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Improving the Survival of Grafted Dopaminergic Neurons: A Review Over Current Approaches

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Neural transplantation is developing into a therapeutic alternative in Parkinson's disease. A major limiting factor is that only 3–20% of grafted dopamine neurons survive the procedure. Recent advances regarding how and when the neurons die indicate that events preceding actual tissue implantation and during the first week thereafter are crucial, and that apoptosis plays a pivotal role. Triggers that may initiate neuronal death in grafts include donor tissue hypoxia and hypoglycemia, mechanical trauma, free radicals, growth factor deprivation, and excessive extracellular concentrations of excitatory amino acids in the host brain. Four distinct phases during grafting that can involve cell death have been identified: retrieval of the embryo; dissection and preparation of the donor tissue; implantation procedure followed by the immediate period after graft injection; and later stages of graft maturation. During these phases, cell death processes involving free radicals and caspase activation (leading to apoptosis) may be triggered, possibly involving an increase in intracellular calcium. We review different approaches that reduce cell death and increase survival of grafted neurons, typically by a factor of 2–4. For example, changes in transplantation procedure such as improved media and implantation technique can be beneficial. Calcium channel antagonists such as nimodipine and flunarizine improve nigral graft survival. Agents that counteract oxidative stress and its consequences, such as superoxide dismutase overexpression, and lazaroids can significantly increase the survival of transplanted dopamine neurons. Also, the inhibition of apoptosis by a caspase inhibitor has marked positive effects. Finally, basic fibroblast growth factor and members of the transforming growth factor-beta superfamily, such as glial cell line-derived neurotrophic factor, significantly improve the outcome of nigral transplants. These recent advances provide hope for improved survival of transplanted neurons in patients with Parkinson's disease, reducing the need for human embryonic donor tissue and increasing the likelihood of a successful outcome.

Key words: Transplantation; Parkinson's disease; Neuroprotection; Growth factor; Cell death

A LOW SURVIVAL RATE OF GRAFTED DOPAMINE NEURONS: BIOLOGICAL CHALLENGE AND CLINICAL PROBLEM

Already in 1987 (16) it was proposed that only around 10% of grafted rat dopaminergic neurons survive intracerebral transplantation, and several studies since then have confirmed that the survival rate is in the range of 1–20% (Tables 1 and 2). Although these early observations identified an interesting biological problem, the poor survival of grafted dopaminergic neurons did not constitute a major practical problem in experimental settings where it was possible to simply increase the amount of implanted tissue by using more donors. It was not until it was shown that nigral implants from several human embryos gave rise to functional effects in a Parkinson's disease patient (88) that the issue of improving the survival of grafted dopamine neurons attained high priority. Indeed, as evidenced by positron emission tomography using fluorodopa as a ligand, robust clinical improvement in patients receiving transplants is believed to depend upon restoration of a high degree of dopaminergic striatal neurotransmission (64). Of course, other factors may be important for the clinical outcome, such as the age of the patient, disease duration, severity of underlying dopaminergic denervation, and the presence of nondopaminergic lesions. Nonetheless, clinical findings stressing the importance of a restoration of striatal neurotransmission in Parkinson's disease patients receiving grafts are in agreement with results from experiments in rats indicating a correlation between behavioral effects and graft survival [for review see (18)]. It

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Table 1. Attempts to Enhance Survival of Grafted Rat Dopaminergic Neurons by Trophic Factors

	Control	Treatment
GDNF		
Rosenblad et al. (114)[b]	19.7%	37.4%
Sinclair et al. (127)[a]	0.7%	8.1%
Sinclair et al. (127)[b]	1.0%	13.0%
Granholm et al. (59)	no absolute quantification; 2.5-fold increase	
Apostolides et al. (fresh) (4)[b]	2.4%	1.6%
Apostolides et al. (hibernated 6 days) (4)[a]	2.6%	3.4%
Mehta et al. (hibernated 6 days) (96)[b]	1.6%	2.0%
Sautter et al. (117)[a]	5.1%	12.9%
Sullivan et al. (131)[b]	13.6%	36.6%
Yurek et al. (147)[a]	1.9%	5.8%
bFGF		
Mayer et al. (3-week survival) (93)[a]	0.9%	1.6%
Mayer et al. (9-week survival) (93)[a]	1.0%	2.7%
Takayama et al. (135)[a]	1.5%	20.0%
Takayama et al. (135)[b]	6.3%	87.5%
Zeng et al. (151)[b]	10.5%	24.8%
kFGF		
Haque et al. (67)[a]	1.5%	0.8%
GDNF + bFGF + IGF		
Zawada et al. (24-h survival) (150)[a]	8.2%	12.7%
Zawada et al. (7-day survival) (150)[a]	4.1%	6.6%
Neurturin		
Rosenblad et al. (113)[b]	23.3%	41.1%
GDF-5		
Sullivan et al. (131)[b]	13.6%	33.3%
NGF		
Sauer et al. (116)[b]	11.8%	7.3%
Zeng et al. (151)[b]	10.5%	11.7%
BDNF		
Sauer et al. (116)[b]	11.8%	7.3%
Yurek et al. (149)[a]	no quantification of TH+ cell number	
NT-3		
Haque et al. (68)[a]	0.9%	1.6%
NT-4/5		
Haque et al. (68)[a]	0.9%	1.2%

The figures are either those stated by the respective authors or have been computed based on data presented in the publication. The computations are based on either one of the two following assumptions: [a]there are around 35,000 dopamine neurons (tyrosine hydroxylase positive, TH+) in one rat embryonic mesencephalon; [b]around 8% of the cells in dissected embryonic rat mesencephalic tissue are dopaminergic. This implies that there are around 400,000 cells obtained from each dissected ventral mesencephalon.

is believed that significant clinical improvement in Parkinson's disease patients requires that the transplant is derived from ventral mesencephalic tissue from 4–8 aborted embryos per side of the brain. The need for multiple donors for each patient represents a limiting step in many clinical trials (134). Considering that the survival rate of grafted human dopaminergic neurons is believed to be around 5–10%, based on data from xenografts to immunosuppressed rats (23,54) and postmortem findings in Parkinson's disease patients receiving grafts

Table 2. Attempts to Enhance Survival of Grafted Rat Dopaminergic Neurons by Cytoprotective Drugs

	Control	Treatment
Lazaroids		
Nakao et al. (98)[a]	15.7%	41.4%
Grasbon-Frodl et al. (hibernated 8 days) (62)[a]	6.5%	14.0%
Björklund et al. (13)[a]*	0.9%	2.3%
Karlsson et al. (79)[a]	6.4%	10.9%
α-phenyl-*N-tert*-butyl nitrone (PBN)		
Karlsson et al. (78)[a]	8.5%	9.5%
NOS inhibitors		
Van Muiswinkel et al. (140)[b]	2.1%	2.0%
Calcium antagonists		
Finger and Dunnett (52)	increased graft volume (dopamine neurons not quantified)	
Kaminski Schierle et al. (77)[a]	8.2%	21.5%
Caspase inhibitors		
Schierle et al. (119)[a]	10%	36%

Figures have been calculated as detailed in the legend of Table 1.
*Solid grafts placed in the anterior chamber of the eye.

(81,82), an increase in survival of grafted human dopamine neurons would substantially decrease the amount of embryonic tissue needed per patient. Recent years have seen reports of a large number of attempts to improve transplant survival by several different approaches. In this review, we describe possible basic mechanisms involved in death of transplanted neurons and the effects of technical modifications in grafting procedure and pharmacological intervention on the survival of grafted dopaminergic neurons.

HOW CAN THE SURVIVAL RATE OF GRAFTED DOPAMINE NEURONS BE CALCULATED?

When attempting to increase the survival of transplanted dopaminergic neurons, it is essential to estimate the survival rate under basal control conditions. While a given neuroprotective treatment may be effective at improving graft survival when the basal survival rate is low (e.g., due to suboptimal tissue preparation techniques), the same treatment may be without impact once the transplantation method is slightly improved. While this does not necessarily subtract anything from the biological significance of the neuroprotective treatment, it reduces its importance in the clinical setting when the basic transplantation methodology should already be optimized. There are two commonly employed approaches to estimate the survival rate of grafted dopaminergic neurons. In the first approach, there is a direct comparison between the number of surviving dopaminergic neurons in the transplant and the number of dopamine neurons estimated to be present in the initial donor material at the start of the procedure. In the case of the laboratory rat, it has been estimated that there are a total of 30,000–40,000 dopaminergic neurons in the adult ventral mesencephalon, when both the substantia nigra and ventral tegmental area cell groups are included (11,49,56,70,132). Thus, in this model each donor embryo is expected to contribute to the graft tissue preparation with 30,000–40,000 dopamine neurons. By estimating the volume of vehicle in which each donor tissue piece is dissociated when preparing a cell suspension, and monitoring the volume of transplant tissue injected in each implant, it is possible to calculate how many neurons were grafted to each host. There are certain possible shortcomings with this method. There is still no published account of a more precise, stereological assessment of the number of mesencephalic, tyrosine hydroxylase-immunopositive, dopaminergic neurons in different strains of rats. Also there may be significant differences in the number of dopaminergic neurons in male and female rat embryos (10). Moreover, there may be a higher number of dopaminergic neurons in the embryo than estimated for the adult, because some nigral neurons are believed to undergo programmed cell death before the rat reaches adulthood (75,137).

In the second approach, the number of transplanted

mesencephalic cells is monitored by assessing the concentration of cells in the graft suspension (19) and noting the volume of cell suspension grafted to each host. Based on immunocytochemical studies of fresh smears of mesencephalic cell suspensions, it is estimated that the proportion of dopaminergic (tyrosine hydroxylase-positive) neurons is 8–10% in embryonic rat (115) and mouse (99,121) donor tissue. The main shortcomings with this method are that immunocytochemistry on cell smears may not accurately reflect the true proportion of dopaminergic neurons (not all dopaminergic neurons may express immunocytochemically detectable levels of tyrosine hydroxylase under these conditions), and cells may be lost in earlier steps when preparing the tissue (resulting in an overestimate of survival of grafted neurons). Moreover, this second approach is dependent on the size of the dissected tissue piece, which adds yet another source of variance between laboratories. On balance, the first method to calculate cell survival may prove more accurate if reliable data become available on the number of dopamine neurons normally present in the ventral mesencephalon of each donor embryo. Both models for assessing graft survival have been used in the studies presented in this review. In Tables 1 and 2 we state the method we have used to calculate survival of transplanted dopamine neurons, based on information about the grafting procedure presented in each respective study.

WHEN DO TRANSPLANTED NEURONS DIE?

Grafted embryonic dopamine neurons may die during one of at least four phases that encompass the transplantation procedure and time during which the nigral transplant develops in the host brain (Fig. 1). First, the hypoxic and hypoglycemic insult caused by removing the donor embryo from its maternal blood supply is likely to cause oxidative damage (58). Second, the axotomy during dissection and the other traumatic damage caused by mechanical dissociation, as well as the hypoxia during the delay before implantation into the host brain, are also possible culprits in the neuronal damage. Third, the actual implantation procedure and the immediate period (first 1–3 days) after graft injection into the new adult host environment may constitute a period of further death. During this phase, the new environment may reduce survival of grafted neurons via excessive extracellular glutamate and oxidative stress, caused by damage to the adult nervous system (5,89), and the expression of potentially damaging inflammatory cytokines as a result of the brain damage (57). Fourth, it is conceivable that there is cell death due to a lack of appropriate neurotrophic support at a later stage when the embryonic neurons slowly mature in the new host environment (45).

Based on results from in vitro culture experiments, Fawcett et al. (49) proposed that around 30% of the embryonic dopamine neurons die already during the mechanical dissociation step of the donor tissue preparation (phase 2, Fig. 1). This is consistent with accumulating experimental evidence indicating that the majority of dopaminergic neurons grafted as dissociated cell suspensions die before implantation and within the first week after surgery (8,37,42,81,102). This evidence stems from quantitative assessment of the number of surviving tyrosine hydroxylase-immunopositive neurons present in different grafts that were prepared in an identical fashion and implanted in rats killed at various time points, from 4 days up to 12 weeks after graft surgery. The results indicate no major changes in the number of surviving grafted dopaminergic neurons after the first week following surgery. An earlier study (90) labeled dying neurons in grafts of solid pieces of mesencephalic tissue by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end (TUNEL) method, which reveals fragmented chromatin. The solid nigral grafts displayed progressively fewer TUNEL positive cells (90) and it indicated that neuronal death may be most marked within the first 2 weeks after grafting. Recently, Zawada and coworkers (150) have more closely pinpointed the time of cell death in nigral transplants. Using solid strands of embryonic mesencephalic tissue for transplantation, they have shown that the majority of TUNEL-positive, apoptotic neurons appear in grafts as early as 24 h after implantation. We recently also observed a large number of TUNEL-positive, apoptotic neurons in nigral suspension grafts, 4 days after surgery (120). Using a different morphological technique, we recently obtained even further support for the idea that cell death is most marked soon after transplantation. We studied the number of Fluoro-Jade-labeled neurons within grafts of dissociated nigral tissue (42). Fluoro-Jade was recently launched as a marker for degenerating neurons by Schmued et al. (123). We examined four different time points after transplantation (6, 10, 14, and 42 days) and stained adjacent sections through the host striatum with Fluoro-Jade and tyrosine hydroxylase antiserum. We found numerous Fluoro-Jade-stained neurons in the grafts at 6 and 10 days after implantation surgery. The number of labeled neurons was reduced at 14 days and there were no Fluoro-Jade-positive neurons by the 42-day time point. Importantly, the number of surviving dopaminergic neurons in the grafts did not change between 6 and 42 days, indicating that the major cell death had occurred prior to 6 days. Therefore, neuroprotective strategies should primarily focus on the transplantation procedure itself and on the first week after implantation.

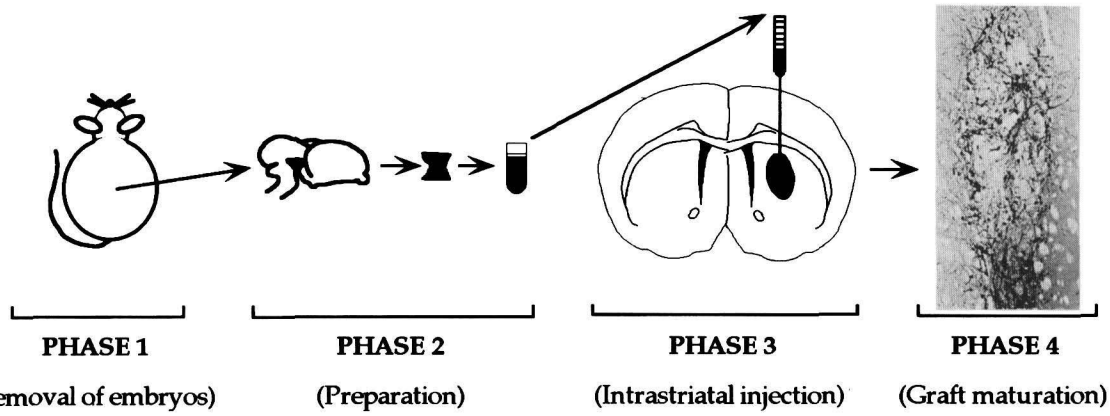

Figure 1. Scheme summarizing four phases, from the donor tissue stage to the maturing implant, when transplanted dopaminergic neurons may die. Phase 1: hypoxic and hypoglycemic insult caused by removing the donor embryo from its maternal blood supply. Phase 2: axotomy during dissection and other traumatic damage caused by mechanical dissociation, as well as the hypoxia during cell suspension storage. Phase 3: implantation procedure and the immediate period (first 1–3 days) after graft injection into the new adult host environment, when also toxic factors present in the traumatized host striatum may affect the graft. Phase 4: maturation in the new host environment that may involve dopaminergic neuron death due to a lack of appropriate neurotrophic support.

CHANGES IN TRANSPLANTATION TECHNIQUE TO IMPROVE TRANSPLANT SURVIVAL

This review is primarily focused on studies aimed at improving mesencephalic transplant survival by direct addition of growth factors and neuroprotective agents to nigral donor tissue. Therefore, we mostly describe studies where the donor embryos were of an optimal developmental stage for obtaining nigral tissue. In rats, this is equivalent to an embryonic age of around 13–15 days (with the day of mating considered as day 0) when grafting dissociated tissue [for review see (22)], and can be extended up to day 17 when grafting solid pieces of mesencephalon (125). However, even when using embryonic tissue of an optimal donor age, there are several modifications in basic transplantation methodology that can affect graft survival and that deserve to be mentioned briefly. First, choice of medium for dissection and tissue preparation is of importance. During the 1980s, most experiments involving nigral cell suspension transplantation employed a simple, unbuffered glucose–saline medium to prepare and dissociate the transplant tissue (12). Over recent years there has been a move towards using more balanced media, such as Hank's balanced salt solution (HBSS). When nigral cell suspensions are stored at room temperature and in vitro cell viability is assessed, it is evident that a high cell viability is maintained for longer time periods when the cells are kept in HBSS instead of glucose–saline (101). Recently, Björklund et al. (13) found that solid nigral transplants placed in the anterior chamber of the eye survive better if the culture medium Dulbecco's modified Eagle medium (DMEM), as opposed to HBSS, is used when harvesting and injecting the tissue. Congruent with these findings, it has recently been demonstrated that grafts of dissociated mesencephalic tissue survive implantation into the striatum significantly better when they are prepared in DMEM than in HBSS (141). With respect to cell suspension transplants, the degree of tissue dissociation is another important factor influencing graft survival (9,19). In a direct comparison, Watts et al. (142) observed improved survival when the nigral donor tissue was grafted intrastriatally as small tissue blocks, compared to when it was mechanically dissociated into a cell suspension. It seems that excessive mechanical dissociation of donor tissue by repeated and vigorous trituration through the tip of a pipette, until a single cell suspension is obtained, can cause irreparable damage to the cells, and that a mixture of small aggregates and single cells may be a better alternative (9). Furthermore, it is conceivable that maintaining some degree of the normal structure within the grafted tissue piece may promote the formation of appropriate and functional neuronal connections within the graft and with the host brain. Finally, as mentioned previously, Fawcett and coworkers (49) have estimated that around 30% of the embryonic dopamine neurons die when the mesencephalon is dissociated into a single cell suspension.

In an attempt to minimize host brain damage, Nikkhah and coworkers modified a micrografting technique (44) and injected nigral cell suspensions into, for example, the striatum. In their detailed methodological de-

scription of the technique (102), the survival rate of dopaminergic neurons grafted as a single cell suspension using a conventional metal cannula was about 0.5–1%, which is relatively low compared to the survival rates in the untreated control groups presented in Tables 1 and 2. The poor survival is probably related primarily to the excessive mechanical trauma incurred upon the embryonic donor tissue, and, possibly, the inclusion of a centrifugation step during tissue preparation. While graft survival was relatively poor following tissue dissociation into single cell suspensions, Nikkhah et al. (102) did observe an improved survival of dopaminergic neurons, a yield up to about 5–6%, if the cells were implanted into the striatum through a fine glass capillary (outer diameter around 0.05 mm) as opposed to a conventional metal cannula (outer diameter 0.5 mm). This observation suggests that it is beneficial to minimize host brain trauma at the implantation site and thereby reduce the cell death that we propose occurs acutely after implantation in "phase 3" of Figure 1. Further support for this concept comes from an experiment showing that nigral grafts survive less well when the implantation cannula has an outer diameter of 2.5 mm than when it is 0.5–1.0 mm in size (17). In a very recent innovative study, Sinclair and coworkers (126) have directly addressed the issue of the importance of host brain trauma. They inflicted a delay of varying duration (20 min, 1 or 3 h) between insertion of the injection cannula into the striatum and actual ejection of the graft tissue, and compared the survival with grafts that were ejected immediately. A 1- or 3-h delay resulted in two to three times as many surviving dopaminergic neurons, while a 20-min delay had no effect. These findings identify the immediate postoperative period as an interesting phase during which pharmacological interventions aimed at minimizing oxidative stress and excitotoxicity may reduce the death of implanted dopaminergic neurons.

MECHANISMS THAT MEDIATE CELL DEATH IN TRANSPLANTED NEURONS AND EFFECTS OF PHARMACOLOGICAL INTERVENTIONS

Cell death is currently considered to occur according to one of two patterns: necrosis or apoptosis, although intermediate forms exist (110). Both processes are likely to play an important role in cell death that accompanies embryonic neuronal transplantation (Fig. 2). Necrotic cell death is typically the result of an pathological event and follows an insult severe enough to cause irreversible cellular dysfunction and energy deprivation. Usually necrotic cell death is a fast process, occurring in minutes to hours and is finally characterized by cell and organelle swelling and plasma membrane permeabilization, followed by an inflammatory reaction in the surrounding tissue [for review see (84)]. In contrast, apoptotic cell death (including programmed cell death) is typically a slow, controlled, energy- and protein synthesis-dependent process lasting a period of hours to days [for review see (84)]. Apoptosis occurs naturally during the development of the nervous system (107,112) but has also been implicated in ischemia and many neurodegenerative diseases (15 85). Apoptosis can be initiated by binding of ligands to the death receptor Fas/Apo[apoptosis]-1, or the trigger of apoptosis may also be a mild chemical or metabolic insult (63). Although the sequences of intracellular signals differ somewhat, in both cases there is caspase activation, resulting in intranucleosomal DNA fragmentation, chromatin condensation, cell shrinkage, and disassembly into membrane-enclosed vesicles (apoptotic bodies) [for review see (63)] (Fig. 2). Subsequently, there is phagocytosis of the remnants of the dead apoptotic cell, preventing a local inflammatory reaction. Permeabilization of the inner mitochondrial membrane, caused by oxidative damage to mitochondrial membrane lipids or proteins, can result in impairment of ATP production, energy deprivation, and cell necrosis. In apoptosis, mitochondria can contribute by releasing pro-apoptotic factors, such as cytochrome c, apoptosis inducer factor (AIF), and pro-caspases [for review see (84)]. Under situations of excitotoxicity and oxidative stress, which are described in more detail below, neurons may undergo both necrosis and apoptosis, depending on the severity of the insult (3).

Neuronal injury is often associated with a sustained elevation of intracellular calcium and an increased oxidative stress (27,85,108), conditions that are likely to prevail also in grafted neurons. Normally, cytosolic calcium is buffered by mitochondria and the endoplasmic reticulum, as well as by several calcium binding proteins (71,108). Calcium homeostasis is further controlled by extrusion mechanisms over the outer plasma membrane. A shortage of energy, as may occur in neural transplants, can lead to reduced calcium extrusion and make it impossible for the cell to uphold a plasma membrane potential, which in turn results in release of glutamate from the depolarized neuron into the surrounding extracellular space (86). As a result, there is excessive stimulation of ionotropic excitatory amino acid receptors with concomitant increased calcium entry and overload of mitochondria, free radical generation, and activation of several potentially detrimental enzymes, including phospholipases, proteases, and nitric oxide synthase (NOS) (86) (Fig. 2). This form of neuronal cell death has been termed "excitotoxicity" (106) and will be addressed in more detail in a later section.

Oxidative stress is a consequence of disturbed homeostasis between production and scavenging of re-

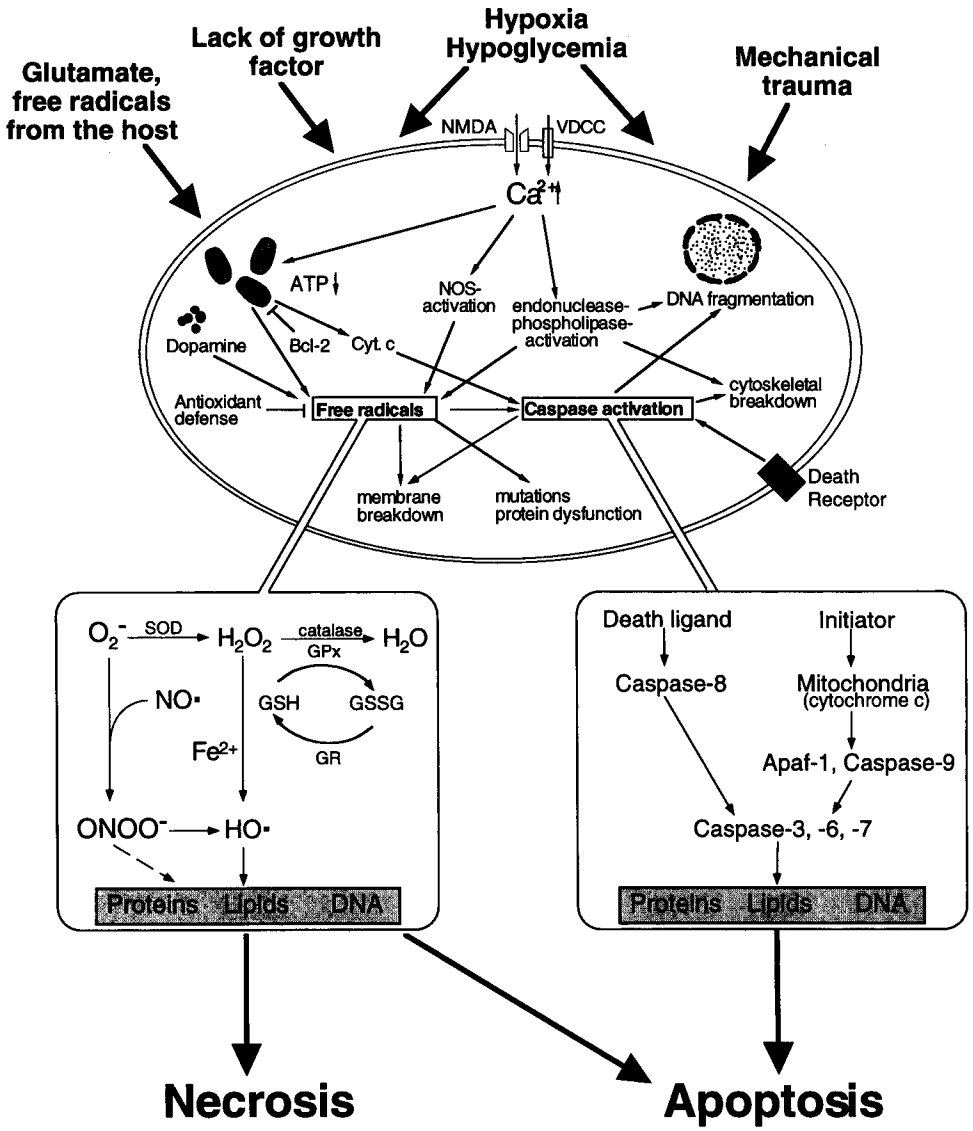

Figure 2. Scheme summarizing suggested cell death mechanisms in transplanted mesencephalic cells. The diagram schematically depicts: sources of cell stress; intracellular mechanisms that can lead to cell death, with emphasis to reactions involving reactive oxygen species and caspases activation; and, in more detail, pathways that lead to cell death by necrosis or apoptosis. Descriptions of the various mechanisms can be found in the text and cited references. Abbreviations: Apaf-1, apoptotic protease activating factor-1; ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma-2, anti-apoptotic proto-oncogene product; cyt.c, cytochrome c; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSH, glutathione disulfide; NMDA, *N*-methyl-D-aspartate; $NO^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; $ONOO^-$, peroxynitrite; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; VDCC, voltage-dependent calcium channel.

active oxygen species (e.g., superoxide). The major cellular production of superoxide radicals occurs at mitochondrial respiratory chain complexes I and III [reviewed in (128)]. Superoxide radicals are also formed by, for example, activated microglia (30). Superoxide can generate new reactive oxygen species via dismutation to hydrogen peroxide followed by subsequent reaction with iron to form hydroxyl radicals. Alternatively, superoxide reacts with nitric oxide to form peroxynitrite, which is highly toxic to several cellular components (111). Nitric oxide is produced by nitric oxide synthase (NOS), which is a calcium-activated enzyme. Interest-

ingly, auto- and enzymatic oxidation of dopamine also leads to the generation of superoxide and hydrogen peroxide (76). Therefore, it has been proposed that dopaminergic neurons are more likely to be subjected to oxidative stress than other neuronal cell types (104).

During normal development and adult life, dopaminergic neurons are known to respond to several types of growth factors (45). Loss of neurotrophic support can lead to neuronal death both in developing and adult brains, and may be an important trigger of cell stress in grafted embryonic dopamine neurons. Intracellular signaling leading to death of neurons deprived of growth factors is not fully understood, but a complex series of events is likely to lead to the apoptosis in deprived cells (35,36).

In the following four sections of this article we review interventions aimed at reducing neuronal death in grafts and that have focused on different, but still connected, cell death mechanisms: a) calcium and excitotoxicity; b) oxidative stress; c) caspase activation and apoptosis; d) lack of trophic support.

Excitotoxicity and Neuronal Death in Nigral Transplants

Of the three major classes of ionotropic glutamate receptors known in the brain: *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methylisoxazole-4-proprionate (AMPA)/kainate (KA) receptors, and metabotropic receptors, it is believed NMDA receptors play the most prominent role in excitotoxicity, although the other receptor subtypes may also contribute to cell death (32). Furthermore, there are three different types of voltage-dependent calcium channels (VDCC) present in neurons, called T-, L-, and N-channels (94), that can also be involved in calcium-mediated toxicity.

Excitotoxic neuronal death may be induced in nigral transplants during the retrieval and dissection of embryonic donor brain tissue (phases 1 and 2 in Fig. 1) and by the surgical procedure itself (phase 3 in Fig. 1) (28,95). Glutamate receptors of the NMDA subtype are present in the adult substantia nigra (146) and dizocilpine hydrogen maleate (MK-801), a potent inhibitor of the NMDA receptor, can protect cultivated dopaminergic neurons against stress induced by withdrawal of serum from the culture medium (118). However, treatment of embryonic mesencephalic donor tissue with MK-801 does not improve dopaminergic neuron survival in grafts (120). These findings suggest that cell death occurring during phase 2 (Fig. 1) of the nigral transplantation protocol is not primarily due to excitotoxicity mediated via the NMDA receptor.

Nimodipine, a calcium channel blocker, has been shown to enhance the growth and vascularization of intrastriatal nigral grafts when administered intragastrically to transplant recipients (52). However, this positive effect on graft survival was only apparent either when the donors were older than considered optimal, or, alternatively, donor tissue of an optimal age had been stored for several hours at room temperature before implantation. More recently, we have found that flunarizine, an antagonist of L-, T-, and N-type calcium channels (2,139), can protect dopamine neurons from death induced by serum removal in rat embryonic mesencephalic cultures (118). When adding flunarizine to cell suspensions of embryonic mesencephalic tissue prior to transplantation, we observed increased survival of dopaminergic neurons to 260% of control values. Furthermore, there was a 200% increase in graft volume, indicating that the effect of flunarizine was not specific for dopaminergic neurons (77). To test whether flunarizine is capable of interfering with cell death mechanisms occurring during phase 2 (Fig. 1) of the transplantation protocol, we incubated cell suspensions at 20–37°C for a few hours with 1 μM flunarizine present. Flunarizine significantly inhibited overall cell lysis, as measured by lactate dehydrogenase (LDH) release into the medium from dying cells, indicating that a relatively high percentage of the initial cells (40%) are lost at a very early stage of the transplantation protocol (i.e., during phase 2, Fig. 1). Furthermore, we observed a high degree of DNA fragmentation, an indicator of apoptosis, that was almost completely inhibited by the addition of flunarizine (77).

The neuroprotective effects of flunarizine on dopaminergic neurons may not only be related to the drug acting as a calcium channel antagonist. First, the HBSS medium in which the nigral cell suspensions are prepared contains only a very low concentration of calcium. Second, Eichler et al. (39) have demonstrated that flunarizine can prevent death of cultured dorsal root ganglion cells subjected to trophic factor withdrawal even in the absence of extracellular calcium. Flunarizine has also been shown to act as an inhibitor of the mitochondrial permeability transition pore (mitochondrial megachannel) (40), which can participate in the initiation of apoptosis. Furthermore, flunarizine has previously been shown to protect against oxidative stress and lipid peroxidation in brain cells (136).

Reducing Oxidative Stress in Nigral Transplants

Most reactive oxygen species are free radicals, such as hydroxyl ($HO^{\bullet}$), superoxide ($O_2^{\bullet -}$), and nitric oxide ($NO^{\bullet}$) radicals, but there are also nonradical species such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (Fig. 2). The hydroxyl radical is the most reactive oxygen species and is the main mediator of oxidative damage. For example, hydroxyl attack on DNA leads to strand breaks and base modifications, and attack on pro-

teins can lead to impaired cellular function (53). Finally, attack by reactive oxygen species on lipid membranes leads to generation of lipid radicals and a chain reaction of lipid peroxidation is initiated (66). We will describe two principally different methods to counteract oxidative stress in grafted neurons. First, we discuss interference with the formation of specific radicals (e.g., by enhancing naturally occurring antioxidant systems). Second, we describe agents that either disarm reactive oxygen species in a nonspecific fashion or inhibit the propagation of radical reactions that mediate cell-damaging processes.

Superoxide dismutase (SOD) catalyses the conversion of superoxide to hydrogen peroxide. There are three isoforms of SOD, of which Cu/Zn SOD has been suggested to contribute the most to the dismutase activity in the brain. In order to evaluate the role of Cu/Zn SOD in transplantation of dopaminergic neurons, we used embryonic ventral mesencephalic tissue from transgenic mice overexpressing human Cu/Zn SOD as donor tissue (99). Tissue from transgenic or nontransgenic littermates was transplanted into adult rats. We observed an average increase in the survival of dopaminergic neurons to 400% of mean control value, and the striatal area in the host reinnervated by the grafts was found to be 6.5 times higher in the transgenic group compared with the control (99). However, using adenoviral transfer of the human gene for Cu/Zn SOD, as an alternative approach to deliver SOD, did not significantly increase the survival of grafted rat nigral neurons (7). This was possibly due to relatively low proportion of the grafted cells being transduced by the adenoviral infection (7).

The cell permeant synthetic compound, Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), has been suggested to have similar dismutase action as endogenous SOD (48,80). However, in addition to scavenging superoxide, MnTBAP has been reported to also scavenge hydrogen peroxide (33) and peroxynitrite anion (133). In a recent pilot study, we observed an approximate doubling in the number of surviving dopaminergic neurons in mesencephalic transplants when MnTBAP was added to the implanted nigral cell suspension during phase 2 of the procedure (Fig. 1) (41). In the cell, both glutathione peroxidase and catalase can further process the product of superoxide dismutation and convert hydrogen peroxide to water. In a pilot study, we recently examined the effects of catalase as a potentially protective agent in grafts. Indeed, the addition of catalase to the cell suspension resulted in more than twice as many surviving dopamine neurons in the grafts (41).

In order to evaluate the role of nitric oxide in transplants, Van Muiswinkel and coworkers treated mesencephalic cell suspensions and graft recipients with the NOS inhibitor N^{ω}-nitro-*l*-arginine methylester (L-NAME) (140). It was found that L-NAME given to cell suspensions alone (phase 2) or to both cell suspension and graft recipients (phases 2–4) did not enhance the survival of transplanted dopaminergic neurons in comparison to control transplanted rats. Taken together, these experiments indicate that superoxide and hydrogen peroxide contribute to death of transplanted dopaminergic neurons. Because inhibition of NOS did not have an effect on neuronal survival, but treatment with catalase did, it could be speculated that superoxide radical primarily exerts its toxic effects via conversion to hydrogen peroxide and not via peroxynitrite formation.

While the experiments described above may eventually help to elucidate which radical-forming pathways are most important during the death of grafted dopamine neurons, it is also possible that a nonspecific inhibition of radical reactions could be beneficial to graft survival. Spin-trap agents are a class of agents that react with free radicals to form more stable and less reactive adducts. The spin-trap agent most commonly used to prevent neuronal damage is α-phenyl-*N-tert*-butyl nitrone (PBN), which, for example, can reduce ischemic damage in gerbils and rats (26,105,145) and protect striatal neurons from excitotoxic lesions (100,124). However, PBN does not increase the survival of transplanted dopaminergic neurons when given to the graft recipient before and during the first 24 h after transplantation (phase 3, Fig. 1), or when added to the cell suspension (phase 2, Fig. 1) (78).

Lipid peroxidation is another radical-mediated process that is possible to pharmacologically inhibit. The main initiator of lipid peroxidation is the hydroxyl radical, which abstracts a hydrogen from membrane lipids, generating lipid radicals and starting a chain reaction of free radical damage [see (66)]. This leads to formation of toxic by-products (such as malondialdehyde and 4-hydroxynonenal), disruption of membrane integrity, inability to maintain ion homeostasis, and ultimately cell death (92). Lazaroids are a group of compounds specifically designed to inhibit lipid peroxidation [for review see (65)]. The first generation of lazaroids, 21-aminosteroids, scavenge radicals and also interact directly with cell membranes to decrease membrane fluidity (14). The most known 21-aminosteroid, tirilazad mesylate (U-74006F), has been investigated in a transplantation paradigm where intact pieces of ventral mesencephalon were grafted into the anterior chamber of the eye (13). Whereas tirilazad treatment during dissection only (phase 2) failed to increase the survival of dopaminergic neurons in these solid grafts of mesencephalic tissue, lazaroid treatment during dissection and once weekly after transplantation (phase 2–4) afforded an increase in dopaminergic neuronal survival to 150–250% of control grafts (13). Tirilazad mesylate has not yet been evalu-

ated in cell suspension grafts in animal experiments. However, another related 21-aminosteroid (U-74389G) was shown to increase the survival of dopaminergic neurons, as well as the graft volume, to 260% of control values (98). Tirilazad mesylate also increases survival of human and rat mesencephalic neurons in vitro in cell suspensions (109) and is currently used in clinical transplantation in Parkinson's disease patients at Lund University Hospital, Sweden.

In second-generation lazaroids (the 2-methylaminochromans), antioxidant capacity was further increased by replacing the steroid portion of tirilazad with an α-tocopherol (vitamin E) ring. One of these new lazaroids, U-83836E, has been widely investigated on cultured and grafted embryonic mesencephalic neurons. For example, U-83836E protects cultured dopaminergic neurons from oxidative stress induced by glutathione depletion (60), nitric oxide toxicity (61), and serum deprivation (55,78). In a transplantation experiment, U-83836E was injected to the pregnant rat, during phase 1 of the transplantation procedure (Fig. 1), and added to all the solutions during tissue dissociation and immediately thereafter, equivalent to phase 2 (Fig. 1). This treatment increased the survival of transplanted dopaminergic neurons to 265% of control values (98). In another transplantation study, U-83836E increased the survival to 170% of mean control values when present only during the dissection procedure and onwards (i.e., phase 2) (Fig. 1) (79). Pyrrolopyrimidines are a novel group of lazaroids specifically designed to penetrate the blood–brain barrier more easily than tirilazad and U-83836E. Moreover, pyrrolopyrimidines are less membrane-bound than the other lazaroids and can therefore more effectively get access to the intracellular space (65). Even though the pyrrolopyrimidine U-101033E affords greater protection than tirilazad in focal ischemia (65,122), iron-induced lipid peroxidation (65), and peroxynitrite toxicity (51), it does not increase the survival of grafted dopaminergic neurons when added to the cell suspension (5 μM) (J. Karlsson and P. Brundin, unpublished result). Thus, first- and second-generation lazaroids can improve the survival of transplanted dopaminergic neurons, but the novel pyrrolopyrimidines are not effective. This difference may be related to the physical properties of the compounds, where tirilazad and U-83836E are more likely to insert into the membranes than pyrrolopyrimidines.

Reducing Apoptosis in Nigral Transplants

As previously mentioned, most of the dopaminergic neurons in nigral grafts die during tissue preparation or within 1–4 days after transplantation (37,42,77,102,150) and it has been shown that at least part of this cell death is apoptotic (90,119,150).

A family of proteases, called caspases, has been shown to play a central role in the apoptotic process (Fig. 2) (110,130). Hitherto, 14 different caspases have been identified. Caspases participate in a cascade of events that is triggered in response to pro-apoptotic signals and culminates in cleavage of specific proteins, including proteins involved in fragmentation and repair of DNA, and maintenance of cell morphology (Fig. 2) (110,130). Caspases can be divided into two groups: effector caspases that function in cell disassembly, and initiator caspases that are important in initiating this disassembly in response to pro-apoptotic signals. Inhibitors of caspases have been shown to effectively block neuronal apoptosis in many different experimental paradigms, including growth factor withdrawal (97), excitotoxicity (85), axotomy (34), cerebral ischemia (69), and brain trauma (143).

In a recent study we investigated the effects of the caspase inhibitor Ac-YVAD-cmk on the survival of dopaminergic neurons (119). In a first set of experiments we showed that Ac-YVAD-cmk significantly increased the survival of cultured dopaminergic neurons. In a second step, embryonic nigral cell suspensions were prepared as for transplantation and activity of caspase 3, an important effector caspase (Fig. 2), and DNA fragmentation were evaluated. Both caspase 3 activity and DNA fragmentation were increased already a few hours after dissociation of cell suspension, indicating that cell death is initiated before implantation surgery, at least in a subpopulation of cells. Interestingly, treatment of nigral cell suspensions with Ac-YVAD-cmk drastically reduced both caspase 3 activity and DNA fragmentation in the cell suspensions and consequently reduced the amount of apoptotic cells in nigral grafts 4 days after transplantation. Finally, we determined whether the addition of Ac-YVAD-cmk to transplant suspensions would result in augmented long-term transplant survival in rats. Treatment of nigral cell suspensions with Ac-YVAD-cmk increased the survival of grafted dopaminergic neurons to around 350% of control. In addition, both graft volume and innervation of the striatum were increased in Ac-YVAD-cmk-treated grafts. These results imply that at least a part of the cell death in nigral transplants is dependent on caspase activation.

Proteins of the bcl-2 family play a central role in the regulation of caspase activation and therefore in the apoptotic process (1,110). The members of the bcl-2 family are either anti-apoptotic, like Bcl-2 (Fig. 2) and Bcl-X_L, or pro-apoptotic, like Bax. Indeed, transgenic mice expressing human Bcl-2 exhibit reduced apoptosis due to axotomy (38,47), ischemia (91), or neurotoxins (103,144). In a recent study we found that cultured dopaminergic neurons from transgenic mice expressing human Bcl-2 were protected from death induced by serum deprivation or exposure to staurosporine (121). How-

ever, expression of human Bcl-2 did not increase the survival of dopaminergic neurons transplanted to rats. The absence of protection of nigral grafts by overexpression of Bcl-2 may be due to one of the following mechanisms: (a) Bcl-2 may be functionally inactivated in transplanted neurons; (b) an increased expression of Bcl-2 may not be enough to inhibit the activation of caspases normally regulated by Bcl-2 due to very strong pro-apoptotic signals; (c) cell death mechanisms that are unique to transplanted neurons may bypass a potential block by Bcl-2 (Fig. 2). Taken together, these results imply that at least a part of the cell death of transplanted dopamine neurons is apoptotic and dependent on activation of caspases.

Enhancing Nigral Transplant Survival by Growth Factor Treatment

Prior to the modern era of neurotrophic factors and the identification of several growth factors that are active specifically on dopaminergic neurons, several studies used striatal target cells as a source of trophic support for nigral dopaminergic neurons. Cell culture studies [for review see (6)] revealed that striatal cells can influence the maturation and biochemistry of embryonic dopamine neurons. When this approach is applied to the neural transplantation paradigm, it is reported that addition of embryonic striatal cells to the implants can induce trophic effects, expressed as an enhanced fiber outgrowth from grafted dopaminergic neurons (20,31,43, 148). Nevertheless, the vast majority of studies indicate that the survival of the grafted dopaminergic neurons is not improved (20,31,43,148), although there are exceptions to this view (129). In conclusion, it seems that the addition of embryonic striatal target tissue is not a reliable way to increase the survival of transplanted dopamine neurons.

Three different families of growth factors have been investigated regarding their effects on transplanted dopamine neurons: the fibroblast growth factor family, the transforming growth factor-beta (TGF-β) family, and the neurotrophins.

Basic fibroblast growth factor (bFGF) was found in 1989 to promote the survival and development of mesencephalic neurons in vitro (50). The effect is considered to be mediated via glial cells (46), but the precise underlying mechanism for the survival-promoting effect on dopaminergic neurons is not known. In a pioneering article on the effects of bFGF on nigral grafts, the growth factor was administered either as pretreatment (phase 2, Fig. 1) in the cell suspension or given to the host as repeated intrastriatal infusions (phases 3 and 4, Fig. 1) (93). Pretreatment with bFGF produced significant effects on survival of grafted dopaminergic neurons. There was a slightly greater relative effect, up to about 270% of control (Table 2), when pretreatment of the donor tissue was combined with repeated intrastriatal bFGF infusions in the host over 20 days following transplantation. Even greater increases in the number of surviving tyrosine hydroxylase-positive neurons have been reported when growth factor was continuously delivered from co-implanted fibroblasts genetically modified to produce and release bFGF (135) (Table 2). Although the results suggest that a continuous supply of bFGF to the graft, even after implantation, is superior, one study has demonstrated that pretreatment with bFGF is sufficient to elicit an increase in dopamine neuron survival up to over 200% of control (151). In this case, the bFGF-induced enhancement of survival was accompanied by a marked increase in the number of activated astrocytes in the implants, suggesting that bFGF does indeed exert its effect via stimulation of glial cells, also in nigral grafts (151). A secretory form of FGF, kFGF, has been found not to enhance the survival of grafted dopaminergic neurons (67).

Glial cell line-derived neurotrophic factor (GDNF), a member of the TGF-β superfamily, was found in 1993 to promote the survival and differentiation of embryonic midbrain cultures (87). GDNF acts via GDNF family of receptors (GFR) α1 (25), but the precise mechanism of action of GDNF on dopaminergic neurons is not known. Data from in vitro studies indicate that GDNF reduces the amount of apoptosis of embryonic (29) and postnatal dopaminergic neurons (24). Repeated injections of GDNF adjacent to the intrastriatal nigral grafts every 1–3 days for 10–21 days increase the survival and fiber outgrowth of the dopaminergic neurons (114,127). Several other protocols for delivery of GDNF to grafts have also been employed successfully. Sullivan and coworkers (131) only added GDNF to the cell suspension (phase 2, Fig. 1) and still observed a significant increase in the number of surviving dopamine neurons. There is also increased survival of dopaminergic neurons within mesencephalic grafts following pretreatment with GDNF combined with intrastriatal GDNF infusions at 10 days and 4 weeks (59). Moreover, constant delivery of GDNF from an osmotic pump for 2 weeks (147) or from implanted polymer-encapsulated genetically modified cells for 6 weeks (117) results in improved survival. Typically these studies have demonstrated an increase in survival from 200% to 1300% (partly dependent on the survival rate in the control group, Table 2) of control grafts. However, when mesencephalic neurons are pretreated with GDNF in a hibernation medium at 4°C for 6 days prior to dissociation and then are grafted either in the presence (4) or absence (96) of GDNF in the medium, there are no major effects of GDNF on graft survival. These results indicate that GDNF is not effective when the cell metabolism is reduced due to the lowered tem-

perature (21). Another member of the TGF-β superfamily, growth/differentiation factor 5, has recently been shown to be as effective as GDNF at enhancing the survival of mesencephalic grafts when added to the cell suspension (131). Finally, neurturin, a new member of the GDNF ligand family (83), has been shown to increase the survival of dopaminergic neurons both in cultures (72) and in grafts (113).

Also, members of the neurotrophin family [e.g., brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT4/5)] have been shown to promote the survival of dopaminergic neurons in vitro (73,130). These neurotrophic factors act through stimulation of the receptors of the trk family, which have been shown to be present on dopaminergic neurons (74). In the case of nigral transplants none of the neurotrophin family members tested [i.e., nerve growth factor (NGF) (116,151), BDNF (116,149), NT3 (68), and NT4/5 (68)] have been found to enhance the survival of implanted mesencephalic dopamine neurons (Table 2). However, BDNF (116,149) and NT4/5 (68) increase the fiber outgrowth from grafted nigral neurons. Even though there is no increase in the survival of grafted dopaminergic neurons following treatment with these factors, their ability to enhance fiber outgrowth may still be important in attempts to improve nigral transplant function.

To summarize, the growth factors bFGF, GDNF, growth/differentiation factor 5, and neurturin are all capable of increasing the survival of transplanted dopaminergic neurons. Although growth factors can exert survival promoting effects when added to the graft preparation (93,131,151), it may be more effective to supply the trophic factor to the graft not only during preparatory stages (phase 2, Fig. 1) but also once it has been transferred to the host brain (phases 3 and 4 , Fig. 1).

CONCLUDING REMARKS

The past 5 years have seen considerable advances regarding our understanding of when and how grafted dopaminergic neurons die, and also on different methods to improve their survival. One ultimate goal of these studies is the development of a clinical protocol that requires that only one human donor embryo is necessary for each transplant recipient patient. This would require an increase in the survival beyond that observed so far with any single one of the treatments described in this review. Needless to say, such a protocol must use drugs/growth factors and a delivery technology that are safe in a clinical setting.

Currently there appear to exist three main options when trying to attain the goal of "one donor–one patient." First, it is possible to continue to search for better neuroprotective agents, although it is not certain that there exist more effective compounds. Second, it is valid to test combinations of treatments already found to be effective, but it is unlikely that effects will be additive in many cases. It seems that the combinations that are most likely to exert additive, positive effects are those that bring together factors with principally different modes of actions, such as antioxidants and anti-apoptotic agents. Third, increased emphasis should be placed on understanding the changes in the host brain that take place during graft implantation (phase 3, Fig. 1) and inhibiting their detrimental effects on transplant survival. With several active research groups contributing to all of these options, there is cause for optimism for the future.

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