the two locations that have been explored most extensively as target sites for grafting of VM tissue. Interestingly, survival of DA neurons in grafts placed in the midbrain is substantially poorer than when grafts are placed in the striatum. Nikkhah and colleagues report a yield of around 1.2% DA neurons from VM cell suspensions placed in the striatum (Nikkhah et al., 1994b) compared to only 0.17% following grafting into the midbrain of DA-depleted rats (Nikkhah et al., 1994a). Similarly, Thompson et al. have reported 3.5% (Thompson et al., 2005) compared to 1% (Thompson et al., 2009) yields in the striatum and substantia nigra, respectively. These results may reflect a generally less trophic environment in the midbrain to support the survival and growth of grafted DA neurons and/or a lack of striatal target sites for the outgrowing axons. A conspicuous feature of intranigral grafts is the small size of the surviving DA neurons compared to those in intrastriatal grafts (L. Thompson, unpublished observations). This is reminiscent of studies showing that loss of the striatal target in adult rats following excitotoxic lesion causes a reduction in soma size of the DA neurons in the midbrain (Lundberg et al., 1994). For developing DA neurons placed in the adult midbrain, the lack of a readily accessible striatal target may result in a small soma size, and may additionally lead to the death of a subset of these cells. The programmed cell death of neurons that fail to establish synaptic connectivity during development of the central nervous system is a well-described phenomenon (Llambi et al., 2001; Mehlen and Mazelin, 2003), although it has not been described specifically for midbrain DA neurons.

3 DIFFERENTIATION AND COMPOSITION OF VM GRAFTS

3.1 Nondopaminergic cells in VM grafts

Grafts of primary VM tissue are highly heterogeneous with respect to cell type. The DA neuron component, in fact, represents only a minor fraction of the total cell population in mature grafts. The neuronal population in VM grafts will include serotonin-,

γ-aminobutyric acid (GABA)-, enkephalin-, and substance P-containing neurons, as well as many that cannot be readily identified based on neurochemical phenotype (Bolam et al., 1987; Dunnett et al., 1988; Kordower et al., 1996; Mahalik and Clayton, 1991; Thompson et al., 2008). Xenografting studies of murine (Thompson et al., 2008) or porcine (Isacson and Deacon, 1996) VM into rat hosts have allowed for detailed characterization of the morphological features of the grafts using species-specific antibodies. The mouse-specific M2 and M6 antigens are expressed almost ubiquitously throughout cells contained in VM grafts and therefore form a kind of graft-specific "counterstain" in immunohistochemical studies using a mix of M2 and M6 (M2M6) antibodies (Fig. 3), against which it is possible to assess the shape and volume of the graft and also the relative contribution of specific cell types.

Double staining for markers of neurochemical identity shows that the grafts contain DA and serotonin neurons as relatively minor subpopulations, while the vast majority of neurons in the grafts have a GABA phenotype (Fig. 3). The serotonin neurons result from the inclusion of the rostral part of the adjacent raphé nucleus, which sits just behind the mid-hind brain border normally used as the caudal limit of standard VM dissections. The relative contribution of serotonin neurons may thus vary depending on the dissection parameters. Using standard E12 mouse or E14 rat VM dissections, the number of serotonin neurons is typically in order of 15–20% of the number of DA neurons (Bye et al., 2012; Carlsson et al., 2007) although this can reach up to 50% of the DA component when using wider dissection limits, including a greater proportion of the caudal part of the VM (Carlsson et al., 2007; Garcia et al., 2011). This may be important to consider in light of transplantation studies in rodents showing that grafted serotonin (5HT) neurons can worsen dyskinetic behavior (Carlsson et al., 2007, 2009), possibly by storing and releasing DA as a kind of "false transmitter" in an unregulated fashion (Carta et al., 2007; for reviews, see Lane et al., 2010 and this volume). Studies in rodents suggest that 5HT neuron-mediated dyskinesia is likely to be most problematic in the absence of a dopaminergic system that can effectively buffer dopaminergic overflow in the striatum (Carlsson et al., 2009; Garcia et al., 2011). Thus, the ratio of 5HT to DA neurons, rather than the absolute number of 5HT neurons *per se*, may be more important to consider in VM grafting studies, and variables affecting this ratio should be identified and monitored. Work from Bye et al. (2012) highlights donor age as a relevant parameter in this context. A comparison of graft composition following transplantation of mouse VM at either E10 or E12 showed a dramatic shift in the 5HT to DA neuron ratio from 1:5 at E12 to 1:20 for the younger E10 donor preparation. Together with the findings of Torres et al. (2007), showing increased yield of DA neurons using younger donor tissue, these results speak in favor of a systematic analysis of human VM graft composition across different donor ages.

The large number of GABA neurons in VM grafts are likely derived from progenitors for various GABA-rich nuclei contained in the VM pieces, including the substantia nigra pars reticulata which contains GABAergic projection neurons that innervate the thalamus. As discussed in the next section on *connectivity*, these GABAergic neurons are capable of extensive axonal growth throughout the host brain.

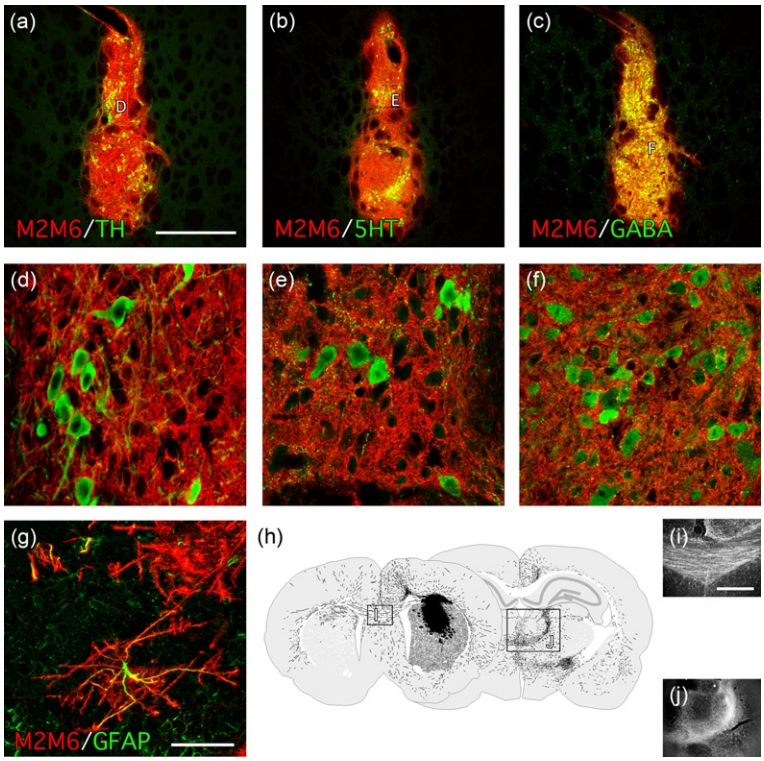

FIGURE 3

VM grafts are comprised of a heterogeneous mix of different neuronal and glial cell types. Six weeks after transplantation of 1×10^5 E12.5 mouse VM cells into the rat striatum, immunohistochemistry for the mouse-specific M2 and M6 antigens (red) illustrate the gross morphology of the grafts (A–C). Double labeling for markers of neurochemical identity (green) reveal the presence of DA (TH+; A—boxed area expanded as D), serotonin (5HT; B—boxed area expanded as E), and GABA (C—boxed area expanded as F) containing neurons. The grafts are also rich in glial cells, some of which have astrocytic morphology and express glial fibrillary acidic protein (GFAP; G). The M2M6 antigens can be detected throughout the axonal and dendritic processes of many of the grafted neurons, particularly GABA-ergic neurons. Careful tracing over darkfield photomontages of coronal sections labeled with M2M6 antibodies illustrates the extensive nature of M2M6+ fiber growth throughout the host brain (H, representative sections from animals grafted as neonates). Darkfield images corresponding to the boxed areas in (H) illustrate M2M6+ fibers extending through the corpus callosum at the midline (I) and innervating midline and intralaminer thalamic nuclei caudal to the graft. Scale bars: (A–C), 500 μm; (D), 50 μm; I, 500 μm.

(*Some of the images shown here are modified reproductions from Thompson et al., 2008.*)

In addition to the neuronal populations, primary VM grafts are also rich in glia. The glial component is particularly conspicuous when visualized in mouse-to-rat studies using the M2M6 antibodies, which intensely label cells with glial morphology (Fig. 3G), some of which co-label with glial fibrillary acidic protein (Thompson et al., 2008). Interestingly, although it is well established that glial cells provide significant trophic support for cultured midbrain DA neurons (Takeshima et al., 1994), cell-sorting experiments in which the glial component is eliminated from the cell preparation prior to grafting suggest that glial cells are more or less dispensable for DA neuron survival, differentiation, and functional integration *in vivo* after transplantation (Jonsson et al., 2009; Thompson et al., 2006). The finding that the composition of VM grafts can be significantly manipulated without adversely affecting the survival and integration of grafted DA neurons is particularly encouraging in light of current efforts to develop cell-sorting strategies to isolate relatively pure, and therefore standardizable, populations of transplantable DA progenitors from stem cells.

3.2 Midbrain DA neuron subtypes in VM grafts

The midbrain DA neurons are a heterogeneous population that can be further divided into distinct cell groups based on morphological, anatomical, and functional features. Cytoarchitectural location in the midbrain and efferent projection patterns define three major cell groups as A8, A9, and A10, according to the classification of cerebral monoamine neurons originally introduced by Dahlstrom and Fuxe (1964) (Fig. 4). The A10 neurons are located in a medial position spanning the midline and send projections to cortical and limbic structures including the nucleus accumbens, amygdala, hippocampus, and the prefrontal and cingulate cortex to form the mesocorticolimbic pathway. These neurons are relatively resistant in PD pathology and are among the last to degenerate. The A9 neurons form a compact layer of cells extending further laterally from the lateral border of the A10 group and send projections which predominately innervate the dorsolateral striatum to form the nigrostriatal pathway and, to a lesser extent, innervate extrastriatal areas including cortex. The A9 neurons are some of the first to degenerate in PD, resulting in deafferentation of their striatal targets and a significant loss of DA levels in these areas. The A8 neurons lie caudal to the A9 cell group and innervate both limbic and striatal areas as well as provide a local innervation of A9 and A10 neurons. For a detailed review of DA systems in the mammalian brain, see Björklund and Dunnett (2007). These neuroanatomical features correlate well with certain molecular and morphological attributes that can be used to distinguish between A9 and A10 DA neurons. For example, the A9 neurons have a large (20–30 μm in mouse), angular morphology and express the G protein-coupled potassium channel subunit Girk2, while the A10 neurons are spherical and smaller in size (10–15 μm in mouse) and more commonly express calbindin, rather than Girk2 (Mendez et al., 2005; Reyes et al., 2012; Thompson et al., 2005)—Fig. 4.

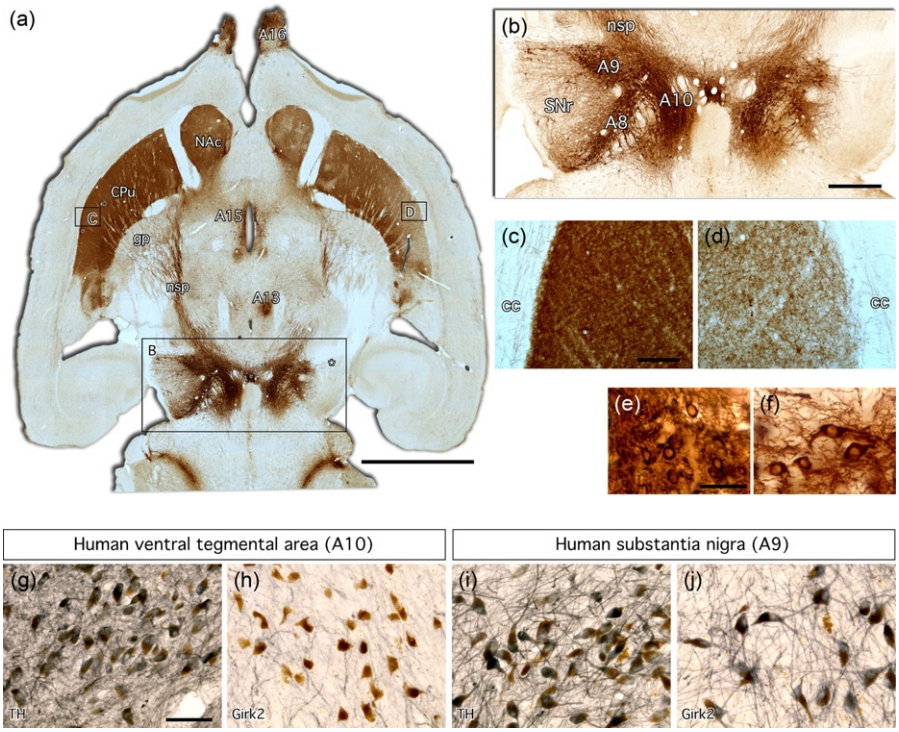

FIGURE 4

Basic neuroanatomical and morphological features of the midbrain dopamine neuron projection system. (A) Immunohistochemistry for tyrosine hydroxylase in a horizontal section through the adult mouse brain shows the midbrain DA neurons and their efferent projection patterns. The major DA cell groups—A8, A9, and A10—are shown in (B) at higher magnification. Note this animal has had a partial lesion of the DA projection system through injection of 6-hydroxydopamine into the midbrain. This is evident as a loss of the A9 cell group on the right-hand side (marked as "*") as well as the corresponding nigrostriatal projection (nsp) to the forebrain and also local innervation of the substantia nigra pars reticulata (SNr). Boxed areas in (A) at the level of the caudate putamen show a substantial loss in the density of the TH+ terminal network on the lesioned side (D) compared to the intact side (C) of the brain. Subtypes of DA neurons differ in their morphological features. The A10 cells tend to be smaller with a spherical shape (E) while the A9 neurons are generally larger with a more elongated shape (F). Similarly, immunohistochemistry for TH (G and I) and Girk2 (H and J) in human midbrain shows that the smaller A10 cells are TH+ (G; TH is shown in black, neuromelanin can be seen as a brown deposit in the DA neurons) but are largely devoid of Girk2 (H), while the larger TH+, A9 neurons (I) express Girk2 throughout the cell bodies and also the basal dendrites (J). Scale bars: (A), 2 mm; (B), 500 μm; (C–D), 100 μm; (E–F), 50 μm; (G–J), 50 μm. Abbreviations: cc, corpus callosum; CPu, caudate putamen unit; gp, globus pallidus; NAc, nucleus accumbens; nsp, nigrostriatal pathway; SNr, substantia nigra pars reticulata.

Interestingly, many of the parameters that distinguish between different DA neuron subtypes in the intact midbrain can also be used to identify subsets of DA neurons in VM grafts. Intrastriatal grafts of primary VM tissue have a predictable cytoarchitectural arrangement whereby the large, Girk2+ A9 neurons are distributed mainly throughout the periphery of the graft, while the smaller, calbindin+ A10 neurons are preferentially located closer to the center of the graft (Grealish et al., 2010; Mendez et al., 2005; Thompson et al., 2005)—Fig. 5. This is reminiscent of the medial, A10, and lateral, A9, locations of each population in the intact midbrain, and suggests that the integrity of signaling mechanisms that guide positioning of these cell types relative to one another during normal development is maintained in VM cell suspensions.

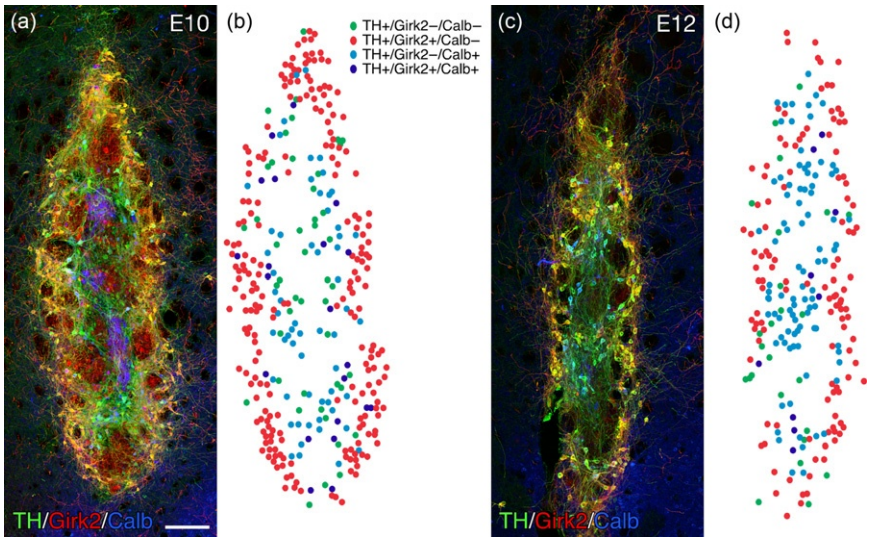

FIGURE 5

Dopamine neuronal subtype composition of VM grafts from different donor ages. Immunohistochemistry for tyrosine hydroxylase (green), Girk2 (red), and calbindin (blue) 10 weeks after intrastriatal transplantation of 1×10^5 cells prepared from either E10 (A, B) or E12 (C, D) mouse VM shows the typical distribution of A9 (Girk2+) neurons around the periphery of the graft, while the A10 (calbindin+) are preferentially located closer to the center of the graft. The proportion and distribution of different DA subtypes according to Girk2+ and calbindin+ phenotype is also illustrated schematically for the E10 (B) and E12 (D) grafts, highlighting the greater number of DA neurons overall in grafts generated from E10 donors, as well as the greater proportion of A9 (Girk2+) subtypes in these grafts compared to those derived from E12 VM. Scale bar: (A), 100 μm.

(*The images shown here are modified reproductions from Bye et al., 2012.*)

Quantification of Girk2- and calbindin-expressing DA neurons in intrastriatal grafts of mouse VM show that around 60–70% are Girk2+, 30–40% calbindin+, and 10–15% express both proteins (Bye et al., 2012; Grealish et al., 2010). While these proteins are by no means absolute markers of A9 and A10 phenotype, they serve as useful tools for identification and quantification of DA neuronal subpopulations in transplantation studies. The study from Bye et al. (2012) shows that donor age is a variable that can influence the relative contribution of Girk2+ and Calbindin+ DA subtypes in intrastriatal grafts of mouse VM, with a greater proportion of Girk2+ DA neurons (~75%) in grafts derived from younger E10 donor tissue compared to grafts derived from older E12 VM (60%)—Fig. 5. This likely reflects the relative staging of the birth and differentiation of specific DA subpopulations in the embryonic midbrain. Birth-dating studies show that peak production of the A9 neurons initially precedes development of the A10 population, while at later stages of midbrain neurogenesis, both A9 and A10 subtypes are generated (Bye et al., 2012; Gates et al., 2006; Joksimovic et al., 2009). Despite a relatively modest difference in the proportion of Girk2+ DA neurons between ages in the study from Bye et al. (2012), the difference in the absolute numbers may be substantially greater with an accompanying increase in the overall yield of DA neurons from younger donor tissue, as reported by Torres et al. (2007). Thus, when grafting the same number of cells (1×10^5), Bye et al. (2012) report an average of around 4000 Girk2+ DA neurons in intrastriatal grafts derived from E10 donors compared to only 1200 derived from E12 VM. Given that the A9 component in VM grafts is likely to be particularly important for functional recovery (Grealish et al., 2010; and see discussion below), variables that impact on the proportion and number of A9 neurons will be important to identify and monitor as part of efforts to establish more effective and consistent therapeutic outcomes following VM grafting in PD patients.

4 CONNECTIVITY OF VM GRAFTS

Intrastriatal grafts of primary VM are capable of establishing extensive afferent and efferent connectivity with the host brain. Fundamental to the functional impact of the grafts is the capacity of the grafted DA neurons to form a functional terminal network with the host striatum. An extensive body of work in this area shows that transplanted midbrain DA neurons possess an intrinsic capacity for innervation of the adult striatum (Björklund et al., 1983b; Brundin and Björklund, 1987; Dunnett et al., 1988; Freund et al., 1985; Isacson et al., 1995; Nikkhah et al., 1994b; Thompson et al., 2005; for reviews, see Herman and Abrous, 1994; Winkler et al., 2000). Pre-clinical studies as well as observations from grafted patients suggest that the volume, density, and specific areas of the striatum innervated by grafted DA neurons play a role in determining the overall functional impact (Hagell and Brundin, 2001). Experiments in rodents (Dunnett et al., 1983, 1988; Grealish et al., 2010; Nakao et al., 1995) and primates (Redmond et al., 2008) have shown a relationship between the degree of striatal innervation and improvement in some aspects of motor function. In the work by Grealish

et al. (2010), the ability of the grafted DA neurons to innervate the dorsolateral part of the host striatum, in fact, had a decisive impact on improvement turning behavior and forelimb use in the cylinder test (see further discussion below). This is analogous to observations in grafted PD patients suggesting that favorable clinical outcomes are associated with a reinnervation of 30–50% of the putamen (Kordower et al., 1996, 1998; Piccini et al., 2005). While the association between the degree of graft-derived striatal innervation and functional impact is likely to be more complicated than a simple linear relationship, it is clear that a threshold level must be reached within the functionally relevant striatal area to obtain a meaningful level of motor improvement. Understanding and improving the capacity for DA neurons in VM grafts to innervate the host striatum therefore remain important areas of research.

4.1 Variables affecting outgrowth of DA neurons

Both donor- and host-specific variables, as well as the method of implantation, can affect the level of striatal innervation following VM transplantation. Studies in rats show that the density of fiber outgrowth from single deposits of VM is greatest in the immediate vicinity of the graft (<0.5 mm) and declines sharply over the next 1–2 mm. Notably, although increasing the size and cell number can improve the volume and density of innervation up to a point, there is a threshold where increasing graft size no longer improves the level of innervation. This limitation can be overcome to some degree by placement of multiple deposits throughout the striatum (Nikkhah et al., 1993, 1994b; Winkler et al., 1999, 2003). These studies show that the volume and density of the terminal network in the striatum can be increased dramatically by distributing the same amount of VM tissue throughout the striatum as multiple small "micrografts" rather than a larger single deposit. Notably, this approach also resulted in the improvement of certain sensorimotor functions previously resistant to correction by single graft placements (Nikkhah et al., 1993).

The degree of fiber outgrowth from grafted DA neurons is also influenced by the status of host DA system. Specifically, a greater level of host-derived denervation of the striatum facilitates a more pronounced innervation from grafted DA neurons (Doucet et al., 1990; Gage et al., 1983; Kirik et al., 2001; Thompson et al., 2005). This is particularly well exemplified in studies using donor VM tissue from mice where GFP is expressed under control of the tyrosine hydoxylase promoter and therefore in all DA neurons (Thompson et al., 2005; Fig. 6). By using GFP to distinguish between graft- and host- derived DA fiber patterns, it is possible to unambiguously identify and measure the volume of the terminal network established by DA neurons grafted into the intact striatum and to compare this with measurements from grafts placed in the lesioned striatum (Fig. 6F and G). Results from these experiments show that the fiber outgrowth is significantly greater ($\sim$40%) in the denervated host. This may reflect an increased trophic tone in the striatum due to the upregulation of growth factors such as BDNF and GDNF that occurs following removal of the host DA system (Yurek and Fletcher-Turner, 2001; Zhou et al., 1996) and/or less competition for synaptic contact with the medium spiny striatal neurons from the

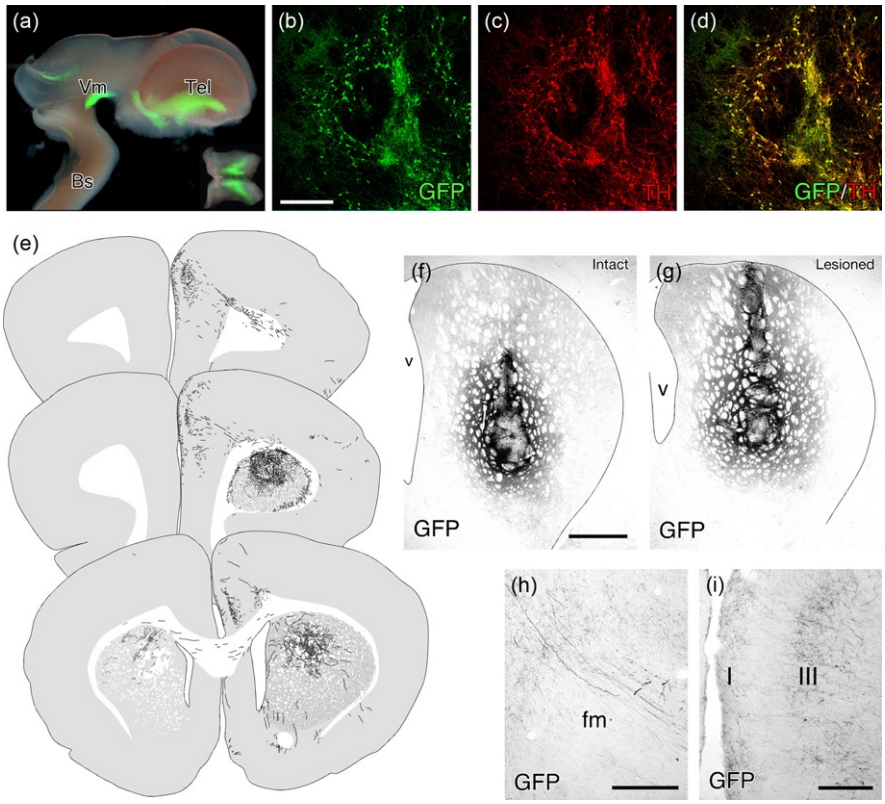

FIGURE 6

Visualizing dopamine-specific connectivity following VM grafting using donor tissue from the *TH*–GFP reporter mouse. (A) The embryonic (E12.5) brain of a transgenic mouse in which green fluorescent protein (GFP) is expressed under control of the promoter sequence for tyrosine hydroxylase (TH), and therefore in all dopamine neurons. (B–C) Immunohistochemistry for GFP (green) and TH (red) in intrastriatal grafts generated from E12.5 VM preparations from *TH*–GFP mice shows that expression of the GFP reporter matches well with TH expression in the grafted cells. This means that GFP+fiber patterns in the host brain can confidently be used as a surrogate marker of connectivity from grafted DA neurons. (E) The schematic drawings shown here illustrate GFP + fiber patterns in the forebrain of hosts grafted with 1×10^5 E12.5 *TH*–GFP VM cells as neonates. These were generated by carefully tracing over GFP + fibers in photomontages of coronal sections labeled with an antibody against GFP. The GFP reporter allows for unambiguous detection of graft-derived DA fibers, even in the presence of an existing host DA fiber network, and can therefore be used to illustrate the more extensive outgrowth of DA fibers from intrastriatal VM grafts in the lesioned (G) compared to intact (F) striatum. Dopamine neurons in intrastriatal VM grafts can extend long-distance projections to extrastriatal areas, including through forceps minor (H) in order to innervate the overlying cortical areas (I), most notably the anterior cingulate cortex. Scale bars: (B), 50 μm; (F–G), 1 mm; (H), 500 μm; (I), 200 μm. Abbreviations: I, cortical layer 1; III cortical layer 3; fm, forceps minor.

(*Some of the images shown here are modified reproductions from Thompson et al., 2005.*)

outgrowing axons. While these studies provide important insights into the basic mechanisms underlying growth and integration of grafted DA neurons, it is worth bearing in mind that there is not necessarily a directly translatable clinical correlate of this scenario, whereby there would be advantages transplanting into patients with more advanced striatal denervation. On the contrary, clinical benefit may be more difficult to achieve when grafting into patients with advanced disease progression (Olanow et al., 2003) or with greater denervation of the striatum outside of the grafted areas (Piccini et al., 2005).

When looking at donor-specific aspects that may influence the efferent connectivity of grafted DA neurons, a study from Bye et al. (2012) suggests that donor age may be an important variable. A comparison of mice grafted with E10 or E12 mouse VM showed that animals receiving younger donor tissue had on average a significantly greater volume and density of innervation of the dorsolateral striatum and also increased levels of DA. It is unclear whether this was from the greater number of cells in the younger grafts or a difference in the intrinsic ability of DA neurons from younger donor tissue to innervate the host striatum. Regardless, it further highlights the potential of younger donor tissue for reconstruction of striatal DA circuitry.

4.2 Host-derived afferent connectivity with intrastriatal VM grafts

Relatively less is known regarding the implications of afferent connectivity from the host for graft function. Intracerebral dialysis studies show that DA is released from intrastriatal VM grafts in an "autoregulated" fashion (Strecker et al., 1987; Zetterstrom et al., 1986). Thus, despite an ectopic location that presumably disrupts the normal pattern of afferent input to midbrain DA neurons, the grafted neurons maintain the capacity for regulated release of DA at synaptic contacts with host striatal neurons. Electrophysiological studies show that the majority of DA neurons in intrastriatal grafts maintain a normal electrophysiological profile, including the persistence of spontaneous pacemaker activity (Sorensen et al., 2005; Chapter 6). These experiments, along with earlier studies (Fisher et al., 1991), additionally show electrophysiological responses in grafted neurons following stimulation of the host striatum or cortex, indicating functional afferent input from these regions.

Evidence for host afferent connectivity with VM grafts also comes from neuroanatomical tracing studies and studies of ultrastructural graft features using electron microscopy (Bolam et al., 1987; Doucet et al., 1989). The results indicate that the grafts can receive afferent input from 5HT-, SP-, and glutamic acid decarboxylase-containing host neurons. This connectivity may, at least in part, modulate the activity of DA as well as non-DA components in VM grafts and therefore contribute to graft function.

4.3 Cell-type specificity of efferent outgrowth from intrastriatal VM grafts

Both DA and non-DA neurons in intrastriatal VM grafts are capable of extensive axonal outgrowth and innervation of adjacent striatal, as well as more distant extrastriatal, targets. The use of tools such as species-specific antibodies (e.g., against

mouse-specific M2M6 or species-specific neurofilament epitopes) and reporter proteins such as GFP has greatly contributed to our understanding of the growth and efferent connectivity of VM grafts (for review, see Thompson and Björklund, 2009).

Sawamoto et al. (2001) reported the generation of a transgenic mouse in which GFP is expressed under control of the rat promoter sequence for tyrosine hydroxylase (TH), and therefore in all DA neurons (Fig. 6A). Transplantation experiments using donor VM from these mice has allowed for a more detailed analysis of patterns of fiber outgrowth from grafted DA neurons than had previously been possible using TH immunohistochemistry alone. Immunohistochemistry for GFP in neonatal or adult rats grafted with E12.5 VM from *TH*–GFP mice revealed an extensive innervation of the host striatum, but notably also the presence of GFP+ fibers in extrastriatal areas, including the overlying frontal cortex, as well as the perirhinal and piriform cortices and the amygdala (Thompson et al., 2005)—Fig. 6E, H, and I. The level of GFP+ fiber outgrowth to both striatal and extrastriatal areas was more pronounced in neonatal compared to adult recipients, and in some cases, extended through the corpus callosum in order to innervate the contralateral striatum. Importantly, retrograde tracing experiments showed that innervation of different host territories by grafted DA neurons occurs in a neuronal subtype-specific manner. Specifically, backfilling from prefrontal cortex identifies DA neurons with A10 phenotype based on morphology, central graft location, and calbindin expression, while tracing from dorsolateral striatum identifies large, Girk2+, A9 neurons in the periphery of the graft. Hence, specific midbrain DA subtypes in VM grafts appear to be intrinsically programmed to innervate their normal developmental targets at the time of implantation. This result extends earlier findings demonstrating the capacity for grafted DA neurons to innervate the striatum as a specific property of midbrain DA neurons, and not one shared by other, non-midbrain DA phenotypes, such as those from the nearby diencephalon (Abrous et al., 1988; Hudson et al., 1994; Zuddas et al., 1991). An implication from these findings is that successful restoration of motor function following VM grafting will require the presence of specific DA subtypes capable of reinnervating the denervated areas. In PD patients, the early loss of A9 neurons innervating the striatum, particularly the putamen, is an important pathological feature underlying motor dysfunction (Kish et al., 1988). Thus, the functional reinstatement of this circuitry will likely require the presence of sufficient numbers of A9 DA neurons capable of providing a robust innervation of the host striatum.

In support of this concept are studies showing that the number of A9 neurons in VM grafts correlates with the level of behavioral recovery in rodents (Kuan et al., 2007), which is, in fact, lost upon removal of the A9 component (Grealish et al., 2010). In the study by Grealish et al. (2010), a comparison of the anatomical and functional properties of VM grafts either (a) containing a typical mix of A9 and A10 neurons or (b) where the A9 neurons were selectively removed, was achieved in transplantation studies using donor tissue from the *Pitx3*-GFP reporter mouse line developed by Zhao et al. (2004). Because the GFP has been "knocked in" to the *Pitx3* gene locus, mice homozygous for GFP ($Pitx3^{GFP/GFP}$) are functional *Pitx3* knockouts and have essentially the same phenotype as aphakia mice (where there is a specific

developmental loss of A9 DA neurons while the A10 population is spared; Smidt et al., 2004). Grafts of VM from heterozygous *Pitx3*$^{GFP/WT}$ mice have a normal complement of A9 and A10 neurons associated with robust striatal innervation and reinstatement of deficits in motor function induced by 6-hydroxyDA lesion. On the other hand, grafts derived from *Pitx3*$^{GFP/GFP}$ VM are composed almost exclusively of DA neurons with A10 phenotype and, despite the same number overall number of DA neurons, fail to innervate the dorsolateral striatum or to reverse motor impairment. Thus, the DA neuronal subtype composition in VM grafts has a major impact on the pattern of host innervation as well as the capacity for the grafts to improve motor function. It is worth noting that while these results highlight the importance of A9-based striatal reinnervation for the reinstatement of certain motor functions, they by no means imply that the A10 neurons are dispensable for the overall functional impact of VM grafts. Studies in rats show that striatal territories normally innervated by A10 neurons, such as the nucleus accumbens, play an important role in motivational aspects of motor function and that VM transplants in animals with selective DA depletion in this more ventral region can improve motor function (Brundin et al., 1987). In summary, this work highlights that the DA subtype composition of VM grafts is an important determinant of efferent connectivity and functional impact. Variables that may influence the DA subtype composition, such as donor age or tissue handling, are therefore highly relevant to consider in VM grafting studies.

Xenografting studies using species-specific antibodies show that also the non-DA neuronal component in VM grafts is characterized by a predictable pattern of efferent connectivity, suggesting target-directed mechanisms of fiber outgrowth also for these cell types (Isacson and Deacon, 1996; Thompson et al., 2008). Non-DA neurons are capable of axonal growth over long distances in order to innervate various extrastriatal targets including the cortex, thalamus, and midbrain (Fig. 3H–J). Retrograde-tracing studies indicate that many of these projections originate from GABAergic neurons in the grafts (Thompson et al., 2008). These neurons likely represent the inclusion of progenitors for midbrain GABAergic projection neurons normally contained in the dissected VM pieces used for transplantation. This might include, for example, GABAergic neurons of the substantia nigra pars reticulata that project to the superior colliculus (Williams and Faull, 1985) and thalamus (Beckstead et al., 1979; Cornwall and Phillipson, 1988) as well as GABAergic neurons in the VTA (Beckstead et al., 1979) and adjacent nuclei, including the mammillary region (Shibata, 1992). Although extensive in nature, it is not certain if and how the connectivity of these GABAergic neurons in VM grafts contributes functional impact (not least because the intrinsic non-DA neurons remain largely intact in the 6-OHDA-lesioned host rat brain).

As discussed above, the functional relevance of connectivity by 5HT neurons as a non-DA component of VM grafts is clearer. There is ample evidence to suggest these neurons may be at least partly responsible for the appearance of graft-induced dyskinesias in PD patients in some cases (Carlsson et al., 2007, 2009). Even as a relatively small fraction of the grafted cells, the 5HT neurons are capable of establishing

an extensive terminal network in the host striatum (Carlsson et al., 2007; Wright et al., 1991). Interestingly, in the DA-depleted, but not the intact, striatum, grafted 5HT neurons give rise to a dense hyperinnervation over and above normal patterns of 5HT innervation in the intact brain (Wright et al., 1991). This "supra-normal" level of 5HT connectivity in the PD-affected striatum may be a contributing factor in the mishandling of DA as a basis for graft-induced dyskinesia. This concept is supported indirectly by imaging studies suggesting serotonergic hyperinnervation of the striatum as a conspicuous feature in some of the patients experiencing unwanted dyskinesias after transplantation (Politis et al., 2010, 2011).

4.4 Connectivity of intranigral VM grafts

As discussed above, clinical benefit following transplantation in patients requires extensive dopaminergic innervation of the putamen through transplantation directly into this target area. However, even in the most impressive cases in patients and animal models, certain aspects of motor function remain insensitive to correction following intrastriatal VM grafting despite extensive striatal reinnervation (for review, see Lindvall and Hagell, 2000; Winkler et al., 2000). The reason for this is not entirely clear. Dopaminergic denervation in areas not extensively innervated by grafted DA neurons, for example, cortical and limbic regions, may be a factor. Other limitations of the ectopic placement of the DA neurons may also be important. Although DA neurons grafted into the forebrain are capable of intrinsically releasing DA, this may occur in a suboptimal manner due to the lack of appropriate afferent input these cells receive in their normal midbrain location. Further, the loss of local DA signaling in the midbrain through dendritic innervation of the substantia nigra pars reticulata by overlying DA neurons (Fig. 4A and B) will not in any way be restored by placement of new neurons in the striatum. Thus, there are well-defined shortcomings of striatal placement of grafted DA neurons that may limit functional efficacy and that drive a continuing interest in this field to explore the possibility of a more accurate reconstruction of the nigrostriatal pathway through placement of grafted DA neurons into their normal midbrain location.

Early studies in this field showed that, although DA neurons survived after transplantation into the midbrain of 6-OHDA-lesioned rats, they failed to extend axons along the nigrostriatal pathway or to induce any level of recovery of motor function (Björklund et al., 1983b; Dunnett et al., 1983). This appeared to be the result of a restrictive host environment rather than a limitation of the growth capacity of the grafted neurons. Experiments using so-called bridge grafts using pieces of peripheral nerve or Schwann cells to provide a permissive growth substrate between the midbrain and striatum showed that the grafted DA neurons had the intrinsic potential to extend axons from the midbrain in order to innervate the host striatum (Aguayo et al., 1984; Brecknell et al., 1996; Gage et al., 1985; Wilby et al., 1999). Xenografting studies also showed that DA neurons from human or porcine VM placed into the midbrain of 6-OHDA-lesioned rats could innervate appropriate forebrain targets, including the striatum (Isacson et al., 1995; Wictorin et al., 1992) leading to the hypothesis that this

was the result of a failure of outgrowing axons to recognize species-specific growth-inhibitory cues. Further, work from Bentlage et al. (1999) showed that growth along the nigrostriatal pathway was permissive in the neonatal rat brain, but this capacity was lost when the host brain reached around 20 days of age.

Taken together, these studies lead to the conclusion that the adult brain is incapable of supporting long-distance growth of axons from allografted DA neurons along the nigrostriatal pathway. However, subsequent experiments in adult mice using donor VM from GFP reporter mice have shown this not to be the case (Gaillard et al., 2009; Thompson et al., 2009). Immunohistochemistry for GFP 16 weeks after intranigral grafts of E12.5 VM from *TH*–GFP donors showed a remarkably specific pattern of axonal growth from grafted DA neurons along the nigrostriatal pathway in order to provide a an elaborate terminal network in the striatum (Thompson et al., 2009; Fig. 7). The growth pattern matched well with the anatomy of the intrinsic system. The GFP+ axons formed a polarized group of loosely bundled, unramified fibers running parallel to the medial forebrain bundle and through the globus pallidus, but abruptly gave rise to a highly elaborated terminal network on reaching the striatum. Some animals also showed substantial normalization of amphetamine-induced rotational behavior, indicating functional connectivity of the grafted neurons. The degree of growth along the nigrostriatal pathway as well as the size of the striatal terminal network and the degree of improvement in rotational scores could be significantly enhanced by overexpression of GDNF in the striatal target. This is consistent with other studies showing that GDNF can stimulate DA fiber outgrowth from intrastriatal (Rosenblad et al., 1996) and intranigral (Redmond et al., 2009; Wilby et al., 1999) VM grafts.

An interesting feature of the pattern of GFP+ fiber outgrowth is the intermingling of graft-derived fibers with remaining fibers from host DA neurons in partially lesioned animals (Fig. 7D). This raises the possibility that a residual host-derived DA fiber pathway can stimulate, or may even be required for significant growth of axons from grafted DA neurons. Although the pattern of GFP+ and host-derived fibers in the medial fiber bundle was not indicative of a contact-mediated interaction, diffusible trophic factors known to be released by host DA neurons, such as BDNF (Bustos et al., 2004), may stimulate and support the extension of axons from grafted DA neurons by forming a kind of "growth-permissive corridor." The concept of "pioneer axons" that support the growth of later growing axons during normal development of the nervous system has been well described (Hidalgo and Brand, 1997; Klose and Bentley, 1989; Lin et al., 1995; McConnell et al., 1989, 1994; Molnar et al., 1998; Pittman et al., 2008). This hypothesis may explain why previous studies failed to detect significant nigrostriatal growth from DA neurons placed in the midbrain. Without the use of a GFP reporter to identify graft-derived fiber patterns, earlier studies had to rely on the detection of TH, which will be expressed by fibers of both graft and host origin. This therefore required complete ablation of the host system in order to confidently identify axons from grafted DA neurons, thus potentially creating an unfavorable environment for growth. Such a scenario may suggest that regrowth along the nigrostriatal pathway from grafted DA neurons may be more challenging in PD

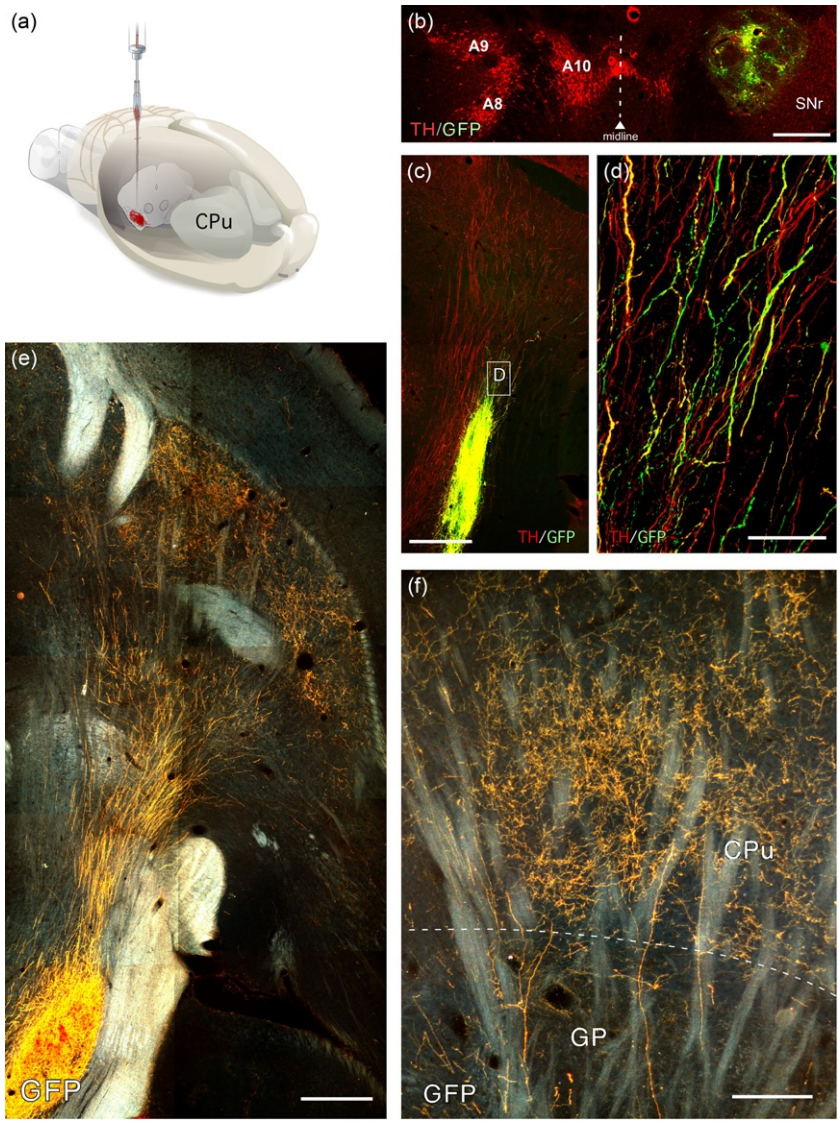

FIGURE 7

Connectivity of allografted dopamine neurons placed into the adult midbrain. Immunohistochemistry for GFP in mice grafted with donor VM tissue from *TH*–GFP mice allows for detailed and unambiguous characterization of fiber outgrowth patterns from DA neurons allografted into the midbrain. (A) A schematic representation illustrates the implantation site in the midbrain and highlights the relatively long distance required for connectivity of grafted DA neurons with the host forebrain, including the striatum. (B) Immunohistochemistry for TH (red) and GFP (green) in a horizontal section through the

patients with advanced diseased states characterized by substantial loss of the intrinsic projection system.

In summary, the overall experience in this field, including new insights from studies using donor tissue from reporter mice, shows that while functional reinstatement of midbrain DA circuitry from VM grafts placed in the midbrain is challenging, there is significant scope for further development of the concept. The proof-of-principle findings that new DA neurons allografted into the adult midbrain can establish functional connectivity with the host forebrain sets the scene for strategies aimed at augmenting this phenomenon in animal models of PD in order to improve the functional impact.

5 CLOSING REMARKS

An extensive body of basic and clinical research has led to a detailed understanding of the growth and connectivity of intracerebral VM grafts and, importantly, how these properties relate to restoration of motor function. This forms an important platform for the refinement and optimization of current transplantation procedures using fetal tissue and, importantly, for the development of new procedures using stem cells.

A major challenge for the establishment of a cell-based therapy as a realistic mainstream option for PD patients is to deliver well-defined procedures that ensure effective and predictable therapeutic outcomes. As reviewed in this chapter, specific variables related to the host, the donor VM preparation, and also the implantation technique can have a significant impact on the survival, composition, and

midbrain shows the placement and survival of an intranigral graft implanted into an animal with a 6-hydroxydopamine lesion, relative to the remaining DA cells groups (A8, A9, and A10) on the intact side of the brain. (C) The GFP reporter allows for detection of the grafted DA neurons (C) and their associated fibers (D, green/yellow) even when intermingled closely with a residual host fiber pathway (red) in partially lesioned animals. (E) A photo-montage of darkfield images throughout a horizontal section labeled with an antibody against GFP and visualized using the chromogen di-amino-benzidine (DAB) shows the extensive growth of GFP+ axons from the anterior part of the graft in order to extend along the nigrostriatal pathway and into the host striatum. (F) The pattern of growth of DA neurons in intranigral VM grafts matches remarkably well with the normal structure of the midbrain DA projection system, including the growth of unramified axons that extend parallel to the medial forebrain bundle and then abruptly give rise to a highly elaborated terminal network on reaching the host striatum (dashed line indicates approximate border between the globus pallidus and the striatum). Scale bars: (B) and (C), 500 μm; (D), 50 μm; (E), 500 μm; and (F), 200 μm. Abbreviations: CPu, caudate putamen unit; GP, glubus pallidus; SNr, sunstantia nigra pars compacta.

(*Some of the images shown here are modified reproductions from Thompson et al., 2009.*)

connectivity of VM grafts. Monitoring and controlling these variables, as well as on-going research aimed at identifying additional factors that affect graft structure and function, will form an important part of strategies to optimize VM grafting procedures. Significant progress has been made along these lines already. Careful analysis of the design and results from failed clinical trials, as well as on-going research in animal models of PD, has led to the identification of key variables related to patient selection and tissue preparation that will need to be monitored closely in order to achieve better outcomes in future trials (for reviews, see Winkler et al., 2005; Chapter 9).

The detailed understanding we have gained on the anatomical and functional properties of DA neurons in VM grafts through more than 30 years of research in this area also forms an important point of reference for the interpretation of transplantation experiments using stem cells. Although there are now robust procedures for the generation of correctly specified midbrain DA neurons from highly expandable populations of stem cells (Chapter 13), the potential of these cells for effective reconstruction of DA circuitry *in vivo* remains largely unexplored. An important goal of research in this area is to determine whether DA neurons derived from stem cells share the remarkable capacity of midbrain DA neurons for highly specific, target-directed growth and functional connectivity after transplantation in the adult brain.

References

Abrous, N., Guy, J., Vigny, A., Calas, A., Le Moal, M., Herman, J.P., 1988. Development of intracerebral dopaminergic grafts: a combined immunohistochemical and autoradiographic study of its time course and environmental influences. J. Comp. Neurol. 273, 26–41.

Aguayo, A.J., Björklund, A., Stenevi, U., Carlstedt, T., 1984. Fetal mesencephalic neurons survive and extend long axons across peripheral nervous system grafts inserted into the adult rat striatum. Neurosci. Lett. 45, 53–58.

Baker-Cairns, B.J., Sloan, D.J., Broadwell, R.D., Puklavec, M., Charlton, H.M., 1996. Contributions of donor and host blood vessels in CNS allografts. Exp. Neurol. 142, 36–46.

Barker, R.A., Fricker, R.A., Abrous, D.N., Fawcett, J., Dunnett, S.B., 1995. A comparative study of preparation techniques for improving the viability of nigral grafts using vital stains, in vitro cultures, and in vivo grafts. Cell Transplant. 4, 173–200.

Barker, R.A., Dunnett, S.B., Faissner, A., Fawcett, J.W., 1996. The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. Exp. Neurol. 141, 79–93.

Beckstead, R.M., Domesick, V.B., Nauta, W.J., 1979. Efferent connections of the substantia nigra and ventral tegmental area in the rat. Brain Res. 175, 191–217.

Bentlage, C., Nikkhah, G., Cunningham, M.G., Björklund, A., 1999. Reformation of the nigrostriatal pathway by fetal dopaminergic micrografts into the substantia nigra is critically dependent on the age of the host. Exp. Neurol. 159, 177–190.

Björklund, A., Dunnett, S.B., 2007. Dopamine neuron systems in the brain: an update. Trends Neurosci. 30, 194–202.

Björklund, A., Stenevi, U., 1979. Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. Brain Res. 177, 555–560.

Björklund, A., Stenevi, U., Schmidt, R.H., Dunnett, S.B., Gage, F.H., 1983a. Intracerebral grafting of neuronal cell suspensions. I. Introduction and general methods of preparation. Acta Physiol. Scand. Suppl. 522, 1–7.

Björklund, A., Stenevi, U., Schmidt, R.H., Dunnett, S.B., Gage, F.H., 1983b. Intracerebral grafting of neuronal cell suspensions. II. Survival and growth of nigral cell suspensions implanted in different brain sites. Acta Physiol. Scand. Suppl. 522, 9–18.

Bolam, J.P., Freund, T.F., Björklund, A., Dunnett, S.B., Smith, A.D., 1987. Synaptic input and local output of dopaminergic neurons in grafts that functionally reinnervate the host neostriatum. Exp. Brain Res. 68, 131–146.

Brecknell, J.E., Haque, N.S., Du, J.S., Muir, E.M., Fidler, P.S., Hlavin, M.L., Fawcett, J.W., Dunnett, S.B., 1996. Functional and anatomical reconstruction of the 6-hydroxydopamine lesioned nigrostriatal system of the adult rat. Neuroscience 71, 913–925.

Broadwell, R.D., Charlton, H.M., Ebert, P., Hickey, W.F., Villegas, J.C., Wolf, A.L., 1990. Angiogenesis and the blood-brain barrier in solid and dissociated cell grafts within the CNS. Prog. Brain Res. 82, 95–101.

Brundin, P., Björklund, A., 1987. Survival, growth and function of dopaminergic neurons grafted to the brain. Prog. Brain Res. 71, 293–308.

Brundin, P., Barbin, G., Isacson, O., Mallat, M., Chamak, B., Prochiantz, A., Gage, F.H., Björklund, A., 1985a. Survival of intracerebrally grafted rat dopamine neurons previously cultured in vitro. Neurosci. Lett. 61, 79–84.

Brundin, P., Isacson, O., Björklund, A., 1985b. Monitoring of cell viability in suspensions of embryonic CNS tissue and its use as a criterion for intracerebral graft survival. Brain Res. 331, 251–259.

Brundin, P., Strecker, R.E., Londos, E., Björklund, A., 1987. Dopamine neurons grafted unilaterally to the nucleus accumbens affect drug-induced circling and locomotion. Exp. Brain Res. 69, 183–194.

Brundin, P., Strecker, R.E., Widner, H., Clarke, D.J., Nilsson, O.G., Astedt, B., Lindvall, O., Björklund, A., 1988. Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour, and dopamine release. Exp. Brain Res. 70, 192–208.

Brundin, P., Björklund, A., Lindvall, O., 1990. Practical aspects of the use of human fetal brain tissue for intracerebral grafting. Prog. Brain Res. 82, 707–714.

Brundin, P., Duan, W.M., Sauer, H., 1994. Functional effects of mesencephalic dopamine neurons and adrenal chromaffin cells grafted to the rodent striatum. In: Dunnett, S.B., Björklund, A. (Eds.), Functional Neural Transplantation. Raven Press, New York, pp. 9–46.

Brundin, P., Karlsson, J., Emgard, M., Schierle, G.S., Hansson, O., Petersen, A., Castilho, R.F., 2000. Improving the survival of grafted dopaminergic neurons: a review over current approaches. Cell Transplant. 9, 179–195.

Bustos, G., Abarca, J., Campusano, J., Bustos, V., Noriega, V., Aliaga, E., 2004. Functional interactions between somatodendritic dopamine release, glutamate receptors and brain-derived neurotrophic factor expression in mesencephalic structures of the brain. Brain Res. Brain Res. Rev. 47, 126–144.

Bye, C.R., Thompson, L.H., Parish, C.L., 2012. Birth dating of midbrain dopamine neurons identifies A9 enriched tissue for transplantation into Parkinsonian mice. Exp. Neurol. 236, 58–68.

Carlsson, T., Carta, M., Winkler, C., Björklund, A., Kirik, D., 2007. Serotonin neuron transplants exacerbate L-DOPA-induced dyskinesias in a rat model of Parkinson's disease. J. Neurosci. 27, 8011–8022.

Carlsson, T., Carta, M., Munoz, A., Mattsson, B., Winkler, C., Kirik, D., Björklund, A., 2009. Impact of grafted serotonin and dopamine neurons on development of L-DOPA-induced dyskinesias in Parkinsonian rats is determined by the extent of dopamine neuron degeneration. Brain 132, 319–335.

Carta, M., Carlsson, T., Kirik, D., Björklund, A., 2007. Dopamine released from 5-HT terminals is the cause of L-DOPA-induced dyskinesia in Parkinsonian rats. Brain 130, 1819–1833.

Collier, T.J., Sortwell, C.E., Daley, B.F., 1999. Diminished viability, growth, and behavioral efficacy of fetal dopamine neuron grafts in aging rats with long-term dopamine depletion: an argument for neurotrophic supplementation. J. Neurosci. 19, 5563–5573.

Cornwall, J., Phillipson, O.T., 1988. Afferent projections to the dorsal thalamus of the rat as shown by retrograde lectin transport—I. The mediodorsal nucleus. Neuroscience 24, 1035–1049.

Dahlstrom, A., Fuxe, K., 1964. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol. Scand. 62 (Suppl. 232), 231–255.

Doucet, G., Murata, Y., Brundin, P., Bosler, O., Mons, N., Geffard, M., Ouimet, C.C., Björklund, A., 1989. Host afferents into intrastriatal transplants of fetal ventral mesencephalon. Exp. Neurol. 106, 1–19.

Doucet, G., Brundin, P., Descarries, L., Björklund, A., 1990. Effect of prior dopamine denervation on survival and fiber outgrowth from intrastriatal fetal mesencephalic grafts. Eur. J. Neurosci. 2, 279–290.

Duan, W.M., Widner, H., Brundin, P., 1995. Temporal pattern of host responses against intrastriatal grafts of syngeneic, allogeneic or xenogeneic embryonic neuronal tissue in rats. Exp. Brain Res. 104, 227–242.

Dunnett, S.B., Björklund, A., Schmidt, R.H., Stenevi, U., Iversen, S.D., 1983. Intracerebral grafting of neuronal cell suspensions. IV. Behavioural recovery in rats with unilateral 6-OHDA lesions following implantation of nigral cell suspensions in different forebrain sites. Acta Physiol. Scand. Suppl. 522, 29–37.

Dunnett, S.B., Hernandez, T.D., Summerfield, A., Jones, G.H., Arbuthnott, G., 1988. Graft-derived recovery from 6-OHDA lesions: specificity of ventral mesencephalic graft tissues. Exp. Brain Res. 71, 411–424.

Dunnett, S.B., Björklund, A., Lindvall, O., 2001. Cell therapy in Parkinson's disease—stop or go? Nat. Rev. Neurosci. 2, 365–369.

Emgard, M., Karlsson, J., Hansson, O., Brundin, P., 1999. Patterns of cell death and dopaminergic neuron survival in intrastriatal nigral grafts. Exp. Neurol. 160, 279–288.

Fallon, J.H., Moore, R.Y., 1978. Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum. J. Comp. Neurol. 180, 545–580.

Fearnley, J.M., Lees, A.J., 1991. Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain 114 (Pt 5), 2283–2301.

Fisher, L.J., Young, S.J., Tepper, J.M., Groves, P.M., Gage, F.H., 1991. Electrophysiological characteristics of cells within mesencephalon suspension grafts. Neuroscience 40, 109–122.

Freed, C.R., Greene, P.E., Breeze, R.E., Tsai, W.Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, H., Culver, S., Trojanowski, J.Q., Eidelberg, D., Fahn, S., 2001. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N. Engl. J. Med. 344, 710–719.

Freund, T.F., Bolam, J.P., Björklund, A., Stenevi, U., Dunnett, S.B., Powell, J.F., Smith, A.D., 1985. Efferent synaptic connections of grafted dopaminergic neurons reinnervating the host neostriatum: a tyrosine hydroxylase immunocytochemical study. J. Neurosci. 5, 603–616.

Frodl, E.M., Duan, W.M., Sauer, H., Kupsch, A., Brundin, P., 1994. Human embryonic dopamine neurons xenografted to the rat: effects of cryopreservation and varying regional source of donor cells on transplant survival, morphology and function. Brain Res. 647, 286–298.

Gage, F.H., Björklund, A., Stenevi, U., Dunnett, S.B., 1983. Intracerebral grafting of neuronal cell suspensions. VIII. Survival and growth of implants of nigral and septal cell suspensions in intact brains of aged rats. Acta Physiol. Scand. Suppl. 522, 67–75.

Gage, F.H., Stenevi, U., Carlstedt, T., Foster, G., Björklund, A., Aguayo, A.J., 1985. Anatomical and functional consequences of grafting mesencephalic neurons into a peripheral nerve "bridge" connected to the denervated striatum. Exp. Brain Res. 60, 584–589.

Gaillard, A., Decressac, M., Frappe, I., Fernagut, P.O., Prestoz, L., Besnard, S., Jaber, M., 2009. Anatomical and functional reconstruction of the nigrostriatal pathway by intranigral transplants. Neurobiol. Dis. 35, 477–488.

Garcia, J., Carlsson, T., Dobrossy, M., Nikkhah, G., Winkler, C., 2011. Impact of dopamine to serotonin cell ratio in transplants on behavioral recovery and L-DOPA-induced dyskinesia. Neurobiol. Dis. 43, 576–587.

Gates, M.A., Torres, E.M., White, A., Fricker-Gates, R.A., Dunnett, S.B., 2006. Re-examining the ontogeny of substantia nigra dopamine neurons. Eur. J. Neurosci. 23, 1384–1390.

German, D.C., Schlusselberg, D.S., Woodward, D.J., 1983. Three-dimensional computer reconstruction of midbrain dopaminergic neuronal populations: from mouse to man. J. Neural Transm. 57, 243–254.

German, D.C., Manaye, K., Smith, W.K., Woodward, D.J., Saper, C.B., 1989. Midbrain dopaminergic cell loss in Parkinson's disease: computer visualization. Ann. Neurol. 26, 507–514.

Grealish, S., Jonsson, M.E., Li, M., Kirik, D., Björklund, A., Thompson, L.H., 2010. The A9 dopamine neuron component in grafts of ventral mesencephalon is an important determinant for recovery of motor function in a rat model of Parkinson's disease. Brain 133, 482–495.

Hagell, P., Brundin, P., 2001. Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease. J. Neuropathol. Exp. Neurol. 60, 741–752.

Herman, J.P., Abrous, N.D., 1994. Dopaminergic neural grafts after fifteen years: results and perspectives. Prog. Neurobiol. 44, 1–35.

Hidalgo, A., Brand, A.H., 1997. Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of Drosophila. Development 124, 3253–3262.

Hornykiewicz, O., 1975. Brain monoamines and parkinsonism. Natl. Inst. Drug Abuse Res. Monogr. Ser. 3, 13–21.

Hudson, J.L., Bickford, P., Johansson, M., Hoffer, B.J., Strömberg, I., 1994. Target and neurotransmitter specificity of fetal central nervous system transplants: importance for functional reinnervation. J. Neurosci. 14, 283–290.

Isacson, O., Deacon, T.W., 1996. Specific axon guidance factors persist in the adult brain as demonstrated by pig neuroblasts transplanted to the rat. Neuroscience 75, 827–837.

Isacson, O., Deacon, T.W., Pakzaban, P., Galpern, W.R., Dinsmore, J., Burns, L.H., 1995. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. Nat. Med. 1, 1189–1194.

Janec, E., Burke, R.E., 1993. Naturally occurring cell death during postnatal development of the substantia nigra pars compacta of rat. Mol. Cell. Neurosci. 4, 30–35.

Joksimovic, M., Anderegg, A., Roy, A., Campochiaro, L., Yun, B., Kittappa, R., McKay, R., Awatramani, R., 2009. Spatiotemporally separable Shh domains in the midbrain define distinct dopaminergic progenitor pools. Proc. Natl. Acad. Sci. USA 106, 19185–19190.

Jonsson, M.E., Ono, Y., Björklund, A., Thompson, L.H., 2009. Identification of transplantable dopamine neuron precursors at different stages of midbrain neurogenesis. Exp. Neurol. 219, 341–354.

Kirik, D., Winkler, C., Björklund, A., 2001. Growth and functional efficacy of intrastriatal nigral transplants depend on the extent of nigrostriatal degeneration. J. Neurosci. 21, 2889–2896.

Kish, S.J., Shannak, K., Hornykiewicz, O., 1988. Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. N. Engl. J. Med. 318, 876–880.

Klose, M., Bentley, D., 1989. Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. Science 245, 982–984.

Kordower, J.H., Rosenstein, J.M., Collier, T.J., Burke, M.A., Chen, E.Y., Li, J.M., Martel, L., Levey, A.E., Mufson, E.J., Freeman, T.B., Olanow, C.W., 1996. Functional fetal nigral grafts in a patient with Parkinson's disease: chemoanatomic, ultrastructural, and metabolic studies. J. Comp. Neurol. 370, 203–230.

Kordower, J.H., Freeman, T.B., Chen, E.Y., Mufson, E.J., Sanberg, P.R., Hauser, R.A., Snow, B., Olanow, C.W., 1998. Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. Mov. Disord. 13, 383–393.

Kornblum, H.I., Hussain, R.J., Bronstein, J.M., Gall, C.M., Lee, D.C., Seroogy, K.B., 1997. Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. J. Comp. Neurol. 380, 243–261.

Kuan, W.L., Lin, R., Tyers, P., Barker, R.A., 2007. The importance of A9 dopaminergic neurons in mediating the functional benefits of fetal ventral mesencephalon transplants and levodopa-induced dyskinesias. Neurobiol. Dis. 25, 594–608.

Lane, E.L., Björklund, A., Dunnett, S.B., Winkler, C., 2010. Neural grafting in Parkinson's disease unraveling the mechanisms underlying graft-induced dyskinesia. Prog. Brain Res. 184, 295–309.

Lin, D.M., Auld, V.J., Goodman, C.S., 1995. Targeted neuronal cell ablation in the Drosophila embryo: pathfinding by follower growth cones in the absence of pioneers. Neuron 14, 707–715.

Lindvall, O., Björklund, A., 2004. Cell therapy in Parkinson's disease. NeuroRx 1, 382–393.

Lindvall, O., Hagell, P., 2000. Clinical observations after neural transplantation in Parkinson's disease. Prog. Brain Res. 127, 299–320.

Llambi, F., Causeret, F., Bloch-Gallego, E., Mehlen, P., 2001. Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. EMBO J. 20, 2715–2722.

Lundberg, C., Wictorin, K., Björklund, A., 1994. Retrograde degenerative changes in the substantia nigra pars compacta following an excitotoxic lesion of the striatum. Brain Res. 644, 205–212.

Mahalik, T.J., Clayton, G.H., 1991. Specific outgrowth from neurons of ventral mesencephalic grafts to the catecholamine-depleted striatum of adult hosts. Exp. Neurol. 113, 18–27.

McConnell, S.K., Ghosh, A., Shatz, C.J., 1989. Subplate neurons pioneer the first axon pathway from the cerebral cortex. Science 245, 978–982.

McConnell, S.K., Ghosh, A., Shatz, C.J., 1994. Subplate pioneers and the formation of descending connections from cerebral cortex. J. Neurosci. 14, 1892–1907.

Mehlen, P., Mazelin, L., 2003. The dependence receptors DCC and UNC5H as a link between neuronal guidance and survival. Biol. cell 95, 425–436.

Mendez, I., Sanchez-Pernaute, R., Cooper, O., Vinuela, A., Ferrari, D., Björklund, L., Dagher, A., Isacson, O., 2005. Cell type analysis of functional fetal dopamine cell suspension transplants in the striatum and substantia nigra of patients with Parkinson's disease. Brain 128, 1498–1510.

Molnar, Z., Adams, R., Blakemore, C., 1998. Mechanisms underlying the early establishment of thalamocortical connections in the rat. J. Neurosci. 18, 5723–5745.

Nakao, N., Frodl, E.M., Duan, W.M., Widner, H., Brundin, P., 1994. Lazaroids improve the survival of grafted rat embryonic dopamine neurons. Proc. Natl. Acad. Sci. USA 91, 12408–12412.

Nakao, N., Frodl, E.M., Widner, H., Carlson, E., Eggerding, F.A., Epstein, C.J., Brundin, P., 1995. Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. Nat. Med. 1, 226–231.

Nikkhah, G., Duan, W.M., Knappe, U., Jodicke, A., Björklund, A., 1993. Restoration of complex sensorimotor behavior and skilled forelimb use by a modified nigral cell suspension transplantation approach in the rat Parkinson model. Neuroscience 56, 33–43.

Nikkhah, G., Bentlage, C., Cunningham, M.G., Björklund, A., 1994a. Intranigral fetal dopamine grafts induce behavioral compensation in the rat Parkinson model. J. Neurosci. 14, 3449–3461.

Nikkhah, G., Cunningham, M.G., Jodicke, A., Knappe, U., Björklund, A., 1994b. Improved graft survival and striatal reinnervation by microtransplantation of fetal nigral cell suspensions in the rat Parkinson model. Brain Res. 633, 133–143.

Nikkhah, G., Winkler, C., Rödter, A., Samii, M., 2000. Microtransplantation of nigral dopamine neurons: a "step by step" recipe. In: Dunnett, S.B., Boulton, A.A., Baker, G.B. (Eds.), Neuromethods: Cell and Tissue Transplantation in the CNS. The Human Press, Totowa, pp. 207–231.

Olanow, C.W., Goetz, C.G., Kordower, J.H., Stoessl, A.J., Sossi, V., Brin, M.F., Shannon, K.M., Nauert, G.M., Perl, D.P., Godbold, J., Freeman, T.B., 2003. A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann. Neurol. 54, 403–414.

Olson, L., Seiger, A., Strömberg, I., 1983. Intraocular Transplantation in Rodents: A Detailed Account of the Procedure and Examples of Its Use in Neurobiology with Special Reference to Brain Tissue Grafting. Academic Press, New York.

Pakkenberg, B., Moller, A., Gundersen, H.J., Mouritzen Dam, A., Pakkenberg, H., 1991. The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. J. Neurol. Neurosurg. Psychiatry 54, 30–33.

Perlow, M.J., Freed, W.J., Hoffer, B.J., Seiger, A., Olson, L., Wyatt, R.J., 1979. Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. Science 204, 643–647.

Piccini, P., Pavese, N., Hagell, P., Reimer, J., Björklund, A., Oertel, W.H., Quinn, N.P., Brooks, D.J., Lindvall, O., 2005. Factors affecting the clinical outcome after neural transplantation in Parkinson's disease. Brain 128, 2977–2986.

Pittman, A.J., Law, M.Y., Chien, C.B., 2008. Pathfinding in a large vertebrate axon tract: isotypic interactions guide retinotectal axons at multiple choice points. Development 135, 2865–2871.

Politis, M., Wu, K., Loane, C., Quinn, N.P., Brooks, D.J., Rehncrona, S., Björklund, A., Lindvall, O., Piccini, P., 2010. Serotonergic neurons mediate dyskinesia side effects in Parkinson's patients with neural transplants. Sci. Transl. Med. 2, 38ra46.

Politis, M., Oertel, W.H., Wu, K., Quinn, N.P., Pogarell, O., Brooks, D.J., Björklund, A., Lindvall, O., Piccini, P., 2011. Graft-induced dyskinesias in Parkinson's disease: high striatal serotonin/dopamine transporter ratio. Mov. Disord. 26, 1997–2003.

Redmond Jr., D.E., Vinuela, A., Kordower, J.H., Isacson, O., 2008. Influence of cell preparation and target location on the behavioral recovery after striatal transplantation of fetal dopaminergic neurons in a primate model of Parkinson's disease. Neurobiol. Dis. 29, 103–116.

Redmond Jr., D.E., Elsworth, J.D., Roth, R.H., Leranth, C., Collier, T.J., Blanchard, B., Bjugstad, K.B., Samulski, R.J., Aebischer, P., Sladek Jr., J.R., 2009. Embryonic substantia nigra grafts in the mesencephalon send neurites to the host striatum in non-human primate after overexpression of GDNF. J. Comp. Neurol. 515, 31–40.

Reyes, S., Fu, Y., Double, K., Thompson, L., Kirik, D., Paxinos, G., Halliday, G.M., 2012. GIRK2 expression in dopamine neurons of the substantia nigra and ventral tegmental area. J. Comp. Neurol. 520, 2591–2607.

Rosenblad, C., Martinez-Serrano, A., Björklund, A., 1996. Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. Neuroscience 75, 979–985.

Sauer, H., Frodl, E.M., Kupsch, A., ten Bruggencate, G., Oertel, W.H., 1992. Cryopreservation, survival and function of intrastriatal fetal mesencephalic grafts in a rat model of Parkinson's disease. Exp. Brain Res. 90, 54–62.

Sautter, J., Tseng, J.L., Braguglia, D., Aebischer, P., Spenger, C., Seiler, R.W., Widmer, H.R., Zurn, A.D., 1998. Implants of polymer-encapsulated genetically modified cells releasing glial cell line-derived neurotrophic factor improve survival, growth, and function of fetal dopaminergic grafts. Exp. Neurol. 149, 230–236.

Sawamoto, K., Nakao, N., Kobayashi, K., Matsushita, N., Takahashi, H., Kakishita, K., Yamamoto, A., Yoshizaki, T., Terashima, T., Murakami, F., Itakura, T., Okano, H., 2001. Visualization, direct isolation, and transplantation of midbrain dopaminergic neurons. Proc. Natl. Acad. Sci. USA 98, 6423–6428.

Schaar, D.G., Sieber, B.A., Dreyfus, C.F., Black, I.B., 1993. Regional and cell-specific expression of GDNF in rat brain. Exp. Neurol. 124, 368–371.

Schierle, G.S., Hansson, O., Leist, M., Nicotera, P., Widner, H., Brundin, P., 1999a. Caspase inhibition reduces apoptosis and increases survival of nigral transplants. Nat. Med. 5, 97–100.

Schierle, G.S., Leist, M., Martinou, J.C., Widner, H., Nicotera, P., Brundin, P., 1999b. Differential effects of Bcl-2 overexpression on fibre outgrowth and survival of embryonic dopaminergic neurons in intracerebral transplants. Eur. J. Neurosci. 11, 3073–3081.

Seroogy, K.B., Lundgren, K.H., Lee, D.C., Guthrie, K.M., Gall, C.M., 1993. Cellular localization of transforming growth factor-alpha mRNA in rat forebrain. J. Neurochem. 60, 1777–1782.
Shibata, H., 1992. Topographic organization of subcortical projections to the anterior thalamic nuclei in the rat. J. Comp. Neurol. 323, 117–127.
Simonds, G.R., Freed, W.J., 1990. Effects of intraventricular substantia nigra allografts as a function of donor age. Brain Res. 530, 12–19.
Sinclair, S.R., Svendsen, C.N., Torres, E.M., Martin, D., Fawcett, J.W., Dunnett, S.B., 1996. GDNF enhances dopaminergic cell survival and fibre outgrowth in embryonic nigral grafts. Neuroreport 7, 2547–2552.
Sinclair, S.R., Fawcett, J.W., Dunnett, S.B., 1999a. Delayed implantation of nigral grafts improves survival of dopamine neurones and rate of functional recovery. Neuroreport 10, 1263–1267.
Sinclair, S.R., Fawcett, J.W., Dunnett, S.B., 1999b. Dopamine cells in nigral grafts differentiate prior to implantation. Eur. J. Neurosci. 11, 4341–4348.
Smidt, M.P., Smits, S.M., Bouwmeester, H., Hamers, F.P., van der Linden, A.J., Hellemons, A.J., Graw, J., Burbach, J.P., 2004. Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. Development 131, 1145–1155.
Sorensen, A.T., Thompson, L., Kirik, D., Björklund, A., Lindvall, O., Kokaia, M., 2005. Functional properties and synaptic integration of genetically labelled dopaminergic neurons in intrastriatal grafts. Eur. J. Neurosci. 21, 2793–2799.
Sortwell, C.E., 2003. Strategies for the augmentation of grafted dopamine neuron survival. Front. Biosci. 8, s522–s532.
Sortwell, C.E., Camargo, M.D., Pitzer, M.R., Gyawali, S., Collier, T.J., 2001. Diminished survival of mesencephalic dopamine neurons grafted into aged hosts occurs during the immediate postgrafting interval. Exp. Neurol. 169, 23–29.
Stenevi, U., Björklund, A., Svendgaard, N.A., 1976. Transplantation of central and peripheral monoamine neurons to the adult rat brain: techniques and conditions for survival. Brain Res. 114, 1–20.
Stenevi, U., Kromer, L.F., Gage, F.H., Björklund, A., 1985. Solid neural grafts in intracerebral transplantation cavities. In: Björklund, A., Stenevi, U. (Eds.), Neural Grafting m the Mammalian CNS. Elsevier, Amsterdam, pp. 41–49.
Strecker, R.E., Sharp, T., Brundin, P., Zetterstrom, T., Ungerstedt, U., Björklund, A., 1987. Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. Neuroscience 22, 169–178.
Strömberg, I., Björklund, L., Johansson, M., Tomac, A., Collins, F., Olson, L., Hoffer, B., Humpel, C., 1993. Glial cell line-derived neurotrophic factor is expressed in the developing but not adult striatum and stimulates developing dopamine neurons in vivo. Exp. Neurol. 124, 401–412.
Sullivan, A.M., Pohl, J., Blunt, S.B., 1998. Growth/differentiation factor 5 and glial cell line-derived neurotrophic factor enhance survival and function of dopaminergic grafts in a rat model of Parkinson's disease. Eur. J. Neurosci. 10, 3681–3688.
Takeshima, T., Johnston, J.M., Commissiong, J.W., 1994. Mesencephalic type 1 astrocytes rescue dopaminergic neurons from death induced by serum deprivation. J. Neurosci. 14, 4769–4779.
Thompson, L.H., Björklund, A., 2009. Transgenic reporter mice as tools for studies of transplantability and connectivity of dopamine neuron precursors in fetal tissue grafts. Prog. Brain Res. 175, 53–79.

Thompson, L., Barraud, P., Andersson, E., Kirik, D., Björklund, A., 2005. Identification of dopaminergic neurons of nigral and ventral tegmental area subtypes in grafts of fetal ventral mesencephalon based on cell morphology, protein expression, and efferent projections. J. Neurosci. 25, 6467–6477.

Thompson, L.H., Andersson, E., Jensen, J.B., Barraud, P., Guillemot, F., Parmar, M., Björklund, A., 2006. Neurogenin2 identifies a transplantable dopamine neuron precursor in the developing ventral mesencephalon. Exp. Neurol. 198, 183–198.

Thompson, L.H., Kirik, D., Björklund, A., 2008. Non-dopaminergic neurons in ventral mesencephalic transplants make widespread axonal connections in the host brain. Exp. Neurol. 213, 220–228.

Thompson, L.H., Grealish, S., Kirik, D., Björklund, A., 2009. Reconstruction of the nigrostriatal dopamine pathway in the adult mouse brain. Eur. J. Neurosci. 30, 625–638.

Torres, E.M., Monville, C., Lowenstein, P.R., Castro, M.G., Dunnett, S.B., 2005. Delivery of sonic hedgehog or glial derived neurotrophic factor to dopamine-rich grafts in a rat model of Parkinson's disease using adenoviral vectors Increased yield of dopamine cells is dependent on embryonic donor age. Brain Res. Bull. 68, 31–41.

Torres, E.M., Monville, C., Gates, M.A., Bagga, V., Dunnett, S.B., 2007. Improved survival of young donor age dopamine grafts in a rat model of Parkinson's disease. Neuroscience 146, 1606–1617.

Wictorin, K., Brundin, P., Sauer, H., Lindvall, O., Björklund, A., 1992. Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. J. Comp. Neurol. 323, 475–494.

Widenfalk, J., Nosrat, C., Tomac, A., Westphal, H., Hoffer, B., Olson, L., 1997. Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J. Neurosci. 17, 8506–8519.

Wilby, M.J., Sinclair, S.R., Muir, E.M., Zietlow, R., Adcock, K.H., Horellou, P., Rogers, J.H., Dunnett, S.B., Fawcett, J.W., 1999. A glial cell line-derived neurotrophic factor-secreting clone of the Schwann cell line SCTM41 enhances survival and fiber outgrowth from embryonic nigral neurons grafted to the striatum and to the lesioned substantia nigra. J. Neurosci. 19, 2301–2312.

Williams, M.N., Faull, R.L., 1985. The striatonigral projection and nigrotectal neurons in the rat. A correlated light and electron microscopic study demonstrating a monosynaptic striatal input to identified nigrotectal neurons using a combined degeneration and horseradish peroxidase procedure. Neuroscience 14, 991–1010.

Winkler, C., Bentlage, C., Nikkhah, G., Samii, M., Björklund, A., 1999. Intranigral transplants of GABA-rich striatal tissue induce behavioral recovery in the rat Parkinson model and promote the effects obtained by intrastriatal dopaminergic transplants. Exp. Neurol. 155, 165–186.

Winkler, C., Kirik, D., Björklund, A., Dunnett, S.B., 2000. Transplantation in the rat model of Parkinson's disease: ectopic versus homotopic graft placement. Prog. Brain Res. 127, 233–265.

Winkler, C., Bentlage, C., Cenci, M.A., Nikkhah, G., Björklund, A., 2003. Regulation of neuropeptide mRNA expression in the basal ganglia by intrastriatal and intranigral transplants in the rat Parkinson model. Neuroscience 118, 1063–1077.

Winkler, C., Kirik, D., Björklund, A., 2005. Cell transplantation in Parkinson's disease: how can we make it work? Trends Neurosci. 28, 86–92.

Wright, A.K., Arbuthnott, G.W., Dunnett, S.B., 1991. Serotonin hyperinnervation after foetal nigra or raphe transplantation in the neostriatum of adult rats. Neurosci. Lett. 128, 281–284.

Yurek, D.M., 1998. Glial cell line-derived neurotrophic factor improves survival of dopaminergic neurons in transplants of fetal ventral mesencephalic tissue. Exp. Neurol. 153, 195–202.

Yurek, D.M., Fletcher-Turner, A., 2001. Differential expression of GDNF, BDNF, and NT-3 in the aging nigrostriatal system following a neurotoxic lesion. Brain Res. 891, 228–235.

Zeng, B.Y., Jenner, P., Marsden, C.D., 1996. Altered motor function and graft survival produced by basic fibroblast growth factor in rats with 6-OHDA lesions and fetal ventral mesencephalic grafts are associated with glial proliferation. Exp. Neurol. 139, 214–226.

Zetterstrom, T., Brundin, P., Gage, F.H., Sharp, T., Isacson, O., Dunnett, S.B., Ungerstedt, U., Björklund, A., 1986. In vivo measurement of spontaneous release and metabolism of dopamine from intrastriatal nigral grafts using intracerebral dialysis. Brain Res. 362, 344–349.

Zhao, S., Maxwell, S., Jimenez-Beristain, A., Vives, J., Kuehner, E., Zhao, J., O'Brien, C., de Felipe, C., Semina, E., Li, M., 2004. Generation of embryonic stem cells and transgenic mice expressing green fluorescence protein in midbrain dopaminergic neurons. Eur. J. Neurosci. 19, 1133–1140.

Zhou, J., Pliego-Rivero, B., Bradford, H.F., Stern, G.M., 1996. The BDNF content of postnatal and adult rat brain: the effects of 6-hydroxydopamine lesions in adult brain. Brain Res. Dev. Brain Res. 97, 297–303.

Zuddas, A., Corsini, G.U., Barker, J.L., Kopin, I.J., Di Porzio, U., 1991. Specific reinnervation of lesioned mouse striatum by grafted mesencephalic dopaminergic neurons. Eur. J. Neurosci. 3, 72–85.