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INTRODUCTION:

The remaining obstacles to achieving reliable therapeutic effects by neurotransplantation for Parkinson's disease (PD) are 1) poor survival of grafted fetal neurons and 2) insufficient axonal outgrowth and functional recovery.

While about 50% of cells born in the CNS will die naturally, up to 99% of fetal cells die after neural transplantation, which suggests that a large fraction of this cell death is preventable. Activation of caspases is one of the final steps before a neuron is committed to die by apoptosis. Since fetal development of neuronal grafts in many ways mimics normal development it is very likely that the caspases play a major role in the control of cell death in transplants. The structural brain repair experiments proposed will use fetal DA cells obtained from ventral mesencephalon (VM) of transgenic mice. In our first objective of preventing neuronal apoptosis, we will study the short-term inhibition by pre-treatment of fetal cells with pharmacological inhibitors of caspases. Our second objective of enhancing axonal growth leading to optimal functional recovery by neuronal transplants, employs transgenic bcl-2 overexpressing donor cells. Evidence now exists that the very potent positive effects by the bcl-2 molecule on axonal outgrowth are distinct from its anti-apoptotic effect. We hypothesized that bcl-2 and similar molecules influence the growth of axons in the fetal and adult CNS, and that controlling such molecules could significantly improve neural transplantation and related regenerative therapies.

This basic research can be translated clinically in neurosurgical transplantation therapy for Parkinson's disease patients, for example by similar transgenic porcine donor cell modifications. In summary, by using donor cell modification of molecules involved in apoptosis and axonal growth, these experiments provide insights and therapeutic strategies for preventing neuronal death and enhancing axonal outgrowth for dopaminergic transplants.

BODY:

STATEMENT OF WORK

We describe below the research accomplishments associated with the approved Statement of Work, which is copied here in bold. The publications and figures referenced are attached in the Appendix.

STEPS 1.1-1.2 Reduction of apoptotic cell death leading to greater cell survival in dopaminergic neuronal grafts can be achieved by permanent deletion of apoptotic effectors such as Caspase 3/CPP32 and JNK3 in transplanted neurons:

In year 1-2, we will start the transplantation experiments of embryonic day 14 (E14) ventral mesencephalon (VM) cells from CPP32 and JNK3 deficient (knock out) mice and wild type (wt) litter mate controls into the striatum of adult parkinson model rats; obtained by neurotoxic lesion using 6-OHDA. Functional recovery will be determined by the decrease observed in amphetamine-induced rotational asymmetry at various time points (4 to 12 weeks) after transplant. Post-mortem microscopic analyses, using markers for tyrosine hydroxylase (TH) and aldehyde dehydrogenase (AHD), will reveal the extent of the increase in surviving TH and TH/AHD. The knock-out donor cells for the dominant Caspase 3 and the stress-induced JNK3 will provide separate evidence for the apoptotic pathways that are involved in neural transplant cell death. These results are compared to those obtained after incubating the wild-type donor tissue short-term (1 hour) with selective and non-specific synthetic caspase inhibitors *prior* to transplantation (since these molecules z-DEVD.CHO/fmk; and BAF (Bocasparyl(OMe)-fluoromethylketone) have poor penetration across the blood-brain barrier).

We started the transplantation experiments of embryonic day 13-14 ventral mesencephalon from CPP32 and JNK3 knock-out mice and their counterpart wild-type. We discovered immediate problems with obtaining a sufficient number of CPP32 knock-out fetuses because the unexpected abnormal formation of the ventral mesencephalon, including dopaminergic neurons. For this reason, we abandoned the knock-out part of the CPP32 and JNK3 experiment but did go ahead and investigated the role of apoptosis in addition to complement mediated cell death in neural cell transplantation. Initial experiments were aimed at determining whether overnight incubation of ventral mesencephalic (VM) cells with Bocasparyl (OMe)-fluoromethylketone (BAF) would lead to greater cell survival. The studies showed a false positive dye exclusion cell viability which led to an overestimation of viable cells prior to transplants. Overnight caspase inhibition treatment did not enhance cell survival as calculated by the post-graft Th-positive cell counts (Figure 1a). However, parallel *in vitro* experiments demonstrated that VM cells are susceptible to complement mediated cell lysis that can be blocked with an anti-C5 complement inhibitor (18A10). This hypothesis was tested in rat VM allografts and animals were transplanted with either a) BAF b) C5 c) a combination of BAF and C5 or d) untreated VM. Interestingly, there was a significant difference between the number of TH-positive cells in the group of combined BAF/C5 versus control at 12 weeks post-transplantation (Figure 1b). The synergistic effects of anti-apoptotic (BAF) and C5 inhibition in transplant survival *in vivo* indicates that initial complement mediated and traumatic cell injury both contribute to post-graft cell death, and possibly through similar intracellular mechanisms.

STEPS 2.1: Transplantation of dopaminergic neurons permanently expressing human bcl-2 leads to enhanced and long-distance axonal outgrowth and improved function of neuronal transplants used for Parkinson's disease:

STEP 2.1.1. Normally, transplantation of ventral mesencephalon (VM) cells to a single site in the striatum will only result in fetal axonal penetration of the tissue immediately surrounding the graft. In year 1-2, we will carry out a conclusive series of experiments with transplant donor tissue of E14 VM from bcl-2 overexpressing mice or wild type (wt) control mice to the striatum of parkinson model rats, with prior neurotoxic 6-OHDA damage. We will investigate functional recovery by amphetamine induced rotation over an 8 to 10 week period. At post-mortem, by immunohistochemistry for TH, AHD and human bcl-2 (hubcl-2) we will determine axonal outgrowth and the extent of axonal outgrowth into the surrounding host striatum.

In step 2.1, we have carried out the transplantation of hubcl-2 cells. This has led to the publication of a manuscript in Neuroscience (Holm et al., 2001). We have shown that bcl-2 overexpressing dopamine neurons have enhanced outgrowth after transplantation into the striatal system.

STEP 2.1.2. Prior work has shown that transplantation of wt VM cells to the substantia nigra does *not* result in long distance growth to the target host striatum. Continuous expression of bcl-2 will allow axons from the bcl-2 transgenic cells to reach distant host targets. We will therefore transplant E14 VM from bcl-2 overexpressing mice to the substantia nigra of the parkinson model rats. Again, we will investigate functional recovery through amphetamine induced rotation over an 8 to 10 week period. By retrograde tracing combined with immunohistochemistry for TH, AHD and specific staining for human bcl-2; we will determine axonal outgrowth and completeness of reconnection of the damaged neuronal circuitry.

In step 2.1.2., we have planned and transplanted bcl-2 overexpressing cells into the substantia nigra for long-distance growth. Early experiments showed surprising limited survival of

both wild-type and transgenic mice in that location. We have thus limited our experiments to the study of axonal outgrowth from the original transplant site (striatum) and published this results in Holm et al., 2001 (see above).

STEP 2.1.3. (Deleted by Reviewers)

STEP 2.1.4. The time course of bcl-2 expression is correlated with axonal extension from neurons in primary cell culture, similar to expression and outgrowth patterns seen in normal development. Bcl-2 overexpressing cells will continue to extend axons after 8 days in vitro (DIV) when the axonal outgrowth from normal cells has stopped. In this step, we will investigate the expression of bcl-2 in cultured VM neurons from wt controls and bcl-2 overexpressing mice and measure axonal growth in both, following double immunohistochemical labeling for TH, AHD, mouse- and hubcl-2. The cultures will be fixed at various time points from 2 to 12 DIV. In primary wt cell cultures, we will also examine if cells from different parts of the CNS with different rate of maturation will have variable but coinciding patterns of bcl-2 expression and axonal outgrowth. The expression of bcl-2 in VM neuronal cultures will then be compared to its expression in neuronal cultures derived from other parts of the developing CNS such as basal forebrain (septum), cerebellum and lateral ganglionic eminence, from bcl-2 overexpressors and wt controls and stained with bcl-2 and markers for specific subpopulations of these neuronal regions. These experiments will provide axonal growth data from different anatomical regions and specific neuronal phenotypes; with different rate of maturation will and corresponding bcl-2 expression. These data will be applied to neural transplantation surgery requiring extensive or long-distance axonal growth for therapeutic repair or regeneration.

We carried out cell culture studies for expression of various outgrowths of the dopaminergic neurons and have stained for human bcl-2 (Holm et al., 2001).

STEP 2.2. The cell cycle regulating molecules p21 and p53 bring the neuron to a terminal differentiation stage. Bcl-2 appears to be involved in the signals to block these molecules, thereby keeping the neuron in an elongation stage. In our final experiments of this series, bcl-2 overexpressing cells will be used as tools to investigate *other* molecules that act on axonal growth. The expression of the molecules FKBP12, calcineurin, p21, and p53, will be investigated in primary cell cultures of dopaminergic neurons from bcl-2 transgenic or wt mice, via western blot and immunostaining. This final step will lead us to additional molecules; relevant for future approaches for enhanced axonal growth and regeneration in therapies for patients with sustained neuronal damage or degeneration.

We have carried out cell culture experiments of FKBP12 and calcineurin in primary dopaminergic cultures to see how this molecule may change outgrowth. We have found data that support a mechanistic role of phosphorylation signals in the axonal elongation stage (Costantini and Isacson, 2000).

KEY RESEARCH ACCOMPLISHMENTS:

- The synergistic effects of anti-apoptotic (BAF) and C5 inhibition in transplant survival *in vivo* indicates that initial complement mediated and traumatic cell injury both contribute to post-graft cell death, possibly through similar intracellular mechanisms.
- Transgenic donor cells with caspase 3 and JNK3 deficiency may not develop appropriate ventral mesencephalic substantia nigra dopaminergic regions and therefore not be useful for transplantation.

- Overexpression of the bcl-2 gene in dopaminergic neurons can enhance their axonal growth capacity. After cell transplantation of mice hubcl-2 expressing fetal dopaminergic neurons to 6-OHDA lesioned rat striatum, an enhanced axonal outgrowth was seen compared to control wild-type cells.
- Axonal elongation and branching in dopaminergic neurons may be controlled by different intercellular signalling involving at times FKBP-12, calcineurin, p21 and p53.

REPORTABLE OUTCOMES:

Manuscripts:

1. Holm, K. and Isacson, O. (1999) Factors intrinsic to the neuron can induce and maintain the ability for neurite outgrowth: a role for bcl-2? *Trends Neurosci.* 22, 269-273.
2. Boonman, Z. and Isacson, O. (1999) Apoptosis in neuronal development and transplantation: role of caspases and trophic factors. *Exp. Neurol.* 156, 1-15.
3. Costantini, L.C. and Isacson, O. (1999) Dopamine neuron grafts: development and molecular biology. In: *Dopamine Neuron Development*, U. di Porzio, R. Parnas-Alonso and C. Perone-Capano, eds., R.G. Landes Company, Georgetown, pp. 123-137.
4. Isacson, O. and Sladek, J. (1999) Cellular and Molecular Treatments of Neurological Diseases. *Exp. Neurol.* 159, 1-3.
5. Costantini, L.C., Isacson, O. (2000) Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons. *Exp. Neurol.* 164, 60-70.
6. Costantini, L.C., Isacson, O. (2000) Neuroimmunophilin ligand enhances neurite outgrowth and effect of fetal dopamine transplants. *Neuroscience* 100, 515-520.
7. Isacson, O., Costantini, L., Schumacher, J.M., Cicchetti, F., Chung, S. and Kim, K.-S. (2001) Cell Implantation Therapies for Parkinson's Disease Using Neural Stem, Transgenic or Xenogenic Donor Cells. *Parkinson's Disease and Related Disorders* (D. Calne, A. Deutch, eds.) Elsevier Science Ltd. 7, 205-212.
8. Isacson, O., van Horne, C., Schumacher, J.M., Brownell, A.-L. (2001) Improved surgical cell therapy in Parkinson's disease: physiological basis and new transplantation methodology. In: *Parkinson's Disease, Advances in Neurology*, D. Calne, ed. Lippincott Williams Wilkins, Philadelphia, PA, 86: 447-454.
9. Kang, U.J. and Isacson, O. (2001) The Potential of Gene Therapy for Treatment of Parkinson's Disease. In: *Principles of Surgery for Parkinson's Disease and Movement Disorders*, Krauss, K., Jankovic, J., Grossman, R. eds. Lippincott-Raven, in press.
10. Holm, K.H., Cicchetti, F., Bjorklund, L., Boonman, Z., Tandon, P., Costantini, L.C., Deacon, T.W., Chen, D.F., Isacson, O. (2001) Enhanced axonal growth from hubcl2 transgenic mouse dopamine neurons transplanted to the adult rat striatum. *Neuroscience* 104, 397-405.
11. Cicchetti, F., Costantini, L., Belizaire, R., Burton, W., Isacson, O., Fodor, W. Combined apoptosis and complement inhibition improve neural graft survival in the rat brain. Manuscript in preparation.

Abstracts:

1. L.C. Costantini, D. Cole, O. Isacson. Neurophilin ligand enhances reinnervation of host striatum from fetal dopamine transplants. American Society for Neural Transplantation and Repair, 2000.
2. F. Cicchetti, L. Costantini, A. Moore, R. Belizaire, W. Burton, W. Fodor & O. Isacson. Combined Apoptosis and Complement Inhibitors Improve Porcine Neural Xenotransplant Survival in the Rat Brain. American Society for Neural Transplantation and Repair, 2000.
3. O. Isacson. Neural cell transplantation in neurodegenerative diseases. XVIII Intl. Congress of the Transplantation Society, Rome, 2000.
4. O. Isacson. Development of neuronal repair and reconstruction strategies against neurodegenerative disease. Intl. Workshop on Stem Cell Biology and Cellular Molecular Treatment, Tokyo, 2000.
5. O. Isacson, Primary Neuronal Cell Transplantation for Parkinson's Disease, The Cell Transplant Society, Montreux, Switzerland, Mar. 21-24, 1999.
6. L.C. Constantini, D. Cole, O. Isacson. Neurophilin ligands: neurotrophic effects in models of Parkinson's disease. American Society for Neural Transplantation, 1999.
7. O. Isacson. Dopamine neuron transplantation: pharmacological and behavioral aspects. Behavioral Pharmacology Meeting, Sept. 1-4, 1999.
8. O. Isacson. Neural Transplantation in Neurodegenerative Diseases. Year of the Brain Intl. Symp. Oct. 1-3, 1999.

Presentations:

- 1999 Cornell Medical School/New York Hospital "Developing nerve cells against neurodegeneration" (grand rounds & lecture)
- 1999 Montreux, Switzerland, The International Cell Transplant Society, "Primary neuronal cell transplantation for Parkinson's disease (lecture)
- 1999 Keystone Symposia, "Neural xenotransplantation for neurodegenerative disease" (lecture)
- 1999 Dalhousie University, Halifax, Clinical Neuroscience (rounds) and Dept. of Anatomy and Neurobiology (lecture)
- 1999 University of Pittsburgh Medical Center, Dept. of Pathology (lecture)
- 1999 University of Rochester, Experimental Therapeutics Workshop (lecture) and Neurology Grand Rounds
- 1999 Vancouver, BC, XIIIth Intl. Congress on Parkinson's Disease (lecture)
- 1999 Odense, Denmark, 7th Intl. Neural Transplantation Meeting (lecture)
- 1999 Boston, European Behavioral Pharmacology Society and Behavioral Pharmacology Society Conference (lecture)
- 1999 Austrian Parkinson Society, Vienna (lecture)
- 1999 Bonn, Intl. Neuroscience Symposium "Molecular Basis of CNS Disorders" (lecture)
- 1999 London, The Novartis Foundation "Neural Transplantation in Neurodegenerative Disease"
- 1999 Miami, 6th National Parkinson's Foundation Intl. Symposium on Parkinson's Research (lecture)
- 2000 Louisville, "The Neuroscience of Developing Cell Therapies for Parkinson's Disease" (lecture)
- 2000 Zurich, Intl. Study Group on the Pharmacology of Memory, (lecture)

- 2000 Tokyo, Intl. Workshop: Stem Cell Biology & Cellular Molecular Treatment (lecture)
- 2000 Il Ciocco, Italy, Gordon Research Conference (lecture)
- 2000 Rome, Intl. Cong. of the Transplantation Society (lecture)
- 2000 Turin, Italy, Cellular & Molecular Mechanisms of Brain Repair (lecture)
- 2000 Stockholm, Karolinska Institute, Neural Donor Cells for Transplantation (lecture)
- 2001 Colorado, Winter Conf. on Brain Repair, Cell and Gene Therapy for Basal Ganglia Disorders (panel organizer)
- 2001 San Francisco, AAAS, Stem Cell Biology and Parkinson's Disease (session lecture)
- 2001 Paris, Association pour la Neuro-Psycho-Pharmacologie, Huntington a Model Disease (lecture)
- 2001 Valencia, Spain, Fundacion Valenciana de Estudios Avanzados, The Impact of Stem Cell Research on Neural Transplantation (lecture)
- 2001 Potomac, MD, Workshop on Department of Defense Sponsored Parkinson's Related Research (Session Chair)
- 2001 Philadelphia, World Parkinson's Day Symposium, Thomas Jefferson University (lecture)
- 2001 Detroit, MI, Wayne State University Center for Molecular Medicine and Genetics (lecture)
- 2001 Osaka, Japanese Society for Neural Growth, Regeneration and Transplantation (lecture)
- 2001 Halifax, NS, Canadian Congress of Neurological Sciences (lecture)
- 2001 Princeton, NJ, Intl. Neurodegeneration Conference (lecture)
- 2001 Colorado Springs, CO, Intl. Neurotoxicology Meeting (lecture)

CONCLUSIONS:

Our findings support the idea of using anti-apoptotic agents to prevent otherwise healthy neurons from demise after transplantation. In addition, we have discovered a role of complement mediated cell damage after transplantation. Our data in this project also indicate that the bcl-2 molecule can modify the growth state of the axon of the neuron. The experiments have demonstrated that the implantation of hubcl-2 overexpressing mouse neurons into the rat dopamine denervated striatum can lead to an improvement in donor axonal outgrowth. We have also shown that anti-apoptotic and C5 inhibition can lead to greater cell survival in neural transplantation. Our experiments also indicate that neurotrophic factors such as GDNF and immunophilin ligands also can modify axonal elongation and branching patterns at least of cultured dopaminergic neurons. The effort to derive a better treatment for patients and U.S. service personnel with Parkinson's disease is supported by these cell transplantation studies. The transgenic modification of dopaminergic neurons and the successful transplantation of a number of these dopamine-producing cells may lead to improved functional recovery in surgical transplantation therapies. The neuroprotective measures for neurons being transplanted (anti-apoptosis, neurotrophic factors, and complement-inhibition) appear useful and necessary to enhance procedures and outcomes of grafting protocols.

BIBLIOGRAPHY: See Curriculum Vitae for Dr. Isacson (appendix)

PERSONNEL: (Who received salary support from this grant)

Dr. Ole Isacson, principal investigator
Dr. Sangmi Chung, postdoctoral fellow
Mark Gorman, research assistant
Andrew Ferree, research assistant

APPENDICES:

Figure and Figure Legend

Publications:

Manuscripts:

1. Holm, K. and Isacson, O. (1999) Factors intrinsic to the neuron can induce and maintain the ability for neurite outgrowth: a role for bcl-2? *Trends Neurosci.* 22, 269-273.
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6. Costantini, L.C., Isacson, O. (2000) Neuroimmunophilin ligand enhances neurite outgrowth and effect of fetal dopamine transplants. *Neuroscience* 100, 515-520.
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1. F. Cicchetti, L. Costantini, A. Moore, R. Belizaire, W. Burton, W. Fodor & O. Isacson. Combined Apoptosis and Complement Inhibitors Improve Porcine Neural Xenotransplant Survival in the Rat Brain. *American Society for Neural Transplantation and Repair*, 2000.

Curriculum Vitae: Dr. Ole Isacson

Figure 1

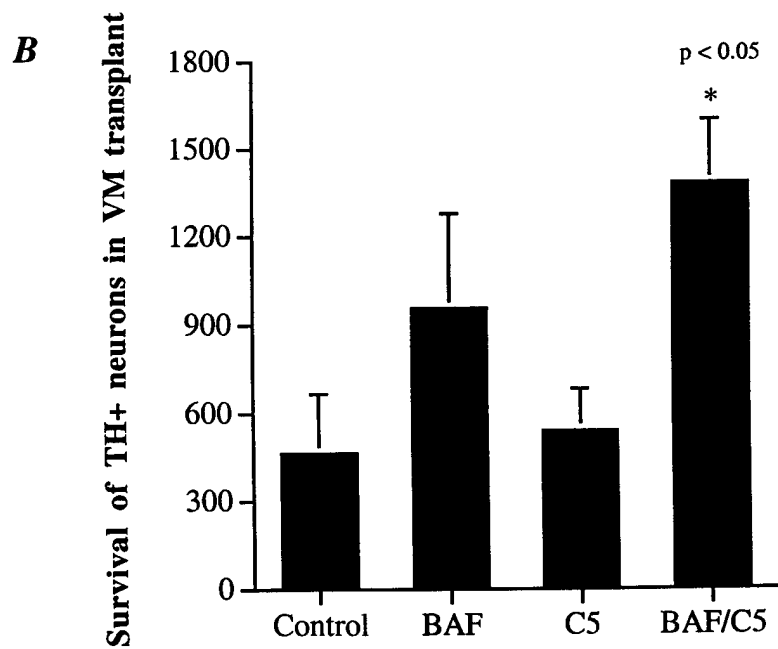
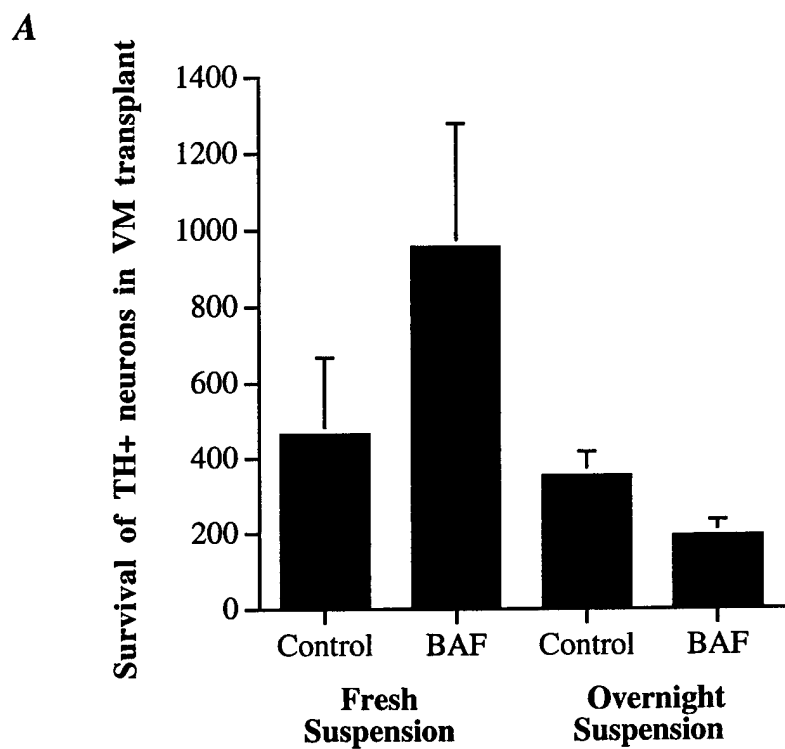


Figure legend:

A) In Vivo Assessment of Caspase Inhibition: Fresh vs. Overnight Treatment Conditions.

There was no significant difference between overnight incubation versus fresh in the control groups. Rat recipients receiving VM cell suspension that were treated with BAF just prior to transplant resulted in a greater number of TH positive neurons compared to control. In contrast, VM cell suspension that were incubated with BAF overnight showed less TH positive cells than the control group. BAF treatment just prior to transplantation enhanced TH cell survival in comparison to the fresh isolation control group but did not reach statistical significance. However fresh BAF treatment resulted in significantly greater TH cell survival as compared to the overnight BAF and overnight control treatment groups.

B) Effects of Caspase and Complement Inhibition on Allograft Survival.

The anti-C5 treatment had no affect on enhancing TH cell survival and had comparable TH cell counts as compared to the control group. There was a 2.5 fold increase in TH cell counts in the BAF group versus control that did not reach statistical significance. The average TH cell number was enhanced by the addition of C5 to the BAF treatment. The BAF/C5 treatment resulted in a 3 fold increase in TH positive cell numbers. This combination treatment had a synergistic effect showing a greater number of surviving TH positive cells then the added effect of the single treatments.

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Factors intrinsic to the neuron can induce and maintain its ability to promote axonal outgrowth: a role for BCL2?

Karin Holm and Ole Isacson

The adult CNS provides a poor environment for axonal growth and regeneration. The question of to what extent the loss of axonal growth occurring as the brain matures is dependent on factors intrinsic or extrinsic to the growing neuron is still unanswered. Examination of axonal growth from neural transplants provides insight into the roles of growth factors, inhibitory molecules, growth-promoting substrates and the differences between CNS and PNS environments in the regulation of neurite extension. The data that imply a role for BCL2 and related molecules in such processes are reviewed in this article, which analyzes the factors intrinsic to the neuron that control its capacity for axonal growth.

Trends Neurosci. (1999) 22, 269–273

IMPLANTATION OF NEURONS into the brain provides an *in vivo* assay for measuring permissive and non-permissive axonal-growth substrates, as well as differences in the capacity of neurons to extend their axons. In a recent experiment Davies and colleagues¹ asked the question of whether, in the absence of glial scarring, adult white matter can be growth permissive for axons from an adult neuron and, moreover, whether local production of chondroitin sulfate in the host will decrease the ability of grafted neurons to extend their axons. Using a microtransplantation technique to minimize scarring, they reported rapid axonal extension by grafted adult dorsal-root-ganglion neurons (DRG) for long distances in the host white matter. Grafts with no outgrowth were surrounded by a border of chondroitin sulfate, whereas no increase in proteoglycans was detected in grafts that extended their axons (see Fig. 1A,B). The researchers' interpretation of these data is that the adult CNS environment can still be growth supporting for adult neurons. However, the adult PNS (which contains DRG neurons) normally retains the ability to regenerate axons and, thus, raises the question of whether DRG neurons might also possess intracellular properties that allow them to extend axons for long distances. An alternative interpretation, therefore, is that intrinsic properties of the transplanted PNS neurons, like fetal CNS neurons, can

overcome the growth inhibition⁴ by the adult CNS environment. In fact, in an experiment in 1977, Björklund and Stenevi² transplanted adult sympathetic-ganglion neurons to a cavity in the septo-hippocampal junction of bilaterally sympathectomized adult rats (Fig. 1A,C). They demonstrated that axonal growth was produced by these transplanted nonadrenergic PNS neurons, which extensively reinnervated the host hippocampus across the necrosis of the lesion, and that continuous axonal extension occurred up to three months after transplantation. Axons grew for more than 4 mm into the hippocampus. As spontaneous regeneration by the host-brain noradrenergic axons was prevented by toxic lesion of nonadrenergic neurons located in the locus coeruleus, these experiments demonstrate that PNS neurons have the ability to extend axons for considerable distances in the adult CNS. Given that PNS neurons and some CNS neuronal phenotypes, such as nonadrenergic and cholinergic^{2,5}, retain their ability to regenerate throughout adulthood, there must be molecules and intrinsic processes that allow such confirmed axonal growth. These intrinsic properties might involve the same growth-promoting molecules that are active during the short time when embryonic transplanted CNS neurons are able to extend axons, despite the inhibitory properties of the adult CNS (Ref. 4).

Karin Holm and Ole Isacson are at the Neuroregeneration Laboratory, Harvard Medical School, Program in Neuroscience, McLean Hospital, Belmont, MA 02178-9106, USA.

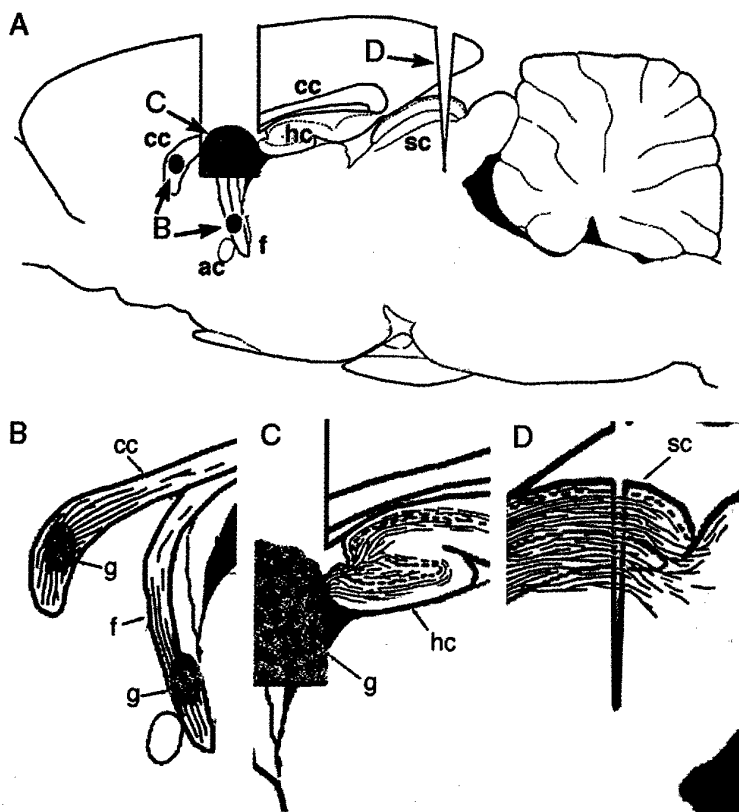


Fig. 1. In vivo paradigms that illustrate conditions and factors involved in maintaining capacity for long-distance axonal growth in the adult brain. By transplanting adult dorsal-root ganglia (DRG) to the white-matter tracts of adult rats [see red arrows in (A)], Davies et al.¹ demonstrated that long-distance axonal growth occurred from the grafted adult DRG neurons into the adult host, unless excessive proteoglycan-rich barriers prevented axonal penetration. The placements of the grafts (red) are illustrated in (A) (the upper sagittal view of the rat brain) and in more detail in (B). The axons growing within the white-matter tracts are detailed in (B). In an earlier but related experimental paradigm (C), Björklund and Stenevi² managed to transplant adult superior cervical ganglia (green) to the site of an adult fimbria-fornix transection in rats. Axons from the adult noradrenergic neurons (green) penetrated the adult dorsal hippocampus (hc) and these axons grew for several millimeters into the adult hippocampus. A third example (D) of a capacity for axonal growth into CNS territory, which is not normally permissive to penetration, is provided by the experiments of Chen et al.³ In mice that overexpress Bcl2, at an age when axonal regeneration in the retino-tectal system is normally abortive (postnatal day 4), these authors demonstrated optic-tract axonal regrowth [dark-blue fibers in (D)] through a transected area into the tectum by postnatal day 10. The three in vivo examples of regenerative or regeneration-like growth all illustrate situations in which factors intrinsic to neurons can extend the capacity for axonal growth into relatively non-permissive adult neural substrates. Abbreviations: ac, anterior commissure; cc, corpus callosum; f, fornix; g, grafted neurons; hc, hippocampus; sc, superior colliculus.

In normal development, axonal growth occurs in spurts that are interrupted by growth-cone collapse⁶⁻⁸. It appears that molecular gradients of attractive and inhibitory cues provide axonal orientation by inducing asymmetric growth-cone collapse and extension⁶. Axons from transplanted fetal neurons can grow extensively in neonatal host brain, but less so adult host brain^{9,10}. Therefore, the adult CNS environment is likely to cause more frequent growth-cone collapse of transplanted neurons, which increases the time needed for the axon to reach its target. Most transplanted neuronal phenotypes only persist in a growing state for a limited time. In fetal allotransplantation to the adult host brain, this time is usually too short for the axon to reach distant targets. Xenotransplantation experiments that use donor species with slower embryonic development than the host, for example,

pig¹¹ or human¹², transplanted to adult rats, show that the longer time window for outgrowth in this paradigm allows the axons to reach long-distance targets. One frequently used explanation for this is that axons from the xenotransplants could be less responsive to growth-inhibiting molecules produced in the host brain. This is certainly possible, but less likely as we have preliminary observations that the growth from xenotransplants in the adult is relatively impeded compared with growth in developing postnatal-day 7 (P7) brain¹⁰, which indicates that growth inhibition seen in allotransplants is also present in xenotransplants. Also, mouse fetal tissue that is xenotransplanted to the rat striatum does not show the increase in axonal growth relative to rat donor tissue that is seen when human tissue is xenotransplanted into the rat¹⁰.

Another reason for the ability of embryonic neurons to extend axons in the adult brain could be that they have yet to produce receptors for certain growth-inhibiting factors. There is, in fact, partial evidence of upregulation of the synthesis of these receptors in developing neurons around the time of myelination¹³. An intrinsic program for axonal outgrowth would, thus, be correlated with a low responsiveness to inhibitory signals from the environment. As the neuron matures, the increased production of the receptors for growth inhibitors would retard its growth. The ability for long-distance axonal growth from xenotransplants could, therefore, be due to the greater length of time that elapses before these receptors are produced, coupled to the delay while certain growth-promoting molecules become active within the neuron.

Fetal neuronal transplantation as a model system: factors extrinsic to the neuron that might be involved in regulation of axonal outgrowth

The adult CNS is a relatively poor environment for axonal growth and regeneration. Nevertheless, fetal neurons are capable of extending axons to some degree, even in the adult brain, especially when transplanted to their axonal target zones. For example, fetal rat ventral-mesencephalic (VM) cells that are allotransplanted to the adult striatum of an animal model of Parkinson's disease will form connections and create behavioral recovery in the recipients¹⁴. However, in most cases, histological analysis shows that only a part of the target, that which immediately surrounds the graft, is innervated using this transplantation method. Conversely, when transplanted to neonatal brain, these fetal neurons migrate into the surrounding brain and extend axons for long distances^{9,10}. Interestingly, the host age at which homotopically transplanted embryonic VM neurons fail to extend axons for a long distance to the striatum appears to be between P8 and P20.

What are the factors involved in this transition of the host environment from one that is permissive to growth to one that inhibits growth? High levels of growth factors are present in the brain during development and correlate in time and location with enhanced growth of specific groups of neurons. For example, the gene encoding NGF is highly expressed in the hippocampus and cortex during development¹⁵, and likewise the gene encoding glial-derived neurotrophic factor (GDNF) is expressed in the developing striatum¹⁶. There are several studies that show improved innervation of tissue surrounding the graft

when the graft is exposed to growth factors. Granholm and colleagues showed more extensive innervation from dopaminergic cells transplanted to the striatum after pretreatment of the fetal tissue with GDNF (Ref. 17). In addition to improved innervation of nearby target tissue, long-distance growth has also been achieved using growth factors. For example, Wang *et al.*¹⁸ created a track of GDNF along which axons from grafted dopaminergic neurons placed in the substantia nigra (SN) could grow to the target striatum. Furthermore, productions of growth factors could be the cause of more-extensive or long-distance axonal outgrowth from grafted neurons in a number of experiments that use co-transplantation of embryonic tissue. Transplantation of a mixture of fetal VM and striatal cells into adult 6-hydroxy-dopamine-lesioned striatum results in a larger area of dense innervation surrounding the graft¹⁹. When transplanting embryonic VM cells to the lesioned SN and creating a bridge of embryonic cortical tissue along an oblique needle track from the SN to the striatum, Dunnett and colleagues²⁰ showed that dopaminergic fibers from the graft could grow along the bridge graft all the way to the striatum. Mendez and colleagues²¹ transplanted fetal VM cells simultaneously to the SN and the striatum of adult rats and, surprisingly, growth of dopaminergic axons from the graft in the SN to the VM graft in the striatum was observed, using retrograde labeling with flourogold. As a control, fetal cortical neurons transplanted into the striatum did not cause the homotopically placed dopaminergic neurons to grow to the striatum. A possible explanation for this long-distance axonal growth could be the release of appropriate trophic factors by fetal cells, which thereby stimulate long-distance directional growth of axons, as appropriate axon orienting (tropic) factors are still provided by the adult CNS environment¹¹.

A number of factors and conditions that inhibit growing neurites have been described, many of which are present in the adult CNS (Ref. 4). Blocking these factors is one approach that can be used to make the adult CNS more permissive for axonal growth and regeneration. Several experiments that used antibodies to growth-inhibiting proteins, such as myelin-associated neurite-growth inhibitors, to treat lesioned axons in the CNS, demonstrate a moderate increase in regeneration. Thus, it seems that these proteins are, to some extent, involved in the inhibition of axonal growth in the CNS. Inhibitors of neurite growth and the application of antibodies directed to these inhibitors are reviewed by Schwab⁴. The growth-inhibiting properties of the adult CNS surroundings are not seen in the PNS. Instead, the PNS environment usually provides excellent conditions for regeneration and axonal growth. For example, peripheral myelin has been shown to promote axonal growth, even for CNS neurons²². In early transplantation experiments, Aguayo *et al.*²³ used grafted sciatic nerve to connect dopaminergic grafts in the SN to their striatal target. They observed remarkable long-distance growth of dopaminergic fibers through the whole length of the sciatic graft (outside the skull and back into the striatum). In summary, these studies demonstrate the importance of factors present in the surrounding CNS for the ability of neurons to extend and regenerate axons (Table 1).

TABLE 1. Factors and conditions that are involved in axonal outgrowth mainly as studied in transplantation experiments

	Growth promoting	Growth inhibiting
Extrinsic	Fetal host ⁹ Fetal tissue at target ^{19,21} Minimal scarring from transplantation ¹ Peripheral environment ^{22,23} Denervated target area ²⁴ Growth-factor stimulation ^{17,18}	Adult host ¹⁰ Proteoglycans (chondroitin sulfate) surrounding the transplant ¹ Myelin-associated growth inhibitors ⁴ Intact target area ²⁴
Intrinsic	BCL2 (Refs 24,25) Factors present during certain time of fetal development ^{1,12} Factors in fetal and adult PNS neurons ¹²	Receptors for growth inhibitors ¹³ Inhibitory factors or lack of growth-promoting factors in adult CNS neurons ²⁶

Undoubtedly, the CNS becomes less permissive for axonal growth as the brain matures. Nevertheless, as already described, fetal neurons transplanted to the adult CNS have the ability to extend axons in gray and white matter. What are the factors involved in this ability of the fetal neuron to overcome the growth inhibition by the surrounding adult brain?

In search for intrinsic factors: does BCL2 regulate the potential of a neuron to extend its axon?

As for extrinsic factors that regulate axonal extension, several molecules and conditions intrinsic to the neuron appear to control axonal growth; such as growth-associated proteins, activated cytoskeletal proteins, Ca²⁺, cAMP and other second-messenger systems^{7,8,27-29}. BCL2 is among the factors that have been discussed in the context of the induction and maintenance axonal growth²³. The *Bcl2* gene was originally discovered as the proto-oncogene at the breakpoint of the follicular lymphoma translocation t(14;18). It has been shown to be protective against cell death induced by a variety of apoptotic and necrotic stimuli, such as ischemia³⁰, traumatic brain injury³¹, growth-factor deprivation and generation of free radicals³². *Bcl2* is expressed in the CNS during the middle to late gestational period and its expression is downregulated at the time of birth, persisting in regions with late differentiation, such as the dentate gyrus^{25,33}. The highest levels of BCL2 seen during the embryonic period are found in the neuroblasts of the ventricular zones and in post-mitotic neurons during the period of naturally occurring cell death (NOCD). With aging, BCL2 levels decline in most CNS neurons, but remain elevated in neurons of the PNS. *Bcl2* expression is high in the cortical plate: most prominently around E16. Residual *Bcl2* expression in the adult brain occurs mainly in microglia²⁵. However, this interpretation was challenged by a recent study in squirrel monkeys that described two patterns of neuronal *Bcl2* expression³³: (1) a uniform immunostaining of the entire cell including its processes; and (2) a granular immunostaining that excluded the processes. Over time the first pattern is gradually replaced by the second pattern, which persists in the adult brain. The authors suggest that the former pattern of BCL2 immunostaining is seen in neurons undergoing developmental differentiation while the latter pattern might represent immature undifferentiated neurons that persist in the adult brain³³.

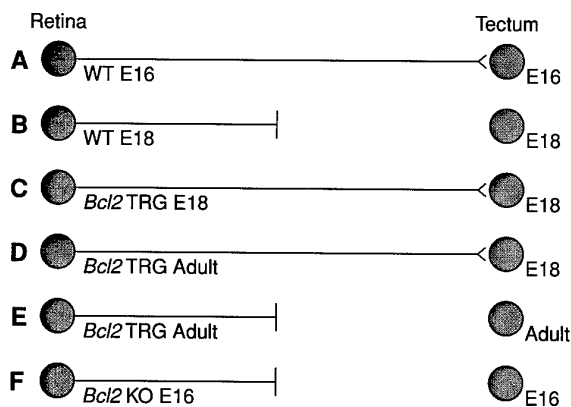


Fig. 2. Schematic representation of co-cultures of retina and tectum at various developmental timepoints. Wild-type control embryonic-day 16 (WT E16) retinal ganglion cells (RGCs) show extensive innervation of the tectal target (A). At E18 (WT E18), there is a complete failure to innervate the tectum (B). Conversely, the RGCs of E18 mice that overexpress *Bcl2* (*Bcl2* TRG E18) still show extensive target innervation of the tectum (C), as do the same RGCs in adults (*Bcl2* TRG Adult) (D). These RGCs also fail to innervate adult tectum (E). RGCs from *Bcl2* knockout mice (*Bcl2* KO E16) have lost their ability to innervate tectal tissue already by E16 (F). Data for the figure taken from Chen *et al.*³

Naturally occurring cell death is a prominent feature of the developing CNS. It can involve as many as 80% of the neurons, and one of the major functions of histogenetic cell death (a form of NOCD) in the nervous system seems to be the efficient innervation by cells, as a result of competition for growth factors, at the time the cells reach their target³⁴. The period of *Bcl2* expression in the developing nervous system coincides closely with the period of NOCD at the time when the axons reach their target³⁰ and could, thus, be involved in selective sparing of neurons during this period. Mice that overexpress *Bcl2* show reduced neuronal loss during NOCD, which leads to hypertrophy of the nervous system³⁰. Interestingly, high levels of *Bcl2* expression also correspond to the entire phase of axonal elongation²⁵. Evidence now exists that BCL2 is indeed involved in the process of axonal extension, which is a function that is distinct from its anti-apoptotic activity. In a recent experiment, Chen *et al.*³ used mice that overexpressed human *BCL2*, coupled to a neuron-specific-enolase promoter. Retino-tectal co-cultures prepared from these mice showed continuous innervation of the tectum at E18, in contrast to wild-type retinal controls (Fig. 2). Incubation with ZVAD-FMK, which is a wide-spectrum caspase inhibitor of apoptosis, prevented cell death as did overexpression of *Bcl2*, but no significant increase in axonal outgrowth was observed²³. This indicates that effects on axonal outgrowth produced by BCL2 are distinct from its anti-apoptotic effect. Moreover, reduced levels of BCL2 decrease the axonal growth-promoting ability of sensory neurons³⁵ and in retino-tectal co-cultures prepared from *Bcl2* knockouts at embryonic day 16 (E16), homozygote cultures showed an 80% decrease in innervation of the tectum compared to wild-type controls. This corresponds to the effects observed in wild-type retina co-cultured with tectum at E18, the time when axonal extension has come to an end. Immunohistochemistry of embryonic retinal tissue showed that there was a total loss of BCL2 immunoreactivity in retinas from E18

wild-type mice³. This demonstrates that BCL2 levels are directly correlated to the ability of the retinal neuron to extend axons. Axotomized retinal axons in a P4 mouse that overexpressed *Bcl2* grew in large numbers across the lesion site and innervated the tectum at a site that was caudal to the injury³ (see Fig. 1A,D). Using a dopaminergic cell line, Oh and colleagues³⁶ showed that vector induction of human *BCL2* leads to robust neurite formations when compared with a control vector. Increased time in culture (more than four days) led to increased neurite length. Zhang and colleagues³⁷ used a human neural-crest-derived cell line that undergoes spontaneous differentiation and normally expresses moderate levels of BCL2. They showed that manipulations of BCL2 levels with antisense cDNA resulted in a lack of differentiation and that overexpression of *Bcl2* produced by adding sense cDNA resulted in increased axonal outgrowth³⁷. The ability of the PNS to regenerate axons and the persistence of BCL2 levels in these cells also suggest that BCL2 has a growth-promoting effect. Notably, *Bcl2* expression has been described in the superior cervical ganglion and the DRG in mice at five months²⁵.

Concluding remarks

Several extracellular and intracellular factors have been discussed that influence axonal growth in the context of neuron-target interactions *in vitro*, after *in vivo* CNS lesions and by fetal neuronal transplants placed in the adult brain. It can be concluded that, even in the adult brain, it is possible to induce the production of factors intrinsic to the neuron that increase and maintain axonal growth. Further studies of BCL2 and other similar molecules that influence the growth capacity of axons in the adult CNS should, therefore, be instructive.

Selected references

- 1 Davies, S.J. *et al.* (1997) *Nature* 390, 680-683
- 2 Björklund, A. and Stenevi, U. (1977) *Brain Res.* 138, 259-270
- 3 Chen, D.F. *et al.* (1997) *Nature* 385, 434-439
- 4 Schwab, M.E., Kapfhammer, J.P. and Bandtlow, C.E. (1993) *Annu. Rev. Neurosci.* 16, 565-595
- 5 Björklund, A. and Stenevi, U. (1979) *Physiol. Rev.* 59, 62-100
- 6 Keynes, R.J. and Cook, G.M. (1995) *Curr. Opin. Neurobiol.* 5, 75-82
- 7 Igarashi, M. *et al.* (1995) *J. Neurosci.* 15, 5660-5667
- 8 Loschinger, J. *et al.* (1997) *J. Neurobiol.* 33, 825-834
- 9 Nikkash, G. *et al.* (1995) *J. Neurosci.* 15, 3548-3561
- 10 Isacson, O. and Deacon, T. (1997) *Trends Neurosci.* 20, 477-482
- 11 Isacson, O. *et al.* (1995) *Nat. Med.* 1, 1189-1194
- 12 Victorin, K. *et al.* (1990) *Nature* 347, 556-558
- 13 Shewan, D., Berry, M. and Cohen, J. (1995) *J. Neurosci.* 15, 2057-2062
- 14 Dunnett, S.B. and Björklund, A. (1994) *Functional Neurotransplantation*, Raven Press
- 15 Kaisho, Y. *et al.* (1991) *Biochem. Biophys. Res. Commun.* 174, 379-385
- 16 Strömberg, I. *et al.* (1993) *Exp. Neurol.* 124, 401-412
- 17 Granholm, A.C. *et al.* (1997) *Exp. Brain Res.* 116, 29-38
- 18 Wang, Y. *et al.* (1996) *Cell Tissue Res.* 286, 225-233
- 19 Brundin, P. *et al.* (1986) *Brain Res.* 389, 77-84
- 20 Dunnett, S.B., Rogers, D.C. and Richards, S.J. (1989) *Exp. Brain Res.* 75, 523-535
- 21 Mendez, I., Sadi, D. and Hong, M. (1996) *J. Neurosci.* 16, 7216-7227
- 22 Kromer, L.F. and Cornbrooks, C.J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 6330-6334
- 23 Aguayo, A.J. *et al.* (1984) *Neurosci. Lett.* 45, 53-58
- 24 Doucet, G. *et al.* (1990) *Eur. J. Neurosci.* 279-290
- 25 Merry, D.E. *et al.* (1994) *Development* 120, 301-311
- 26 Chen, D.F., Jhaveri, S. and Schneider, G.E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7287-7291
- 27 Nikolic, M. *et al.* (1998) *Nature* 395, 194-198
- 28 Lnenicka, G.A., Arcaro, K.F. and Calabro, J.M. (1998) *J. Neurosci.* 18, 4966-4972

-
- 29 Wang, Q. and Zheng, J.Q. (1998) *J. Neurosci.* 18, 4973-4984
30 Martinou, J.C. *et al.* (1994) *Neuron* 13, 1017-1030
31 Clark, R. *et al.* (1997) *J. Neurosci.* 17, 9172-9182
32 Merry, D.E. and Korsmeyer, S.J. (1997) *Annu. Rev. Neurosci.* 20, 245-267
33 Bernier, P.J. and Parent, A. (1998) *J. Neurosci.* 18, 2486-2497
34 Oppenheim, R.W. (1991) *Annu. Rev. Neurosci.* 14, 453-501
35 Hilton, M., Middleton, G. and Davies, A.M. (1997) *Curr. Biol.* 7, 798-800
36 Oh, Y.J., Swarzenski, B.C. and O'Malley, K.L. (1996) *Neurosci. Lett.* 202, 161-164
37 Zhang, K.Z. *et al.* (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 4504-4508
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REVIEW

Apoptosis in Neuronal Development and Transplantation: Role of Caspases and Trophic Factors

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Fetal ventral mesencephalic (VM) transplants have been studied in the context of dopaminergic (DA) replacement therapy for Parkinson's disease (PD). DA neurons from VM transplants will grow axons and form functional synapses in the adult host central nervous system (CNS). Recently, studies have demonstrated that most of the transplanted DA neurons die in grafts within the first week after implantation. An important feature of neural development, also in transplanted developing fetal neural tissue, is cell death. However, while about 50% of cells born in the CNS will die naturally, up to 99% of fetal cells die after neural transplantation. It has been shown that VM grafts contain many apoptotic cells even at 14 days after transplantation. The interleukin-1 β converting enzyme (ICE) cysteine protease and 11 other ICE-like-related proteases have been identified, now named caspases. Activation of caspases is one of the final steps before a neuron is committed to die by apoptosis. Here we review this cell death process in detail: Since the growth of fetal neural grafts placed in the adult brain in many ways mimics normal development, it is likely that the caspases also play a functional role in transplants. Pharmacological inhibitors of caspases and genetically modified mice are now available for the study of neuronal death in fetal neuronal transplants. Understanding cell death mechanisms involved in acute cellular injury, necrosis, and programmed cell death (PCD) is useful in improving future neuronal transplantation methodology, as well as in neuroprotection, for patients with neurodegenerative diseases. © 1999 Academic Press

Transplanting fetal VM neurons has proved that structural brain repair is possible for patients with PD. However, while necessary functional and structural

repair is possible using fetal neurons (61, 66), current transplant preparation and procedures are associated with a low yield of surviving DA neurons after transplantation and this limits the potential utility of this treatment (65). During brain development, cell death is believed to occur as a result of the adjustment of neuron number to their trophic factor producing targets (105). Depending on the cellular system, in the range of 20–80% (9, 11, 13, 14, 81, 105) of born neurons die during normal CNS development. However, up to 99% (9, 11, 13, 14) of the transplanted neurons die during graft development. Recent discoveries have provided insight into at least two different kinds of cell death seen in fetal graft development: apoptosis and necrosis (1, 23, 43, 86, 145). A large proportion of cell death in transplants appears to be apoptotic (86, 145). Although many studies show behavioral recovery in rodent models of PD with only 1–4% (97, 98, 112) of transplanted dopaminergic neurons surviving, preventing the massive cell loss seen in neural transplants could improve functional effects as well as reduce inflammation and the presence of immunological stimuli that could lead to transplant rejection. The major current obstacle to transplanting a large number of PD patients with VM grafts is insufficient access to VM DA neurons for grafting. By increasing cell survival in a graft, less tissue would need to be transplanted for functional effects to occur.

Apoptosis, Programmed Cell Death, and Necrosis

Apoptosis or programmed cell death (PCD) is a fundamental biological process in eukaryotes in which individual cells die by activating their own genetically programmed cell death mechanisms (70). The term apoptosis (etymologically from Greek apo-from, detached, separate, and ptosis-falling) was introduced after the discovery that a similar cell death cascade as that seen during PCD also occurs when mature cells are dying as a consequence of some pathological circumstances (70, 135). In the developing nervous system, a large number of cells die before birth by PCD (81, 105).

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PCD is therefore believed to be molding the nervous system's cellular structure and function. The surviving cells retain their ability to undergo apoptosis in adulthood and can display this process during aging and in neurodegenerative diseases (34, 95), such as amyotrophic lateral sclerosis (ALS) (69, 128, 129), Parkinson's disease (94), Huntington's disease (108), Alzheimer's disease (28, 96), and spinal muscular atrophy (68, 126). Neuronal apoptosis is also seen to some degree after acute injuries: trauma (21), ischemia (53), and stroke (67, 79).

There is evidence that both apoptosis and necrosis can occur after similar tissue injury (109, 121). Apoptotic death is distinguished from necrosis by a number of morphological and biochemical criteria (Table 1) (70, 135). The main criterion for apoptosis is the presence of internucleosomal DNA cleavage, seen as a "DNA ladder" on gel electrophoresis, although this is considered a relatively late event (25, 127, 134). However, cases have been described in which apoptosis occurs without demonstrable DNA laddering (25, 26, 103, 127), as in tissues containing diverse cell types where only a subset of cells die by apoptosis. This may be relevant to grafted VM, where *in situ* labeling techniques could be preferable to DNA gel electrophoresis. For VM transplants, double labeling for apoptotic nuclei and dopamine neurons would define the PCD of the therapeutically relevant cell type. Such methodology has been applied in the study of apoptotic dopamine neurons in culture (22, 93, 144). Apoptotic dopamine neurons *in vivo* were clearly described by Macaya *et al.* (85) in a developmental striatal target loss in rodents. *In situ* techniques have proven reliable in studies of susceptibility of developing dopaminergic neurons to undergo apoptosis in response to both 6-OHDA and hypoxic-ischemic injury (88, 104). However, DNA fragmentation

TABLE 1

Morphological Differences between Apoptosis and Necrosis

Apoptosis (70, 135)	Necrosis (70, 135)
Preservation of membrane integrity	Loss of membrane integrity
Cytoplasmic and nuclear fragmentation (internucleosomal cleavage of DNA/"DNA ladder"), condensed chromatin remains in nucleus	Nuclear flocculation
Diminution of cellular volume	Loss of lysosomal contents, loss of cellular content
Plasma membrane blebbing/constriction	Cellular swelling
Morphological conservation of organellar structure, retained lysosomal contents	Morphological signs of organellar damage
Apoptotic bodies (budding off of cellular fragments) recognition and phagocytosis, no inflammatory response	Cell lysis, inflammatory response

TABLE 2

A. NGF Deprivation: Different Phases of PCD and Apoptotic Events in Dying Neurons (36, 71, 76)

Healthy neuron		→		Degraded neuron	
1. Activation	2. Propagation	3. Commitment	4. Execution		
Apoptotic trigger-like NGF ↓	ROS ↑ Gene expression Glucose uptake ↓ Purine efflux ↑ RNA and protein synthesis ↓	Caspases	Substrate cleavage-like, PARP, and lamins DNA degradation		

B. Time Course of Events in Sympathetic Neurons Deprived of NGF (32)

Hours after NGF deprivation	Apoptotic event after NGF deprivation
Propagation phase	
3-8	ROS activation
5-10 (maximum 12-18)	c-jun, c-myc, mdk-1, and cyclin D1 expression
6	Decreased glucose uptake
10	Increased purine efflux
10 (maximum 15-20)	c-fos, fos-b, and NGFI-A expression
12	Decreased RNA and protein synthesis
12-18	No morphological changes
17-20	DNA fragmentation
Commitment phase	
22	"Commitment point"; depending on neuron's age; the older, the more resistant
18-24	Shrinkage and condensation of nuclei
24-30	Significant irregularities
24-36	Atrophied neurons
48	95% of neurons dead with degeneration of cellular organelles

may not be an absolute criterion for apoptosis, since certain neuronal populations as exemplified by rat cerebellar granule neurons (118) appear to be able to withstand such a process or show stereotypic biochemical and morphological changes of apoptosis, without clear evidence of internucleosomal DNA cleavage.

Apoptosis can be divided into four phases (Tables 2A and 2B): First, activation of the cell death program is induced by several apoptotic triggers such as deprivation of trophic support. Second, this path toward cell death coincides with metabolic changes such as decreased glucose uptake and reduced protein and RNA synthesis. Beyond a certain point, these changes appear irreversible and the cell reaches the point of "no return," characterized by stereotypic morphological changes in cell structure. Finally, the execution phase is characterized by lysis of the cell (31, 32, 70, 135). The distinction between necrosis and apoptosis is crucial

because the latter is under active cell control (39). PCD was first studied in the nematode *Caenorhabditis elegans* (58, 123). Pro- and antiapoptotic genes were found, for which there are mammalian homologues (Table 3), indicating that apoptosis is evolutionary and genetically conserved (141). One such gene is *ced-3* which encodes a member of the ICE cysteine protease family and is crucial for the execution of all PCDs in mammals (141). ICE, the first of 12 members of a larger proteolytic family, was initially identified as the protease responsible for proteolytic activation of the interleukin-1 β (IL-1 β) precursor (24, 100, 115, 130–132). This family of molecules are now known as caspases, standing for cysteine proteases ("c") with cleavage sites after aspartic acid residues ("aspase"). Caspases are synthesized as inactive proenzymes in the cytoplasm and are activated by cleavage at internally specified conserved aspartate residues, in cells undergoing apoptosis. The caspases thus initiate a cascade of proteolytic cleavage leading to activation of downstream caspases with cellular substrates, such as poly(ADP-ribose) polymerase (PARP) and lamins. Cellular apoptotic mechanisms can involve the Bcl2 family members, mitochondrial release of cytochrome *c*, and the activation of caspases (see Fig. 2): A biochemical sequence for such a process would be the release of cytochrome *c* after opening of mitochondrial permeability pores, which would then activate pro-caspase-3. It has recently been shown that Bcl2 interferes with the activation of caspase-3 by preventing the release of cytochrome *c* (36, 71, 111, 139, 140). BclXL, an antiapoptotic member of the Bcl2 family, may act by holding the proapoptotic Apaf-1/caspase-3 complex inactively bound to the mitochondrial membrane (18, 45, 57, 111). Bax is a proapoptotic homologue of Bcl2 also bound to the mitochondria, which induces the release of cytochrome *c* by heterodimerizing with BclXL and displaces BclXL from the inactive Apaf-1/caspase-3 complex. This activates caspase-3, which cleaves other caspases in the death cascade (18, 45, 111, 114) (see Fig. 2). Neither caspase inhibitors nor Bcl2 has been shown to prevent Bax-induced cytochrome *c* release. Nonetheless, cells overexpressing both Bcl2 and Bax show no signs of caspase activation and survive even though they have signifi-

cant amounts of cytochrome *c* in the cytoplasm, indicating that Bcl2 can prevent Bax-induced apoptosis by other mechanisms (114) (see Fig. 2).

Apoptosis and Its Role in Cell Death of Neuronal Transplants and Fetal Development

It may be more appropriate to use the term PCD rather than apoptosis when describing cell death in developing grafts, since the term PCD was originally introduced to describe cell death that occurs specifically during embryonic, fetal, and early postnatal development and thus is a physiological process (82). PCD timing varies from region to region in the brain and is species-specific, though stereotypical. Most probably, PCD is normally the result of competition for a limited amount of target-derived trophic factors and occurs mainly in neurons with axons that have reached their targets (16, 81, 105). This suggests that to some extent synaptic interactions control the survival of neurons during PCD (16, 81, 105). In the developing CNS the survival of neurons depends on access to appropriate amounts of trophic factors (105). During neurogenesis and maturation one-half or more of the number of neurons born are eliminated by PCD (81, 105). During this process, it is believed that the number of neurons in connecting regions is matched to obtain a well-functioning system (59, 105, 125).

The trophic conditions for transplanted fetal cells are different from normal development. For example, the levels of target-derived trophic factors are reduced in the adult compared to the fetal/neonatal brain. Nonetheless, glioblasts contained in the fetal cell preparations develop and produce a glial environment not unlike that seen in normal development. Although many adverse factors influence cells in the process of fetal neural transplantation, such as trauma, oxidative stress, ischemia (144), and lack of growth factors (105), we believe it is reasonable to assume that the process of PCD would continue in the developing transplanted tissue, as it would during normal development. Likewise, the function of PCD in developing VM transplants would be to eliminate cells that are not able to integrate in the developing nervous system, because these cells are possibly unhealthy or maladapted.

The extent of PCD in transplanted fetal substantia nigra is probably more severe than during ordinary development, since there will be greater competition for fewer neurotrophic factors. Moreover, not all of the cells in the fetal VM are dopaminergic and therefore may not respond to the same trophic factors or those available in atypical or ectopic targets (17, 64). The time course of neuronal cell death in nigral transplants has been studied by Barker *et al.* (5). They showed that the majority of DA neurons die during the first 7 days after transplantation. The survival rate of DA neurons is higher in three-dimensional *in vitro* cultures (with the

TABLE 3

Function of Genes Modulating PCD/Apoptosis in *C. elegans* and Mammals (39, 58, 136, 141)

Gene	Protein	Function	Mammalian homologue
<i>ced-3</i>	CED-3	Vital for cell death	Caspase-3-like-caspases (19, 141)
<i>ced-4</i>	CED-4	Vital for cell death	Apaf-1 (146)
<i>ced-9</i>	CED-9	Prevents cell death	Bcl2-family (80)

same dimensions as an *in vivo* graft made out of identical cell suspensions), indicating that features in the tissue environment surrounding a graft are unfavorable to the DA neurons.

Interestingly, Zawada *et al.* (145) showed that growth factors (GDNF, IGF1, and FGF2) reduce apoptosis in VM grafts and thus increase dopaminergic neuron survival. The growth factors appear most effective the first 24 h after grafting, since cell death after the first week was proportional to the number of cells surviving at the first day; 49% versus 32% at 24 h and 26% versus 16% dopaminergic neuron survival at 7 days after grafting in trophic factor-treated and control animals, respectively (see Fig. 1). The number of apoptotic cells was five times greater in 1-day-old grafts compared to 7-day-old grafts in both groups, as assessed by TUNEL technique, indicating that most apoptosis occurs shortly after transplantation. Since only 0.5–10% of the originally transplanted neurons survive after approximately 3 months (9, 12–14) and 16% of dopaminergic cells survive after 1 week, it seems that most cell death takes place in the first week after transplantation (see Fig. 1). Mahalik *et al.* observed many apoptotic cells in 2-week-old fetal mesencephalic grafts and very few apoptotic cells in 4-week-old grafts (86). However, many questions remain. For example, will the rescue of dopaminergic neurons from apoptotic death by trophic factors in the first week of graft development (145) endure? Is apoptosis in the first 2 weeks of graft development fully responsible for the final number of surviving neurons?

Most likely, neuronal death in fetal VM transplants can occur by both necrosis and apoptosis since both mechanisms were observed in developing grafts (1, 23, 43, 86, 145). Previously it was believed that the major mechanism of cell death in developing grafts was necrosis (1, 23). Although neural transplants may exhibit characteristics of both necrosis and apoptosis, one process may dominate (1, 23, 86, 145). Recent studies have demonstrated a continuum between apoptosis and necrosis (36, 109, 121). They show that the same type of stimulus can lead to either apoptosis or necrosis, depending on the concentration of the toxic agent, such as glutamate receptor agonists (35, 109). Low concentrations induce apoptosis; high concentrations induce necrosis. Of note, Bcl-2 is able to block both apoptosis and necrosis, suggesting that some events underlying apoptosis and necrosis may be similar (119).

STRATEGIES TO DECREASE CELL DEATH IN FETAL NEURONAL TRANSPLANTS

Cell Preparation Techniques in Transplantation Paradigms

The first fetal nigral grafts were implanted as solid pieces into adult rats with DA loss of the striatum (10)

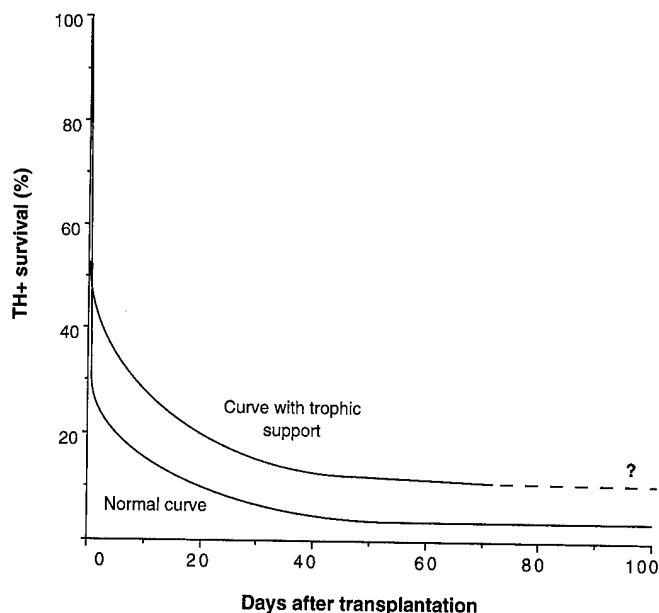


FIG. 1. An estimated *in vivo* dopaminergic neuronal survival rate in ventral mesencephalic (VM) grafts placed in the adult rat brain from several independent research groups. Notably, there is considerable (up to 99%) cell death associated with the transplantation paradigms. Since approximately 50% of neurons die during normal development, it seems reasonable that optimization of neuronal survival in fetal transplants is possible. Based on data from Zawada *et al.* (145), major cell loss occurs during the first days after transplantation (26% survival after day 1). At 7 days, this value had decreased to 16% in that study. Survival rates at 4–5 weeks vary considerably (1.5 to 7%) (98, 112). At longer survival times, the percentage survival ranges between circa 0.5 and 10% (8, 9, 13, 14). In similar fetal ventral mesencephalic cell preparations treated with trophic factors (GDNF, FGF2), the survival rates are higher. Zawada *et al.* (145) found a 49% survival of TH+ neurons 1 day after transplantation with a combination treatment of GDNF, IGF1, and FGF2. These grafts displayed a 32% survival at 7 days. Estimates from studies at 4 weeks by Rosenblad *et al.* (112) indicated a 2-fold increase in DA survival of cell suspension grafts in the presence of GDNF (although the total percentage survival was lower than in previously cited studies). In VM solid tissue grafts, Granholm *et al.* (47) also found a 2.6-fold increase in cell survival at 6 weeks after transplantation with GDNF treatment compared to untreated tissue, which would suggest an 18% survival of TH+ neurons based on the estimated 7% survival rate of TH+ neurons in similar untreated solid VM tissue grafts after 10 weeks according to Haque *et al.* (52).

or as tissue pieces in the ventricle adjacent to the caudate nucleus (107). Subsequently, methodology for transplantation of dissociated cell suspensions of fetal VM was developed, providing many alternative techniques for preparing the tissue prior to transplantation (37). Many basic variables such as gestational age, dissection procedures, and dissociation media can influence the viability of the grafted neurons. For example, variations in proteolytic enzymes such as trypsin, use of DNase, and amount of trituration can influence the outcome. Barker *et al.* (6) demonstrated that optimal neuron survival scores were obtained in VM tissue from rat embryonic day 13 and 14 (E13–E14) fetuses when

incubated in purified 0.1% trypsin solution for 60 min and triturated using a fire-polished Pasteur pipette. We estimate that the survival rate of tyrosine hydroxylase positive (TH+) neurons in their studies ranged from approximately 2.3 to 18% in 4-month-old grafts depending on which brands, concentrations, and incubation times of trypsin were used (6). As far as surgical procedures influence outcome, the micrografting technique is one of the most recent modifications of experimental CNS transplantation techniques. This technique allows precise and reproducible injection of VM cell suspensions at single or multiple sites with minimal trauma, which yields improved graft survival and integration of the grafted neurons (101, 102).

Trophic Factors

Since with current neurotransplantation procedures only 0.5–10% of transplanted fetal neurons survive (9, 12–14), it is of interest to enhance this fraction of living cells. For practical purposes, current transplant protocols for PD patients require as many as 10–15 fetuses to obtain a set of transplantable VMs to provide a sufficient number of DA cells to help the patient (9, 13). Neurotrophic factors are able to promote the survival of DA neurons (see Fig. 2). For example, pretreating VM neurons with basic fibroblast growth factor (FGF2) followed by repeated FGF2 intrastriatal injections increases the survival of TH+ neurons 100% (90). In addition, cointegration of FGF2-transfected fibroblasts together with fetal dopamine cells causes augmented survival and fiber outgrowth of transplanted DA cells (124). Infusions of brain-derived neurotrophic factor (BDNF) in VM grafts produce enhanced striatal DA innervation of the host (143). Intrastriatal infusions of glial cell line-derived neurotrophic factor (GDNF) dose-dependently enhance DA cell survival and fiber outgrowth from VM grafts (120). Granholm *et al.* (47) showed increased size of transplanted VM tissue pieces, dopaminergic cells, and fiber outgrowth after pretreating VM tissue GDNF. Furthermore, Rosenblad *et al.* (112) showed that infusions of GDNF adjacent to intrastriatal VM grafts improve TH+ neuron survival and TH+ fiber outgrowth. Sautter *et al.* (116) showed that genetically modified GDNF-releasing capsules implanted near intrastriatal dopaminergic cell grafts show increased DA neuron survival and fiber growth toward the trophic factor-releasing capsule. As mentioned previously, Zawada *et al.* (145) demonstrated that GDNF, IGF1, and FGF2 prevent apoptosis in mesencephalic grafts. The most substantial effects are seen in grafts that are treated continuously with trophic factors after transplantation (20, 30, 90, 113, 116, 124, 143). Trophic factors cannot be given systematically because of the near impermeable blood-brain barrier (BBB). Infusions of trophic factors can, however, be made directly into the CNS area of transplanta-

tion (90, 143). To overcome the BBB, trophic factors can be conjugated with antibodies against the transferrin receptor, cross the BBB, and result in increased graft survival (2, 3, 41, 46). Such transferrin receptor antibody-based delivery systems may also be useful in transporting other large molecules, such as pharmacological caspase inhibitors.

Another way of providing trophic factors to fetal neural grafts is by cotransplantation of genetically modified cells to produce increased graft survival (20, 30, 116, 124). Analogous to trophic factor enhancement is cointegration of fetal cells with their fetal target tissue. In cointegration of VM and striatum (LGE), a denser innervation area in the striatum of the host brain can be seen compared to VM cells alone (15). Yurek *et al.* also found larger TH+ cell bodies and more fibers in the VM/LGE cointegrations implanted into the striatum of rats with unilateral 6-OHDA lesions (142). Such cotransplants increased function, cell survival, and reinnervation of the lesioned adult striatum (27). BDNF-producing E18 striatal astrocytes cotransplanted with fetal VM tissue also promoted the survival of dopaminergic neurons in the denervated striatum. However, postnatal day 0 (P0) striatal BDNF-secreting astrocytes transplanted together with fetal nigral cells impaired graft survival. BDNF was not able to overcome the influence of the astrocytes' age on cell survival (74). As a corollary, cotransplantation of human NGF-producing fibroblasts and adrenal chromaffin cells in the striatum of hemiparkinsonian rats increased long-term functional survival (29).

Overall, the observations that a significant proportion of transplanted neurons die by PCD are consistent with the neurotrophic theory, which states that developing neurons are initially overproduced and then compete for limited amounts of target-derived survival-promoting factors as to prevent their death (17, 63, 64, 81, 105).

Lazaroids, Antioxidants, cAMP, Ascorbic Acid, and Calcium Blockers

Lazaroids are 21-amino steroids with antioxidant activity, which inhibit free radical generation and lipid peroxidation. Treating VM cell cultures with lazarooids U-74389G and U-83836E enhanced the survival of TH+ neurons (44). Moreover, identical lazarooids prolonged cell viability in dissociated fetal VM cell suspensions prior to grafting and increased the survival of dopaminergic neurons 2.6-fold following transplantation (97). Utilizing lazarooid U-83836E in medium at 4°C for 8 days in fetal VM tissue before culturing and striatal transplantation enhances the survival of dopaminergic neurons compared to untreated control VM tissue (50). U-83836E, used at a very low concentration, protected against oxidative stress in cultured cortical neurons exposed to beta-amyloid toxicity (84). In addi-

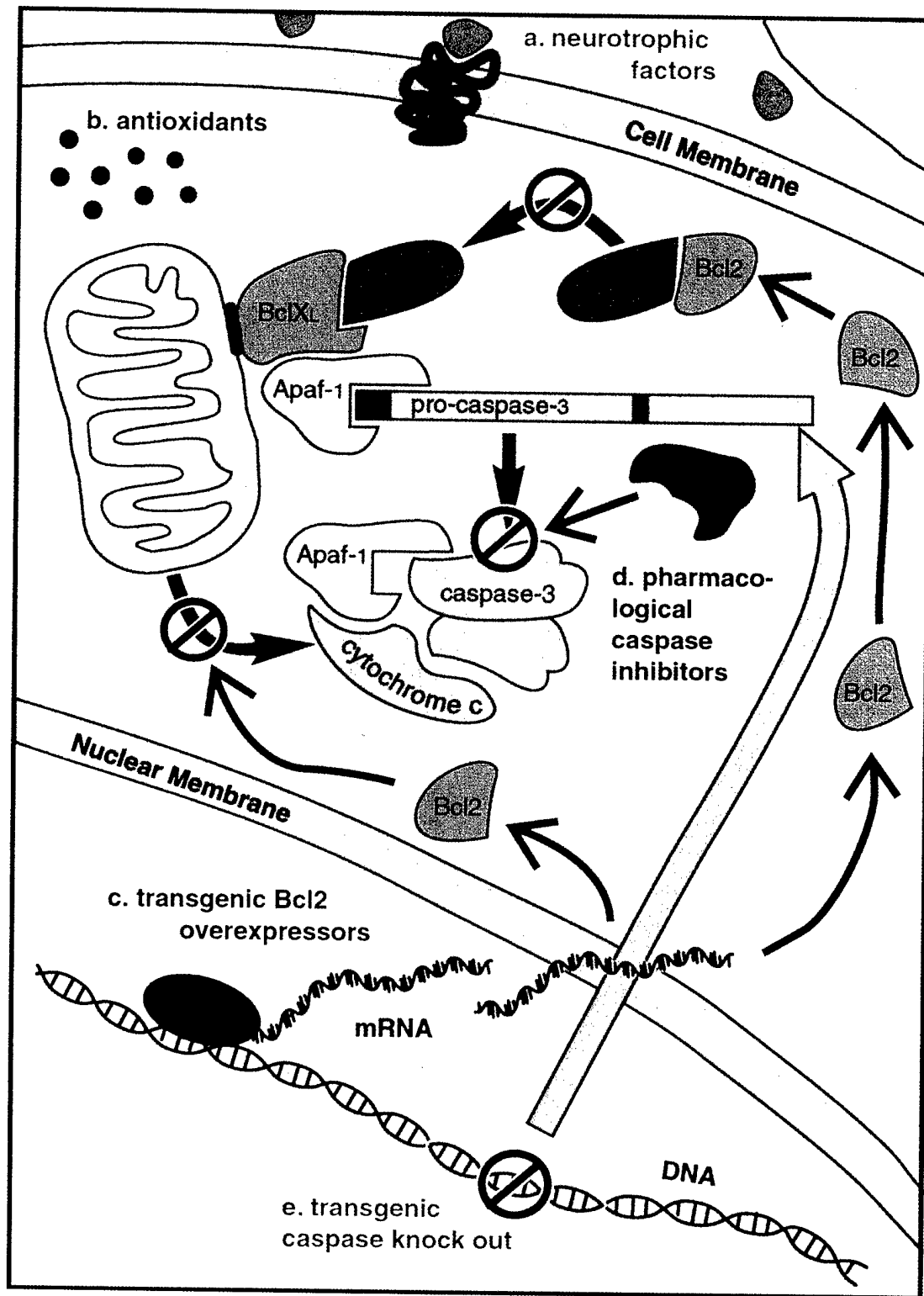


FIG. 2. Possible mechanisms that increase TH⁺ neuron survival in fetal mesencephalic grafts. (a) Grafts pretreated with trophic factors before transplantation and/or after grafting show increased dopaminergic neuron survival. Cografting of neurons with their target increases graft survival presumably by target-derived trophic factors. (b) Treatment with lazardol and other antioxidants increased neuron survival in grafts. (c) Transplantation of neuronal grafts overexpressing Bcl2 may increase survival since Bcl2 plays an important role in preventing apoptosis. The Bcl2 family has a double role in preventing activation of caspase-3, one of the most effective executioners of apoptosis. BclXL keeps the apoptotic Apaf-1 (apoptosis protease activating factor)/pro-caspase-3 complex inactively bound to the mitochondrion and Bcl2 prevents permeability from cytochrome c (presumed, Apaf-2) of the mitochondrion so that its binding to Apaf-1/pro-caspase-3 is prevented and

tion, U-83836E is neuroprotective to glutathione-depleted embryonic mesencephalic neurons (48) and prevented their death induced by congeners of nitrogen monoxide (49). Tirilazad mesylate, the single lazaroid approved for human use, has been found to improve the survival of both rat and human fetal VM cells *in vitro* (106).

In experiments in which neurons were taken from either transgenic mice overexpressing superoxide dismutase (SOD) (98) or adenovirus modified to overexpress SOD (4), similar survival effects to those observed in the lazaroid experiments were observed. Grafting of tissue derived from the SOD transgenics resulted in improved cell viability prior to grafting and a fourfold increase in TH+ neuronal survival following transplantation (98). However, the adenovirus-modified SOD-expressing grafts failed to show significant increase in dopaminergic neuron survival, although a trend was seen (4).

Dibutyryl cyclic AMP (dbcAMP), a cell membrane-permeable analogue of cyclic AMP (cAMP), attenuates apoptotic death and promotes maturation of cultured TH+ mesencephalic dopaminergic neurons (54, 93). dbcAMP was most effective when added immediately after cell plating, but delayed treatment also prevented degenerative processes (93). These results suggest that additional factors can be tailored for neuroprotective effects; for example, the survival-promoting action of GDNF on DA neurons appears to require activation of cAMP-dependent signaling pathways (40).

Pharmacological Inhibition of Caspases

A powerful strategy to decrease cell death in dissociated cell suspension or dissected tissue pieces would be treatment with caspase inhibitors prior to transplanting these fetal cells into the host brain (see Fig. 2). There are several potent caspase inhibitors available and we will first describe a few current examples of how they have been applied to neural cells in preventing apoptosis. There are several examples of induction of caspase(s) during apoptosis in neurons *in vitro* and *in vivo*.

Intracerebroventricular (icv) administration of an irreversible ICE/caspase-1 inhibitor, z-VAD-DCB, markedly reduces brain damage after focal cerebral ischemia in the rat (83). Yakovlev *et al.* found that caspase-3-like proteases are activated after traumatic brain injury in rats and demonstrated that blockade of these caspases

by inducing z-DEVD-fmk (a relatively specific inhibitor of caspase-3) icv before and after injury markedly reduced posttraumatic apoptosis as shown by DNA electrophoresis, TUNEL staining, and improved neurological recovery (137). Haviv *et al.* (55) studied apoptosis in neuronal PC12 cells deprived of trophic factor and found that caspase-3 or caspase-3-like proteases, but not caspase-1, are induced during the process. Both the viral caspase inhibitor gene p35 and broad spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) inhibit the death of neuronal PC12 cells (55). Definitive proof of caspase-3 involvement was obtained by Northern and Western blotting, demonstrating that PC12 cells express caspase-3 and that cleavage of caspase-3 substrates occurs in cell lysates prepared from trophic factor-deprived PC12 cells. Consistent with these findings, a previous study had shown that the viral apoptosis suppressor gene p35 was neuroprotective in an immortalized mesencephalic cell line (110). Moreover, caspase-1-specific inhibitors, namely Ac-Try-Val-Ala-Asp-chloromethylketone (Y-VAD.CMK), Ac-Try-Val-Ala-Asp-aldehyde, and crmA, a cytokine response modifier gene and a viral caspase inhibitor, were much less potent in inhibiting cell death (55). Of note, Y-VAD.CMK is relatively specific for caspase-1, but to a certain extent also blocks caspase-4 (87).

In the context of oxidative stress, Du *et al.* (36) found that exposure of cerebellar granule cells to low concentrations of MPP+ results in apoptosis and that the specific caspase-3 inhibitor acetyl-DEVD-CHO markedly attenuates such apoptotic death. Interestingly, necrosis of sucrose-cultured granule neurons occurred when the level of oxidative stress was increased by elevation of MPP+ concentrations (36). Moreover, cytoplasmic extracts from low-dose MPP+-treated cells contained protease activity that cleaved acetyl-DEVD-p-nitroaniline, a caspase-3 substrate (36). The more specific inhibitor of caspase-1, acetyl-YVAD-CHO, was ineffective against low-dose MPP+ neurotoxicity (36). Notably, cytochrome *c*, also known as Apaf-2 [one of the three apoptosis protease-activating factors (57)], was increased just before onset of apoptosis (36).

NGF-deprived sympathetic rat neurons in culture show reduced apoptosis when treated with Bocasparyl(OMe)-fluoromethylketone (BAF), an inhibitor of both caspases-1 and -3 (33). BAF did not prevent decreased protein synthesis or increased expression of c-jun, c-fos,

activation of caspase-3 will not occur. The following may happen when a death stimulus activates the apoptotic pathway. Bax will displace BclXL from Apaf-1/pro-caspase-3 and cytochrome *c* will bind to Apaf-1/pro-caspase-3. This will result in activation of pro-caspase-3 and activation of the caspase cascade will lead to cleavage of critical cellular substrates resulting in dissolution of the cell. The Bcl2 family is able to inhibit this process. (d) Pharmacological caspase inhibitors obstruct caspase activation and therefore cell death. Pretreating fetal mesencephalic grafts with caspase inhibitors may increase dopaminergic neuron survival. (e) Transplanting neural tissue from transgenic knock out (ko) mice may also increase neuron survival.

and other mRNAs that occur in the process of neuronal apoptosis (Tables 2A and 2B). This indicates that the caspases function downstream of several transcriptional events during apoptosis (33). Addition of either NGF or BAF prevented cell death of NGF-deprived sympathetic neurons at similar time points (33).

Thus, there are a number of factors to be determined for obtaining effective pharmacological caspase inhibition for fetal neural cell transplantation. Generally, it is useful to define which specific caspase(s) is involved in cell death of the specific cell type transplanted (54, 91, 93).

Inhibition of Caspases: Gene Deletion in Mice

An alternative to the pharmacological strategy of caspase inhibition is to transplant neural fetal tissue from mice lacking genes encoding caspases (see Fig. 2). Many different mice with a targeted endogenous gene disruption for one of the caspases have been developed over the past few years. For example, ICE/caspase-1-deficient mice (76) (Table 4) show no phenotypic abnormalities in the brain (76). Mice that express a dominant negative mutant of caspase-1 (42) and caspase-1-deficient mice (117) are protected against ischemia-induced brain damage. Thus, in some cells caspase-1 depression helps prevent apoptosis, while its role in PCD in brain development does not appear significant (76). CPP32-, Yama-, and apopain/caspase-3-deficient mice (77) (Table 4) show deviant brain development as a consequence of decreased developmental apoptosis, indicating that caspase-3 plays an essential role during morphogenetic cell death in the mammalian brain. In this study, the entire brain was larger, and in such caspase-3-deficient mice, there were significant hyperplasias, ectopic cell masses (in cortex, cerebellum, striatum, and hippocampus), double brain structures (in cortex and optic stalk), and disordered cell layering. No apoptotic cells were found along ventricular walls, optic stalk, or spinal cord, locations ordinarily packed with apoptotic cells during CNS neurogenesis. Caspase-3-deficient mice suffer from premature lethality and are not fertile. Surprisingly, caspase-3-deficient thymocytes were evenly sensitive to various apoptotic stimuli compared to the wild-type (wt) thymocytes, indicating that caspase-3 is specifically activated in the brain. Woo *et al.* (133) found evidence that caspase-3 activation is stimulus- and tissue-dependent. Moreover, caspase-3 is constitutively expressed in adult brain and is activated after ischemic attacks (99). However, note that one study found that most types of human brain and spinal cord neurons had little or no caspase-3 expression (73).

ICH-1/caspase-2 is highly expressed during embryonic days 9 and 16 in the brain and various other tissues. ICH-1/caspase-2-deficient mice (7) (Table 4) show no obvious cellular or histological brain abnormali-

ties. However, fewer neurons were found in the facial nuclei in the caspase-2-deficient newborns compared to the wt mice. At embryonic day 16.5 (at a time when PCD still occurs), the facial neuron number was equal in knock-out (ko) and wild-type mice, indicating that accelerated apoptosis had occurred rather than a decreased production of neurons.

Recently, a new caspase ko mouse has been introduced by Kuida *et al.* (76), namely the ICE-LAP6/Mch6 or caspase-9-deficient mouse. The majority of caspase-9 ko mice die perinatally with a marked enlarged and malformed brain, most severe within the cortex and forebrain, as a result of reduced apoptosis during development of the nervous system. No apparent malformations are found in spinal cord and other parts of the fetal body. At different embryonic stages there are abnormalities in the brain, generally a larger proliferative zone devoid of pyknotic clusters and fewer apoptotic cells in both forebrain and midbrain. For example, at E16.5, the ko brain shows an increased number of proliferative cells as well as protrusion of the midbrain from the skull. The ectopic brain masses are enlarged and irregular on the surface. Caspase-9^{-/-} mice are smaller than control littermates, and most die 3 days after birth. Caspase-9 deficiency prevents caspase-3 activation in fetal mice brains. In addition, cytochrome *c*-mediated cleavage of caspase-3 is absent in the cytosol of caspase-9-deficient cells but is restored after addition of caspase-9, suggesting that caspase-9 acts upstream of caspase-3 in certain apoptotic pathways (75). Moreover, these observations may confirm that caspase-9 is APAF-3 in the proapoptotic Apaf-1/cytochrome *c* (APAF-2) multiprotein complex which activates pro-caspase-3 during apoptotic death (see Fig. 2) (78). The caspase-9-deficient thymocytes are resistant to a variety of apoptotic stimuli, for example, anti-CD3 plus anti-CD28, etoposide, γ -irradiation, and dexamethasone (51, 75), but are equally susceptible to apoptosis mediated by Fas (75), UV irradiation, and anti-CD95 (51) compared with wt thymocytes. The caspase-9-deficient thymocytes' resistance to some apoptotic stimuli is consistent with the delayed DNA fragmentation and absence of caspase-3-like cleavage (75). Caspase-9-deficient embryonic stem cells and embryonic fibroblasts are also resistant to UV light and γ -irradiation, although cytochrome *c* is translocated in the cytosol of caspase-9-deficient embryonic stem cells after UV stimulation, suggesting that caspase-9 acts downstream of cytochrome *c* (51). Moreover, caspase processing is halted in embryonic stem cells but not in thymocytes or splenocytes. These studies show that the requirement for (caspase-3 and) caspase-9 in apoptosis is cell type- and stimulus-specific, indicating the existence of multiple apoptotic pathways (Table 4) (51).

Thus, reducing apoptosis in neural transplantation may be possible by using fetal dopaminergic cells from

TABLE 4

Different Caspase-Deficient Mice, Their Morphology, Function, and Protection against Induced Apoptosis and Trauma

Mouse deficient for	Brain morphology	Body morphology	Altered functions	Induced apoptosis/ trauma
Caspase-1 (87)	Normal	Normal	IL-1 β , IL-1 α , TNF- α , and IL-6 production after LPS induction	Monocytes (87) and mice (90) resistant to endotoxic agent LPS Thymocytes sensitive to dexamethasone and ionizing radiation, resistant to anti-Fas antibody (87) Decreased brain damage and faster functional recovery in ischemic models (51, 65)
Caspase-2 (8)	Normal, except for less motor neurons in facial nucleus at E 16.5	Excessive germ cells in ovaries	Oocyte death	Oocytes resistant to chemotherapeutic drugs B-cells resistant to granzyme B and perforin but sensitive to several agents ^a (8), accelerated cell death in NGF-deprived sympathetic neurons
Caspase-3 (88)	Larger brain with hyperplasias, ectopic cell masses, disorganized cell deployment, double brain structures, and less pyknotic cells	Smaller, die at 1–3 weeks of age	PCD in CNS	Thymocytes are sensitive to the following stimuli ^c (88) Lymphocytes are resistant to anti-CD95 plus anti-CD3, UV, and γ -irradiation (159) Splenocytes are sensitive to UV and γ -irradiation (63) Embryonic fibroblasts are resistant to ^b (159)
Caspase-9 (86)	Enlarged malformed brain-like protrusions of brain mass, stenosis of ventricles, heterotopias, invagination, and interruption of the telencephalic wall with less pyknotic cells	Smaller, most die before postnatal day 3	PCD in CNS Activation of caspase-3	Thymocytes are resistant to the following stimuli: dexamethasone, γ -irradiation, etoposide, and anti-CD3 plus anti-CD28 (63) and are sensitive to α -CD95, TNF- α , UV, shock, sorbitol (63), and Fas (86) Splenocytes are resistant to γ -irradiation and are sensitive to UV, sorbitol, adriamycin, etoposide, and <i>cis</i> -platinum (63) Embryonic stem cells are resistant to all stimuli used in splenocytes (63) Embryonic fibroblasts are resistant to ^a (159)
Caspase-11 (158)	Normal	Normal	IL-1 β and IL-1 α secretion after LPS induction	Mice resistant to LPS Fibroblasts resistant to caspase-1 overexpression

^a Anti-Fas antibody, doxorubicin, etoposide, γ -irradiation, and staurosporine.^b UV, γ -irradiation, adriamycin, and etoposide.^c Anti-Fas antibody, dexamethasone, C2-ceramide, staurosporin, and γ -irradiation.

transgenic mice deficient for one particular caspase. There are currently at least five different caspase ko mice, respectively caspases-1, -2, -3, -9, and -11 (Table 4). In caspase-3 ko, the brain is predominantly affected relative to other body regions as a result of failed neural apoptosis during CNS development. Although the brain abnormalities observed in caspase-3 and -9 kos are strikingly similar, it appears that caspase-9 ko brain is more severely affected than caspase-3-deficient brain.

The similarities of the phenotypes indicate that caspases-3 and -9 may act along the same biological pathway during brain development. Furthermore, it has been shown that caspase-9 is an upstream activator of caspase-3 (76) and this may be why caspase-9 ko brain is more affected than caspase-3 ko brain. Nonetheless, the other mutant caspase mice (caspases-1, -2, and -11) have normal phenotypes, indicating a redundant role for these caspases during CNS development. How-

ever, it is not known if these neurons are functionally altered. Modifying these caspases may still be protective for neurons in a fetal neural transplant and may therefore serve as alternative donor sources.

The differences in brain development and other characteristics seen between caspase-3 ko and caspase-1, -2, and -11 kos may be due to, first, the fact that caspase-3 and caspases-1, -2, and -11 are functionally different: respectively "effector" caspases (caspases-3, -6, -7, and -9) and "regulator" caspases (caspases-1, -2, -3, -4, -5, -8, and -10). This distinction is based on the different lengths of the caspases' N-terminal prodomains, assuming that this may be of functional importance. Effector caspases have short prodomains while regulator caspases have long prodomains. In a hypothetical cascade, the regulator caspases operate upstream and the effector caspases act downstream to the cascade cleaving crucial substrates (100, 130). *In vitro* studies have demonstrated the existence of such a caspase cascade but to what extent this occurs *in vivo* is still unknown (115, 122). There may also be tissue-specific activation of caspases. Moreover, not all caspases may be involved in developmental PCD, although they could still play a role in nondevelopmental cell death.

There are other genetically modified mice with decreased apoptosis that could be useful as donor tissue for fetal dopaminergic transplants, for example Bcl2 overexpressors (see Fig. 2) and JNK3 (c-Jun amino-terminal kinase) ko mice. Bcl2 overexpressing mice show reduced neuronal loss during PCD, leading to hypertrophy of the CNS. Adult mice are less susceptible to ischemia; they show reduced brain infarction after middle cerebral artery occlusion (89). Additional evidence for Bcl2's regulator role in neuronal development and PCD is that Bcl2-deficient mice showed progressive degeneration of several neurons in the CNS after the physiological PCD period (92). In SOD mice with overexpressed Bcl2 in their genome, onset of ALS-like degeneration was delayed (72). Another enzyme involved in stress-induced cell apoptosis is JNK3. Adult JNK3-deficient mice are healthy and fertile, look normal, and show no apparent abnormalities in different brain regions (138). JNK3^{-/-} mice are protected against glutamate neurotoxicity (138). We are currently investigating survival and axonal growth of fetal VM tissue obtained from Bcl2 overexpressors in animal models of PD (62).

Pharmacological Caspase Inhibitors and/or Caspase Gene-Deficient Tissue Transplantation with Trophic Factors

Pretreating neural grafts with pharmacological caspase inhibitors or transplanting VM tissue deficient for a specific caspase in the deafferented striatum appears to increase the survival of DA neurons in

transplants (56). However, it is not yet known if the neurons saved by inhibiting caspases are unhealthy or dysfunctional, since they are prevented from dying in the last stages of the apoptotic pathway. It is questionable whether it is always beneficial to block a cell death program since cells may be already dysfunctional before the caspases are actually activated and execute cells. It might be beneficial to lose these unhealthy neurons in order to obtain a well-functioning integration between the grafted fetal neurons and the host brain. An example that may illustrate this point is the neurodegenerative disease ataxia-telangiectasia (AT), where it appears that the lack of appropriate cell death mechanisms of damaged neurons can lead to accumulation of dysfunctional neurons, which then degenerate at a later stage (60). Thus, it is conceivable that in some situations preventing physiological PCD may lead to unforeseen disturbances in normal graft development and experiments are needed to illuminate these issues. It has been shown that treating fetal mesencephalic transplants with trophic factors also increases survival, fiber outgrowth, and functional recovery in models with PD (see above). Adding NGF to trophic factor-dependent neurons prevents the neurons from dying by blocking a crucial posttranslationally late event in neuronal PCD (32, 33, 38). However, NGF can only abort PCD before the neurons are irreversibly committed to die (31–33). Presumably, the caspases are activated during the commitment phase or just before the execution phase of PCD (Tables 2A and 2B).

In conclusion, to maximize the use of the protective strategies discussed, it may therefore be possible to combine pharmacological caspase inhibitors and trophic factors in neural transplants. The trophic factors can prevent neuronal death by providing a growth stimulatory effect for the neurons, while caspase inhibitors prevent neurons from reaching the irreversible biochemical executive point for a cell death process to occur. This suggests that combinations of antiapoptotic agents and trophic factors might act synergistically in blocking cell death.

The current understanding of neuronal PCD in normal brain development now includes knowledge of intricate intracellular cascade mechanisms, including caspases, that serve in the larger bioadaptive context to control the size of neuronal brain cell populations. This understanding can now be experimentally advanced in neurotherapeutic efforts to treat neurodegenerative disease by neural transplantation.

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REFERENCES

1. Abrous, N., J. Guy, A. Vigny, A. Calas, M. Le Moal, and J. P. Herman. 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.
2. Backman, C., G. M. Rose, R. T. Bartus, B. J. Hoffer, E. J. Mufson, and A. C. Granholm. 1997. Carrier mediated delivery of NGF: Alterations in basal forebrain neurons in aged rats revealed using antibodies against low and high affinity NGF receptors. *J. Comp. Neurol.* **387**: 1–11.
3. Backman, C., G. M. Rose, B. J. Hoffer, M. A. Henry, R. T. Bartus, P. Friden, and A. C. Granholm. 1996. Systemic administration of a nerve growth factor conjugate reverses age-related cognitive dysfunction and prevents cholinergic neuron atrophy. *J. Neurosci.* **16**: 5437–5442.
4. Barkats, M., N. Nakao, E. M. Grasbon-Frodl, A. Bilanz-Bleuel, F. Revah, J. Mallet, and P. Brundin. 1997. Intrastriatal grafts of embryonic mesencephalic rat neurons genetically modified using an adenovirus encoding human Cu/Zn superoxide dismutase. *Neuroscience* **78**: 703–713.
5. Barker, R. A., S. B. Dunnett, A. Faissner, and J. W. Fawcett. 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.
6. Barker, R. A., R. A. Fricker, D. N. Abrous, J. Fawcett, and S. B. Dunnett. 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.
7. Bergeron, L., G. I. Perez, G. Macdonald, L. Shi, Y. Sun, A. Jurisicova, S. Varmuza, K. E. Latham, J. A. Flaws, J. C. Salter, H. Hara, M. A. Moskowitz, E. Li, A. Greenberg, J. L. Tilly, and J. Yuan. 1998. Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes Dev.* **12**: 1304–1314.
8. Bjorklund, A. 1993. Intracerebral transplantation: Prospects for neuronal replacement in neurodegenerative diseases. *Res. Publ. Assoc. Res. Nerv. Ment. Dis.* **71**: 361–374.
9. Bjorklund, A. 1993. Neurobiology. Better cells for brain repair. *Nature* **362**: 414–415.
10. Bjorklund, A., and U. Stenevi. 1979. Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. *Brain Res.* **177**: 555–560.
11. Bjorklund, A., U. Stenevi, R. H. Schmidt, S. B. Dunnett, and F. H. Gage. 1983. Intracerebral grafting of neuronal cell suspensions. I. Introduction and general methods of preparation. *Acta Physiol. Scand. Suppl.* **522**: 1–7.
12. Bjorklund, A., U. Stenevi, R. H. Schmidt, S. B. Dunnett, and F. H. Gage. 1983. 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.
13. Brundin, P., G. Barbin, O. Isacson, M. Mallat, B. Chamak, A. Prochiantz, F. H. Gage, and A. Bjorklund. 1985. Survival of intracerebrally grafted rat dopamine neurons previously cultured *in vitro*. *Neurosci Lett.* **61**: 79–84.
14. Brundin, P., O. Isacson, and A. Bjorklund. 1985. 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.
15. Brundin, P., O. Isacson, F. H. Gage, and A. Bjorklund. 1986. Intrastriatal grafting of dopamine-containing neuronal cell suspensions: Effects of mixing with target or non-target cells. *Brain Res.* **389**: 77–84.
16. Burek, M. J., and R. W. Oppenheim. 1996. Programmed cell death in the developing nervous system. *Brain Pathol.* **6**: 427–446.
17. Caldero, J., D. Prevette, X. Mei, R. A. Oakley, L. Li, C. Milligan, L. Houenou, M. Burek, and R. W. Oppenheim. 1998. Peripheral target regulation of the development and survival of spinal sensory and motor neurons in the chick embryo. *J. Neurosci.* **18**: 356–370.
18. Chinnaiyan, A. M., K. O'Rourke, B. R. Lane, and V. M. Dixit. 1997. Interaction of CED-4 with CED-3 and CED-9: A molecular framework for cell death. *Science* **275**: 1122–1126.
19. Chinnaiyan, A. M., K. Orth, K. O'Rourke, H. Duan, G. G. Poirier, and V. M. Dixit. 1996. Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. *J. Biol. Chem.* **271**: 4573–4576.
20. Choi-Lundberg, D. L., Q. Lin, Y. N. Chang, Y. L. Chiang, C. M. Hay, H. Mohajeri, B. L. Davidson, and M. C. Bohn. 1997. Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* **275**: 838–841.
21. Clark, R. S., J. Chen, S. C. Watkins, P. M. Kochanek, M. Chen, R. A. Stetler, J. E. Loeffert, and S. H. Graham. 1997. Apoptosis-suppressor gene bcl-2 expression after traumatic brain injury in rats. *J. Neurosci.* **17**: 9172–9182.
22. Clarkson, E. D., W. M. Zawada, and C. R. Freed. 1995. GDNF reduces apoptosis in dopaminergic neurons *in vitro*. *Neuroreport* **7**: 145–149.
23. Clayton, G. H., T. J. Mahalik, and T. E. Finger. 1991. GAP-43 and 5B4-CAM immunoreactivity during the development of transplanted fetal mesencephalic neurons. *Exp. Neurol.* **114**: 1–10.
24. Cohen, G. M. 1997. Caspases: The executioners of apoptosis. *Biochem. J.* **326**: 1–16.
25. Cohen, G. M., X. M. Sun, R. T. Snowden, D. Dinsdale, and D. N. Skilleter. 1992. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J.* **286**: 331–334.
26. Collins, R. J., B. V. Harmon, G. C. Gobe, and J. F. Kerr. 1992. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int. J. Radiat. Biol.* **61**: 451–453.
27. Costantini, L. C., and A. Snyder-Keller. 1997. Co-transplantation of fetal lateral ganglionic eminence and ventral mesencephalon can augment function and development of intrastriatal transplants. *Exp. Neurol.* **145**: 214–227.
28. Cotman, C. W., E. R. Whittemore, J. A. Watt, A. J. Anderson, and D. T. Loo. 1994. Possible role of apoptosis in Alzheimer's disease. *Ann. N.Y. Acad. Sci.* **747**: 36–49.
29. Date, I., T. Shingo, T. Ohmoto, and D. F. Emerich. 1997. Long-term enhanced chromaffin cell survival and behavioral recovery in hemiparkinsonian rats with co-grafted polymer-encapsulated human NGF-secreting cells. *Exp. Neurol.* **147**: 10–17.
30. Date, I., Y. Yoshimoto, Y. Gohda, T. Furuta, S. Asari, and T. Ohmoto. 1993. Long-term effects of cogafts of pretransected peripheral nerve with adrenal medulla in animal models of Parkinson's disease. *Neurosurgery* **33**: 685–690.
31. Deckwerth, T. L., and E. Johnson, Jr. 1993. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J. Cell Biol.* **123**: 1207–1222.
32. Deshmukh, M., and E. Johnson, Jr. 1997. Programmed cell death in neurons: Focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. *Mol. Pharmacol.* **51**: 897–906.

33. Deshmukh, M., J. Vasilakos, T. L. Deckwerth, P. A. Lampe, B. D. Shivers, and E. Johnson, Jr. 1996. Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. *J. Cell Biol.* **135**: 1341–1354.
34. Dragunow, M., G. A. MacGibbon, P. Lawlor, N. Butterworth, B. Connor, C. Henderson, M. Walton, A. Woodgate, P. Hughes, and R. L. Faull. 1997. Apoptosis, neurotrophic factors and neurodegeneration. *Rev. Neurosci.* **8**: 223–265.
35. Du, Y., K. R. Bales, R. C. Dodel, E. Hamilton-Byrd, J. W. Horn, D. L. Czilli, L. K. Simmons, B. Ni, and S. M. Paul. 1997. Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. *Proc. Natl. Acad. Sci. USA* **94**: 11657–11662.
36. Du, Y., R. C. Dodel, K. R. Bales, R. Jemmerson, E. Hamilton-Byrd, and S. M. Paul. 1997. Involvement of a caspase-3-like cysteine protease in 1-methyl-4-phenylpyridinium-mediated apoptosis of cultured cerebellar granule neurons. *J. Neurochem.* **69**: 1382–1388.
37. Dunnett, S. B., and A. Bjorklund. 1997. Basic neural transplantation techniques. I. Dissociated cell suspension grafts of embryonic ventral mesencephalon in the adult rat brain. *Brain Res. Brain Res. Protoc.* **1**: 91–99.
38. Edwards, S. N., A. E. Buckmaster, and A. M. Tolkovsky. 1991. The death programme in cultured sympathetic neurones can be suppressed at the posttranslational level by nerve growth factor, cyclic AMP, and depolarization. *J. Neurochem.* **57**: 2140–2143.
39. Ellis, H. M., and H. R. Horvitz. 1986. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**: 817–829.
40. Engele, J., and B. Franke. 1996. Effects of glial cell line-derived neurotrophic factor (GDNF) on dopaminergic neurons require concurrent activation of cAMP-dependent signaling pathways. *Cell Tissue Res.* **286**: 235–240.
41. Friden, P. M., L. R. Walus, P. Watson, S. R. Doctrow, J. W. Kozarich, C. Backman, H. Bergman, B. Hoffer, F. Bloom, and A. C. Granholm. 1993. Blood-brain barrier penetration and *in vivo* activity of an NGF conjugate. *Science* **259**: 373–377.
42. Friedlander, R. M., V. Gagliardini, H. Hara, K. B. Fink, W. Li, G. MacDonald, M. C. Fishman, A. H. Greenberg, M. A. Moskowitz, and J. Yuan. 1997. Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. *J. Exp. Med.* **185**: 933–940.
43. Friel, L. A., B. Blanchard, T. J. Collier, J. D. Elsworth, J. R. Taylor, R. H. Roth, D. E. Redmond, Jr., and J. R. Sladek, Jr. 1998. Effects of the calcium channel blocker nimodipine on the survival of intrastriatal ventral mesencephalic grafts *in vitro* and *in vivo*. *Am. Soc. Transplant. Abstr.* **4:0R 48**.
44. Frodl, E. M., N. Nakao, and P. Brundin. 1994. Lazaroids improve the survival of cultured rat embryonic mesencephalic neurones. *Neuroreport* **5**: 2393–2396.
45. Golstein, P. 1997. Controlling cell death. *Science* **275**: 1081–1082.
46. Granholm, A. C., C. Backman, F. Bloom, T. Ebendal, G. A. Gerhardt, B. Hoffer, L. Mackerlova, L. Olson, S. Soderstrom, L. R. Walus *et al.* 1994. NGF and anti-transferrin receptor antibody conjugate: Short- and long-term effects on survival of cholinergic neurons in intraocular septal transplants. *J. Pharmacol. Exp. Ther.* **268**: 448–459.
47. Granholm, A. C., J. L. Mott, K. Bowenkamp, S. Eken, S. Henry, B. J. Hoffer, P. A. Lapchak, M. R. Palmer, C. van Horne, and G. A. Gerhardt. 1997. Glial cell line-derived neurotrophic factor improves survival of ventral mesencephalic grafts to the 6-hydroxydopamine lesioned striatum. *Exp. Brain Res.* **116**: 29–38.
48. Grasbon-Frodl, E. M., A. Andersson, and P. Brundin. 1996. Lazaroid treatment prevents death of cultured rat embryonic mesencephalic neurons following glutathione depletion. *J. Neurochem.* **67**: 1653–1660.
49. Grasbon-Frodl, E. M., and P. Brundin. 1997. Mesencephalic neuron death induced by congeners of nitrogen monoxide is prevented by the lazard U-83836E. *Exp. Brain Res.* **113**: 138–143.
50. Grasbon-Frodl, E. M., N. Nakao, and P. Brundin. 1996. The lazard U-83836E improves the survival of rat embryonic mesencephalic tissue stored at 4°C and subsequently used for cultures or intracerebral transplantation. *Brain Res. Bull.* **39**: 341–347.
51. Hakem, R., A. Hakem, G. S. Duncan, J. T. Henderson, M. Woo, M. S. Soengas, A. Elia, J. L. de la Pompa, D. Kagi, W. Khoo, J. Potter, R. Yoshida, S. A. Kaufman, S. W. Lowe, J. M. Penninger, and T. W. Mak. 1998. Differential requirement for caspase 9 in apoptotic pathways *in vivo*. *Cell* **94**: 339–352.
52. Haque, N. S., C. J. LeBlanc, and O. Isacson. 1997. Differential dissection of the rat E16 ventral mesencephalon and survival and reinnervation of the 6-OHDA-lesioned striatum by a subset of aldehyde dehydrogenase-positive TH neurons. *Cell Transplant.* **6**: 239–248.
53. Hara, H., R. M. Friedlander, V. Gagliardini, C. Ayata, K. Fink, Z. Huang, M. Shimizu-Sasamata, J. Yuan, and M. A. Moskowitz. 1997. Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc. Natl. Acad. Sci. USA* **94**: 2007–2012.
54. Hartikka, J., M. Staufienbiel, and H. Lubbert. 1992. Cyclic AMP, but not basic FGF, increases the *in vitro* survival of mesencephalic dopaminergic neurons and protects them from MPP(+)-induced degeneration. *J. Neurosci. Res.* **32**: 190–201.
55. Haviv, R., L. Lindenboim, H. Li, J. Yuan, and R. Stein. 1997. Need for caspases in apoptosis of trophic factor-deprived PC12 cells. *J. Neurosci. Res.* **50**: 69–80.
56. Helt, C., G. Hoernig, L. Sanders, K. Giardina, G. Gerhardt, and A.-C. Granholm. 1998. Effects of caspase inhibitor BOS-ASP-CH₂F on fetal ventral mesencephalic transplants. *Soc. Neurosci. Abstr.* **24:0R 1056**.
57. Hengartner, M. O. 1997. Apoptosis. CED-4 is a stranger no more. *Nature* **388**: 714–715.
58. Hengartner, M. O., and H. R. Horvitz. 1994. Programmed cell death in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.* **4**: 581–586.
59. Herrup, K., H. Shojaeian-Zanjani, L. Panzini, K. Sunter, and J. Mariani. 1996. The numerical matching of source and target populations in the CNS: The inferior olive to Purkinje cell projection. *Brain Res. Dev. Brain Res.* **96**: 28–35.
60. Herzog, K. H., M. J. Chong, M. Kapsetaki, J. I. Morgan, and P. J. McKinnon. 1998. Requirement for atm in ionizing radiation-induced cell death in the developing central nervous system. *Science* **280**: 1089–1091.
61. Holm, K., and O. Isacson. 1999. Factors intrinsic to the neuron can induce and maintain an ability for neurite outgrowth: A role for bcl-2? *Trends Neurosci.*, in press.
62. Holm, K. H., Z. Boonman, P. Tandon, L. C. Costantini, T. W. Deacon, D. F. Chen, and O. Isacson. 1998. Specific graft-host synaptic connections and long distance axonal growth in fetal neurotransplantation to adult CNS, 223.6. *Soc. Neurosci. Abstr.* **24**: 557.

63. Houenou, L. J., R. W. Oppenheim, L. Li, A. C. Lo, and D. Prevette. 1996. Regulation of spinal motoneuron survival by GDNF during development and following injury. *Cell Tissue Res.* **286**: 219–223.
64. Hudson, J. L., P. Bickford, M. Johansson, B. J. Hoffer, and I. Stromberg. 1994. Target and neurotransmitter specificity of fetal central nervous system transplants: Importance for functional reinnervation. *J. Neurosci.* **14**: 283–290.
65. Isacson, O., and X. O. Breakefield. 1997. Benefits and risks of hosting animal cells in the human brain. *Nature Med.* **3**: 964–969.
66. Isacson, O., and T. Deacon. 1997. Neural transplantation studies reveal the brain's capacity for continuous reconstruction. *Trends Neurosci.* **20**: 477–482.
67. Isenmann, S., G. Stoll, M. Schroeter, S. Krajewski, J. C. Reed, and M. Bahr. 1998. Differential regulation of Bax, Bcl-2, and Bcl-X proteins in focal cortical ischemia in the rat. *Brain Pathol.* **8**: 49–62.
68. Iwahashi, H., Y. Eguchi, N. Yasuhara, T. Hanafusa, Y. Matsuzawa, and Y. Tsujimoto. 1997. Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. *Nature* **390**: 413–417.
69. Kaal, E. C., E. A. Joosten, and P. R. Bar. 1997. Prevention of apoptotic motoneuron death *in vitro* by neurotrophins and muscle extract. *Neurochem. Int.* **31**: 193–201.
70. Kerr, J. F., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**: 239–257.
71. Kluck, R. M., S. J. Martin, B. M. Hoffman, J. S. Zhou, D. R. Green, and D. D. Newmeyer. 1997. Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. *EMBO J.* **16**: 4639–4649.
72. Kostic, V., V. Jackson-Lewis, F. de Bilbao, M. Dubois-Dauphin, and S. Przedborski. 1997. Bcl-2: Prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* **277**: 559–562.
73. Krajewska, M., H. G. Wang, S. Krajewski, J. M. Zapata, A. Shabaik, R. Gascoyne, and J. C. Reed. 1997. Immunohistochemical analysis of *in vivo* patterns of expression of CPP32 (caspase-3), a cell death protease. *Cancer Res.* **57**: 1605–1613.
74. Krobert, K., I. Lopez-Colberg, and L. A. Cunningham. 1997. Astrocytes promote or impair the survival and function of embryonic ventral mesencephalon co-grafts: Effects of astrocyte age and expression of recombinant brain-derived neurotrophic factor. *Exp. Neurol.* **145**: 511–523.
75. Kuida, K., T. F. Haydar, C. Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M. S. Su, P. Rakic, and R. A. Flavell. 1998. Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. *Cell* **94**: 325–327.
76. Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S. Su, and R. A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* **267**: 2000–2003.
77. Kuida, K., T. S. Zheng, S. Na, C. Kuan, D. Yang, H. Karasuyama, P. Rakic, and R. A. Flavell. 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**: 368–372.
78. Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. 1997. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**: 479–489.
79. Li, Y., M. Chopp, C. Powers, and N. Jiang. 1997. Apoptosis and protein expression after focal cerebral ischemia in rat. *Brain Res.* **765**: 301–312.
80. Lithgow, T., R. van Driel, J. F. Bertram, and A. Strasser. 1994. The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth Differ.* **5**: 411–417.
81. Lo, A. C., L. J. Houenou, and R. W. Oppenheim. 1995. Apoptosis in the nervous system: Morphological features, methods, pathology, and prevention. *Arch. Histol. Cytol.* **58**: 139–149.
82. Lockshin, R. A., and J. Beaulaton. 1974. Programmed cell death. *Life Sci.* **15**: 1549–1565.
83. Loddick, S. A., A. MacKenzie, and N. J. Rothwell. 1996. An ICE inhibitor, zVAD-DCB, attenuates ischaemic brain damage in the rat. *Neuroreport* **7**: 1465–1468.
84. Lucca, E., N. Angeretti, and G. Forloni. 1997. Influence of cell culture conditions on the protective effect of antioxidants against beta-amyloid toxicity: Studies with lazaroids. *Brain Res.* **764**: 293–298.
85. Macaya, A., F. Munell, R. M. Gubits, and R. E. Burke. 1994. Apoptosis in substantia nigra following developmental striatal excitotoxic injury. *Proc. Natl. Acad. Sci. USA* **91**: 8117–8121.
86. Mahalik, T. J., W. E. Hahn, G. H. Clayton, and G. P. Owens. 1994. Programmed cell death in developing grafts of fetal substantia nigra. *Exp. Neurol.* **129**: 27–36.
87. Margolin, N., S. A. Raybuck, K. P. Wilson, W. Chen, T. Fox, Y. Gu, and D. J. Livingston. 1997. Substrate and inhibitor specificity of interleukin-1 beta-converting enzyme and related caspases. *J. Biol. Chem.* **272**: 7223–7228.
88. Marti, M. J., C. J. James, T. F. W. J. K. Oo, and R. E. Burke. 1997. Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra. *J. Neurosci.* **17**: 2030–2039.
89. Martinou, J. C., M. Dubois-Dauphin, J. K. Staple, I. Rodriguez, H. Frankowski, M. Missotten, P. Albertini, D. Talabot, S. Catsicas, C. Pietra *et al.* 1994. Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* **13**: 1017–1030.
90. Mayer, E., J. W. Fawcett, and S. B. Dunnett. 1993. Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons—II. Effects on nigral transplants *in vivo*. *Neuroscience* **56**: 389–398.
91. Mentz, F., M. D. Mossalayi, F. Ouaz, and P. Debre. 1995. Involvement of cAMP in CD3 T cell receptor complex- and CD2-mediated apoptosis of human thymocytes. *Eur. J. Immunol.* **25**: 1798–1801.
92. Michaelidis, T. M., M. Sendtner, J. D. Cooper, M. S. Airaksinen, B. Holtmann, M. Meyer, and H. Thoenen. 1996. Inactivation of bcl-2 results in progressive degeneration of motoneurons, sympathetic and sensory neurons during early postnatal development. *Neuron* **17**: 75–89.
93. Michel, P. P., and Y. Agid. 1996. Chronic activation of the cyclic AMP signaling pathway promotes development and long-term survival of mesencephalic dopaminergic neurons. *J. Neurochem.* **67**: 1633–1642.
94. Mochizuki, H., K. Goto, H. Mori, and Y. Mizuno. 1996. Histochemical detection of apoptosis in Parkinson's disease. *J. Neurol. Sci.* **137**: 120–123.
95. Mochizuki, H., H. Mori, and Y. Mizuno. 1997. Apoptosis in neurodegenerative disorders. *J. Neural Transm. Suppl.* **50**: 125–140.
96. Nagy, Z. S., and M. M. Esiri. 1997. Apoptosis-related protein expression in the hippocampus in Alzheimer's disease. *Neurobiol. Aging* **18**: 565–571.

97. Nakao, N., E. M. Frodl, W. M. Duan, H. Widner, and P. Brundin. 1994. Lazaroids improve the survival of grafted rat embryonic dopamine neurons. *Proc. Natl. Acad. Sci. USA* **91**: 12408-12412.
98. Nakao, N., E. M. Frodl, H. Widner, E. Carlson, F. A. Eggerding, C. J. Epstein, and P. Brundin. 1995. Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. *Nat. Med.* **1**: 226-231.
99. Namura, S., J. Zhu, K. Fink, M. Endres, A. Srinivasan, K. J. Tomaselli, J. Yuan, and M. A. Moskowitz. 1998. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J. Neurosci.* **18**: 3659-3668.
100. Nicholson, D. W., and N. A. Thornberry. 1997. Caspases: Killer proteases. *Trends Biochem. Sci.* **22**: 299-306.
101. Nikkah, G., M. G. Cunningham, A. Jodicke, U. Knappe, and A. Bjorklund. 1994. Improved graft survival and striatal reinnervation using a microtransplantation approach in the rat Parkinson model. *Brain Res.* **633**: 133-143.
102. Nikkah, G., M. Olsson, J. Eberhard, C. Bentlage, M. G. Cunningham, and A. Bjorklund. 1994. A microtransplantation approach for cell suspension grafting in the rat Parkinson model: A detailed account of the methodology. *Neuroscience* **63**: 57-72.
103. Oberhammer, R. W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**: 453-501.
104. Oo, T. F., C. Henschliffe, and R. E. Burke. 1995. Apoptosis in substantia nigra following developmental hypoxic-ischemic injury. *Neuroscience* **69**: 893-901.
105. Oppenheim, R. W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**: 453-501.
106. Othberg, A., M. Keep, P. Brundin, and O. Lindvall. 1997. Tirilazad mesylate improves survival of rat and human embryonic mesencephalic neurons *in vitro*. *Exp. Neurol.* **147**: 498-502.
107. Perlow, M. J., W. J. Freed, B. J. Hoffer, A. Seiger, L. Olson, and R. J. Wyatt. 1979. Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. *Science* **204**: 643-647.
108. Portera-Cailliau, C., J. C. Hedreen, D. L. Price, and V. E. Koliatsos. 1995. Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. *J. Neurosci.* **15**: 3775-3787.
109. Portera-Cailliau, C., D. L. Price, and L. J. Martin. 1997. Non-NMDA and NMDA receptor-mediated excitotoxic neuronal deaths in adult brain are morphologically distinct: Further evidence for an apoptosis-necrosis continuum. *J. Comp. Neurol.* **378**: 88-104.
110. Rabizadeh, S., D. J. LaCount, P. D. Friesen, and D. E. Bredesen. 1993. Expression of the baculovirus p35 gene inhibits mammalian neural cell death. *J. Neurochem.* **61**: 2318-2321.
111. Reed, J. C. 1997. Double identity for proteins of the Bcl-2 family. *Nature* **387**: 773-776.
112. Rosenblad, C., A. Martinez-Serrano, and A. Bjorklund. 1996. Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience* **75**: 979-985.
113. Rosenblad, C., A. Martinez-Serrano, and A. Bjorklund. 1998. Intrastriatal glial cell line-derived neurotrophic factor promotes sprouting of spared nigrostriatal dopaminergic afferents and induces recovery of function in a rat model of Parkinson's disease. *Neuroscience* **82**: 129-137.
114. Rosse, T., R. Olivier, L. Monney, M. Rager, S. Conus, I. Fellay, B. Jansen, and C. Borner. 1998. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature* **391**: 496-499.
115. Salvesen, G. S., and V. M. Dixit. 1997. Caspases: Intracellular signaling by proteolysis. *Cell* **91**: 443-446.
116. Sautter, J., J. L. Tseng, D. Bragaglia, P. Aebischer, C. Spenger, R. W. Seiler, H. R. Widmer, and A. D. Zurn. 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.
117. Schielke, G. P., G. Y. Yang, B. D. Shivers, and A. L. Betz. 1998. Reduced ischemic brain injury in interleukin-1 beta converting enzyme-deficient mice. *J. Cereb. Blood Flow Metab.* **18**: 180-185.
118. Schulz, J. B., S. Beinroth, M. Weller, U. Wullner, and T. Klockgether. 1998. Endonucleolytic DNA fragmentation is not required for apoptosis of cultured rat cerebellar granule neurons. *Neurosci. Lett.* **245**: 9-12.
119. Shimizu, S., Y. Eguchi, W. Kamiike, Y. Itoh, J. Hasegawa, K. Yamabe, Y. Otsuki, H. Matsuda, and Y. Tsujimoto. 1996. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. *Cancer Res.* **56**: 2161-2166.
120. Sinclair, S. R., C. N. Svendsen, E. M. Torres, D. Martin, J. W. Fawcett, and S. B. Dunnett. 1996. GDNF enhances dopaminergic cell survival and fibre outgrowth in embryonic nigral grafts. *Neuroreport* **7**: 2547-2552.
121. Sloviter, R. S., E. Dean, A. L. Sollas, and J. H. Goodman. 1996. Apoptosis and necrosis induced in different hippocampal neuron populations by repetitive perforant path stimulation in the rat. *J. Comp. Neurol.* **366**: 516-533.
122. Srinivasula, S. M., M. Ahmad, T. Fernandes-Alnemri, G. Litwack, and E. S. Alnemri. 1996. Molecular ordering of the Fas-apoptotic pathway: The Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proc. Natl. Acad. Sci. USA* **93**: 14486-14491.
123. Sulston, J. E., and H. R. Horvitz. 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56**: 110-156.
124. Takayama, H., J. Ray, H. K. Raymon, A. Baird, J. Hogg, L. J. Fisher, and F. H. Gage. 1995. Basic fibroblast growth factor increases dopaminergic graft survival and function in a rat model of Parkinson's disease. *Nat. Med.* **1**: 53-58.
125. Tanaka, H., and L. T. Landmesser. 1986. Cell death of lumbosacral motoneurons in chick, quail, and chick-quail chimera embryos: A test of the quantitative matching hypothesis of neuronal cell death. *J. Neurosci.* **6**: 2889-2899.
126. Tews, D. S., H. H. Goebel, and H. M. Meinck. 1997. DNA-fragmentation and apoptosis-related proteins of muscle cells in motor neuron disorders. *Acta Neurol. Scand.* **96**: 380-386.
127. Tomei, L. D., J. P. Shapiro, and F. O. Cope. 1993. Apoptosis in C3H/10T1/2 mouse embryonic cells: Evidence for internucleosomal DNA modification in the absence of double-strand cleavage. *Proc. Natl. Acad. Sci. USA* **90**: 853-857.
128. Troost, D., J. Aten, F. Morsink, and J. M. de Jong. 1995. Apoptosis in ALS is not restricted to motoneurons: Bcl-2 expression is increased in post-central cortex, adjacent to the affected motor cortex. *J. Neurol. Sci.* **129**: 79-80.
129. Troost, D., J. Aten, F. Morsink, and J. M. de Jong. 1995. Apoptosis in amyotrophic lateral sclerosis is not restricted to motor neurons. Bcl-2 expression is increased in unaffected post-central gyrus. *Neuropathol. Appl. Neurobiol.* **21**: 498-504.
130. Villa, P., S. H. Kaufmann, and W. C. Earnshaw. 1997. Caspases and caspase inhibitors. *Trends Biochem. Sci.* **22**: 388-393.

131. Wang, S., M. Miura, Y. Jung, H. Zhu, V. Gagliardini, L. Shi, A. H. Greenberg, and J. Yuan. 1996. Identification and characterization of Ich-3, a member of the interleukin-1 β converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *J. Biol. Chem.* **271**: 20580–20587.
132. Wang, S., M. Miura, Y. K. Jung, H. Zhu, E. Li, and J. Yuan. 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell* **92**: 501–509.
133. Woo, M., R. Hakem, M. S. Soengas, G. S. Duncan, A. Shahinian, D. Kagi, A. Hakem, M. McCurrach, W. Khoo, S. A. Kaufman, G. Senaldi, T. Howard, S. W. Lowe, and T. W. Mak. 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev.* **12**: 806–819.
134. Wyllie, A. H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**: 555–556.
135. Wyllie, A. H. 1997. Apoptosis: An overview. *Br. Med. Bull.* **53**: 451–465.
136. Xue, D., S. Shaham, and H. R. Horvitz. 1996. The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes Dev.* **10**: 1073–1083.
137. Yakovlev, A. G., S. M. Knoblach, L. Fan, G. B. Fox, R. Goodnight, and A. I. Faden. 1997. Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. *J. Neurosci.* **17**: 7415–7424.
138. Yang, D. D., C. Y. Kuan, A. J. Whitmarsh, M. Rincon, T. S. Zheng, R. J. Davis, P. Rakic, and R. A. Flavell. 1997. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* **389**: 865–870.
139. Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: Release of cytochrome *c* from mitochondria blocked. *Science* **275**: 1129–1132.
140. Yang, J. C., and G. A. Cortopassi. 1998. Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome *c*. *Free Radical Biol. Med.* **24**: 624–631.
141. Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β -converting enzyme. *Cell* **75**: 641–652.
142. Yurek, D. M., T. J. Collier, and J. R. Sladek, Jr. 1990. Embryonic mesencephalic and striatal co-grafts: Development of grafted dopamine neurons and functional recovery. *Exp. Neurol.* **109**: 191–199.
143. Yurek, D. M., W. Lu, S. Hipkens, and S. J. Wiegand. 1996. BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons. *Exp. Neurol.* **137**: 105–118.
144. Zawada, W. M., D. L. Kirschman, J. J. Cohen, K. A. Heidenreich, and C. R. Freed. 1996. Growth factors rescue embryonic dopamine neurons from programmed cell death. *Exp. Neurol.* **140**: 60–67.
145. Zawada, W. M., D. J. Zastrow, E. D. Clarkson, F. S. Adams, K. P. Bell, and C. R. Freed. 1998. Growth factors improve immediate survival of embryonic dopamine neurons after transplantation into rats. *Brain Res.* **786**: 96–103.
146. Zou, H., W. J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell* **90**: 405–413.

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CHAPTER 8

Dopamine Neuron Grafts: Development and Molecular Biology

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Development of Dopaminergic Neurons

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CHAPTER 8

Dopamine Neuron Grafts: Development and Molecular Biology

Lauren C. Costantini and Ole Isacson

The introduction of grafting dopaminergic (DA) neurons into rodent host brains illustrated the potential of this technique for both experimental and clinical applications.^{1,2} Basic research utilizing these transplants has revealed information regarding development and connectivity of CNS neurons, while studies aimed at therapeutic strategies for Parkinson's disease (PD) have shown the potential of this procedure for "biological replacements", reconstructing the circuits within a degenerated brain.³ The current experiments involving fetal neural grafts provide information about mechanisms and processes involved in phenotypic DA neuron development, and serve as a guide to alternative cell sources for clinical neural transplantation.

Establishment of Surviving Dopamine Neuron Grafts

Studies have demonstrated that embryonic day (E) 14 for rodent tissue⁴ and 6.5-9 weeks post-conception for human tissue⁵ are the optimal ages of ventral mesencephalic (VM) donor tissue for DA neuronal survival and functional effects when transplanted into the DA-denervated striatum. The minimum number of surviving transplanted DA neurons required for functional effects to be revealed in animal models is approximately 100-200.⁶ Since only 10% of the transplanted VM cells are DA, and only 1-10% of these DA neurons survive,⁷⁻⁹ as many as 10-15 fetal VM per patient may be required for sufficient survival and reinnervation.¹⁰ Strategies to improve the survival of the DA neurons within these grafts are being considered, including treatment with growth factors, antioxidants, cotransplantation, and modified implantation procedures.¹¹⁻¹⁵

Enhancing Cell Survival with Growth Factors and Target Tissue

Methods to sustain the development and function of embryonic VM DA cells after transplantation into DA-depleted striatum are currently under investigation. Elements which are crucial for the maturation and connectivity of neurons during normal development of the brain may also play a role in the development and integration of grafted embryonic tissue. Based upon the observations that several neurotrophic factors affect the innervation of targets and the survival of neurons during development, administration of these factors along with transplanted "developing" fetal DA neurons has been examined. Among the trophic factors that can enhance development of DA neurons after grafting into rodent are brain-derived neurotrophic factor,^{11,16} basic fibroblast growth factor,¹⁷ and glial cell line-derived neurotrophic factor.¹⁸⁻²⁰ Technical strategies have included exposure of the fetal cells to neurotrophic factors prior to transplantation^{16,17,21} or by administration after transplantation.^{11,18-20}

The target-derived neurotrophic factor hypothesis predicts the presence of DA-trophic activity in striatal tissue, and several studies support this hypothesis. Based on in vitro and in vivo observations of the enhancing effects of striatal tissue on nigral DA cell development and survival,²²⁻²⁴ we have demonstrated that inclusion of embryonic striatal cells,¹⁴ specifically from the lateral ganglionic eminence, enhanced the survival of DA neurons in VM transplants, and also required the transplantation of fewer nigral cells to produce a marked behavioral effect.¹³

Protecting Transplanted Cells with Antioxidants

The poor survival of DA neurons after transplantation may be caused by damage to the VM tissue during preparation, or via cell death after transplantation such as that seen during development.²⁵ A large proportion of DA cell death occurs during the first two weeks after transplantation due to these factors, and/or due to suboptimal conditions in the host brain during the early phases after transplantation.²⁶ One hypothesis for the mechanism of this acute cell death is formation of free radicals during the process of dissociation. This is supported by evidence that VM from transgenic mice overexpressing Cu/Zn superoxide dismutase, the major free radical scavenging enzyme, produces transplants with four-fold greater survival of DA neurons and more extensive functional recovery.¹² Treatment of transplanted cells with antioxidants increased the yield of surviving DA neurons, which correlated with an earlier onset of graft-induced functional effects.^{12,27} These findings have recently been taken into the clinical setting: One inhibitor of lipid peroxidation, tirilazad, has been included in the solutions for storage and preparation of donor tissue in the clinic; in addition, the patients are treated with this lazaroid for 72 hours after transplantation.²⁸

Utilizing Medial VM for Dopamine Grafts

Since several studies investigating optimal donor age have concluded that E13/14 (rat) yields most successful transplants,²⁹ other issues seem critical. The selection of the most appropriate region for dissection is therefore of great importance. Presently, the embryonic dissections utilized for transplantation typically contain 2-10% DA neurons while the remainder are of other neuronal phenotypes, such as GABAergic.^{7,29} Other neurons within a developing transplant may hinder the survival and growth of the small proportion of DA neurons, since neurons compete for the limited supplies of trophic support for survival during development.³⁰ Therefore, obtaining an initially enriched suspension of DA neurons may enhance the development of the transplants.

Cells generated from the ventricular zone at E11 migrate ventrally and then move laterally to form the substantia nigra pars compacta (SNc) and ventral tegmental area. Tyrosine hydroxylase (TH; a synthetic enzyme involved in production of DA) immunoreactivity can be detected at E12.5 in the medio-basal region of the mesencephalon. By E14, TH⁺ cells are located laterally along the ventral surface to form the primordia of the SN.^{31,32} In order to enhance the relative proportion of DA neurons, we compared numbers of DA neurons from tissue dissected from the medial portion of the VM versus the lateral VM (Fig. 8.1). A higher proportion of TH⁺ neurons were observed in primary cultures of medial VM when compared with lateral or whole VM.³³ Evaluation of E16 solid VM transplants revealed a larger number of surviving TH⁺ cells in grafts from medial region of VM compared to lateral VM.³⁴

This higher proportion of DA neurons in medial VM can be attributed to the presence of a higher number of DA neurons in the medial region of the VM at E14. Alternatively, the higher proportion of TH⁺ neurons in medial VM may represent enhanced survival of DA neurons in medial VM. Dopamine neurons are responsive to a variety of trophic factors such as GDNF,³⁵ bFGF,³⁶ TGF β ,³⁷ and BDNF.³⁸ A more pure population of DA neurons present

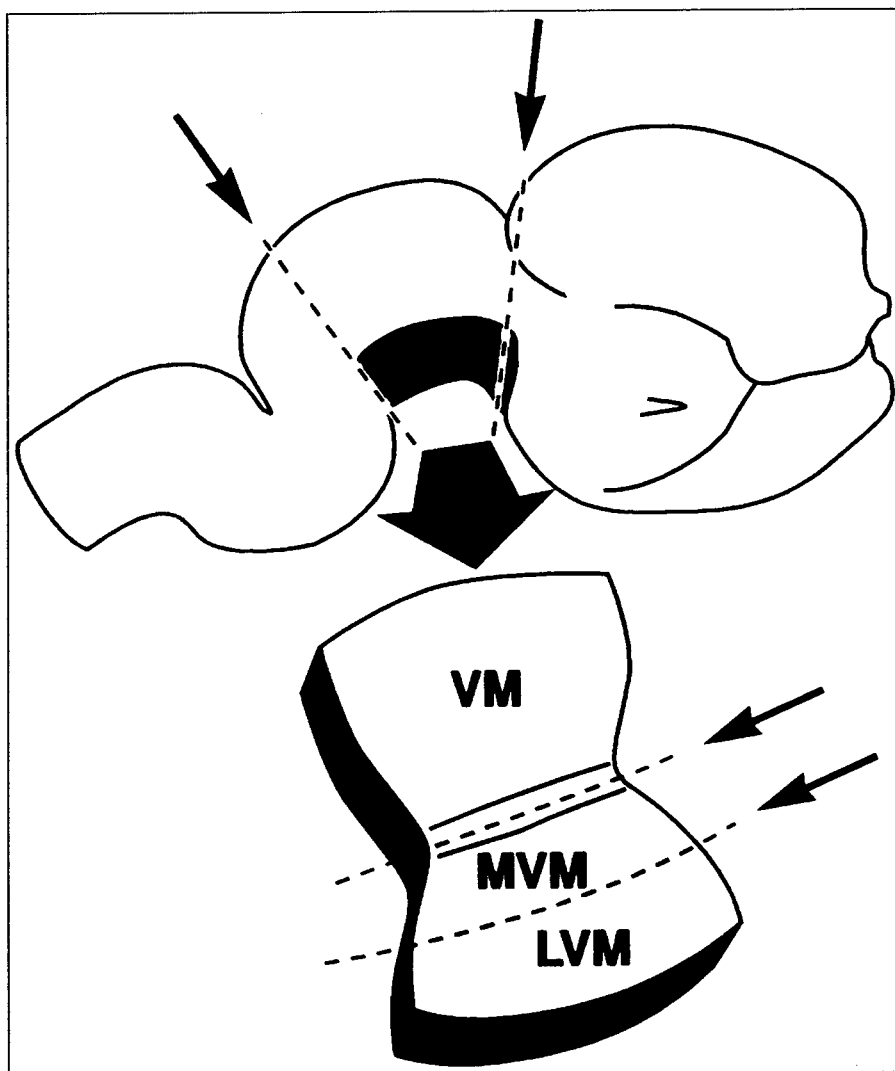


Fig 8.1. Dissection of E14 medial and lateral ventral mesencephalon (VM). Dashed lines through cartoon of entire brain show dissection of VM; dashed lines in enlarged VM region show differential dissection of medial VM (MVM) and lateral VM (LVM). Reprinted with permission from *Neuroreport* 1997; 8:2253-2257, ©Rapid Sciences.

in medial VM may yield less competition from other cell types for trophic factors, and thus enhance the survival of the relatively small proportion of DA neurons present, since receptors for some growth factors have been detected throughout the SN.³⁹ If this is the case, the development of DA neurons after transplantation would be hindered by the presence of other cells. This hypothesis is consistent with findings in nigro-striatal cotransplantation studies: When equal numbers of nigral and striatal cells are cotransplanted, enhanced DA cell survival is obtained.¹³ However, low DA cell survival results when a relatively low number

of embryonic striatal cells are cotransplanted, possibly due to competition among the DA neurons for the limited amount of trophic support provided by the cotransplanted striatal cells.¹³

In addition to a higher proportion of TH⁺ neurons in medial dissections of VM, we also found that a higher proportion of these DA neurons expressed aldehyde dehydrogenase (AHD), a retinoic acid-generating enzyme.^{33,34} Dopamine neurons of the midbrain can be divided into subpopulations based upon expression of neuropeptides and enzymes,⁴⁰ and AHD is expressed in this subpopulation of DA neurons early in development,^{41,42} shortly after the appearance of TH.^{32,43} The retinoid-synthesizing actions of AHD may play a role in development of DA neurons: Mice deficient in the nuclear receptor Nurr-1, which promotes signaling through heterodimerization with a 9-cis-retinoic acid receptor,⁴⁴ failed to produce midbrain DA neurons.⁴² In addition, there is a prevalence of AHD-containing neurons among the SNc DA population which project to the dorsal-lateral and rostral regions of the striatum, previously shown to be regions involved in functional recovery after grafting of VM tissue (see below: Regional and phenotypic specification of dopamine neurons).⁴⁵

Inducing Stem Cells and Other Progenitor Cells

The quest for an unlimited cell source for DA transplantation has yielded not only a wide array of potential cells for this use, but also has provided information on the development of DA phenotypes. Totipotent stem cells, adrenal medulla and peripheral nerve cotransplants, carotid body cell aggregates, testis-derived sertoli cells, and cells obtained from transgenic animals are currently being analyzed both for future clinical use and for probing developmental questions.

Pluripotent cells, used both in studies of neural differentiation and as future therapeutic tools, consist of growth factor-expanded neural progenitors, immortalized cell lines, embryonal carcinoma cells, and embryonic stem cells. Growth factor-expanded cells have been transplanted into the adult brain, forming small grafts which exhibit some migration of cells away from the implantation site.⁴⁶⁻⁴⁸ After growth-factor expanded stem cells isolated from developing CS were transplanted into adult striatum, Svendsen et al observed low cell survival within small grafts, with few differentiated cells expressing neuronal markers.⁴⁷ Immortalized cell lines also exhibit a capacity to differentiate into a number of region-specific neuronal morphologies when transplanted into brain (for review, see ref. 49). The transplantation of these cells into neonatal brain resulted in differentiation into neurons and glia with region-specific morphology.⁵⁰⁻⁵³ However, when transplanted into adult brain, Lundberg et al observed that the plasticity of immortalized cells (generated from embryonic striatum or hippocampus) was more restricted: A majority of these cells differentiated into glia in the adult environment.⁵⁴ Embryonic carcinoma cell lines can differentiate into terminal, nonproliferating neural phenotypes after pretreatment with retinoic acid and subsequent intracerebral transplantation,⁵⁵⁻⁵⁸ and in some cases produce TH⁺ cells.^{56,57} Kleppner et al⁵⁵ observed differentiated neuron-like cells which exhibited different patterns of innervation into the host brain depending on the region of the mouse brain in which they were implanted.

Our laboratory has utilized transplantation as a means to investigate DA neuron development: We tested the potential of blastocyst-derived embryonic stem (ES) cells to differentiate into DA neurons.⁵⁹ These totipotent cells were transplanted into adult mouse striatum and adult DA-lesioned striatum. The grafts developed large numbers of cells exhibiting neuronal morphology and immunoreactivity for neurofilament, neuron specific enolase, TH, and 5-hydroxytryptamine (Fig. 8.2). Though graft size and histology were variable, typical grafts of 5-10 mm³ contained 10-20,000 TH⁺ neurons, whereas dopamine- β -hydroxylase⁺ cells were rare. Most grafts also included non-neuronal regions,

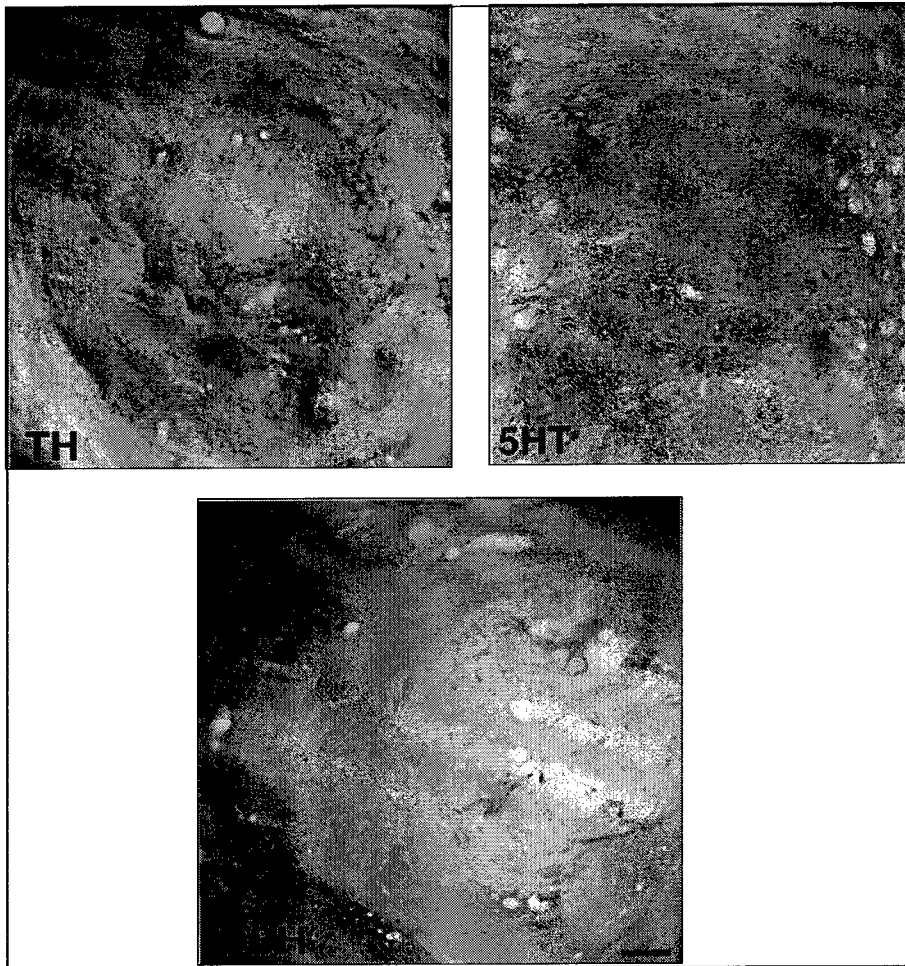


Fig. 8.2. Phenotype of blastocyst-derived embryonic stem cells after transplantation to rat striatum. The grafts develop large numbers of cells exhibiting neuronal morphology and immunoreactivity for TH and 5-hydroxytryptamine (5HT). Dopamine- β -hydroxylase⁺ cells are rare. Scale bar, 200 μ m. Reprinted with permission from *Experimental Neurology* 1998; 149:28-41, © Academic Press.

immunoreactive for glial fibrillary acidic protein. Both monoaminergic neuronal cell types extended axons into the graft and into the surrounding host brain: TH⁺ graft axons grew preferentially into gray matter of the DA-denervated rat striatum, as is typical of endogenous striatal DA innervation. This specific innervation pattern has also been observed for DA axons growing into the host striatum from fetal ventral mesencephalic grafts, but is not exhibited by non-DA fibers from the same grafts,^{60,61} nor by axons from fetal cortical or striatal grafts to adult striatum.⁶⁰ In contrast, 5-HT⁺ axons from ES cell grafts extended equally into white and gray matter regions of the host striatum. Thus, the difference between

growth patterns of TH⁺ and 5-HT⁺ axons reflects characteristics that are typical of these mature CNS cell types.

In contrast to results with other cell lines, the ES cells in our study did not seem to be dependent upon the site of transplantation for differentiation into neurons: We transplanted ES cells into mouse kidney capsule to determine the influence that brain-specific environment may have on the differentiation of these totipotent cells. Kidney capsule grafts also developed large numbers of cells exhibiting neuronal morphology and immunoreactivity for neurofilament, neuron specific enolase, TH, 5-HT, and glial fibrillary acidic protein. Neural induction regardless of transplant site in our paradigm is consistent with recent evidence suggesting that neuralization is a default pathway, and occurs spontaneously if pregastrula cells do not receive other inducing signals to form epidermal, mesodermal, or endodermal cells.⁶² This was first suggested by experiments showing that cells of the early gastrula ectodermal animal cap, that normally develop into epidermal tissue, all form neural tissue if dissociated.⁶³ Bone morphogenic protein (BMP-4) and activin have been implicated as the major inducers of epidermal differentiation during gastrulation. Ectopic application of BMP-4 is sufficient to induce epiderm formation in dissociated animal pole cap cells,⁶⁴ and homozygous knockout mice lacking functional BMP receptor (BMPRI) die in gastrulation,⁶⁵ a time when epidermis would otherwise form. Also, antagonists of BMP-4 or activin signaling, such as noggin, follistatin, and chordin, which are produced in the Spemann organizer region, can induce the ectopic formation of neural tissue.⁶⁶⁻⁶⁸ Thus, any manipulation that disrupts these epidermis-inducing signals results in neural differentiation. In our experiments, transplanting cells that have been dissociated and expanded at the pre-gastrula stage may disrupt the localized cell-cell communications which otherwise inhibit neuralization. Alternatively, the kidney has been shown to contain many neurotrophic factors and also to enhance the development of TH⁺ neurons when cografted with VM.⁶⁹ The lack of kidney structure in GDNF knockout mice also suggests the presence of dopaminotrophic factors present in the kidney which may have induced the totipotent ES cells to differentiate along the DA phenotype.⁷⁰

These findings demonstrate that transplantation to the brain or kidney capsule results in a significant fraction of totipotent ES cells developing into putative DA or serotonergic neurons and that, when transplanted to the brain, these neurons are capable of innervating the adult host striatum.

Other Cell Sources

Recent explorations of alternative cell sources have also contributed to the list of cells utilized for the study of DA neuron transplantation. Donor tissue from other species is an attractive alternative to human fetal tissue, particularly from a donor species that breeds in large litters, such as pig. The porcine DA system contains cell groups resembling A8, A9, and A10 of the rat, and differentiate into the homologous cell groups of human.⁷¹ In pig embryos of 28 days, cells of the VM are committed DA neurons expressing TH, yet have not extended processes.⁷¹ When a suspension of fetal pig VM was transplanted into the striatum of immunosuppressed DA-lesioned rats, these animals showed significant reduction in amphetamine-induced rotation, whereas animals not immunosuppressed showed transient behavioral recovery.⁶ Behavioral recovery was reversed after animals were removed from cyclosporin, suggesting that the grafts were rejected upon cessation of cyclosporin A treatment.⁷² TH⁺ neurons were observed within grafts in animals displaying a high degree of rotational correction. In addition to its obvious potential for use in the clinical setting (see below: "Recent clinical progress and developments"), xenotransplantation also provides a tool to analyze the development of various components of transplanted cells, to be discussed below (see below: "The inhibitory environment of adult brain").

Recently, somatic cell cloning, specifically the production of transgenic bovine embryos, has produced an alternative supply of embryonic VM tissue; these VM cells have improved motor function when transplanted into immunosuppressed parkinsonian rats.⁷³

In order to circumvent the need for immunosuppression after transplantation, several cell sources have been introduced. Testis-derived sertoli cells have been shown to secrete trophic, tropic, and immunosuppressive factors; culturing these cells with embryonic neurons increased DA neuron survival and outgrowth.⁷⁴ Transplantation of these cells produced behavioral recovery in hemiparkinsonian rats, with an increase in TH⁺ immunoreactivity in the zone around the transplant.⁷⁴ A two month survival period of xenotransplanted porcine sertoli cells into rat brain in the absence of systemic immunosuppression indicates production of sufficient local immunosuppression at the site of transplantation, and may be an alternative method for protecting xenotransplanted cells.⁷⁵ Transplantation of chromaffin-like carotid body glomus cells into DA-denervated rat striatum developed into clusters of TH⁺ cells with neuronal morphology which extended fibers out into the host striatum, and produced behavioral recovery in turning behavior and sensorimotor orientation three months post-transplantation.⁷⁶ And finally, revisiting the adrenal medulla grafts, animal studies utilizing co-grafts of adrenal medulla and peripheral nerve have indicated that this procedure can overcome the major problems encountered with adrenal grafts alone, such as limited survival and transient behavioral effects.⁷⁷ Attachment of human fetal VM cells to microcarriers (Cytodex) allowed xenotransplantation into rat host without the need for immunosuppression; however, there was no evidence of TH fiber outgrowth into host striatum, and no functional results were reported.⁷⁸

Regulation of Axonal Outgrowth from Dopamine Grafts

The ability of fetal neurons to be placed into an ectopic region of an adult brain, survive, and extend neurites within this region is remarkable. The functional effects of VM transplants into DA-depleted striatum is often correlated with degree of striatal reinnervation.^{4, 13} However, there is some limitation in the ability of the transplanted neurons to extend neurites in the mature brain. Even though the graft-induced elevations in tissue DA concentrations are substantial,⁷⁹ values taken distant from the graft suggest that reinnervation of the whole striatum does not occur. The hypothesis for this sharp decline in density of TH⁺ fiber outgrowth is that age-dependent characteristics within the host brain alter outgrowth, since extensive outgrowth can be achieved when transplanted into immature (neonatal) host brain. Expression levels and patterns of adhesion molecules expressed by mature host brain are thought to be the culprits of this innervation-inhibitory effect.

The Inhibitory Environment of Adult Brain

The limited regeneration in adult CNS and limited ability of nigral neurons to extend neurites in the mature host brain is also thought to be related to suboptimal properties of the mature striatum as a substrate for the extension of DA neurites.⁸⁰ Allografts into immature host brain show robust neuronal and glial migration away from the transplant site, and a high degree of integration and target-directed neurite outgrowth (Fig. 8.3).³ Fetal cells transplanted into mature brains show neuronal reaggregation around the implant site and limited axonal outgrowth into host brain, suggesting an age-dependent increase in inhibitory or decrease in growth-promoting processes. Since both promoting and repulsive activities influence axonal guidance and extension, alteration of the host brain "substrate" has been examined to obtain more extensive outgrowth from grafts.

The cell-adhesion molecules (CAMs) are involved in promoting neurite extension, by their incorporation into the extracellular matrix and subsequent binding to cell surfaces.⁸¹ Neurite outgrowth from fetal VM cells in culture is enhanced when plated on various cell

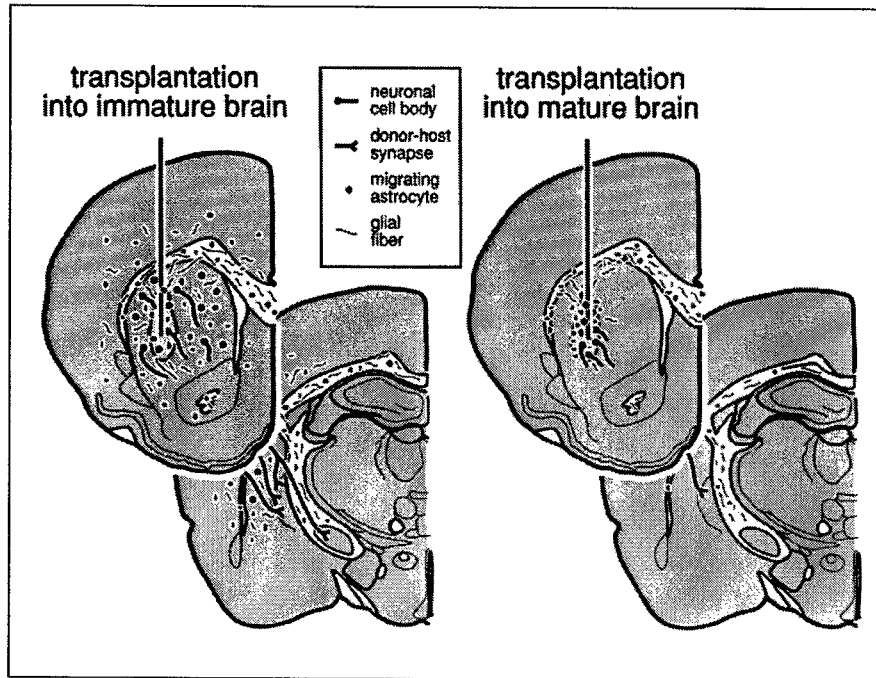


Fig. 8.3. Integration and axonal outgrowth from fetal grafts. Fetal cells transplanted into immature host brain show robust neuronal (black) and glial (grey) migration away from the transplant site, and a high degree of integration and target-directed neurite outgrowth. Fetal cells transplanted into mature brain remain around the implant site and exhibit limited axonal outgrowth into host brain, suggesting an age-dependent increase in inhibitory or decrease in growth-promoting processes. Reprinted with permission from *Trends in Neurosciences* 1997; 20:477-482, ©Elsevier.

adhesion molecules.⁸⁰ Intranigral transplants of VM do not successfully reinnervate striatum unless axonal growth is provoked by bridging the striatum and transplanted tissue via peripheral nerve tissue,⁸² laminin track,⁸³ striatal cells,⁸⁴ or grafts of fibroblast growth factor-transfected schwannoma cells.⁸⁵ The transient expression of these molecules (GAP-43, NCAM and L1) within VM grafts only during the phase of axon elongation further suggests their contribution to outgrowth of developing neurons.^{86,87} Studies utilizing antibodies against growth-inhibiting factors, such as the IN-1 antibody raised against myelin-associated neurite growth inhibitor NT-35/250, have shown enhanced innervation of fetal neocortical innervation into adult host brain.⁸⁸

The argument against outgrowth-inhibitory properties of adult brain stems from studies showing long-distance and target-specific axonal growth from human embryonic transplants into adult rat brain,⁸⁹ as well as from porcine embryonic transplants into adult rat brain.⁶¹ The species-specific markers used in our studies of fetal porcine transplants into adult immunosuppressed rat brain allowed comparison of donor glial fiber and donor axonal growth in different host brain regions, demonstrating their distinct trophic characteristics. Target zones in adult host gray matter were selectively innervated by embryonic donor axons

normally destined to form synapses there, whereas donor glial fibers grew irrespective of any target orientation within white matter tracts (Fig. 8.4A).⁶¹ Neuronal axons branched profusely in gray matter target region and only rarely penetrated or crossed white matter tracts. TH⁺ fibers from transplants placed into the SN were found coursing up toward the striatum through myelinated fiber bundles, then branching into host gray matter, as also shown by Wictorin from human xenotransplants into rat SN.⁸⁹ Interestingly, the non-DA VM cells also grew toward distant gray matter target zones, such as medio-dorsal and ventral anterior thalamus. These data suggest that directional cues for axons, whether diffusible or substrate-bound, are provided by adult host target regions. Since porcine neural development continues four to five times longer than mouse, these axons may develop over a longer time course than that seen in rat-to-rat studies, as illustrated in the development of functional recovery in porcine-transplant recipients (8 weeks post-transplantation) as compared with allografts (6 weeks post-transplantation),

Another aspect of host brain environment which can influence outgrowth from DA grafts is the lesion status: Denervation of the host does seem to promote fiber outgrowth, but has little effect on their survival. Two hypotheses have evolved to explain the apparent increase in outgrowth from grafted cells when transplanted into lesioned versus intact brain: First, outgrowth may be limited due to availability of sites for synaptic contacts, which are increased soon after lesion;⁹⁰ alternatively, injury-induced neurotrophic factors have also been suggested,⁹¹ since graft development is enhanced after administration of extracts from injured brain into the implant site.⁹² Zhou and Chang⁹³ showed a bridging method created by a "trophic track" formed by low-dose injection of excitatory amino acids; they observed TH⁺ fibers streaming along this lesion track from the intranigral VM graft into the striatum, and hypothesize that the lesion induces trophic molecules, extracellular matrices, and vasculature which support reinnervation by TH⁺ fibers.

Regional and Phenotypic Specification of Dopamine Neurons

Different regions of the striatum are associated with specific behaviors in rat; the dorsal striatum receives primary afferents from the motor areas of neocortex, and has been shown to be preferentially involved in rotational recovery after DA neuron transplantation.⁴⁵ In the intact rat, the subpopulation of nigral DA neurons from A9 SNc which co-express AHD project their axons to the dorsal-lateral and rostral regions of the striatum (Fig. 8.4B). As described above (see above: "Utilizing medial VM for dopamine grafts"), the enriched population of DA neurons obtained from a medial versus lateral VM dissection also preferentially expresses AHD; when transplanted into adult DA-denervated rat striatum, these AHD/TH neurons innervate this region of the DA-depleted striatum^{3,34} (Fig. 8.4C), showing a preferential reinnervation of the dorso-lateral striatum corresponding to the normal projection pattern of AHD/TH neurons. Specific innervation by subsets of transplanted DA neurons was also demonstrated by Schultzberg, revealing reinnervation of the DA-depleted striatum by the population of grafted VM neurons lacking cholecystokinin (CCK).⁴⁰ The CCK⁺ fibers were found in a narrow zone immediately adjoining the graft. These data suggest the presence of mechanisms which selectively favor the ingrowth of fibers from the appropriate DA neuronal subset. Thus, enrichment of the DA neuron subpopulation which specifically expresses AHD may allow more appropriate reinnervation of striatum after transplantation, and influence the degree of functional recovery in PD, possibly defined by tropic mechanisms intrinsic to the host brain.

Reconstructing Synaptic Connections with Dopamine Grafts

Functional effects of intrastriatal grafts of fetal DA cells have been illustrated in a range of animal behavioral tests.⁹⁴⁻⁹⁶ The behavioral effects observed are dependent on the survival of DA neurons within the striatum, since grafting of other tissue produces no behavioral effects^{97,98} and removal of transplanted tissue⁹⁹ or immune rejection of transplanted functional analyses of DA grafts.

Many groups have used unilateral, intraparenchymal injection of 6-hydroxydopamine (6-OHDA) as the means of producing a unilateral DA denervation of the striatum, then transplanting DA neurons into this denervated striatum. The spontaneous behavior induced by lesion is improved as tested in several parameters, and depends on graft placement, cell number, and density of reinnervation. Due to the imbalance in DA after unilateral lesion, the animal begins to rotate in response to DA-releasing drugs such as amphetamine.¹⁰¹ The transplantation of DA cells and subsequent reinnervation of the denervated striatum causes the animal to decrease its rotations in response to amphetamine,¹⁰² thus reversing the lesion-induced behavioral abnormalities. Compensation of other lesion-induced changes, such as lesion-induced increases in DA receptor binding,¹⁰³ increased levels of enkephalin, and decreased levels of substance P¹⁰⁴ demonstrate the capacity of these DA cells to affect postsynaptic and presynaptic mechanisms.^{4,102} However, more complex movements (such as food pellet retrieval, stair case and stepping tests) have exhibited limited responses to DA transplants.^{105,106} A microtransplantation procedure which increases the area of striatal reinnervation has shown improved paw reaching in addition to greater striatal reinnervation,¹⁵ suggesting that the limited behavioral recovery of some complex movements so often seen in previous studies may be due to inadequate striatal reinnervation.

Regulated DA Release from Fetal DA Grafts

Methods to improve the number of DA cells that survive transplantation, and enhance the area of the striatum which becomes reinnervated by these cells, are continually being tested; however, the most important factor in obtaining complete and sustained functional effects is the successful formation of synapses between the transplanted cells and the host brain. The use of autologous adrenal cells, fibroblasts transfected with DA-producing enzymes, and other non-neuronal cell types which can secrete DA can perhaps circumvent the problems of limited availability and ethical issues associated with the use of fetal DA neurons. However, functional analyses from these studies indicate that placing a "DA pump" into the striatum may not be as effective in ameliorating the motor symptom of PD as the regulated, synaptic release obtained with transplanted DA neurons;¹⁰ in fact, when DA is directly administered into the ventricle of PD patients, serious psychoses develop,¹⁰⁷ and recent data from differential display has shown the abnormal upregulation of over 10 genes within the striatum.¹⁰⁸ Complications associated with unregulated DA levels are obvious when observing effects of long term L-dopa administration: As PD progresses and the DA neuron degeneration continues, the unregulated formation of DA within the striatum can lead to motor abnormalities such as dyskinesias. The physiological incorporation and regulation of DA release can only be achieved by DA neurons themselves, or by cells which express the complete set of feedback elements required to regulate the release and uptake of DA.

Embryonic DA neurons are not "designed" to produce new connections with mature, established striatal neurons. However, synaptic connections between transplanted VM cells and host cells, as well as afferents from host neurons to transplanted cells, have been illustrated.^{109,110} The inclusion of fetal striatal tissue, specifically lateral ganglionic eminence, within VM transplants not only produced increased DA cell survival and extent of reinnervation into the DA-depleted host striatum, but also showed an increased number of

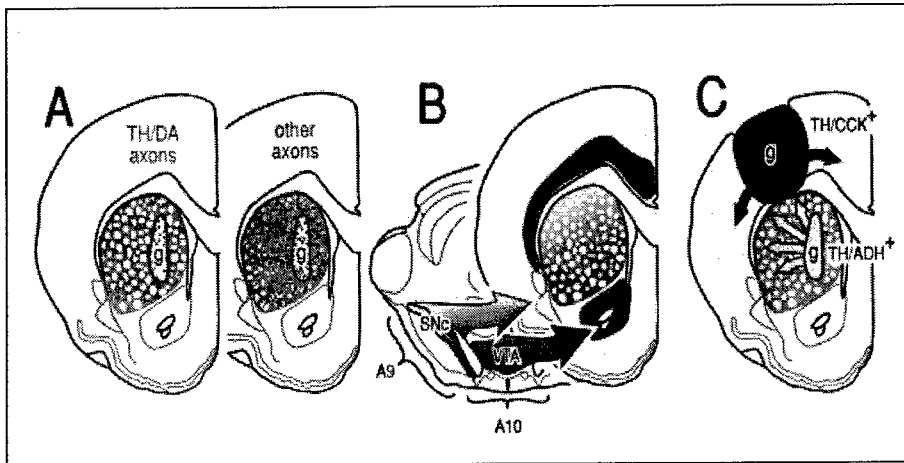


Fig. 8.4. Target-specific innervation by grafted fetal cells. (A) Target zones in adult host gray matter are selectively innervated by embryonic pig donor DA axons normally destined to form synapses there, whereas non-DA donor fibers grow into host myelinated bundles. (B) In the intact rat, the subpopulation of nigral DA neurons from A9 SNc, which coexpress AHD, project their axons to the gray matter of dorso-lateral regions of the striatum. The ventral tegmental area (VTA) neurons from A10 coexpress CCK, and project to ventromedial striatum, nucleus accumbens, neocortex and limbic regions. (C) When the enriched population of TH/AHD neurons obtained from a medial (versus lateral) VM dissection is transplanted into DA-lesioned adult rat striatum, these neurons preferentially reinnervate their normal dorso-lateral striatal target, shown to be involved in rotational recovery after DA neuron transplantation. TH/CCK neurons from VM show different patterns of outgrowth when placed into cortex.⁴⁰ Reprinted with permission from Trends in Neurosciences 1997; 20:477-482, ©Elsevier.

host striatal cells which induced the immediate-early gene *c-fos*, indicating a higher degree of host cell activation.¹³

Several studies have shown normalized activity throughout the basal ganglia after transplantation. Nakao et al¹¹¹ utilized cytochrome oxidase histochemistry to quantify neuronal activity in the 6-OHDA-lesioned rat; the lesion-induced increases in activity of the entopeduncular nucleus and SN reticulata were reversed by intrastriatal VM grafts, whereas the lesion-induced increases in globus pallidus and subthalamic nucleus were not affected by grafting.¹¹¹ The same technique has been used in MPTP-treated monkey receiving VM transplants as well. Dopaminergic grafts increased the metabolic activity of the implanted striatum, particularly in the region of grafts containing greater numbers of DA neurons.¹¹² Also of interest is the finding that the DA neurons exhibit the highest rate of metabolic activity among all cell types contained in the VM grafts.¹¹² Positron emission tomography (PET) and carbon-11 labeled 2B-carbomethoxy-3B-(4-fluorophenyl)tropane (11C-CFT) have been utilized as markers for striatal presynaptic DA transporters in a unilateral lesion model in rat. In the lesioned striatum, the binding ratio was reduced to 15% to 35% of the intact side. After DA neuronal transplantation, behavioral recovery occurred only after the 11C-CFT binding ratio had increased to 75% to 85% of the intact side, revealing a threshold for functional recovery in the lesioned nigrostriatal system after neural transplantation.⁹⁸

Autoregulation of DA release and metabolism by intrastriatal grafts has been shown by microdialysis. Treatment with the DA receptor agonist apomorphine produced a decrease in DA in the grafted striatum.^{6,113} Correct regulation of DA levels by transplanted striatum is also suggested by the observation that behavioral recovery plateaus at high levels of cell survival, with further neuronal survival providing no additional behavioral effects.¹¹³ Further evidence for the formation of functional synapses and autoregulation from transplanted fetal neurons comes from the observation that dyskinesias, expressed either as contraversive circling after repeated L-dopa injections in rodents¹¹⁴ or L-dopa-induced dyskinesias in monkey (Widner H, personal communication) are reduced after transplantation. In patients, however, more variable results have been observed.^{115,116} These data suggest that even a high amount of extracellular DA within the grafted striatum (either through improved cell survival, increased DA release, or addition of L-dopa) will be regulated in a physiological manner by the transplanted DA neurons.

A more recent approach manipulates the striatal cells themselves to produce L-dopa or DA. Various vector systems have been used to deliver TH, GTP-cyclohydrolase I (the rate-limiting enzyme for tetrahydrobiopterin synthesis), and aromatic L-amino acid decarboxylase to striatal neurons and glia, essentially turning them into DA-producing cells.¹¹⁷⁻¹²¹ While these approaches bypass the requirement for synapse formation between transplanted VM neurons and striatal neurons, and apomorphine-induced rotation has been decreased in unilaterally-lesioned animals in some studies, the release of DA from these striatal neurons has not been shown to be regulated, and the normal phenotype of these striatal neurons may also be altered. In addition, the drawbacks typically encountered with gene transfer (such as low transduction rates and limited gene expression) also arise.

Clinical Relevance

The Urgency for an Improved Therapy for Parkinson's Disease

The simple concept of replacing lost neurons by inserting new cells was introduced as a new therapeutic strategy for treatment of PD two decades ago. The progressive loss of DA neurons in the SN and resultant decrease of DA levels in its targets produce the signs of PD: tremors, akinesias, muscle rigidity, and postural instability. Although the cause of this cell loss is not understood, therapeutic strategies to correct the impaired motor function due to the unbalanced basal ganglia circuitry have included pharmacological (L-dopa) as well as surgical (pallidotomy,^{122,123} thalamotomy,¹²⁴ subthalamic nucleus stimulation¹²⁵) approaches. A major obstacle with long term L-dopa treatment is the appearance of severe side effects such as "on-off" phenomena and dyskinesias. Although capable of relieving some parkinsonian motor symptoms (notably tremor and dyskinesias), the surgical approaches each have potential complications, such as cognitive disorders after thalamotomy¹²⁶ and hemiparesis, frontal lobe syndromes and hemorrhage after pallidotomy.^{122,123}

The specificity of cellular degeneration which occurs in PD (DA neurons of the SN), as well as the well defined target of these degenerating cells (the caudate and putamen), have contributed to the direct application of neural transplantation for this disorder. Early clinical transplantation studies involved the use of catecholamine-secreting adrenal medulla cells.^{127,128} The variable and transient alleviation of symptoms, as well as the poor adrenal medulla graft survival and high morbidity of patients, contributed to a transition to fetal cells for PD transplantation. Yet this cell source is not without its drawbacks, specifically the requirement for several fetuses per patient, all within the desired gestation age. However, more recent animal studies utilizing corgrafts of adrenal medulla and peripheral nerve have indicated that this procedure can overcome the major problems encountered with adrenal grafts alone as discussed above.⁷⁷ Several PD patients have received these grafts, and

studies report their success in relieving motor symptoms, through 24 months post-transplantation.^{129,130}

Application of Dopamine Transplants to Parkinson's Patients

The successful data from rodent studies with fetal DA cells, as discussed throughout this chapter, were extended to nonhuman primate models of parkinsonism, induced either by the selective DA neurotoxin MPTP or by 6-OHDA.¹³¹ By demonstrating the capacity of these grafts to improve more complex movements and behavioral tasks, and correlating these improvements with histology and PET imaging of the grafts, these studies then led to the first clinical trials in humans. The initial (and in some cases ongoing) issues regarding the technical aspects of the procedure were assessed, such as patient monitoring, implantation technique, donor tissue properties, immunology of allografts, risk assessment of disease transmission, and ethical considerations. The first two patients to be transplanted were middle-aged females each with about a 14 year history of PD, and symptoms consisting of bradykinesia, rigidity, and severe "off" phases.¹³² Each patient received four VM regions from aborted fetuses, implanted unilaterally into the caudate and putamen, and were immunosuppressed with cyclosporin. Each showed modest clinical improvement, including improved gait, which lasted several years in one patient and which was lost in the other after 11-13 months,¹³² suggesting an immunological rejection of the graft (immunosuppression was withdrawn after 24 months). At nine years, no functional effects persisted, suggesting that cell survival or development was lower than expected.

Examination of the implantation procedure yielded the following improvements: decreasing time between abortion and dissection, and dissection and implantation; buffering of storage/dissection media; adding DNase to final step; reduced cannula size; more tissue. Encouraging results were found with the subsequent two patients receiving fetal VM suspensions: decreasing rigidity, bradykinesia, and number and length of daily "off" periods, which were apparent 6-12 weeks post-transplantation.¹³³ Three years later, both patients showed near normal 18F-fluorodopa uptake in the grafted region, while the contralateral striatum showed decreased uptake when compared with that one year post-transplantation, indicative of progressive degeneration associated with the underlying disease process.¹³⁴ A three year study has shown therapeutically valuable improvement in four out of six patients: Rigidity and hypokinesia improved bilaterally; however, no consistent changes in dyskinesias were observed.¹¹⁶ One of these patients was without L-dopa from 32 months and had normal fluorodopa uptake in the grafted putamen at six years.¹¹⁶ Two additional patients were transplanted utilizing the same protocol, with similar clinical improvements, as well as positive PET results.¹³⁵ Bilateral caudate/putamen grafts into two MPTP-exposed patients have produced marked motor improvement in both patients, correlating with increased uptake of fluorodopa.¹³⁶ The transplantation of solid pieces of VM have also produced clinical improvement with increased fluorodopa in many (but not all) patients 6 and 46 months post-transplantation.^{5,137}

Two instructive clinical studies have provided information on basic parameters. One compared clinical improvements with graft volume: One group of PD patients was transplanted with VM from one to two donors (volume of approximately 20 mm³), while a second group received tissue from three or more donors (approximate volume of 24 mm³).¹³⁸ Both groups demonstrated significant improvement over presurgical baseline scores; however, the high volume group had significantly greater improvement on all UPDRS scores, suggesting that amount of donor tissue may influence clinical outcome. The second study correlated clinical improvement with immunosuppression: After over two years of immunosuppressive treatment, withdrawal from the cyclosporin treatment produced a decline in

the graft-induced motor improvements, implicating a rejection-induced decline in function.¹³⁹

Recent Clinical Progress and Developments

As mentioned previously (see above: "Other cell sources"), xenotransplantation allows the acquisition of large quantities of accurately aged fetal tissue. The T cell-mediated rejection of xenografts can be inhibited by immune suppression,¹⁴⁰ and studies have shown survival, function, and afferent/efferent connections of xenogeneic cells when transplanted into animal hosts,^{6,61} (and see reviews in refs. 141,142). The transplantation of E27 porcine VM unilaterally into the caudate and putamen of twelve immunosuppressed PD patients has produced clinical improvements: UPDRS "off" scores improved 16.9 points in ten evaluable patients at 12 months.¹⁴³ One patient from this study died seven months after surgery from a pulmonary embolism; histological analyses using species-specific markers revealed porcine cells and axonal projections from the grafts into host brain. All three identified grafts contained TH⁺ neurons (630 TH⁺ neurons in all), and non-TH⁺ neurons expressing pig-specific neurofilament protein were also observed within, and extending axons out of, the grafts.⁸ Microglial and T cell markers showed low reactivity in and around the pig cell graft perimeter.

In addition to this histological study, autopsy data has been published from one other laboratory, who bilaterally transplanted 6.5-9 week human fetal VM into postcommisural putamen of several PD patients. Details from two of these patients who died 18-19 months after surgery of events unrelated to the grafting procedure have been reported.^{9,144-146} Both patients showed improved motor function and increases in fluorodopa uptake in the putamen on PET scanning. Histological analysis has shown over 200,000 surviving TH⁺ neurons in the male patient (12 sites) which reinnervated over 53% of the right putamen and 23% of the left putamen in a patch-matrix pattern.⁹ Electron microscopy revealed axo-dendritic and occasional axo-axonic synapses between graft and host, and analysis of TH mRNA revealed higher expression within the fetal neurons than within the residual host nigral cells.⁹ Autopsy of the second patient showed over 130,000 surviving TH⁺ neurons, reinnervating 78% of the putamen.¹⁴⁶ Even in these healthy-appearing grafts and a six month regimen of cyclosporin treatment, pan macrophages and T and B cells were observed within the graft sites.¹⁴⁵

These results have demonstrated the potential usefulness of neuronal replacement therapy for PD, relieving rigidity, akinesia, peak-dose dyskinesias, gait stability, speech, and swallowing. There is no indication that the disease process is negatively affecting the transplanted cells, although the endogenous DA system continues its progressive degeneration.^{116,134} The striatum has a remarkable capacity to compensate for very low levels of DA, as evidenced by the lack of parkinsonian symptomology until 80% of DA is lost. Thus a substantial, though perhaps incomplete, reinnervation may allow maximal functional outcome. However, the basic mechanistic problems with these grafts as outlined in the above review, specifically the limited development and reafferentation of host brain by these grafts, require the continued efforts of investigators in this field. Other issues such as graft location,¹⁴⁷ immunologic questions,^{145,148} further progression of the disease, and continued exposure of fetal cells to L-dopa, remain under intense investigation.

Conclusion

The current understanding of the normal in situ maturation and phenotypic specializations of DA neurons located in the adult substantia nigra parallels the observations made of the development of committed fetal dopamine neurons placed as grafts into the adult CNS. The molecular signaling necessary for the final morphological specializations and connectivity of the nigro-striatal DA system must therefore be largely intrinsic to the developing DA neurons, or, alternatively, present in significant detail in the adult brain for this process to be completed in a normal way.

These findings may be clinically applied to further improvements in DA neuron "replacement" in the PD brain, and provide functional restitution to patients with neurodegenerative diseases.

References

1. Bjorklund A, Stenevi U. Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. *Brain Research* 1979; 177:555-560.
2. Perlow M, Freed W, Hoffer B et al. Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. *Science* 1979; 204:643-647.
3. Isacson O, Deacon TW. Neural transplantation studies reveal the brain's capacity for continuous reconstruction. *Trends in Neuroscience* 1997; 20:477-482.
4. Bjorklund A, Lindvall O, Isacson O et al. Mechanisms of action of intracerebral neural implants: Studies on nigral and striatal grafts to the lesioned striatum. *Trends in Neuroscience* 1987; 10:509-516.
5. Freeman TB, Olanow CW, Hauser RA et al. Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. *Annals of Neurology* 1995; 38:379-388.
6. Galpern WR, Burns LH, Deacon TW et al. Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: Functional recovery and graft morphology. *Experimental Neurology* 1996; 140:1-13.
7. Olanow CW, Kordower JH, Freeman TB. Fetal nigral transplantation as a therapy for Parkinson's disease. *Trends in Neuroscience* 1996; 19:102-109.
8. Deacon T, Schumacher J, Dinsmore J et al. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nature Medicine* 1997; 130:350-353.
9. Kordower J, Rosenstein J, Collier T et al. Functional fetal nigral grafts in a patient with Parkinson's disease: Chemoanatomic, ultrastructural, and metabolic studies. *Journal of Comparative Neurology* 1996; 370:203-230.
10. Bjorklund A. Better cells for brain repair. *Nature* 1993; 362:414-415.
11. Yurek D, Lu W, Hipkens S et al. BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons. *Experimental Neurology* 1996; 137:105-118.
12. Nakao N, Frodl E, Duan WM et al. Lazaroids improve the survival of grafted embryonic dopamine neurons. *Proc Natl Acad Sci* 1994; 91:12408-12412.
13. Costantini LC, Snyder-Keller A. Co-transplantation of fetal lateral ganglionic eminence and ventral mesencephalon can augment function and development of intrastriatal transplants. *Experimental Neurology* 1997; 145:214-227.
14. Brundin P, Isacson O, Gage F et al. Intrastriatal grafting of dopamine-containing neuronal cell suspensions: Effects of mixing with target or non-target cells. *Brain Research* 1986; 389:77-84.
15. Nikkiah G, Olsson M, Eberhard J et al. A microtransplantation approach for cell suspension grafting in the rat Parkinson model: A detailed account of the methodology. *Neuroscience* 1994; 53:57-72.
16. Zhou J, Bradford H, Stern G. Influence of BDNF on the expression of the dopaminergic phenotype of tissue used for brain transplants. *Developmental Brain Research* 1997; 100:43-51.

17. Mayer E, Dunnett S, Fawcett J. Mitogenic effect of basic fibroblast growth factor on embryonic ventral mesencephalic dopaminergic neurone precursors. *Developmental Brain Research* 1993; 72:253-258.
18. Johansson M, Friedemann M, Hoffer B et al. Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts in oculo. *Experimental Neurology* 1995; 134:25-34.
19. Wang Y, Tien L, Lapchak P et al. GDNF triggers fiber outgrowth of fetal ventral mesencephalic grafts from nigra to striatum in 6-OHDA-lesioned rats. *Cell Tissue Research* 1996; 286:225-233.
20. Rosenblad C, Martinez-Serrano A, Bjorklund A. Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience* 1996; 75:979-985.
21. Zawada W, Zastrow D, Clarkson E et al. Growth factors improve immediate survival of embryonic dopamine neurons after transplantation into rats. *Brain Research* 1998; 786:96-103.
22. Prochiantz A, di Porzio U, Kato A et al. In vitro maturation of mesencephalic dopaminergic neurons from mouse embryos is enhanced in presence of their striatal target cells. *Proc Natl Acad Sci USA* 1979; 76:5387-5391.
23. Prochiantz A, Daguet M, Herbert A et al. Specific stimulation of in vivo maturation of mesencephalic dopaminergic neurons by striatal membranes. *Nature* 1981; 293:570-572.
24. di Porzio U, Daguet J, Glowinski J et al. Effect of striatal cells on in vitro maturation of mesencephalic dopaminergic neurons grown in serum-free conditions. *Nature* 1980; 288:370-373.
25. Oo T, Burke R. The time course of developmental cell death in phenotypically defined dopaminergic neurons of the substantia nigra. *Brain Res Dev Brain Res* 1997; 98:191-196.
26. Mahalik T, Hahn W, Clayton G et al. Programmed cell death in developing grafts of fetal substantia nigra. *Experimental Neurology* 1994; 129:27-36.
27. Bjorklund L, Spenger C, Stromberg I. Tirilazad mesylate increases dopaminergic neuronal survival in the in oculo grafting model. *Experimental Neurology* 1997; 148:324-333.
28. Widner H. The Lund Transplant Program for Parkinson's Disease and patients with MPTP-induced Parkinsonism. In: Freeman T, Widner, H., eds. *Cell Transplantation for Neurological Disorders*. Totowa, NJ: Humana Press, 1998:1-19.
29. Brundin P, Isacson O, Bjorklund A. Monitoring of cell viability in suspensions of embryonic CNS tissue and its use as a criterion for intracerebral graft survival. *Brain Res* 1985; 331:251-259.
30. Purves D, Snider W, Voyvodic J. Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature* 1988; 336:123-128.
31. Kawano S, Okajima S, Mizoguchi A et al. Immunocytochemical distribution of Ca(2+)-independent protein kinase C subtypes (delta, epsilon, and zeta) in regenerating axonal growth cones of rat peripheral nerve. *Neuroscience* 1997; 81:263-273.
32. Shults C, Hashimoto R, Brady R et al. Dopaminergic cells align along radial glia in the developing mesencephalon of the rat. *Neuroscience* 1990; 38:427-436.
33. Costantini LC, Lin L, Isacson O. Medial fetal ventral mesencephalon: A preferred source for dopamine neuron grafts. *Neuroreport* 1997; 8:2253-2257.
34. Haque N, LeBlanc C, Isacson O. Differential dissection of the rat E16 ventral mesencephalon and survival and reinnervation of the 6-OHDA-lesioned striatum by a subset of aldehyde dehydrogenase-positive TH neurons. *Cell Transplantation* 1997; 6:239-248.
35. Lin L, Doherty D, Lile J et al. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 1993; 260:1130-1132.
36. Engele J, Bohn M. The neurotrophic effects of fibroblast growth factors on dopaminergic neurons in vitro are mediated by mesencephalic glia. *Journal of Neuroscience* 1991; 11:3070-3078.
37. Widmer H, Alexi T, Valverde J et al. TGF alpha stimulation of phosphatidylinositol hydrolysis in mesencephalic cultures requires neuron-glia interactions. *Neuroreport* 1993; 4:407-410.

38. Hyman C, Goffe M, Barde Y et al. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* 1991; 350:230-232.
39. Lindsay R, Altar C, Cedarbaum J et al. The therapeutic potential of neurotrophic factors in the treatment of Parkinson's disease. *Experimental Neurology* 1993; 124:103-118.
40. Schultzberg M, Dunnett S, Bjorklund A et al. Dopamine and cholecystokinin immunoreactive neurons in mesencephalic grafts reinnervating the neostriatum: Evidence for selective growth regulation. *Neuroscience* 1984; 12:17-32.
41. McCaffery P, Drager U. High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system. *Proc Natl Acad Sci USA* 1994; 91:7772-7776.
42. Zetterstrom R, Solomin L, Jansson L et al. Dopamine neuron agenesis in *Nurr1*-deficient mice. *Science* 1997; 176:248-250.
43. Burgunder J, Young W. Ontogeny of tyrosine hydroxylase and cholecystokinin gene expression in the rat mesencephalon. *Developmental Brain Research* 1990; 52:85-93.
44. Forman B, Umesono K, Chen J et al. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* 1995; 81:541-550.
45. Dunnet S, Bjorklund A, Schmidt R et al. Intracerebral grafting of neuronal cell suspensions. IV. Behavioral recovery in rats with unilateral 6-OHDA lesions in different brain sites. *Acta Physiol Scand* 1983; 522:29-37.
46. Svendsen C, Caldwell M, Shen J et al. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. *Experimental Neurology* 1997; 148:135-146.
47. Svendsen C, Clarke D, Rosser A et al. Survival and differentiation of rat and human epidermal growth factor-responsive precursor cells following grafting into the lesioned adult central nervous system. *Experimental Neurology* 1996; 137:376-388.
48. Minger S, Fisher L, Ray J et al. Long-term survival of transplanted basal forebrain cells following in vitro propagation with fibroblast growth factor-2. *Experimental Neurology* 1996; 141:12-24.
49. Martinez-Serrano A, Bjorklund A. Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends in Neuroscience* 1997; 20:530-538.
50. Renfranz PJ, Cunningham MG, McKay RDG. Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* 1991; 66:713-729.
51. Ryder EF, Snyder EY, Cepko CL. Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer. *Journal of Neurobiology* 1990; 21:356-375.
52. Snyder EY, Deitcher DL, Walsh C et al. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* 1992; 68:33-51.
53. Cattaneo E, Magrassi L, Butti G et al. A short term analysis of the behavior of conditionally immortalized neuronal progenitors and primary neuroepithelial cells implanted in the fetal rat brain. *Developmental Brain Research* 1994; 83:197-208.
54. Lundberg C, Martinez-Serrano A, Cattaneo E et al. Survival, integration and differentiation of neural stem cell lines after transplantation to the adult rat striatum. *Experimental Neurology* 1997; 145:342-360.
55. Kleppner SR, Robinson KA, Trojanowski JQ et al. Transplanted human neurons derived from a teratocarcinoma cell line (NTera-2) mature, integrate, and survive for over 1 year in the nude mouse brain. *Journal of Comparative Neurology* 1995; 357:618-632.
56. Morassutti DJ, Staines WA, Magnuson DSK et al. Murine embryonal carcinoma-derived neurons survive and mature following transplantation into adult rat striatum. *Neuroscience* 1994; 58:753-763.
57. Wojcik BE, Nothias F, Lazar M, et al. Catecholaminergic neurons result from the intracerebral implantation of embryonal carcinoma cells. *Proc Natl Acad Sci USA* 1993; 90:1305-1309.
58. Miyazono M, Lee VM-Y, Trojanowski JQ. Proliferation, cell death, and neuronal differentiation in transplanted human embryonal carcinoma (NTera2) cells depend on the graft

- site in nude and severe combined immunodeficient mice. *Laboratory Investigation* 1995; 73:273-283.
59. Deacon T, Dinsmore J, Costantini LC et al. Blastula-derived stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. *Experimental Neurology* 1998; 149:28-41.
60. Isacson O, Deacon TW. Specific axon guidance factors persist in the mature rat brain: Evidence from fetal neuronal xenografts. *Neuroscience* 1996; 75:827-837.
61. Isacson O, Deacon TW, Pakzaban P et al. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. *Nature Medicine* 1995; 1:1189-1194.
62. Hemmati-Brivanlou A, Melton D. Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell* 1997; 88:13-17.
63. Grunz H, Tacke L. Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Diff Dev* 1989; 28:211-218.
64. Wilson PA, Hemmati-Brivanlou A. Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* 1995; 376:331-333.
65. Hogan B. Bone morphogenic proteins: Multifunctional regulators of vertebrate development. *Genes Dev* 1996; 10:1580-1594.
66. Hemmati-Brivanlou A, Kelley O, Melton D. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* 1994; 77:283-295.
67. Sasai Y, Lu B, Steinbeisser H et al. *Xenopus* chordin: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 1994; 79:779-790.
68. Zimmerman L, De Jesus-Escobar J, Harland R. The Spemann organizer signal noggin binds and inactivates bone morphogenic protein. *Cell* 1996; 86:599-606.
69. Granholm A, Henry S, Herbert M et al. Kidney cogafts enhance fiber outgrowth from ventral mesencephalic grafts to the 6-OHDA-lesioned striatum, and improve behavioral recovery. *Cell Transplantation* 1998; 7:197-212.
70. Pichel J, Shen L, Sheng H et al. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 1996; 382:73-76.
71. Molenaar G, Hogenesch R, Sprengers M et al. Ontogenesis of embryonic porcine ventral mesencephalon in the perspective of its potential use as a xenograft in Parkinson's disease. *Journal of Comparative Neurology* 1997; 382:19-28.
72. Huffaker T, Boss B, Morgan A et al. Xenografting of fetal pig ventral mesencephalon corrects motor asymmetry in the rat model of Parkinson's disease. *Experimental Brain Research* 1989; 77:329-336.
73. Zawada W, Cibelli J, Choi P et al. Somatic cell cloned transgenic bovine neurons for transplantation in parkinsonian rats. *Nature Medicine* 1998; 4:569-574.
74. Sanberg P, Borlongan C, Othberg A et al. Testis-derived Sertoli cells have a trophic effect on dopamine neurons and alleviate hemiparkinsonism in rats. *Nature Medicine* 1997; 3:1129-1132.
75. Saporta S, Cameron D, Borlongan C et al. Survival of rat and porcine sertoli cell transplants in the rat striatum without cyclosporin-A immunosuppression. *Experimental Neurology* 1997; 146:299-304.
76. Espejo E, Montoro R, Armengo J et al. Cellular and functional recovery of parkinsonian rats after intrastriatal transplantation of carotid body cell aggregates. *Neuron* 1998; 20:197-206.
77. Watts R, Mandir A, Bakay R. Intrastriatal cogafts of autologous adrenal medulla and sural nerve in MPTP-induced parkinsonian macaques: Behavioral and anatomical assessment. *Cell Transplantation* 1995; 4:27-38.
78. Saporta S, Borlongan C, Moore J et al. Microcarrier enhanced survival of human and rat fetal ventral mesencephalon cell implanted in the rat striatum. *Cell Transplantation* 1997; 6:579-584.
79. Rioux L, Gaudin D, Bui L et al. Correlation of functional recovery after 6-OHDA lesion with survival of grafted fetal neurons and release of dopamine in the striatum of the rat. *Neuroscience* 1991 ;40:123-131.

80. Poltorak M, Shimoda K, Freed W. L1 substrate enhances outgrowth of tyrosine hydroxylase immunoreactive neurites in mesencephalic cell culture. *Experimental Neurology* 1992; 117:176-184.
81. Lander A. Understanding the molecules of neural cell contacts: Emerging patterns of structure and function. *Trends in Neuroscience* 1989; 12:189-195.
82. Aguayo A, Bjorklund A, Stenevi U, et al. Fetal mesencephalic neurons survive and extend long axons across peripheral nervous system grafts inserted into the adult rat striatum. *Neurosci Lett* 1984; 45:53-58.
83. Zhou F. Connectivities of the striatal grafts and laminin guiding. *Progress in Brain Research* 1990; 82:441-458.
84. Dunnett S, Rogers D, Richards S. Nigrostriatal reconstruction after 6-OHDA lesions in rats: Combination of dopamine-rich nigral grafts and nigrostriatal "bridge" grafts. *Experimental Brain Research* 1989; 75:523-535.
85. Brecknell J, Haque N, Du J et al. Functional and anatomical reconstruction of the 6-hydroxydopamine lesioned nigrostriatal system of the adult rat. *Neuroscience* 1996; 71:913-925.
86. Gopinath G, Sable V, Sailaja K, et al. Cell surface molecules (NCAM and L1) in intrastriatal transplants of embryonic mesencephalon in rats. *Neuroscience* 1996; 73:161-169.
87. Clayton G, Mahalik T, Finger T. GAP-43 and 5B4-CAM immunoreactivity during the development of transplanted fetal mesencephalic neurons. *Experimental Neurology* 1991; 114:1-10.
88. Schulz M, Schnell L, Castro A, et al. Cholinergic innervation of fetal neocortical transplants is increased after neutralization of myelin-associated neurite growth inhibitors. *Experimental Neurology* 1998; 149:390-397.
89. Wictorin K, Brundin P, Sauer H, et al. Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. *Journal of Comparative Neurology* 1992; 323:475-494.
90. Freed W, Cannon-Spoor, H. Cortical lesions increase reinnervation of the dorsal striatum by substantia nigra grafts. *Brain Research* 1988; 446:133-143.
91. Nieto-Sampedro M, Lewis E, Cotman C, et al. Brain injury causes time-dependent increase in neuronotrophic activity at the lesion site. *Science* 1982; 217:860-861.
92. Nieto-Sampedro M, Whittemore S, Needels D, et al. The survival of brain transplants is enhanced by extracts from injured brain. *Proc Natl Acad Sci USA* 1984; 81:6250-6254.
93. Zhou F, Chiang Y. Excitotoxic-induced trophic bridging directs axonal growth of transplanted neurons to distal target. *Cell Transplantation* 1995; 4:103-112.
94. Brundin P, Duan W, Sauer H. Functional effects of mesencephalic dopamine neurons and adrenal chromaffin cells grafted to the rodent striatum. In: Dunnett S, Bjorklund A, eds. *Functional Neural Transplantation*. 2nd ed. New York:Raven Press, 1994:9-46.
95. Annett L. Functional studies of neural grafts in parkinsonian primates. In: Dunnett S, Bjorklund, A, eds. *Functional Neural Transplantation*. 2nd ed. New York:Raven Press, 1994:71-102.
96. Olsson M, Nikkhah G, Bentlage C et al. Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *Journal of Neuroscience* 1995; 15:3863-3875.
97. Dunnett S, Hernandez T, Summerfield et al. Graft-derived recovery from 6-OHDA lesions: Specificity of ventral mesencephalic graft tissue. *Experimental Brain Research* 1988; 71:411-424.
98. Brownell AL, Livni E, Galpern W et al. In vivo PET imaging in rat of dopamine terminals reveals functional neural transplants. *Annals of Neurology* 1998; 43:387-390.
99. Bjorklund A, Dunnett S, Stenevi U et al. Reinnervation of the denervated striatum by substantia nigra transplants: Functional consequences as revealed by pharmacological and sensorimotor testing. *Brain Research* 1980; 199:307-333.

100. Carder R, Snyder-Keller A, Lund R. Behavioral and anatomical correlates of immunologically induced rejection of nigral xenografts. *Journal of Comparative Neurology* 1988; 277:391-402.
101. Ungerstedt U, Arbuthnott G. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Research* 1970; 24:485-493.
102. Dunnett SB, Whishaw IQ, Jones GH et al. Effects of dopamine-rich grafts on conditioned rotation in rats with unilateral 6-hydroxydopamine lesions. *Neuroscience Letters* 1986; 68:127-135.
103. Freed W, Ko G, Niehoff D et al. Normalization of spiroperidol binding in the denervated rat striatum by homologous grafts of substantia nigra. *Science* 1983; 222:937-939.
104. Cenci M, Campbell K, Bjorklund A. Neuropeptide messenger RNA expression in the 6-hydroxydopamine-lesioned rat striatum reinnervated by fetal dopaminergic transplants: Differential effects of the grafts on preproenkephalin, preprotachykinin and prodynorphin messenger RNA levels. *Neuroscience* 1993; 57:275-296.
105. Abrous D, Shaltot A, Torres E, et al. Dopamine-rich grafts into the neostriatum and/or nucleus accumbens: Effects on drug-induced behaviors and skilled paw reaching. *Neuroscience* 1993; 53:187-197.
106. Dunnett S, Wishaw I, Rogers D et al. Dopamine-rich grafts ameliorate whole body motor asymmetry and sensory neglect but not independent limb use in rats with 6-OHDA lesions. *Brain Research* 1987; 75:63-78.
107. Venna N, Sabin T, Ordia J et al. Treatment of severe Parkinson's disease by intraventricular injection of dopamine. *Applied Neurophysiology* 1984; 47:62-64.
108. Gerfen C, Keefe K, Steiner H. Dopamine-mediated gene regulation in the striatum. *Adv Pharmacol* 1998; 42:670-673.
109. Mahalik T, Finger T, Stromberg I et al. Substantia nigra transplants into denervated striatum of the rat: Ultrastructure of graft and host interconnections. *Journal of Comparative Neurology* 1985; 240:60-70.
110. Doucet G, Murata Y, Brundin P et al. Host afferents into intrastriatal transplants of fetal ventral mesencephalon. *Experimental Neurology* 1989; 106:1-19.
111. Nakao N, Ogura M, Nakai K et al. Intrastriatal mesencephalic grafts affect neuronal activity in basal ganglia nuclei and their target structures in a rat model of Parkinson's disease. *Journal of Neuroscience* 1998; 18:1806-1817.
112. Collier T, Redmond DJ, Roth R et al. Metabolic energy capacity of dopaminergic grafts and the implanted striatum in parkinsonian nonhuman primates as visualized with cytochrome oxidase histochemistry. *Cell Transplantation* 1997; 6:135-140.
113. Strecker R, Sharp T, Brundin P et al. Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. *Neuroscience* 1987; 22:169-178.
114. Gaudin D, Rioux L, Bedard P. Fetal dopamine neuron transplants prevent behavioral supersensitivity induced by repeated administration of L-dopa in the rat. *Brain Research* 1990; 506:166-168.
115. Defer G, Geny C, Ricolfi F et al. Long-term outcome of unilaterally transplanted parkinsonian patients. I. Clinical approach. *Brain* 1996; 119:41-50.
116. Wenning G, Odin P, Morrish P et al. Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. *Annals of Neurology* 1997; 42:95-107.
117. Imaoka T, Date I, Ohmoto T et al. Significant behavioral recovery in Parkinson's disease model by direct intracerebral gene transfer using continuous injection of a plasmid DNA-liposome complex. *Human Gene Therapy* 1998; 9:1093-1102.
118. Mandel R, Rendahl K, Spratt S et al. Characterization of intrastriatal recombinant adeno-associated virus-mediated gene transfer of human tyrosine hydroxylase and human

- GTP-cyclohydrolase I in a rat model of Parkinson's disease. *Journal of Neuroscience* 1998; 18:4271-4284.
119. Horellou P, Vigne E, Castel M et al. Direct intracerebral gene transfer of an adenoviral vector expressing tyrosine hydroxylase in a rat model of Parkinson's disease. *Neuroreport* 1994; 6:49-53.
120. During M, Naegele J, O'Malley K et al. Long-term behavioral recovery in parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. *Science* 1994; 266:1399-1403.
121. Kaplitt M, Leone P, Samulski R et al. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nature Medicine* 1994; 8:148-154.
122. Samuel M, Caputo E, Brooks D et al. A study of medial pallidotomy for Parkinson's disease: Clinical outcome, MRI location and complications. *Brain* 1998; 121:59-75.
123. Shannon K, Penn R, Kroin J et al. Stereotactic pallidotomy for the treatment of Parkinson's disease. Efficacy and adverse effects at 6 months in 26 patients. *Neurology* 1998; 50:434-438.
124. Tasker R. Ablative therapy for movement disorders. Does thalamotomy alter the course of Parkinson's disease? *Neurosurg Clin N Am* 1998; 9:375-380.
125. Benabid A, Pollak P, Gervason C et al. Long-term suppression of tremor by chronic stimulation of the ventral intermediate thalamic nucleus. *Lancet* 1991; 337:403-406.
126. Tasker R. Thalamotomy. *Neurosurg Clin N Am* 1990; 1:841-864.
127. Madrazo I, Leon V, Torres C. Transplantation of fetal substantia nigra and adrenal medulla to the caudate putamen in two patients with Parkinson's disease. *New England Journal of Medicine* 1988; 318:51.
128. Backlund E, Granberg P, Hamberger B. Transplantation of adrenal medullary tissue to striatum in parkinsonism. *Journal of Neurosurgery* 1985; 62:169-173.
129. Date I, Asari S, Ohmoto T. Two-year follow-up study of a patient with Parkinson's disease and severe motor fluctuations treated by co-grafts of adrenal medulla and peripheral nerve into bilateral caudate nuclei: Case report. *Neurosurgery* 1995; 37:515-518.
130. Watts R, Subramanian T, Freeman A et al. Effect of stereotaxic intrastriatal cogafts of autologous adrenal medulla and peripheral nerve in Parkinson's disease: Two-year follow-up study. *Experimental Neurology* 1997; 147:510-517.
131. Dunnett S, Annett L, Lindvall O. Nigral transplants in primate models of parkinsonism. In: Bjorklund A, Widner H, eds. *Intracerebral Transplantation in Movement Disorders*. Amsterdam:Elsevier, 1991:27-51.
132. Lindvall O, Rehnström S, Brundin P et al. Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease: A detailed account of methodology and a 6-month follow-up. *Arch Neurol* 1989; 46:615-631.
133. Lindvall O, Brundin P, Widner H et al. Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* 1990; 247:574-577.
134. Lindvall O, Sawle G, Widner H et al. Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. *Annals of Neurology* 1994; 2:172-180.
135. Peschanski M, Defer G, N'Guyen J et al. Bilateral motor improvement and alteration of L-dopa effect in two patients with Parkinson's disease following intrastriatal transplantation of foetal ventral mesencephalon. *Brain* 1994; 117:487-499.
136. Widner H, Tetrad J, Rehnström S et al. Bilateral fetal mesencephalic grafting in two patients with severe parkinsonism induced by MPTP. *New England Journal of Medicine* 1992; 327:1556-1563.
137. Freed CR, Breeze RE, Rosenberg NL et al. Survival of implanted fetal dopamine cells and neurologic improvement 12 and 46 months after transplantation for Parkinson's disease. *New England Journal of Medicine* 1992; 327:1549-1555.
138. Kopyov O, Jacques D, Lieberman A et al. Outcome following intrastriatal fetal mesencephalic grafts for Parkinson's patients is directly related to the volume of grafted tissue. *Experimental Neurology* 1997; 146:536-545.
139. Lopez-Lozano J, Bravo G, Brera B et al. Long-term improvement in patients with severe Parkinson's disease after implantation of fetal ventral mesencephalic tissue in a cavity of

- the caudate nucleus: 5-year follow up in 10 patients. *Journal of Neurosurgery* 1997; 86:931-942.
140. Pedersen E, Poulsen F, Zimmer J et al. Prevention of mouse-rat brain xenograft rejection by a combination therapy of cyclosporin A, prednisolone and azathioprine. *Experimental Brain Research* 1995; 106:181-186.
141. Pakzaban P, Isacson O. Neural xenotransplantation: Reconstruction of neuronal circuitry across species barriers. *Neuroscience* 1994; 62:989-1001.
142. Isacson O, Breakefield XO. Benefits and risks of hosting animal cells in the human brain. *Nature Medicine* 1997; 3:964-969.
143. Ellias S, Palmer E, Kott S et al. Transplantation of fetal porcine ventral mesencephalic cells for treatment of Parkinson's disease: One year safety and efficacy results. *AAN Abstract* 1998; 1:13.
144. Kordower JH, Freeman TB, Snow BJ et al. Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. *New England Journal of Medicine* 1995; 332:1118-24.
145. Kordower J, Styren S, Clarke M, et al. Fetal grafting for Parkinson's disease: Expression of immune markers in two patients with functional fetal nigral implants. *Cell Transplantation* 1997; 6:213-219.
146. Kordower J, Freeman T, Chen E et al. Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. *Movement Disorders* 1998; 13:383-393.
147. Palfi S, Nguyen J, Brugieres P et al. MRI-stereotactical approach for neural grafting in basal ganglia disorders. *Experimental Neurology* 1998; 150:272-281.
148. Bakay R, Boyer K, Freed C et al. Immunological responses to injury and grafting in the central nervous system of nonhuman primates. *Cell Transplantation* 1998; 7:109-120.

Cellular and Molecular Treatments of Neurological Diseases

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This special issue on cellular and molecular research for novel treatments of neurological disease is the result of the Second Cellular and Molecular Treatments of Neurological Diseases (CMT) Conference held in Cambridge, Massachusetts, at the American Academy of Arts and Sciences in October 1998. The meeting brought together over 150 scientists and students working in the field and approached new treatments through three paradigms. First, neural transplantation as a cell replacement therapy was evaluated. The ground-breaking work of Olle Lindvall and others in the field was described by him and other speakers. The neural transplantation field is now beyond the proof of principle and much work remains to develop and optimize these procedures. The "stone age of neural transplantation" may soon be past; we look forward to many new discoveries and procedural improvements to make this procedure available to many patients with neural degenerative diseases. In this small and novel branch of neuroscience, we find ground-breaking work on very specialized systems that can be repaired by neural cell transplants. Professor Ray Lund described his work on the retinotectal system and how it can be repaired. Dr. Lund and colleagues previously demonstrated neural circuitry replacement in the retinotectal system and at this conference described recent work on photoreceptor layer reconstruction in mice with dystrophic photoreceptors of the retina.

At the Second CMT Conference at the AAAS in Cambridge, Massachusetts, the next two themes involved progenitor cell biology and cell differentiation, as well as gene therapy for neurological diseases. In the progenitor cell biology section, a number of eminent speakers, including Dr. McKay, Dr. van der Kooy, Dr. Whittemore, Dr. Mehler, Dr. Brüstle, and Dr. Svendsen described new ideas and conceptual developments of this field. Dr. Clive Svendsen and Oliver Brüstle demonstrated how progenitor cells can be implanted in various systems and used as therapeutic vehicles.

In the third theme of the conference, gene therapy for neurological diseases, a number of distinguished speakers described their work. The engineering of herpesvi-

ral vectors by Dr. Joseph Glorioso was a *tour de force* describing the stepwise approach to improved vector design. Dr. Ron Mandel elaborated on his elegant work on using gene therapy for the treatment of parkinsonism in animal models using recombinant AAV vectors. And finally, Dr. Howard Federoff described advanced vector technology and functional neurobiology necessary for transcriptional activation of posttranscriptional control in gene delivery and gene therapy. These communications at the conference provided a worthwhile learning experience, and we hope that the readers of *Experimental Neurology*, through this special issue, can enjoy some of this work as well.

This special issue begins with a paper by Kordower *et al.* demonstrating that cellular delivery of trophic factors can prevent degeneration typically seen in the striatum of Huntington's disease. These studies are based on findings of extensive neuroprotection caused by a number of factors in the striatum such as NGF, basic FGF, BDNF, and CNTF. The distinction between pharmacological and cellular delivery of factors is also elaborated by Kordower and coauthors. The initial finding of extensive neuroprotection by implanted cells producing NGF in the striatum by Schumacher *et al.* (13) was confirmed by a number of other studies, including genetically engineered progenitor cells producing NGF (12). A number of other studies have also shown that pharmacological treatment with NGF, while creating neuroprotection, was not as effective as the cell-based NGF delivery seen in other work (3, 9, 14). The extensive neuroprotection created by NGF and CNTF (4-7) indicates that the cell-based delivery of neurotrophic factors, while very specific, may also include dynamic receptor inductions, as well as improved efficacy through cofactors. The review by Kordower *et al.* discusses the extensive neuroprotection possible in a variety of animal models with relevance to Huntington's disease.

With regard to another part of the brain, Dr. Lund and colleagues have over a number of years shown us how complex circuitry reconstruction is possible using the transplant paradigm. Using transplantation as a

tool to investigate the plasticity of the nervous system, the group led by Dr. Lund has determined how adaptive the visual system can be with such procedures. In the current review by Dr. Kwan *et al.*, they demonstrate how the photoreceptor layer of the retina can be reconstructed through implantation of postnatal retina fragments. The dystrophic retinal layer, as seen in retinitis pigmentosa, is therefore a target for their research. The finding of simple light/darkness discrimination in the transplanted mice suggests that there is some functional rebuilding of the circuitry. These authors argue that logical circuits that are typical of peripheral nerve stimulation in the retina may also be a therapeutic target of future transplantation work.

The review by Wolfe *et al.* describes the steps necessary to engineer one class of viruses, the herpes simplex viruses (HSV), as vehicles for gene delivery and gene therapy. The systematic approach of determining the biology of the virus by these investigators illustrates that understanding of the genome is necessary prior to accomplishing effective gene delivery. In particular, the HSV vectors have been reengineered to control and use the latency promoter and regulatory regions. Moreover, in the elimination of toxic and replication-competent sequences, the HSV has been structured to maximize its transgene-carrying capacity while not eliminating its specific neurotropic characteristics. The experiments were designed to address the cell-targeting issues necessary for effective gene therapy in the CNS. Wolfe *et al.* also evaluate and discuss the neurodegenerative applications of such viruses as well as the potential application to cancer conditions (15, 16).

The recent work by Mandel *et al.* (10, 11) has demonstrated important new steps toward functional gene delivery in the striatum involving both dopamine-related production and neurotransmission, as well as neuroprotection first described by Bohn and Choi-Lundberg (1). The review reasons that the eventual engineering and development of viral vectors may provide a more continuous release of drugs in the brain. This obviously is still an unaccomplished goal but Mandel *et al.* argue that some of their work on recombinant engineering of adeno-associated viruses illustrates this point. Mandel and colleagues have shown that the co-infection of MD vectors containing the tyrosine hydroxylase gene, as well as the cofactor producing GTP cyclohydrolase, can improve the production of L-dopa in striatum through co-infection of cells. They demonstrated gene expression for up to 6 months. This work forms a data base for future studies toward more effective gene delivery and gene therapy paradigms in that system.

In Halterman and Federoff's work in this special issue, neurodegeneration caused by ischemic conditions is discussed. Their work elaborates on the molecular mechanisms involved in this damage and demon-

strates that the p53 transcriptional activators, as well as the PAS family proteins (here, HIF-1 α), can drive ischemia-induced changes toward degeneration of neurons in the brain. Their data and thinking support the idea that the pathologic gene expression seen under stressors can be modified by adaptive gene expression toward neuronal protection. In particular, Halterman and Federoff elucidate gene delivery as a tool in cell culture and *in vivo* applications to modify gene expression or pro-death gene expression (2, 8).

The CMT Conference at the American Academy of Arts and Sciences was an academic initiative through the Departments of Neurology and Psychiatry at Harvard and included a number of eminent lecturers and was organized by Drs. Ole Isacson and Xandra Breakefield. We hope that this conference series will continue and look forward to the next meeting in the year 2001. Meanwhile, the pages available in this forum of *Experimental Neurology* will surely provide communication on these and related issues.

REFERENCES

1. Bohn, M. C., and D. L. Choi-Lundberg. 1998. Gene therapies for Parkinson's disease. Pages 377-395 in E. A. Chiocca and X. O. Breakefield, Eds., *Gene Therapy for Neurological Disorders and Brain Tumors*. Humana Press, Totowa, NJ.
2. Carmeliet, P., Y. Dor, J.-M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, C. J. Koch, P. Ratcliffe, L. Moons, R. K. Jain, D. Collen, and E. Keshet. 1998. Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**: 485-490.
3. Davies, S. W., and K. Beardsall. 1992. Nerve growth factor selectively prevents excitotoxin induced degeneration of striatal cholinergic neurones. *Neurosci. Lett.* **140**: 161-164.
4. Emerich, D. F., S. Bruhn, Y. Chu, and J. H. Kordower. 1998. Cellular delivery of CNTF, but not NT-4/5, prevents degeneration of striatal neurons in a rodent model of Huntington's disease. *Cell Transplant.* **7**: 213-225.
5. Emerich, D. F., J. P. Hamang, E. E. Baetge, and S. R. Winn. 1994. Implantation of polymer-encapsulated human nerve growth factor-secreting fibroblasts attenuates the behavioral and neuropathological consequences of quinolinic acid injections into rodent striatum. *Exp. Neurol.* **130**: 141-150.
6. Emerich, D. F., S. R. Winn, P. M. Hantraye, M. Peschanski, E. Y. Chen, Y. Chu, P. McDermott, E. E. Baetge, and J. H. Kordower. 1997. Protective effect of encapsulated cells producing neurotrophic factor CNTF in a monkey model of Huntington's disease. *Nature* **386**: 395-399.
7. Emerich, D. R., M. D. Lindner, S. R. Winn, E. Y. Chen, B. R. Frydel, and J. H. Kordower. 1996. Implants of encapsulated human CNTF-producing fibroblasts prevent behavioral deficits and striatal degeneration in a rodent model of Huntington's disease. *J. Neurosci.* **16**: 5168-5181.
8. Endres, M., S. Namura, M. Shimizu-Sasamata, C. Waeber, L. Zhang, T. Gomez-Isla, B. Hyman, and M. Moskowitz. 1998. Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. *J. Cereb. Blood Flow Metab.* **18**: 238-247.
9. Kordower, J. H., V. Charles, R. Bayer, R. T. Bartus, S. Putney, L. R. Walus, and P. M. Friden. 1994. Intravenous administration

- of a transferrin receptor antibody-nerve growth factor conjugate prevents the degeneration of cholinergic neurons in a model of Huntington's disease. *Proc. Natl. Acad. Sci. USA* **91**: 9077-9080.
10. Mandel, R. J., K. G. Rendahl, K. S. Spratt, R. O. Snyder, L. K. Cohen, and S. E. Leff. 1998. Characterization of intrastriatal recombinant adeno-associated virus mediated gene transfer of human tyrosine hydroxylase and human GTP-cyclohydroxylase I in a rat model of Parkinson's disease. *J. Neurosci.* **18**: 4271-4284.
 11. Mandel, R. J., S. K. Spratt, R. O. Snyder, and S. E. Leff. 1997. Midgrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc. Natl. Acad. Sci. USA* **94**: 14083-14088.
 12. Martinez-Serrano, A., and A. Bjorklund. 1996. Protection of the neostriatum against excitotoxic damage by neurotrophin-producing, genetically modified neural stem cells. *J. Neurosci.* **16**: 4604-4616.
 13. Schumacher, J. M., M. P. Short, B. T. Hyman, X. O. Breakefield, and O. Isacson. 1991. Intracerebral implantation of nerve growth factor-producing fibroblasts protects striatum against neurotoxic levels of excitatory amino acids. *Neuroscience* **45**: 561-570.
 14. Venero, J. L., K. D. Beck, and F. Hefti. 1994. Intrastriatal infusion of nerve growth factor after quinolinic acid prevents reduction of cellular expression of choline acetyltransferase messenger RNA and trkA messenger RNA, but not glutamate decarboxylase messenger RNA. *Neuroscience* **61**: 257-268.
 15. Wilson, S. P., D. C. Yeomans, M. A. Bender, Y. Lu, W. F. Goins, and J. C. Glorioso. 1999. Antihyperalgesic effects of infection with a preproenkephalin encoding herpes virus. *Proc. Natl. Acad. Sci. USA* **96**: 3211-3216.
 16. Yamada, M., T. Oligino, M. Mata, J. R. Goss, J. C. Glorioso, and D. J. Fink. 1999. HSV vector-mediated expression of Bcl-2 prevents 6-hydroxydopamine induced degeneration of neurons in the substantia nigra in vivo. *Proc. Natl. Acad. Sci. USA* **96**: 4078-4083.

Immunophilin Ligands and GDNF Enhance Neurite Branching or Elongation from Developing Dopamine Neurons in Culture

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Neurotrophic effects of immunophilin ligands have been shown in animal models of peripheral and central nervous system insult. To investigate the specific growth-promoting effects of these compounds, we examined the effects of various immunophilin ligands on primary dopamine (DA) neurons in culture and compared these with a well-known DA trophic factor, glial cell line-derived neurotrophic factor (GDNF). In neuronal cultures from Embryonic Day 14 ventral mesencephalon, enhanced *elongation* of DA neurites was observed with immunophilin ligands, which inhibited the phosphatase activity of calcineurin (FK506 and cyclosporin A) when compared to vehicle-treated cultures. This elongation was also observed with GDNF, known to exert its trophic effects through phosphorylation-dependent pathways. In contrast, immunophilin ligands that do not inhibit calcineurin (rapamycin and V-10,367) increased *branching* of DA neurites, suggesting that elongation is dependent upon maintained phosphorylation while branching is not. In addition, both V-10,367 and rapamycin antagonized the elongation effects of FK506 and induced branching. The antagonism of elongation (and reappearance of branching) illustrates the intrinsic abilities of developing DA neurons to *either* elongate or branch, but not both. We show that the immunophilin FKBP12 (12-kDa FK506-binding protein) is expressed in ventral mesencephalic neuronal cultures and colocalizes with DA neurons. This work elucidates the specific growth-promoting effects by which GDNF and immunophilin ligands modify developmental growth processes of DA neurons, via their interactions with intracellular targets.

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Key Words: immunophilin; FKBP12; neurite; GDNF; calcineurin; dopamine cultures; FK506; rapamycin.

INTRODUCTION

The regulation of neurite elongation and branching is fundamental for establishing neuronal circuitry (49, 59). Recently, immunosuppressive drugs such as

FK506 and cyclosporin A (CsA) have been shown to enhance neurite outgrowth from various cell lines and peripheral nerve preparations (50, 67), as well as primary CNS neurons in culture (10). These compounds also protect against peripheral and central nervous system insult (10, 26, 65, 70), alter long term potentiation, (LTP) and long term depression, (LTD) (21, 73), increase the rate of peripheral nerve regeneration (25), and regulate neurotransmitter release (58, 69). The observed effects are partially or fully mediated by specific intracellular pathways that are affected by these ligands, but that remain unclear in the context of neuronal cell systems. In the immune system, the immunophilin ligand FK506 complexes with the immunophilin FKBP12 (12-kDa FK506-binding protein). This drug-immunophilin complex then binds to and inhibits the phosphatase activity of calcineurin (48), augmenting the phosphorylation of several substrates (12, 28, 42, 52, 64, 68), which leads to inhibition of cytokine synthesis and immunosuppression (20). Cyclosporin A also inhibits calcineurin, but through its interaction with the immunophilin cyclophilin B.

High levels of FKBP12 and cyclophilin in the brain (68) and their colocalization with calcineurin suggested that the neuronal trophic effects may also act through this intracellular pathway. The neuroprotective effects seen with immunophilin ligands in models of neurodegenerative disorders have led to the design of small-molecule ligands that bind to immunophilins, but are not immunosuppressive (do not interact with calcineurin (3)). One of these novel immunophilin ligands, V-10,367, has an affinity to FKBP12 similar to that of FK506 (K_i of 0.5 nM) and does not decrease the phosphatase activity of calcineurin (3). However, trophic effects are retained even in the absence of calcineurin inhibition: V-10,367 potentiates neuronal growth factor (NGF)-induced neurite outgrowth from immortalized cells in culture (26). In addition, neuroprotective and regenerative effects of these novel immunophilin ligands have been obtained in animal models of peripheral and central nervous system insult (10, 26, 70). We

TABLE 1

Immunophilin Ligands

Compound	Immunophilin interaction	Inhibition of calcineurin
FK506	FKBP12	Yes
CsA	Cyclophilin	Yes
Rapamycin	FKBP12	No
V-10,367	FKBP12	No

recently demonstrated neurotrophic effects of V-10,367 in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (10). Striatal dopaminergic (DA) innervation was spared from MPTP-induced degeneration in animals orally treated with V-10,367, while treatment with FK506 did not cause this effect. These studies suggest that inhibition of this phosphatase may not be required for the trophic effects obtained with these ligands in the nervous system.

To determine the specific effects of these compounds on the DA system, we evaluated the growth-promoting effects of these molecules on primary ventral mesencephalic (VM) DA neurons in culture. Comparisons were made between immunophilin ligands that interact with different intracellular targets and the trophic effects of glial cell line-derived neurotrophic factor (GDNF).

MATERIALS AND METHODS

Primary Ventral Mesencephalic Cultures

Primary cultures of DA neurons (previously shown to be 95% neuronal) were obtained from E14 Sprague-Dawley rat (Charles River, MA) ventral mesencephalon (VM) as described previously (10, 11). Briefly, tissue was dissociated by incubation in 0.025% trypsin solution (37°C, 15 min; Sigma) and triturated in a solution of DNase (0.01%; Sigma) and trypsin inhibitor (0.05%; Sigma). Isolated cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY) containing heat-inactivated horse serum (10%), glucose (6.0 mg/ml), penicillin (10,000 U/ml), streptomycin (10 mg/ml; Sigma), and glutamine (2 mM; Gibco). Five hundred microliters of suspension containing 5×10^5 cells/ml was plated into each well of 24-well trays (Falcon), precoated with poly-L-lysine (Sigma), containing 500 μ l of serum-containing (S+) medium. Unattached cells were aspirated after 1 h, and 1 ml of fresh S+ medium containing immunophilin ligands (Table 1), GDNF, or vehicle (DMSO diluted at equivalent concentrations (1:1000) per well) was added. At 1 day in culture, the medium was replaced with defined medium (containing N2 cocktail; Gibco) containing immunophilin ligands or GDNF (using 30 kDa as the molec-

ular weight of GDNF, the doses of 0.001–100 ng/ml convert to 33 pM–3.3 nM). At 2 days *in vitro* (DIV), cultures were fixed for 1 h with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS).

Immunohistochemistry

Cells were incubated in primary antibody against tyrosine hydroxylase (TH; 1:500; Pel-Freez, Rogers, AK) for 48 h at 4°C; antibody binding was visualized with reaction in 0.05% 3,3'-diaminobenzidine (DAB, Sigma). To study the expression of the immunophilin FKBP12 within VM neurons, cultures were double-labeled with antibodies against TH and FKBP12 (1:2000; Pharmingen, CA) and processed for immunocytochemical detection with texas red- and fluorescein-conjugated secondary antibody (1:200; Jackson ImmunoResearch; West Grove, PA). Fluorescent images of double-labeled FKBP12 and TH was visualized using a confocal argon laser scanning microscope (Leika), processed with Leika Physiology Module Software.

Quantification of Neuron Survival and Neurite Outgrowth

For neuron survival, four random fields at each corner of each well were selected for cell counts, as previously described (10): at 200 $\times$ magnification, and using an eyepiece grid, the number of cells in each 200 $\times$ 20- μ m corner and center of the grid was counted (5 counts per field). Counts consisted of the total number of neurons in each grid region for neuronal viability (defined morphologically in phase contrast and confirmed by trypan blue exclusion test), as well as the number of TH+ neurons. For analysis of neurite elongation and branching, two fields from opposite sides of each well were captured in Adobe Photoshop, and 10 randomly chosen TH+ neurons per field were analyzed. Each neurite from a TH+ neuron was traced and measured (in millimeters) to determine *elongation* effects, and the number of neurites extending from a single TH+ neuron was counted to determine *branching* effects (Fig. 1). The measurements from the 10 TH+ neurons per field were averaged to obtain two measurements per well. Each dose of each compound was run in duplicate per experiment, and data are expressed as percentage control from two to four pooled experiments (each dose in duplicate per experiment, for $n = 8$ –16 per dose). All comparisons were evaluated using analysis of variance (ANOVA) in JMP Version 3.1 (SAS Institute, NC). When significance was obtained, post-hoc Tukey-Kramer HSD was performed to compare significant differences between groups ($*P < 0.05$). Error bars represent SEM.

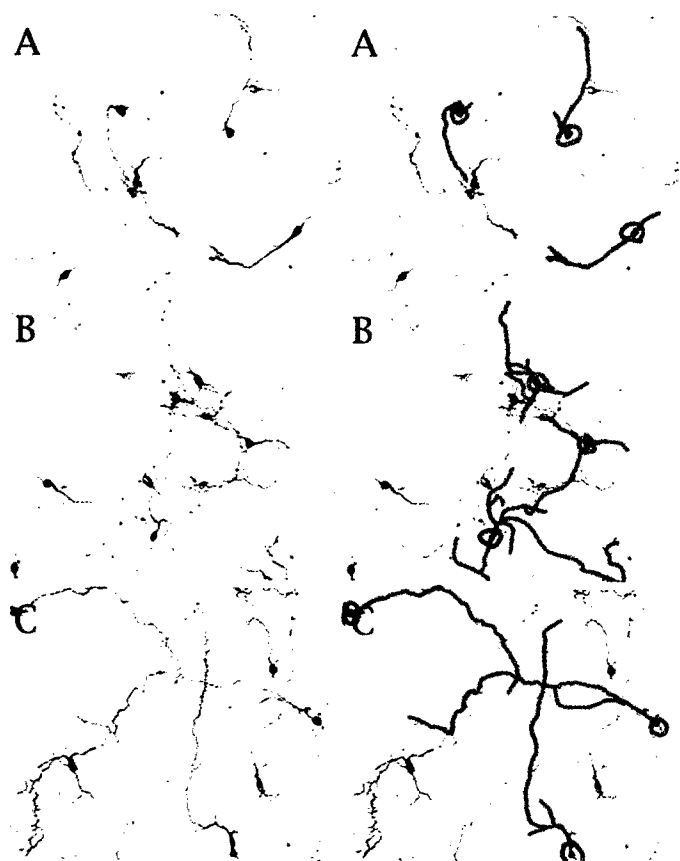


FIG. 1. Measurement of distinct effects of immunophilin ligands on neurite outgrowth of primary DA neurons. Parallel panels illustrate the method of measuring DA neurite outgrowth (stained for TH) in three representative cells (see Materials and Methods, and as quantified in Fig. 2): each TH+ neurite (outlined in red) was traced, counted, and measured from TH+ cell bodies (circled in green). (A) Neurite outgrowth of DA neurons from untreated primary cultures of E14 VM after 2 days in culture. (B) Enhanced *branching* of TH+ neurites from DA neurons after 2-day treatment with 1.0 μ M V-10,367. (C) Enhanced *elongation* of TH+ neurites from DA neurons after 2-day treatment with 1.0 μ M FK506.

RESULTS

To better define intracellular targets and mechanisms of the trophic effects observed with immunophilin ligands, we utilized a system of primary DA neurons from E14 VM. By immunostaining VM cultures for TH (used here as a marker for DA neurons, Fig. 1) we investigated the effects of immunophilin ligands (Table 1) on neurite outgrowth. After 2 days in culture, the vehicle-treated primary DA neurons developed fairly short, unbranched TH+ neurites (Fig. 1A). Treatment of cultures for 2 days with the immunophilin ligand V-10,367 produced a significantly larger number of neurites from each TH+ neuron (Fig. 1B), whereas treatment with FK506 increased the length of neurites from TH+ neurons (Fig. 1C). We quantified these effects as previously described (10). Briefly, each neurite from a TH+ neuron was traced and measured

(in millimeters) to determine its length (elongation), and the number of neurites extending from each TH+ neuron was counted (branching). To determine effects of immunophilin ligands or GDNF on neuronal viability, the total numbers of surviving neurons were analyzed: quantification revealed no effect of treatment with any of the immunophilin ligands or GDNF on total number of neurons (mean number of neurons per field = 128.75; $P > 0.768$) or TH+ neurons (mean number of TH+ neurons per field = 8.31; $P > 0.258$) after 2 days in culture.

Elongation of Developing DA Neurites

To compare the trophic effects of immunophilin ligands to a well-known trophic factor for DA neurons, we first determined the specific effects of GDNF on neurite development. Neurite outgrowth was significantly influenced by 2-day treatment with GDNF (Fig. 2A): the average length of TH+ neurites was significantly longer than that of those in vehicle-treated cultures at a range of doses. We then compared this effect with two immunophilin ligands; FK506 (Fig. 2B) and CsA (Fig. 2C) that bind their respective immunophilins and subsequently inhibit calcineurin. Both FK506 and CsA significantly enhanced elongation of neurites from TH+ neurons. We then analyzed two immunophilin ligands that do not inhibit calcineurin: V-10,367 (Fig. 2D) and rapamycin (Fig. 2E). These compounds had no effect on the length of TH+ neurites. Interestingly, rapamycin showed a significant decrease in the length of neurites at some doses (Fig. 2E).

Branching of Developing DA Neurites

We next determined whether GDNF could affect the number of neurites extending from primary DA neurons. In contrast to elongation, neurite branching was not enhanced with GDNF (Fig. 3A). On the contrary, there tended to be fewer neurites per TH+ neuron than in vehicle-treated cultures at one dose. FK506 did not enhance branching at the doses that caused elongation of neurites (Fig. 3B); however, at one low dose (0.01 μ M), a higher number of neurites was observed. The immunophilin ligand CsA had no effect on branching (Fig. 3C). In contrast, the two compounds that do not inhibit calcineurin (V-10,367 and rapamycin) showed significantly enhanced branching of TH+ neurites (Figs. 3D and 3E, respectively). The most robust effect with V-10,367 was observed at 0.05 μ M, when DA neurons developed 2 $\times$ the number of TH+ neurites that the vehicle-treated cultures developed.

Relationship between Branching and Elongation of Developing DA Neurites

In all cases where a compound produced a significant effect on DA neurite development (as previously ob-

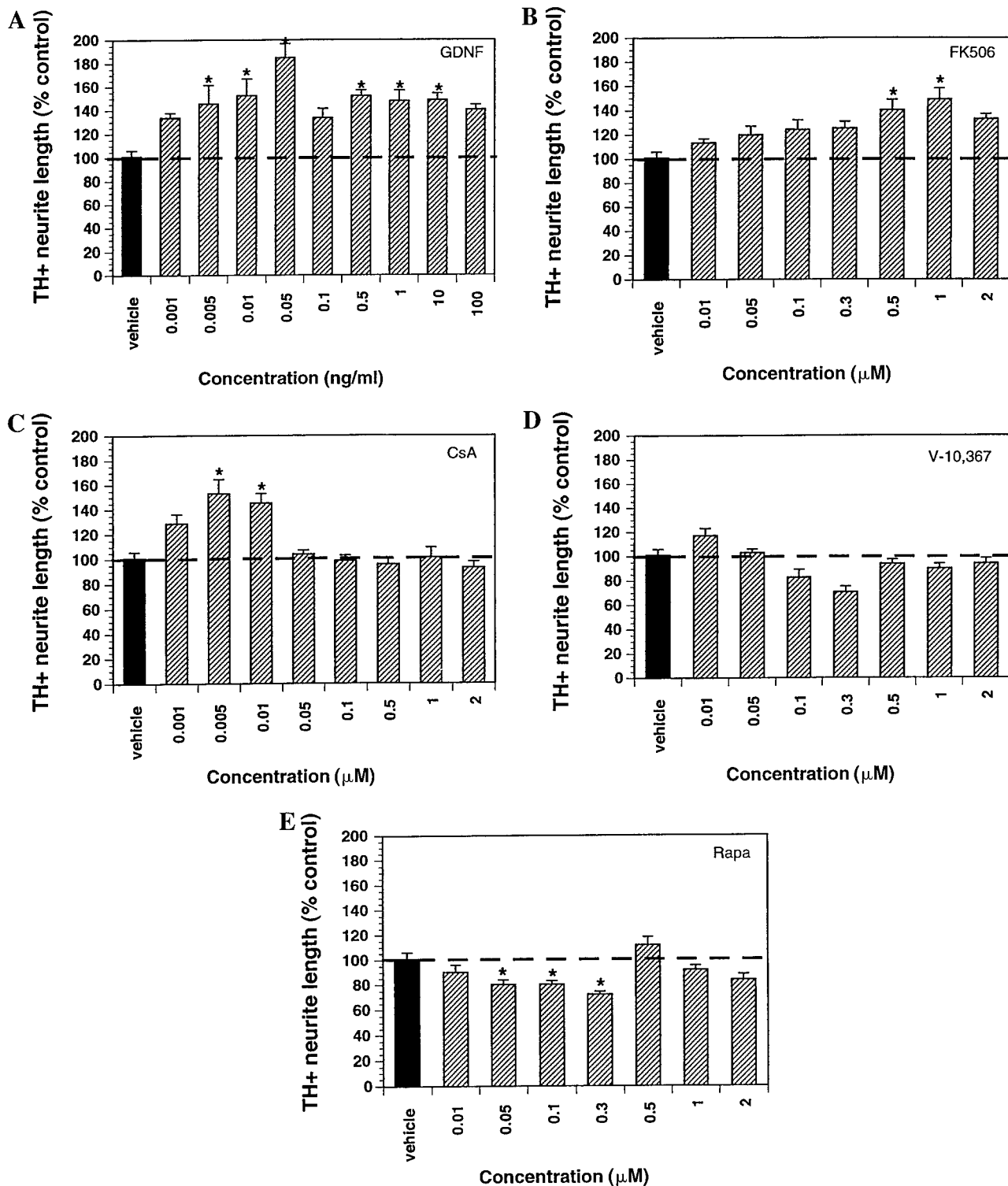


FIG. 2. Elongation of developing DA neurites. Length of TH+ neurites (percentage control) from primary cultures of E14 VM after 2 days of treatment. GDNF produced significantly longer neurites from TH+ neurons (A), as did FK506 (B) and CsA (C). The immunophilin ligand V-10,367, which does not inhibit the phosphatase calcineurin, showed no change in length of TH+ neurites (D), while rapamycin, which also does not inhibit calcineurin, showed a significant decrease in length of TH+ neurites (E). Tukey-Kramer HSD, $*P < 0.05$; error bars represent SEM.

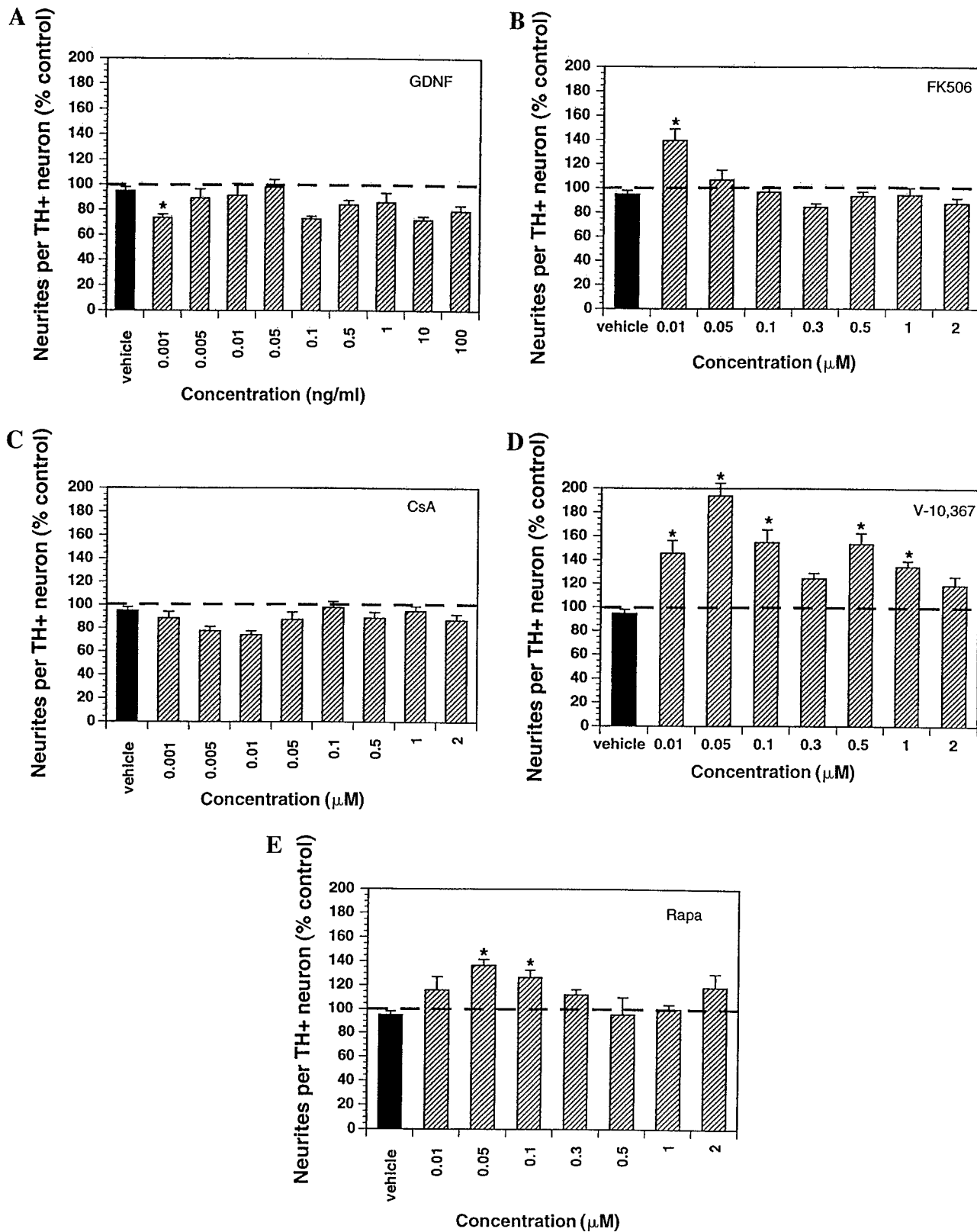


FIG. 3. Branching of developing DA neurites. Number of TH+ neurites (percentage control) from primary cultures of E14 VM after 2 days of treatment. GDNF showed a decrease in branching of TH+ neurites only at the lowest dose (A), while FK506 showed a slight branching effect at its lowest dose (B). CsA showed no effect on branching of TH+ neurites (C). V-10,367 (D) and rapamycin (E), compounds that do not inhibit the phosphatase calcineurin, both showed significantly enhanced branching of TH+ neurites. Tukey-Kramer HSD, $*P < 0.05$; error bars represent SEM.

served with classic neurotrophic factors (36)), a bell-shaped dose-response curve was apparent (see Figs. 2 and 3). In several cases when neurons showed enhanced elongation they did not simultaneously show increased branching, and vice versa. For instance, two doses of rapamycin (0.05 and 0.1 μM) significantly enhanced branching (Fig. 3E), whereas these doses showed significantly shorter TH+ neurites (Fig. 2E). Conversely the elongation produced by 0.005 and 0.01 μM CsA (Fig. 2C) was paralleled by a trend toward decreased branching at these doses (Fig. 3C). Similarly, the only dose of FK506 that did induce branching (0.01 μM , Fig. 3B) showed the lowest value of neurite length (Fig. 2B).

Pharmacological Targets of Trophic Effects

To further define the intracellular mechanisms involved in the elongation and branching observed, we determined the presence of the immunophilin FKBP12 in VM cultures. FKBP12 was expressed in these neurons after 2 days in culture (Fig. 4A) and colocalized with TH+ neurons (Figs. 4B and 4C). We used combinations of V-10,367 or rapamycin with FK506 to determine whether the elongation effect of FK506 involved signaling through calcineurin via its interaction with FKBP12. Rapamycin and V-10,367 also bind FKBP12 and compete with FK506 for FKBP12 binding sites. FK506 (1.0 μM) did not elongate TH+ neurites in the presence of V-10,367 or rapamycin (0.5 and 1.0 μM , Fig. 4D). This effect was observed with 0.5 μM FK506 as well (data not shown). However, V-10,367 and rapamycin were able to induce branching in the presence of FK506 (Fig. 4E).

DISCUSSION

Distinct Growth-Promoting Effects of Immunophilin Ligands

In the present study, we show that immunophilin ligands can enhance branching or elongation of neurite outgrowth from DA neurons. The identification of distinct aspects of neurite development allowed us to pharmacologically dissect specific effects of various immunophilin ligands and compare their effects with those of GDNF. The distinction between branching and elongation of neurites has previously been demonstrated when developing DA neurons are grown in the presence of growth factors (1, 5, 71) or their target striatal cells (14, 34, 62). For instance, exposure of VM neurons to conditioned medium from VM induces growth of dendrite-like neurites (short with a high number of branches), while striatal-conditioned medium stimulates growth of axon-like neurites (62). Though we did not differentiate between axons and dendrites (90% of neurites were MAP+, making selec-

tive analysis unreliable, Costantini *et al.*, unpublished observations), independent regulation of axonal and dendritic initiation and elongation from DA neurons may play a role in the effects observed here. Studies have shown GDNF-induced neurite outgrowth from peripheral (16, 44) and VM neurons in culture (18, 36, 47) or after transplantation of VM tissue into adult brain (2, 29, 40, 61, 63, 80). Though the most prominent influence of GDNF upon developing DA neurons is enhanced survival (17, 43, 46), we did not observe this effect, most likely due to the short time course of our studies.

Several studies have observed enhanced neurite outgrowth with immunophilin ligands that do not inhibit calcineurin (26, 67); however these studies utilized transformed cell lines (dependent on NGF for outgrowth) and DRG explants and did not quantify differences between elongation and branching. The primary DA neuron cultures used in the present study were chosen as a more physiologic system for analyzing neurite outgrowth than growth factor-responsive cell lines or peripheral culture systems.

Generally, growth-promoting effects of neurotrophic factors depend on receptor activation. The primary intracellular receptors for the immunophilin ligands are FKBP and cyclophilins, immunophilins that are highly expressed in the adult rat brain (13, 68). The expression of FKBP12 in fetal VM cultures shown here suggests a physiological role for this protein in developing DA neurons. The observed expression of FKBP12 in all cells in VM cultures suggests that the immunophilin ligands may also affect the GABA neurons present in these cultures. However, these GABA neurons do not extend neurites to the same extent as DA neurons after 2 days in culture. GABA markers stained approximately 90% of the cells and neurites, making analysis of neurites from individual cells unreliable. No obvious changes in neurite outgrowth were observed in this neuronal population after treatment with immunophilin ligands or GDNF (Costantini *et al.*, unpublished observations). Alternatively, the immunophilin ligands may be acting through other immunophilins such as FKBP13 (77), FKBP25 (22), or FKBP52 (24, 72).

The receptor for CsA is the immunophilin cyclophilin (31), also expressed in the substantia nigra (13). Though CsA shows an affinity for its immunophilin that is 10-fold lower than FK506 has for FKBP12 (67), we observed enhanced elongation at lower doses with CsA than with FK506. This suggests that pathways other than the CsA/cyclophilin interaction may be involved. In contrast to our results, CsA prevented axonal elongation in cultured cerebellar neurons with no effect on neurite formation (19) and did not increase axonal regeneration in the sciatic nerve model (78), suggesting that cyclophilin-mediated trophic effects may not be active in these specific neuronal systems.

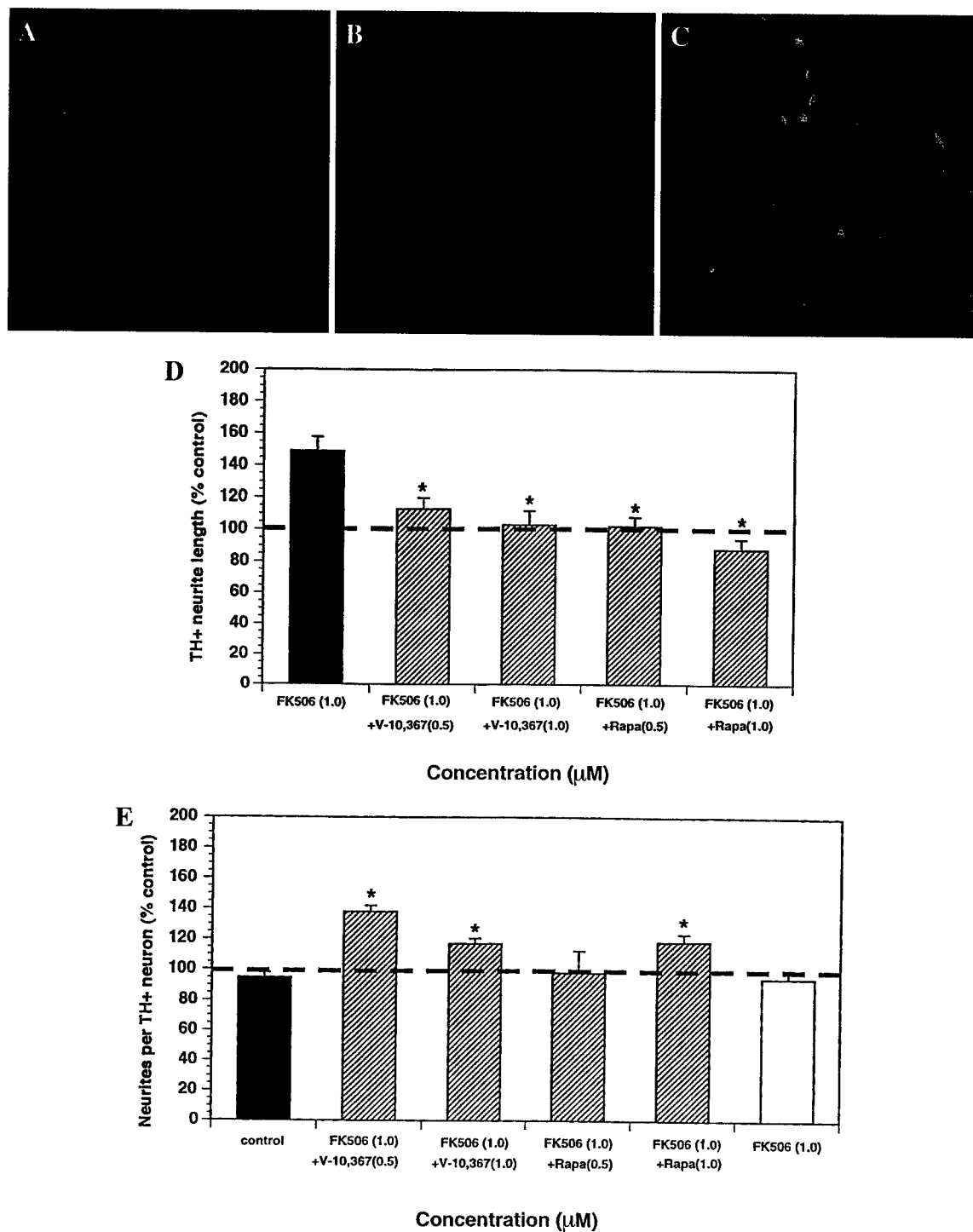


FIG. 4. Intracellular interactions of immunophilin ligands. (A) The immunophilin FKBP12 (green) is expressed in all neurons of primary cultures from E14 VM at 2 DIV. (B) The same field showing TH+ neurons (red). (C) Colocalization of FKBP12 and TH+ neurons (yellow). (D) FK506-induced elongation can be inhibited by compounds that compete for FKBP12-binding sites, such as V-10,367 and rapamycin. (E) Branching can occur with V-10,367 and rapamycin in the presence of FK506. Tukey-Kramer HSD, $*P < 0.05$; error bars represent SEM.

The bell-shaped immunophilin ligand dose-response curves in the present study suggest an optimal immunophilin ligand concentration for maximal trophic effects. Such effects have previously been explained by the "set-point hypothesis" of both growth factors and

Ca²⁺ levels (41, 55), where optimal trophic effects are observed with a mid level of growth factor or Ca²⁺ level and low or high levels produce suboptimal or even toxic effects. Consistent with our present results (though inherent variability may also be responsible for the bell

shapes of these curves), a bell-shaped dose-response curve has been observed for optimal trophic effects of GDNF and FK506 (36, 78). Neurite sprouting and elongation are promoted only when intracellular Ca^{2+} levels are within a permissive range (53–55, 60). Immunophilin ligands have been shown to regulate Ca^{2+} levels via their interactions with two Ca^{2+} channels found in the brain, the ryanodine receptor (RyR), and the inositol triphosphate receptor (IP3R) (7, 8, 37, 38, 66). Furthermore, these receptors are expressed in the primary VM neurons used in the present studies (Costantini, unpublished observations).

Role of Phosphorylation in Elongation or Branching of DA Neurites

We observed enhanced elongation with immunophilin ligands that inhibit the phosphatase activity of calcineurin (48) through their binding to their respective immunophilins (31, 32), as well as with GDNF which requires phosphorylation for its trophic effects (35). Calcineurin is expressed in the substantia nigra (13, 27), and its inhibition maintains the phosphorylation levels of several substrates (12, 28, 42, 52, 64, 68) that are critically involved in neuronal outgrowth (51, 54). Moreover, inhibition of calcineurin by a FK506 analog (ascomycin) stabilized F-actin in cultured hippocampal neurons (30), suggesting a role for calcineurin in cytoskeletal systems during neurite elongation (28).

In our study, the elongation observed with ligands inhibiting calcineurin was also observed with GDNF. The GDNF receptor system consists of GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) and the tyrosine kinase Ret (39), both of which are expressed in developing VM (4, 18, 74, 79). Activation of Ret tyrosine kinase activity by GDNF results in formation of neuritogenesis-associated lamellipodia (75), and tyrosine kinase inhibitors suppress GDNF-induced neurite outgrowth in cell lines (35). Thus, the compounds in our study that showed enhanced elongation of DA neurites are known to maintain phosphorylation levels within the cell, either through kinase activation (GDNF) or through inhibition of calcineurin (FK506 and CsA). Assays determining phosphorylation states of growth-associated proteins, such as GAP43 (6), microtubule-associated proteins (45), nitric oxide synthase (12), actin depolymerization factor (56), and GTPases (23) are required to fully evaluate this hypothesis. Alternatively, the folding of cytoskeletal proteins, receptors, or ion channels (33) through the rotamase-inhibitory effects of immunophilin ligands may alter extension and branching of developing neurites (32, 33).

Given the enhanced branching of DA neurites observed with rapamycin and V-10,367, a mechanism independent of calcineurin inhibition for trophic effects must be in play. Other evidence supporting a cal-

cineurin-independent trophic mechanism is the neuroprotection and regeneration observed with immunophilin ligands that do not inhibit calcineurin (10, 26, 67). We investigated the role of calcineurin in these growth-promoting effects by combining either V-10,367 or rapamycin with FK506. Rapamycin is a competitive inhibitor of FK506 due to its higher affinity for FKBP12 (15) (and we found that rapamycin produced its trophic effects on DA neurites at concentrations lower than those of FK506, reflecting a higher potency). Both rapamycin and V-10,367 antagonized the effects of FK506 on elongation, presumably by competing for FKBP12-binding sites and decreasing the ability of FK506 to inhibit calcineurin. At the lower doses of competing V-10,367 and rapamycin, FK506-induced elongation was blocked but no branching occurred. However, at the higher doses of V-10,367 and rapamycin, branching did occur, even in the presence of FK506. These results support the requirement for maintained phosphorylation to obtain enhanced elongation of DA neurites.

The antagonism of elongation (and reappearance of branching) elucidate the intrinsic abilities of developing DA neurons to *either* elongate or branch, but not both. During development, when DA axons reach their target, they stop elongating and begin to branch extensively (57). Growth cone motility is directly related to neurite branching and stabilizes outgrowth by holding elongation rate at a submaximal level (55). In this way, under conditions of neurite branching (as with V-10,367 and rapamycin), neurites would show reduced elongation.

In contrast to our observations, the enhancement of NGF-induced outgrowth from PC12 cells and DRG by FK506 was not blocked by rapamycin, yet rapamycin alone enhanced outgrowth in these systems (50, 70). In a separate study, FK506 and CsA *inhibited* the NGF-induced neurite extension and neuritogenesis from DRG, while rapamycin inhibited only extension (9). The intracellular pathways present in PC12 and DRG cells are different than those in the primary DA culture system used in the present study, in which we observe rapamycin-associated pathways contributing to increased branching of DA neurites.

In conclusion, this study provides insight into the growth-promoting effects of immunophilin ligands on the DA system. Dopaminergic neurons of the substantia nigra start elongating axons toward their striatal target soon after final differentiation around Embryonic Day 12; then these axons branch extensively to innervate the striatum (76). During this phase, the developing neuron (both in culture and in brain) is expressing and responding to specific proteins that affect neuritogenesis and elongation (6, 17). The contrasting effects we observed on branching and elongation by different immunophilin ligands and GDNF suggest that these molecules differentially modify the

activities, expression, or structural states of proteins involved in neurite outgrowth. These observations may be relevant to recent trophic effects observed *in vivo* with immunophilin ligands (10, 26, 67). Additional studies are required to further elucidate the biochemical and molecular pathways involved in these growth-promoting effects.

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REFERENCES

- Aoyagi, A., K. Nishikawa, H. Saito, and K. Abe. 1994. Characterization of basic fibroblast growth factor-mediated acceleration of axonal branching in cultured rat hippocampal neurons. *Brain Res.* **661**: 117–126.
- Apostilides, C., E. Sanford, M. Hong, and I. Mendez. 1998. Glial cell line-derived neurotrophic factor improves intrastriatal graft survival of stored dopaminergic cells. *Neuroscience* **83**: 363–372.
- Armistead, D. M., M. C. Badia, D. D. Deininger, J. P. Duffy, J. O. Saunders, R. D. Tung, J. A. Thomson, M. T. DeCenzo, O. Futer, D. J. Livingston, M. A. Murcko, M. M. Yamashita, and M. A. Navia. 1995. Design, synthesis, and structure of non-macrocyclic inhibitors of FKBP12, the major binding protein for the immunosuppressant FK506. *Acta. Crystallogr.* **D51**: 522–528.
- Baloh, R., M. Tansey, J. Golden, D. Creedon, R. Heuckeroth, C. Keck, D. Zimonjic, N. Popescu, E. Johnson, and H. Milbrandt. 1997. TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. *Neuron* **18**: 793–802.
- Beck, K. D., B. Knusel, and F. Hefti. 1993. The nature of the trophic action of brain-derived neurotrophic factor, sed(1-3)-insulin-like growth factor-1, and basic fibroblast growth factor on mesencephalic dopaminergic neurons developing in culture. *Neuroscience* **52**: 855–866.
- Benowitz, L. I., and A. Routtenberg. 1997. GAP-43: An intrinsic determinant of neuronal development and plasticity. *Trends Neurosci.* **20**: 84–91.
- Brillantes, A. B., K. Ondrias, A. Scott, E. Kobrinsky, E. Ondriasova, M. C. Moschella, T. Jayaraman, M. Landers, B. E. Ehrlich, and A. R. Marks. 1994. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77**: 513–523.
- Cameron, A. M., J. P. Steiner, D. M. Sabatini, A. I. Kaplin, L. D. Walensky, and S. H. Snyder. 1995. Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc. Natl. Acad. Sci. USA* **92**: 1784–1788.
- Chang, H., K. Takei, A. Sydor, T. Born, F. Rusnak, and D. Jay. 1995. Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin. *Nature* **376**: 686–690.
- Costantini, L., P. Chaturvedi, D. Armistead, P. McCaffrey, T. Deacon, and O. Isacson. 1998. A novel immunophilin ligand: Distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. *Neurobiol. Dis.* **5**: 97–106.
- Costantini, L. C., L. Lin, and O. Isacson. 1997. Medial fetal ventral mesencephalon: A preferred source for dopamine neuron grafts. *Neuroreport* **8**: 2253–2257.
- Dawson, T. M., J. P. Steiner, V. L. Dawson, J. L. Dinerman, G. R. Uhl, and S. H. Snyder. 1993. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate toxicity. *Proc. Natl. Acad. Sci. USA* **90**: 9808–9812.
- Dawson, T. M., J. P. Steiner, W. E. Lyons, M. Fotuhi, M. Blue, and S. H. Snyder. 1994. The immunophilins, FK506 binding protein and cyclophilin, are discretely localized in the brain: Relationship to calcineurin. *Neuroscience* **62**: 569–580.
- Denis-Donini, S., J. Glowinski, and A. Prochiantz. 1983. Specific influence of striatal target neurons on the *in vitro* outgrowth of mesencephalic dopaminergic neurites: A morphological quantitative study. *J. Neurochem.* **3**: 2292–2299.
- Dumont, F., M. Melino, M. Staruch, S. Koprak, P. Fischer, and N. Sigal. 1990. The immunosuppressive macrolides FK506 and rapamycin act as reciprocal antagonists in murine T cells. *J. Immunol.* **144**: 1418–1424.
- Ebendal, T., A. Tomac, B. Hoffer, and L. Olson. 1995. Glial cell line-derived neurotrophic factor stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia. *J. Neurosci. Res.* **40**: 276–284.
- Engel, J. 1998. Spatial and temporal growth factor influences on developing midbrain dopaminergic neurons. *J. Neurosci. Res.* **53**: 405–414.
- Feng, L., C. Wang, H. Jiang, C. Oho, K. Mizuno, M. Dugich-Djordjevic, and B. Lu. 1999. Differential effects of GDNF and BDNF on cultures ventral mesencephalic neurons. *Mol. Brain Res.* **66**: 62–70.
- Ferreira, A., R. Kincaid, and K. Kosik. 1993. Calcineurin is associated with the cytoskeleton of cultured neurons and has a role in the acquisition of polarity. *Mol. Biol. Cell* **4**: 1225–1238.
- Fruman, D., C. Klee, B. Bierer, and S. Burakoff. 1992. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK506 and cyclosporin A. *Proc. Natl. Acad. Sci.* **89**: 3686–3690.
- Funauchi, M., H. Haruta, and T. Tsumoto. 1994. Effects of an inhibitor for calcium/calmodulin-dependent protein phosphatase, calcineurin, on induction of long-term potentiation in rat visual cortex. *Neurosci. Res.* **19**: 269–278.
- Galat, A., W. Lane, R. Standaert, and S. Schreiber. 1992. A rapamycin-selective 25-kDa immunophilin. *Biochemistry* **31**: 2427–2434.
- Gallo, G., and P. Letourneau. 1998. Axon guidance: GTPases help axons reach their targets. *Curr. Biol.* **8**: R80–R82.
- Gold, B., V. Densmore, W. Shou, M. Matzuk, and H. Gordon. 1999. Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. *J. Pharmacol. Exp. Ther.* **289**: 1202–1210.
- Gold, B. G., K. Katoh, and T. Storm-Dickerson. 1995. The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. *J. Neurosci.* **15**: 7509–7516.
- Gold, B. G., M. Zeleny-Pooley, M. S. Wang, P. Chaturvedi, and D. M. Armistead. 1997. A non-immunosuppressive FKBP-12 ligand increases nerve regeneration. *Exp. Neurol.* **147**: 269–278.
- Goto, S., Y. Matsukado, Y. Mihara, N. Inoue, and E. Miyamoto. 1986. The distribution of calcineurin in the rat brain by light and electron microscopic immunohistochemistry and enzyme-immunoassay. *Brain Res.* **397**: 161–172.
- Goto, S., H. Yammamoto, K. Fukunaga, T. Iwasa, Y. Matsukado, and E. Miyamoto. 1985. Dephosphorylation of microtubule-associated protein 2, tau factor, and tubulin by calcineurin. *J. Neurochem.* **45**: 276–283.
- Granholm, A., J. Mott, K. Bowenkamp, S. Eken, S. Henry, B. Hoffer, P. Lapchak, M. Palmer, C. van Horne, and G. Gerhardt. 1997. Glial cell line-derived neurotrophic factor improves sur-

- vival of ventral mesencephalic grafts to the 6-hydroxydopamine lesioned striatum. *Exp. Brain Res.* **116**: 29–38.
30. Halpain, S., A. Hipolito, and L. Saffer. 1998. Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. *J. Neurosci.* **18**: 9835–9844.
31. Handschumacher, R., M. Harding, J. Rice, R. Drugge, and D. Speicher. 1984. Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* **224**: 544–547.
32. Harding, M. W., A. Galat, D. E. Uehling, and S. L. Schreiber. 1989. A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341**: 758–760.
33. Helekar, S. A., D. Char, S. Neff, and J. Patrick. 1994. Prolyl isomerase requirement for the expression of functional homooligomeric ligand-gated ion channels. *Neuron* **12**: 179–189.
34. Hemmendinger, L. M., B. B. Garber, P. C. Hoffmann, and A. Heller. 1981. Target neuron-specific process formation by embryonic mesencephalic dopamine neurons in vitro. *Proc. Natl. Acad. Sci. USA* **78**: 1264–1268.
35. Hiwasa, T., K. Kondo, T. Hishiki, S. Koshizawa, K. Umezawa, and A. Nakagawara. 1997. GDNF-induced neurite formation was stimulated by protein kinase inhibitors and suppressed by Ras inhibitors. *Neurosci. Lett.* **238**: 115–118.
36. Hou, J. G., L. H. Lin, and C. Mytilineou. 1996. Glial cell-line derived neurotrophic factor exerts neurotrophic effects on dopaminergic neurons in vitro and promotes their survival and regrowth after damage by 1-methyl-4-phenylpyridinium. *J. Neurochem.* **66**: 74–82.
37. Jamil, G., R. Hagar, E. J. Kaftan, and B. E. Ehrlich. 1996. Effects of FK506 analogs on ryanodine receptors. *Biophys. J.* **70**: A75.
38. Jayaraman, T., A. Brillantes, A. P. Timerman, S. Fleischer, H. Erdjument-Bromage, P. Tempst, and A. R. Marks. 1992. FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J. Biol. Chem.* **267**: 9474–9477.
39. Jing, S., D. Wen, Y. Yu, P. Holst, Y. Luo, M. Fang, R. Tamir, L. Antonia, Z. Hu, R. Cupples, J. Louis, S. Hu, B. Altrock, and G. Fox. 1996. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR- α , a novel receptor for GDNF. *Cell* **85**: 1113–1124.
40. Johansson, M., M. Friedemann, B. Hoffer, and I. Stromberg. 1995. Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts in oculo. *Exp. Neurol.* **134**: 25–34.
41. Johnson, E. M., T. Koike, and J. Franklin. 1992. The “calcium set-point hypothesis” of neuronal dependence on neurotrophic factor. *Exp. Neurol.* **115**: 163–166.
42. King, M., C. Huang, P. Chock, A. Nairn, H. Hemmings, K. Chan, and P. Greengard. 1984. Mammalian brain phosphoproteins as substrates for calcineurin. *J. Biol. Chem.* **259**: 8080–8083.
43. Kreigstein, K., C. Suter-Crazzolara, W. H. Fischer, and K. Unsicker. 1995. TGF- β superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP⁺ toxicity. *EMBO J.* **14**: 736–742.
44. Leclerc, P., P. Ekstrom, A. Edstrom, J. Priestly, S. Averill, and D. Tonge. 1997. Effects of glial cell line-derived neurotrophic factor on axonal growth and apoptosis in adult mammalian sensory neurons in vitro. *Neuroscience* **82**: 545–558.
45. Letourneau, P. 1996. The cytoskeleton in nerve growth cone motility and axonal pathfinding. *Perspect. Dev. Neurobiol.* **4**: 111–123.
46. Lin, L., D. Doherty, J. Lile, S. Bektess, and F. Collins. 1993. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**: 1130–1132.
47. Lin, L., T. Zhang, F. Collins, and L. Armes. 1994. Purification and initial characterization of rat B49 glial cell line-derived neurotrophic factor. *J. Neurochem.* **63**: 758–768.
48. Liu, J., J. Farmer, W. Lane, J. Friedman, I. Weissman, and S. Schreiber. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**: 807–815.
49. Lo, D. 1998. Instructive roles of neurotrophins in synaptic plasticity. *Prog. Brain Res.* **117**: 65–70.
50. Lyons, W. E., E. B. George, T. M. Dawson, J. P. Steiner, and S. H. Snyder. 1994. Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. *Proc. Natl. Acad. Sci. USA* **91**: 3191–3195.
51. Maness, P., H. Beggs, S. Klinz, and W. Morse. 1996. Selective neural cell adhesion molecule signalling by Src family tyrosine kinases and tyrosine phosphatases. *Perspect. Dev. Neurobiol.* **4**: 169–181.
52. Marcaida, G., E. Kosenko, M. D. Minana, S. Grisolia, and V. Felipo. 1996. Glutamate induces calcineurin-mediated dephosphorylation of Na⁺, K⁺-ATPase that results in its activation in cerebellar neurons in culture. *J. Neurochem.* **66**: 99–104.
53. Mattson, M. P. 1992. Calcium as sculptor and destroyer of neural circuitry. *Exp. Gerontol.* **27**: 29–49.
54. Mattson, M. P., P. B. Guthrie, and S. B. Kater. 1988. Components of neurite outgrowth that determine neuronal cytoarchitecture: Influence of calcium and the growth substrate. *J. Neurosci. Res.* **20**: 331–345.
55. Mattson, M. P., and S. B. Kater. 1987. Calcium regulation of neurite elongation and growth cone motility. *J. Neurosci.* **7**: 4034–4043.
56. Meberg, P., S. Ono, L. Minamide, M. Takahashi, and J. Bamberg. 1998. Actin depolymerization factor and cofilin phosphorylation dynamics: Response to signals that regulate neurite extension. *Cell Motil. Cytoskeleton* **39**: 172–190.
57. Newman-Gage, H., and A. Graybiel. 1988. Expression of calcium/calmodulin-dependent protein kinase in relation to dopamine islands and synaptic maturation in the cat striatum. *J. Neurosci.* **8**: 3360–3375.
58. Nichols, R., G. Suplick, and J. Brown. 1994. Calcineurin-mediated protein dephosphorylation in brain nerve terminals regulates release of glutamate. *J. Biol. Chem.* **269**: 23,817–23,823.
59. O’Leary, D., and S. Koester. 1993. Development of projection neuron types, axon pathways, and patterned connections of the mammalian cortex. *Neuron* **10**: 991–1006.
60. Rehder, V., and S. Kater. 1992. Regulation of neuronal growth cone filopodia by intracellular calcium. *J. Neurosci.* **12**: 1375–1386.
61. Rosenblad, C., A. Martinez-Serrano, and A. Bjorklund. 1996. Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience* **75**: 979–985.
62. Rousselet, A., L. Felter, B. Chamak, and A. Prochiantz. 1988. Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. *Dev. Biol.* **129**: 495–504.
63. Sautter, J., J. Tseng, D. Bragaglia, P. Aebischer, C. Spenger, R. Seiler, H. Widmer, and A. Zurn. 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.
64. Seki, K., H. C. Chen, and K. P. Huang. 1995. Dephosphorylation of protein kinase C substrates, neurogranin, neuromodulin, and MARCKS, by calcineurin and protein phosphatases 1 and 2A. *Arch. Biochem. Biophys.* **316**: 673–679.

65. Sharkey, J., and S. P. Butcher. 1994. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature* **371**: 336–339.
66. Sharp, A., F. Nucifora, O. Blondel, C. Sheppard, C. Zhang, S. Snyder, J. Russell, D. Ryugo, and C. Ross. 1999. Differential cellular expression of isoforms of inositol 1,4,5-triphosphate receptors in neurons and glia in brain. *J. Comp. Neurol.* **406**: 207–220.
67. Steiner, J. P., M. A. Connelly, H. L. Valentine, G. S. Hamilton, T. M. Dawson, L. Hester, and S. H. Snyder. 1997. Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin, and cyclosporin A. *Nat. Med.* **3**: 421–428.
68. Steiner, J. P., T. M. Dawson, M. Fotuhi, C. E. Glatt, A. M. Snowman, N. Cohen, and S. H. Snyder. 1992. High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358**: 584–586.
69. Steiner, J. P., T. M. Dawson, M. Fotuhi, and S. H. Snyder. 1996. Immunophilin regulation of neurotransmitter release. *Mol. Sci.* **2**: 325–333.
70. Steiner, J. P., G. S. Hamilton, D. T. Ross, H. L. Valentine, H. Guo, M. A. Connolly, S. Liang, C. Ramsey, J. J. Li, W. Huang, P. Howorth, R. Soni, M. Fuller, H. Sauer, A. C. Nowotnik, and P. D. Suzdak. 1997. Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proc. Natl. Acad. Sci. USA* **94**: 2019–2024.
71. Studer, L., C. Spenger, R. W. Seiler, C. A. Altar, R. M. Lindsay, and C. Hyman. 1995. Comparison of the effects of the neurotrophins on the morphological structure of dopaminergic neurons in cultures of rat substantia nigra. *Eur. J. Neurosci.* **7**: 223–233.
72. Tai, P., M. Albers, H. Chang, L. Faber, and S. Schreiber. 1992. Association of a 59-kDa immunophilin with the glucocorticoid receptor complex. *Science* **256**: 1315–1318.
73. Torii, N., T. Kamashita, Y. Otsu, and T. Tsumoto. 1995. An inhibitor of calcineurin, FK506, blocks induction of long-term depression in rat visual cortex. *Neuroscience* **185**: 1–4.
74. Treanor, J., L. Goodman, F. deSavauge, D. Stone, K. Poulson, C. Bexk, C. Gray, M. Armanini, R. Pollock, F. Hefti, H. Phillips, A. Goddard, M. Moore, A. Buj-Bello, A. Davies, N. Asai, M. Takahashi, R. Vandlen, C. Henderson, and A. Rosenthal. 1996. Characterization of a multicomponent receptor for GDNF. *Nature* **382**: 80–83.
75. van Weering, D., and J. Bos. 1997. Glial cell line-derived neurotrophic factor induces Ret-mediated lamellipodia formation. *J. Biol. Chem.* **272**: 249–254.
76. Voorn, P., A. Kalsbeek, B. Jorritsma-Byham, and H. Groenewegen. 1988. The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience* **25**: 857–887.
77. Walensky, L., P. Gascard, M. Fields, S. Blackshaw, J. Conboy, N. Mohandas, and S. Snyder. 1998. The 13-kD FK506 binding protein, FKBP13, interacts with a novel homologue of the erythrocyte membrane cytoskeletal protein 4.1. *J. Cell Biol.* **141**: 143–153.
78. Wang, M. S., M. Zeleny-Pooley, and B. G. Gold. 1997. Comparative dose-dependence study of FK506 and cyclosporin A on the rate of axonal regeneration in the rat sciatic nerve. *J. Pharmacol. Exp. Ther.* **282**: 1084–1093.
79. Widenfalk, J., C. Nosrat, A. Tomac, H. Westphal, B. Hoffer, and L. Olson. 1997. Neurturin and glial cell line-derived neurotrophic factor receptor 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.
80. Wilby, M., S. Sinclaire, E. Muir, R. Zietlow, K. Adcock, P. Horellou, J. Rogers, S. Dunnett, and J. Fawcett. 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.



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NEUROIMMUNOPHILIN LIGAND ENHANCES NEURITE OUTGROWTH AND EFFECT OF FETAL DOPAMINE TRANSPLANTS

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Abstract—Neuroimmunophilin ligands have been shown to enhance neurite outgrowth in several neuronal systems in culture, including primary dopaminergic neurons from fetal ventral mesencephalon. We investigated the ability of neuroimmunophilin ligands to enhance outgrowth of transplanted fetal dopamine neurons *in vivo*. Rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal dopamine system were transplanted with rat embryonic day 14 ventral mesencephalon into the striatum, then treated orally with a neuroimmunophilin ligand (15 mg/kg) or vehicle once per day for 14 days. All transplanted animals regained dopamine function over a 10 week behavioral test period, as indicated by decrease and reversal of amphetamine-induced rotation. In addition, neuroimmunophilin ligand-treated animals showed a more pronounced motor response during the first 10 min after amphetamine injection, possibly reflecting increased striatal reinnervation or increased functional capacity. At *post-mortem* analyses, neuroimmunophilin ligand-treated rats showed a significantly higher density of tyrosine hydroxylase-positive fibers reinnervating the lesioned striatum, both immediately surrounding the transplant (92% of unlesioned density in neuroimmunophilin-treated rats vs 67% of unlesioned levels in vehicle-treated rats) and at some distance from the transplant/host interface. The number of tyrosine hydroxylase-positive cells within the transplants was not different between groups. This study demonstrates that short-term oral administration of a neuroimmunophilin ligand can enhance neurite outgrowth from fetal dopamine neuronal transplants. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: behavior, graft, trophic, immunophilin, Parkinson's disease, regeneration.

The discovery that immunosuppressive drugs such as FK506 show trophic effects in several neuronal systems in culture and *in vivo*^{9,12,17,21,25,37,41,44} stimulated the design of compounds that bind the immunophilin FKBP12 (FK506-binding protein, 12 kDa) but do not inhibit the phosphatase calcineurin.³ Investigations of these neuroimmunophilin ligands revealed neurotrophic effects in cell culture and *in vivo*.^{9,10,16} We demonstrated that neuroimmunophilin ligands could increase neurite outgrowth from primary fetal dopamine neurons in culture.^{9,10} Based on these findings, we asked whether such fetal neurons could maintain responsiveness to neuroimmunophilin ligands after transplantation into adult rat brain.

Animal studies have shown that fetal dopamine neuron transplants can survive, develop and functionally reinnervate the dopamine-denervated striatum (reviewed in Ref. 13). Clinical trials of this procedure in Parkinson's disease patients have produced encouraging results,^{14,23,24,29,31,36,46} yet not all parkinsonian signs and motor deficits are relieved. *Post-mortem* studies of current transplant methodologies show low survival of transplanted dopamine fetal cells (approximately 5–10%) and partial reinnervation of the host striatum. Although the survival of transplanted dopamine neurons is critical for this therapy, and several strategies have been utilized to enhance the survival of transplanted cells,^{2,4,7,8,11,18,22,26–28,33,34,38,40,43,48–50} the axonal outgrowth and

synaptic integration of the transplanted neurons may be even more important for complete and sustained symptomatic relief. Although animal studies have shown increased transplant-derived reinnervation with growth factors,^{18,34,38} the utilization of these neurotrophic protocols in the clinical setting has been difficult to accomplish owing to difficulties in administration and pharmacokinetics.³⁰

EXPERIMENTAL PROCEDURES

Lesion surgery

Female Sprague–Dawley rats (Charles River) received unilateral stereotaxic injections of 6-hydroxydopamine (6-OHDA; Sigma, St Louis, MO; 5 µg/µl free base in 0.2% ascorbic acid/saline) into the right medial forebrain bundle (mfb) under ketamine/xylazine anesthesia (60 mg/kg and 3 mg/kg, respectively). Two microliters of 6-OHDA was injected at the following coordinates (calculated from Bregma): AP: –4.4; L: –1.3; V: –7.7; TB: –2.3 at a rate of 0.5 µl/min, with a 2 min period allowed for diffusion prior to slow removal of the needle. Three months after lesion, animals were injected with 3 mg/kg D-amphetamine intraperitoneally, and placed (randomized) into automated rotometer bowls; full-body turns ipsilateral and contralateral to the lesion were monitored over a 90 min period via a computerized activity monitor system, and animals were balanced into two groups by this pretransplant rotation score.

Transplantation and treatment

One month after pretransplant rotational analyses, lesioned animals were transplanted with a cell suspension from the ventral mesencephalon (VM). Embryonic day (E) 14 rat fetuses were removed from pregnant females and placed into ice-cold Ca²⁺/Mg²⁺-free phosphate-buffered saline. VM tissue was dissected, incubated in 0.1% trypsin (Sigma)/0.5 mM EDTA for 20 min at 37°C, washed with Ca²⁺/Mg²⁺-free Hank's buffered saline solution (HBSS) containing 0.1% DNase (Sigma)/20 mM glucose, and triturated in the same solution through a series of fire-polished Pasteur pipettes of diminishing diameters until a milky suspension was obtained. Cell counts and viability were determined by dye-exclusion techniques (acridine orange/ethidium bromide).

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Abbreviations: ANOVA, analysis of variance; E, embryonic day; EDTA, ethylenediaminetetra-acetate; FKBP12, FK506-binding protein, 12 kDa; HBSS, Hank's buffered saline solution; mfb, medial forebrain bundle; OD, optical density; TH, tyrosine hydroxylase; VM, ventral mesencephalon.

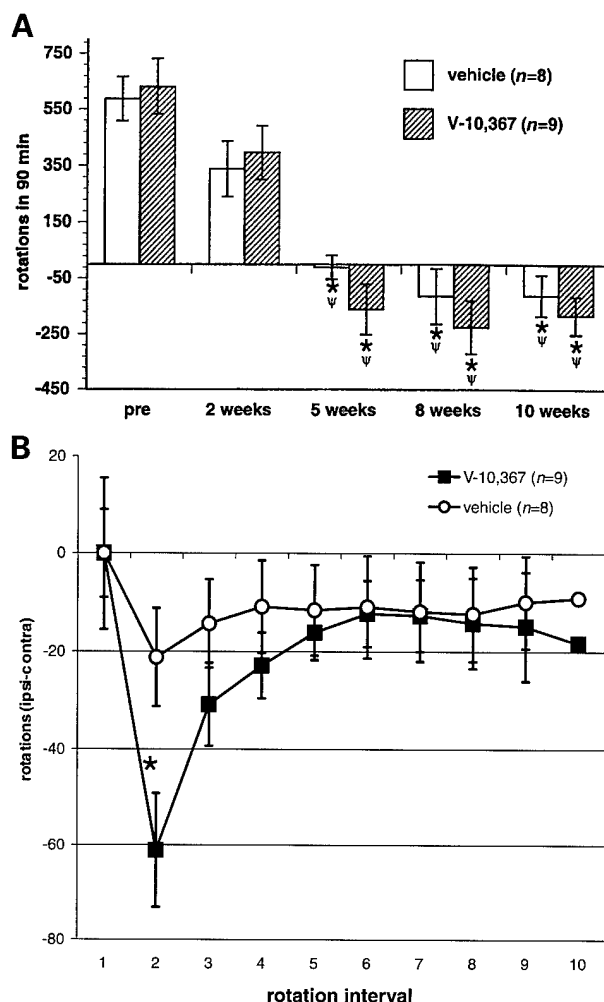


Fig. 1. Amphetamine-induced rotations of transplanted animals. (A) Amphetamine-induced rotation analyses took place prior to transplantation and two, five, eight and 10 weeks post-transplantation. Both groups showed significant decreases in rotation scores by five weeks post-transplant, and maintained this compensation at eight and 10 weeks. Values are given as means $\pm$ S.E.M. of full body turns ipsilaterally (positive values) or contralaterally (negative values) over 90 min. No differences were observed between V-10,367- ($n=9$) and vehicle-treated ($n=8$) groups. * $P < 0.05$ compared to pre-transplant rotation scores; $^{\Psi}P < 0.05$ compared to two weeks post-transplant scores. (B) Amphetamine-induced rotations at 10-min intervals over 90 min (combined eight and 10 weeks post-transplant sessions). Note the high rate of rotation in V-10,367-treated animals during the first 10 min after amphetamine injection (rotation interval 2) compared with vehicle-treated animals. * $P < 0.05$, V-10,367-treated animals significantly different from vehicle-treated animals.

Two microliters of a suspension containing $\sim 100,000$ cells/ μ l (total of 200,000 cells/transplant) were injected into one striatal site of previously lesioned rats at the following coordinates: AP: +1.0; L: -3.0; V: -5.0; TB: 0. Cells were injected using a 10 μ l Hamilton syringe with a Hamilton cannula at a rate of 0.5 μ l/min, with a 2 min period allowed for diffusion prior to slow removal of the needle. Animals were treated with V-10,367 (15 mg/kg/day, qd via oral intubation) or vehicle (water/propylene glycol/ethanol at a ratio of 5:4:1, respectively) beginning the day of transplant, and continuing for 14 days. Numbers of animals per group were $n=10$ in V-10,367-treated group and $n=9$ in vehicle-treated group.

Functional and morphological analyses of dopamine cell transplants

At two, five, eight and 10 weeks post-transplant, animals were again tested for AMPH-induced rotation. Analysis of variance (ANOVA) for repeated measures with correction for multiple

comparisons was used to determine differences over time with post-hoc analyses when significant F ratios were present [in JMP Version 3.1; SAS Institute, NC].

Ten weeks after transplantation, animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed for 6 h then transferred to 20% sucrose for 12 h. Brains were sliced into 40 μ m sections on a freezing microtome and processed for tyrosine hydroxylase (TH) immunocytochemistry. Sections were incubated in primary TH polyclonal antibody (Pel Freeze, 1:500) overnight at room temperature, then incubated with biotinylated goat anti-rabbit secondary antibody and avidin-biotin complex (ABC Elite, Vector), and visualized with 3,3'-diaminobenzidine (DAB, Sigma).

The number of TH⁺ cells in every sixth section of the transplant was counted. These counts were added, multiplied by six and corrected according to Abercrombie.¹ The extent and density of TH⁺ fiber outgrowth into host striatum was quantified using NIH Image 1.61 (adopted from Refs 11, 18, 34 and 47). In brief, two striatal sections per rat, 200 μ m apart within the rostral-to-caudal region of the transplant, were digitally captured by an investigator blinded to treatment. Using calibrated 100 $\times$ 100 μ m digital overlay squares, beginning immediately adjacent to the transplant perimeter and extending in horizontal bands medially and laterally to the edges of the striatum, optical density (OD) measurements were taken at progressively greater distances from the transplant/host interface. No significant differences were seen when medial and lateral measurements were compared with those taken from ventral, medial-ventral, or lateral-ventral bands extending from the transplant perimeter to the edges of host striatum. All lateral and medial measurements were summed for each distance from the transplant/host interface to obtain an average fiber density at each distance from the interface. Measurements of unlesioned contralateral striatum were obtained, and corpus callosum OD measurements were subtracted from each measurement as assay background. Data are expressed as percent unlesioned striatal OD, and analysed by Student's t -test at each interval from the transplant perimeter. One animal from each group was excluded due to absence of surviving transplant; analyses thus included $n=9$ in the V-10,367-treated group, and $n=8$ in the vehicle-treated group. All efforts were made to minimize both the suffering and the number of animals used, and all experiments conformed to the IACUC guidelines on the ethical use of animals.

RESULTS

The effects of orally administered neuroimmunophilin ligand on growth and survival of dopamine transplants into fully lesioned rat striatum were assessed by functional and morphological analyses. Oral neuroimmunophilin ligand treatment (V-10,367 at 15 mg/kg/day, p.o.) began on the day of transplantation and continued for 14 days. Rats were killed 10 weeks after transplantation.

Effects of neuroimmunophilin ligand on the function of dopaminergic transplants

Unilaterally lesioned rats rotate ipsilateral to the lesioned hemisphere when injected with the dopamine-releasing drug amphetamine. A decrease in this rotation after transplantation to the lesioned striatum indicates a capacity for dopamine release within the transplanted striatum, and is a measure of functional efficacy of dopamine transplants. One month prior to transplantation, and at several time-points after transplantation (two, five, eight and 10 weeks), animals were tested for amphetamine-induced rotation. All animals showed high levels of rotation ipsilateral to the lesion prior to transplantation (Fig. 1A, average of 609.9 ipsilateral turns/90 min $\pm$ 62). Two weeks after transplantation, animals in both V-10,367-treated and vehicle-treated groups showed decreases in ipsilateral rotations (Fig. 1A, average of 368.6 ipsilateral turns/90 min $\pm$ 66). By five weeks post-transplant, both groups showed complete normalization (Fig. 1A, average of 89.8

contralateral turns/90 min $\pm$ 54.1), significantly lower than both pretransplant scores and two week post-transplant scores. There was a tendency for increased contralateral rotations at eight weeks (Fig. 1A, average of 172.4 contralateral turns/90 min $\pm$ 68.1) that was maintained at 10 weeks post-transplant (Fig. 1A, average of 148.4 contralateral turns/90 min $\pm$ 49). All animals showed significant functional effects of the transplant regardless of treatment (repeated measures MANOVA, $F_{4,12} = 18.99$; $P < 0.0001$); no differences were observed between vehicle- and V-10,367-treated transplant groups.

Further inspection of rotational behavior within each 90 min session revealed a marked tendency for higher rates of contralateral turning within the first 10 min after amphetamine injection in V-10,367-treated animals, with rotation scores becoming similar between the two groups thereafter for the rest of the 90 min session. This tendency became apparent at five weeks post-transplant, and reached significance between groups at eight weeks ($P = 0.05$), with a strong trend at 10 weeks ($P = 0.09$) post-transplant. To illustrate this effect, rotation scores from eight and 10 weeks were combined (Fig. 1B).

Effects of neuroimmunophilin ligand on the growth of dopaminergic transplants

Four days after final behavioral testing (10 weeks post-transplant), animals were perfused and brain sections immunohistochemically stained for TH. Transplants consisted of aggregates of TH⁺ cell bodies placed within the central or dorsolateral region of the striatum, projecting TH⁺ fibers into the transplant core and out into the host denervated striatum (Fig. 2A, B). The density and extent of reinnervation of the lesioned host striatum from the transplanted cells was significantly higher in V-10,367-treated animals (Fig. 2A) than the vehicle-treated animals (Fig. 2B). Quantification of the density and extent of reinnervation was measured by OD analyses beginning at the transplant perimeter and extending out into host striatum at 100 μ m increments (Fig. 2C). In V-10,367-treated rats, the density of host reinnervation immediately surrounding the transplant was 92% of unlesioned levels, while in vehicle-treated animals this region showed 67% of unlesioned innervation (Fig. 2C). As the distance from the transplant/host interface increased, the levels of reinnervation decreased in both groups; however, V-10,367-treated rats showed significantly higher levels of reinnervation at each distance (Fig. 2C). No increase in host-derived TH⁺ fibers was observed when OD measurements were taken in the region of the nucleus accumbens in either group. Analysis of the number of dopamine neurons within each transplant revealed an average of 1972 ($\pm$ 355) TH⁺ cells in V-10,367-treated rats, and an average of 1872 ($\pm$ 469) TH⁺ cells in vehicle-treated rats, showing no significant differences between groups ($P = 0.85$).

DISCUSSION

We have previously shown that neuroimmunophilin ligands can enhance branching or elongation of neurites from developing fetal dopamine neurons in culture.^{9,10} In the present study, we demonstrate the continued responsiveness of these fetal neurons to neuroimmunophilin ligands following transplantation to the adult brain. Short-term oral

treatment with a neuroimmunophilin ligand increased the axonal outgrowth from fetal dopamine neurons after transplantation to dopamine-denervated striatum. Neuroimmunophilin ligand treatment did not increase the number of TH⁺ neurons, indicating the ability of these compounds to enhance the reinnervation capacity of a limited number of transplanted neurons. V-10,367-treated transplanted animals showed increased initial motor response after amphetamine, possibly reflecting enhanced capacity for dopamine release or enhanced synaptic function.

Enhancing growth of transplanted fetal dopamine neurons by neuroimmunophilin ligand

Several aspects of dopamine cell transplantation require improvements before this technique can be widely utilized as a treatment for Parkinson's disease. Although clinical trials have shown improvements in some motor symptoms,^{14,23,29,31,36,46} a complete cure has not been achieved. In order to enhance the survival and development of the transplanted neurons, several strategies have been utilized, including the use of higher numbers of cells initially grafted,³⁹ free-radical scavengers added to cell suspension or injected after transplantation,^{4,28} inclusion of striatal target tissue,^{7,11,40} addition of growth factors to cells prior to transplantation^{2,8,18,26,27,43,49,50} and injection of growth factors after transplantation.^{22,33,34,38,48} Although impressive results have been obtained in animal models with these procedures, their implementation in a clinical setting has been difficult due to routes of administration (intracranial cannulation, repeated injections and anesthetization prior to each injection) and dosing parameters.³⁰

Although survival of transplanted cells is crucial for behavioral recovery, reinnervation of the dopamine-depleted striatum must also take place. This aspect of transplantation has been difficult to improve, owing to the limited survival of transplanted cells. The present study shows significantly increased reinnervation (92% of unlesioned levels of dopamine innervation) with neuroimmunophilin ligand treatment, even in the presence of no higher number of dopamine cells. The significantly higher density of transplant-derived fibers was observed close to the transplant perimeter and at a distance from the transplant. This increased TH⁺ fiber density could indicate a higher number of TH⁺ fibers extended by the transplanted dopamine neurons, which is consistent with our previous findings in culture: neuroimmunophilin ligands enhanced neurite outgrowth from fetal dopamine neurons without increasing the number of surviving dopamine neurons.^{9,10} Enhancing the degree of outgrowth and thus increasing the likelihood of synaptic contacts in functionally relevant regions of the striatum may play an important role in enhancing and sustaining the function of neuronal transplants. Alternatively, this increased TH⁺ fiber density could indicate a higher expression of TH per axon extended from the transplanted dopamine neurons.

Effects of neuroimmunophilin ligand on motor response in dopamine transplanted animals

All transplanted animals in the present study regained dopamine capacity (decreases in overall amphetamine-induced ipsilateral rotations to zero) by five weeks post-transplant, with contralateral rotations occurring by eight weeks, and

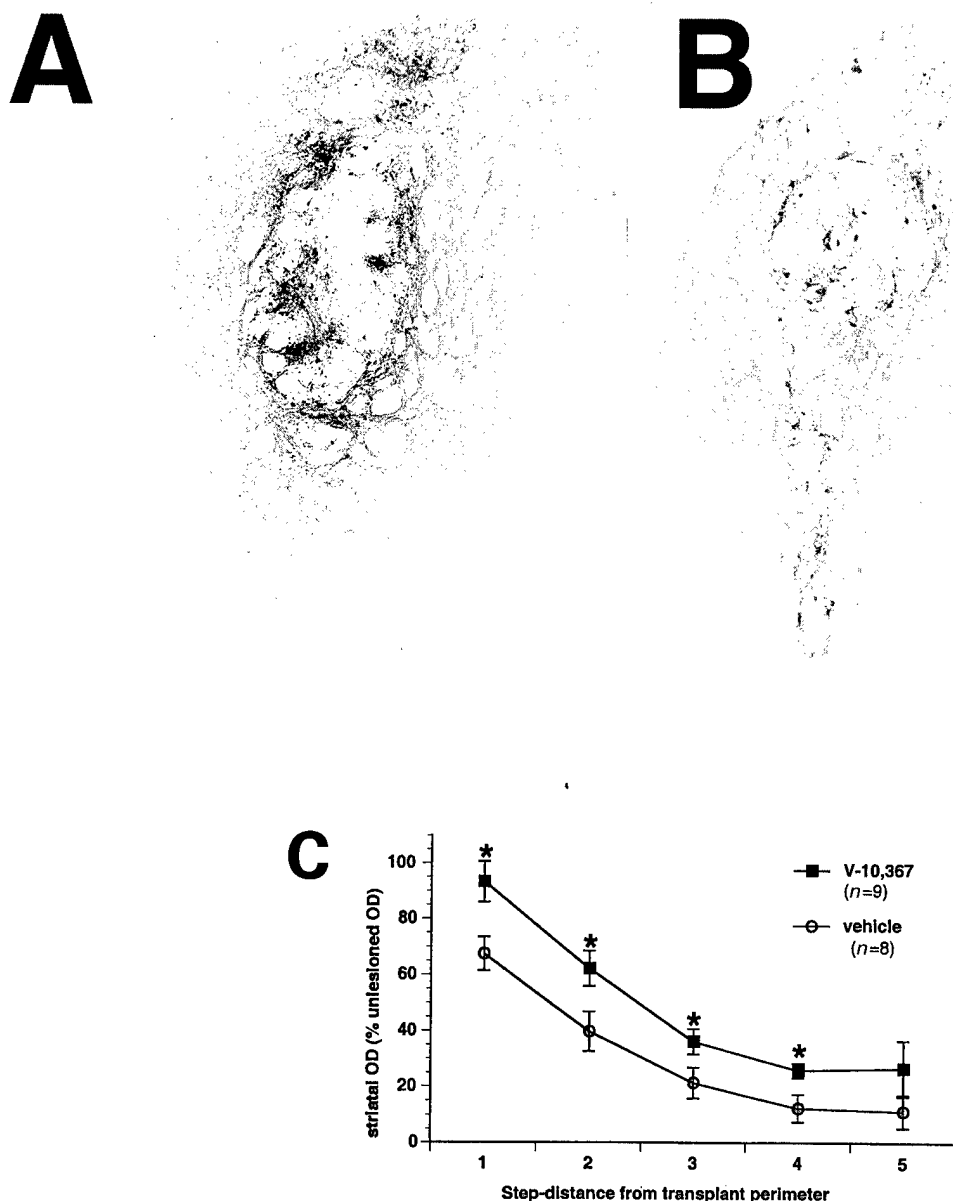


Fig. 2. Transplant survival and host reinnervation. Photomicrographs of representative coronal sections through VM transplants immunostained for TH, 10 weeks post-transplantation. Note the increased fiber density extending into host dopamine-denervated striatum in a V-10,367-treated animal ($n=9$) (A) compared with a vehicle-treated animal ($n=8$) (B). These two animals showed the same number of surviving TH⁺ cells. (C) TH⁺ fiber reinnervation into previously lesioned host striatum from transplants was measured at increasing distances from transplant/host interface. Data are expressed as per cent unlesioned optical density within calibrated 100 μ m intervals from the transplant/host interface to the edge of the striatum. Fiber density declined with distance from the transplant perimeter in both groups; however, the fiber density was significantly higher in V-10,367-treated animals at most points. Error bars represent S.E.M. * $P < 0.05$ compared to vehicle-treated animals.

maintained at this level at 10 weeks post-transplant. There were no differences between neuroimmunophilin ligand-treated animals and vehicle-treated animals in their total number of rotations over the 90 min testing period. However, the higher initial motor response (during the first 10 min after amphetamine injection) observed in V-10,367-treated animals at eight weeks post-transplant may reflect the increased reinnervation observed and/or increased functional capacity. Lesion-induced dopamine receptor supersensitivity within the striatum is not fully normalized following transplantation, and treatments that increase the amount of dopamine released from transplants may contribute to the initially higher level of rotations observed here in V-10,367-treated animals. Alternatively, the increased initial motor response in

V-10,367-treated animals may reflect an effect of this compound on the endogenous host dopamine system. However, OD measurements in the region of the nucleus accumbens (at a distance away from transplant-derived TH⁺ innervation) showed no increase in TH⁺ density between V-10,367- and vehicle-treated animals, indicating that V-10,367 did not induce host dopamine sprouting in this lesion model. These animals were completely dopamine denervated (by mfb lesion) four months prior to transplantation surgery and neuroimmunophilin ligand treatment.

The plateau in overall amphetamine-induced rotation in both V-10,367- and vehicle-treated groups in the present study may reflect a threshold effect. Several studies that have shown growth factor-induced morphological

enhancement of transplanted cells have shown significant decreases in rotational behavior in both treated and untreated groups, with no differences between groups.^{18,33,38,45,47} This reflects a threshold of functional effects in this behavioral test even in the presence of morphological enhancement. Behavioral compensation plateaus at a threshold number of TH⁺ cells, after which further compensation does not occur even in the presence of a higher number of TH⁺ cells. Several threshold numbers have been reported, ranging from 300 to 2500 rat TH⁺ cells,^{5,6,19,32,35} and 850 to 1000 pig TH⁺ cells.^{15,20} The survival of transplanted TH⁺ neurons observed in the present study did not differ between groups, and averaged 1924 TH⁺ cells. Therefore, in the present study, animals may have reached the maximum level of compensation in the amphetamine test (overall scores at each time-point) due to the high number of surviving dopamine cells in both groups, or due to threshold levels of amphetamine-induced dopamine release and autoregulation with the level of reinnervation reached in both groups.⁴²

CONCLUSIONS

These results extend our previous studies showing enhanced neurite outgrowth from fetal dopamine neurons in culture with neuroimmunophilin ligands, by illustrating similar effects of these compounds on fetal dopamine neurons after transplantation to adult brain. The increased density of innervation shown here with two-week oral neuroimmunophilin ligand treatment, in the presence of the same number of surviving dopamine neurons, indicates a potential for these compounds to enhance reconstruction of dopamine-denervated striatal circuitry.

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REFERENCES

1. Abercrombie M. (1946) Estimation of nuclear populations from microtome sections. *Anat. Rec.* **94**, 239–247.
2. Apostilides C., Sanford E., Hong M. and Mendez I. (1998) Glial cell line-derived neurotrophic factor improves intrastriatal graft survival of stored dopaminergic cells. *Neuroscience* **83**, 363–372.
3. Armistead D. M., Badia M. C., Deininger D. D., Duffy J. P., Saunders J. O., Tung R. D., Thomson J. A., DeCenzo M. T., Guter O., Livingston D. J., Mureko M. A., Yamashita M. M. and Navia M. A. (1995) Design, synthesis, and structure of non-macrocyclic inhibitors of FKBP12, the major binding protein for the immunosuppressant FK506. *Acta Cryst.* **D51**, 522–528.
4. Bjorklund L., Spenger C. and Stromberg I. (1997) Tirilazad mesylate increases dopaminergic neuronal survival in the *in oculo* grafting model. *Expl Neurol.* **148**, 324–333.
5. Brundin P., Barbin G., Strecker R., Isacson O., Prochiantz A. and Bjorklund A. (1988) Survival and function of dissociated rat dopamine neurons grafted at different developmental stages or after being cultured *in vitro*. *Devl Brain Res.* **39**, 233–243.
6. Brundin P., Isacson O. and Bjorklund A. (1985) 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.
7. Brundin P., Isacson O., Gage F. and Bjorklund A. (1986) Intrastriatal grafting of dopamine-containing neuronal cell suspensions: effects of mixing with target or non-target cells. *Brain Res.* **389**, 77–84.
8. Clarkson E. D., Zawada W. M., Adams F. S., Bell K. P. and Freed C. R. (1998) Strands of embryonic mesencephalic tissue show greater dopamine neuron survival and better behavioral improvement than cell suspensions after transplantation in parkinsonian rats. *Brain Res.* **806**, 60–68.
9. Costantini L. C., Chaturvedi P., Armistead D., McCaffrey P. G., Deacon T. W. and Isacson O. (1998) A novel immunophilin ligand: distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. *Neurobiol. Dis.* **5**, 97–106.
10. Costantini L. C. and Isacson O. (2000) Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons in culture. *Expl Neurol.* **164**, 60–70.
11. Costantini L. C. and Snyder-Keller A. (1997) Co-transplantation of fetal lateral ganglionic eminence and ventral mesencephalon can augment function and development of intrastriatal transplants. *Expl Neurol.* **145**, 214–227.
12. Dawson T. M., Steiner J. P., Dawson V. L., Dinerman J. L., Uhl G. R. and Snyder S. H. (1993) Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate toxicity. *Proc. natn. Acad. Sci. USA* **90**, 9808–9812.
13. Dunnett S. and Bjorklund A. (1999) Prospects for new restorative and neuroprotective treatments in Parkinson's disease. *Nature* **399**, A32–A39.
14. Freeman T. B., Olanow C. W., Hauser R. A., Nauert M., Smith D. A., Borlongan C. V., Sanberg P. R., Holt D. A., Kordower J. H., Vingerhoets F. J. G., Snow B. J., Calne D. and Gauger L. L. (1995) Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. *Ann. Neurol.* **38**, 379–388.
15. Galpern W. R., Burns L. H., Deacon T. W., Dinsmore J. and Isacson O. (1996) Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. *Expl Neurol.* **140**, 1–13.
16. Gold B. G., Zeleny-Pooley M., Chaturvedi P. and Wang M. S. (1998) Oral administration of a nonimmunosuppressant FKBP12 ligand speeds nerve regeneration. *NeuroReport* **9**, 553–558.
17. Gold B. G., Katoh K. and Storm-Dickerson T. (1995) The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. *J. Neurosci.* **15**, 7509–7516.
18. Granholm A., Mott J., Bowenkamp K., Eken S., Henry S., Hoffer B. J., Lapchak P. A., Palmer M. R., van Horne C. and Gerhardt G. A. (1997) Glial cell line-derived neurotrophic factor improves survival of ventral mesencephalic grafts to the 6-hydroxydopamine lesioned striatum. *Expl Brain Res.* **116**, 29–38.
19. Haque N., LeBlanc C. and Isacson O. (1997) Differential dissection of the rat E16 ventral mesencephalon and survival and reinnervation of the 6-OHdopamine-lesioned striatum by a subset of aldehyde dehydrogenase-positive TH neurons. *Cell Transpl.* **6**, 239–248.
20. Huffaker T., Boss B., Morgan A., Neff N. T., Strecker R. E., Spence M. S. and Miao R. (1989) Xenografting of fetal pig ventral mesencephalon corrects motor asymmetry in the rat model of Parkinson's disease. *Expl Brain Res.* **77**, 329–336.
21. Ide T., Morikawa E. and Kirino T. (1996) An immunosuppressant, FK506, protects hippocampal neurons from forebrain ischemia in the Mongolian gerbil. *Neurosci. Lett.* **204**, 157–160.
22. Johansson M., Friedemann M., Hoffer B. and Stromberg I. (1995) Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts *in oculo*. *Expl Neurol.* **134**, 25–34.
23. Kordower J. H., Freeman T. B., Snow B. J., Vingerhoets F. J. G., Mufson E. J., Sanberg P. R., Hauser R. A., Smith D. A., Nauert M., Perl D. P. and Olanow C. W. (1995) Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. *New Engl. J. Med.* **332**, 1118–1124.

24. Lindvall O., Sawle G., Widner H., Rothwell J. C., Björklund A., Brooks D., Brundin P., Frackowiak R. S., Marsen C. D., Odin P. and Rehnström S. (1994) Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. *Ann. Neurol.* **2**, 172–180.
25. Lyons W. E., George E. B., Dawson T. M., Steiner J. P. and Snyder S. H. (1994) Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. *Proc. natn Acad. Sci. USA* **91**, 3191–3195.
26. Mayer E., Dunnett S. and Fawcett J. (1993) Mitogenic effect of basic fibroblast growth factor on embryonic ventral mesencephalic dopaminergic neuron precursors. *Devl Brain Res.* **72**, 253–258.
27. Mehta V., Hong M., Spears J. and Mendez I. (1998) Enhancement of graft survival and sensorimotor behavioral recovery in rats undergoing transplantation with dopaminergic cells exposed to glial cell line-derived neurotrophic factor. *J. Neurosurg.* **88**, 1088–1095.
28. Nakao N., Frodl E., Duan W. M., Widner H. and Brundin P. (1994) Lazaroids improve the survival of grafted embryonic dopamine neurons. *Proc. natn. Acad. Sci. USA* **91**, 12,408–12,412.
29. Olanow C. W., Kordower J. H. and Freeman T. B. (1996) Fetal nigral transplantation as a therapy for Parkinson's disease. *Trends Neurosci.* **19**, 102–109.
30. Palfi S. (1998) Clinical and pathological evaluation of patient with Parkinson's disease (PD) following intracerebroventricular (icv) GDNF. *Soc. Neurosci. Abstr.* **24**, 42.
31. Peschanski M., Defer G., N'Guyen J., Ricolfi F., Monfort J. C., Remy P., Geny C., Samson Y., Hantraye P., Jeny R., Gaston A., Keravel Y., Degos J. D. and Cesaro P. (1994) Bilateral motor improvement and alteration of L-DOPA effect in two patients with Parkinson's disease following intrastriatal transplantation of foetal ventral mesencephalon. *Brain* **117**, 487–499.
32. Rioux L., Gaudin D., Bui L., Gregoire L., DiPaolo T. and Bedard P. (1991) Correlation of functional recovery after 6-OHDA lesion with survival of grafted fetal neurons and release of dopamine in the striatum of the rat. *Neuroscience* **40**, 123–131.
33. Rosenblad C., Kirik D. and Björklund A. (1999) Neurturin enhances the survival of intrastriatal fetal dopaminergic transplants. *NeuroReport* **10**, 1783–1787.
34. Rosenblad C., Martinez-Serrano A. and Björklund A. (1996) Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience* **75**, 979–985.
35. Sauer H., Frodl E. and Kupsch A. (1992) Cryopreservation, survival and function of intrastriatal fetal mesencephalic grafts on a rat model of Parkinson's disease. *Expl Brain Res.* **90**, 54–62.
36. Schumacher J., Ellias S., Palmer E. P., Kott H. S., Dinsmore J., Dempsey P. K., Fischman A. J., Thomas C., Feldman R. G., Kassissieh S., Raineri R., Manhart C., Fink J. S. and Isacson O. (2000) Transplantation of embryonic porcine mesencephalic tissue in patients with Parkinson's disease. *Neurology* **54**, 1042–1050.
37. Sharkey J. and Butcher S. P. (1994) Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature* **371**, 336–339.
38. Sinclair S. R., Svendsen C. N., Torres E. M., Martin D., Fawcett J. W. and Dunnett S. B. (1996) GDNF enhances dopaminergic cell survival and fibre outgrowth in embryonic nigral grafts. *NeuroReport* **7**, 2547–2552.
39. Sladek J. R., Jr, Collier T. J., Elsworth J. D., Roth R. H., Taylor J. R. and Redmond D. E., Jr (1998) Intrastriatal grafts from multiple donors do not result in a proportional increase in survival of dopamine neurons in nonhuman primates. *Cell Transpl.* **7**, 87–96.
40. Sortwell C., Collier T. and Sladek J. (1998) Co-grafted embryonic striatum increases the survival of grafted embryonic dopamine neurons. *J. comp. Neurol.* **399**, 530–540.
41. Steiner J. P., Hamilton G. S., Ross D. T., Valentine H. L., Guo H., Connolly M. A., Liang S., Ramsey C., Li J. J., Huang W., Howorth P., Soni R., Fuller M., Sauer H., Nowotnik A. C. and Suzdak P. D. (1997) Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proc. natn Acad. Sci. USA* **94**, 2019–2024.
42. Strecker R., Sharp T., Brundin P., Zetterstrom T., Ungerstedt U. and Björklund A. (1987) Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. *Neuroscience* **22**, 169–178.
43. Sullivan A., Pohl J. and Blunt S. (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.
44. Wang M. S., Zeleny-Pooley M. and Gold B. G. (1997) Comparative dose-dependence study of FK506 and cyclosporin A on the rate of axonal regeneration in the rat sciatic nerve. *J. Pharmac. exp. Ther.* **282**, 1084–1093.
45. Wang Y., Tien L. T., Lapchak P. A. and Hoffer B. J. (1996) GDNF triggers fiber outgrowth of fetal ventral mesencephalic grafts from nigra to striatum in 6-OHDA-lesioned rats. *Cell Tiss. Res.* **286**, 225–233.
46. Wenning G. K., Odin P., Morrish P., Rehnström S., Widner H., Brundin P., Rothwell J. C., Brown R., Gustavii B., Hagel P., Jahanshahi M., Sawle G., Björklund A., Brooks D. J., Marsden C. D., Quinn N. P. and Lindvall O. (1997) Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. *Ann. Neurol.* **42**, 95–107.
47. Wilby M. J., Sinclair S. R., Muir E. M., Zietlow R., Adcock K. H., Horellou P., Rogers J. H., Dunnett S. B. and 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.
48. Yurek D. M., Lu W., Hipkens S. and Wiegand S. J. (1996) BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons. *Expl Neurol.* **137**, 105–118.
49. Zawada W. M., Zastrow D. J., Clarkson E. D., Adams F. S., Bell K. P. and Freed C. R. (1998) Growth factors improve immediate survival of embryonic dopamine neurons after transplantation into rats. *Brain Res.* **786**, 96–103.
50. Zhou J., Bradford H. and Stern G. (1997) Influence of BDNF on the expression of the dopaminergic phenotype of tissue used for brain transplants. *Devl Brain Res.* **100**, 43–51.

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ELSEVIER

Cell implantation therapies for Parkinson's disease using neural stem, transgenic or xenogeneic donor cells

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Abstract

A new therapeutic neurological and neurosurgical methodology involves cell implantation into the living brain in order to replace intrinsic neuronal systems, that do not spontaneously regenerate after injury, such as the dopaminergic (DA) system affected in Parkinson's disease (PD) and aging. Current clinical data indicate proof of principle for this cell implantation therapy for PD. Furthermore, the disease process does not appear to negatively affect the transplanted cells, although the patient's endogenous DA system degeneration continues. However, the optimal cells for replacement, such as highly specialized human fetal dopaminergic cells capable of repairing an entire degenerated nigro-striatal system, cannot be reliably obtained or generated in sufficient numbers for a standardized medically effective intervention. Xenogeneic and transgenic cell sources of analogous DA cells have shown great utility in animal models and some promise in early pilot studies in PD patients. The cell implantation treatment discipline, using cell fate committed fetal allo- or xenogeneic dopamine neurons and glia, is currently complemented by research on potential stem cell derived DA neurons. Understanding the cell biological principles and developing methodology necessary to generate functional DA progenitors is currently our focus for obtaining DA cells in sufficient quantities for the unmet cell transplantation need for patients with PD and related disorders. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Dopamine; Fetal cell; Parkinson's disease; Regeneration; Stem cells; Transplantation

1. Introduction

The relatively new concept of replacing large numbers of degenerated neurons by implanting new cells into the adult brain has created a complementary therapeutic strategy to that of traditional pharmacological therapies for Parkinson's disease (PD). The specificity of cellular degeneration which occurs in PD (DA neurons of the SN), and the relatively major synaptic target region of these degenerating DA cells (the caudate, putamen and SN), have made PD the most accessible therapeutic application for neural cell implantation methodology.

Early clinical transplantation studies involved autologous transplantation of catecholamine-containing adrenal medulla cells [1,2]. The absence of objective reductions of PD signs, the low adrenal medulla graft survival and the reported morbidity of patients reinforced the scientific rationale for using fetal neural donor cells instead. Cell implantation for PD using fetal DA cells is likely to improve

greatly by scientific and technical advances. The development of brain cell transplantation with embryonic neurons and glia is innovative both from a technical and biological standpoint and will require much work to optimize. The scaling up of this method from rodents to primates has proved very challenging; particularly in obtaining an acceptable, abundant and reliable cell. In the initial series of clinical pilot transplant experiments performed in Europe, the first two PD patients did not show a meaningful recovery. Parallel technical and cell dose enhancements produced dramatically better results in the next two patients receiving unilateral fetal VM suspensions. MPTP-exposed patients received VM DA cell suspensions bilaterally into the striatum and this caused motor improvement in association with increased fluorodopa uptake [3]. Recent data from the studies of Lindvall and colleagues indicate DA cell survival in patients for almost a decade after surgery, with meaningful clinical improvement [4]. The transplantation of non-dissociated human VM tissue pieces has also provided benefits to many patients [5,6]. In this series of transplantation studies carried out by Olanow and colleagues in the US, autopsy from two bilaterally transplanted (6.5–9 week human fetal

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VM) patients who died 18–19 months after surgery showed over 200,000 surviving DA neurons, which reinnervated about 50% of the right putamen and 25% of the left putamen [7]. Electron microscopy revealed axo-dendritic and occasional axo-axonic synapses between graft and host, and analysis of TH mRNA revealed higher expression within the fetal neurons than within the residual host nigral cells [7]. Autopsy of another patient in this surgical group showed over 130,000 surviving DA neurons, reinnervating almost 80% of the putamen [8]. Notably, both patients had shown major improvements in motor function and increases in fluorodopa uptake in the putamen on PET scanning.

An alternative source of fetal donor cells for clinical cell implantation therapy for neurodegenerative disease is xenogeneic. The remarkable homogeneity in cellular (neurons and glia) basic structure and function suggested that even discordant mammalian species (rodent into non-human primates) could effectively replace local synaptic function after cell loss in the adult brain [9,10]. Such across-species cell transfer (xenotransplantation) allows a more standardized acquisition of larger quantities of appropriate fetal tissue than from human abortions. The immunological reaction of complement activated rejection and T-cell mediated responses leading to rejection of xenografts can in many ways be inhibited by immune suppression [11]. Transplantation studies in animals have shown survival, function, and afferent/efferent connections of xenogeneic cells when transplanted into animal hosts [12,13] (and see reviews [14,15]). In the first pilot-clinical trial, the transplantation of E27 porcine VM into the caudate and putamen on one side of the brain of twelve immunosuppressed PD patients produced some clinical improvements [16]. The overall results indicated that the scaling up problems, also seen with human fetal cells, were significant, further compounded by more vigorous immunological responses in primate and human hosts compared to laboratory rodents. One patient from this study died seven months after surgery from a pulmonary embolism; histological analyses using species-specific markers showed porcine neuron projection axons and forming synapses in the host brain. All three identified transplant sites contained DA neurons (a total of 630 DA neurons), and non-DA neurons expressing pig-specific neurofilament [17]. Pig glial cell, including astrocytes also survived in the patient's brain. Microglial and T-cell markers showed low reactivity in and around the pig cell graft perimeter.

2. The scientific foundation of cell implantation therapy for Parkinson's disease

Basic research involving cell implantation has made it abundantly clear that biological cell replacements strategies can provide the basis for reconstruction and repair of damaged or dysfunctional neuronal connections of the damaged or disease afflicted adult brain [18]. Functional effects of intrastriatal grafts of fetal DA cells have been

illustrated in a range of animal behavioral tests [19–21]. The behavioral effects observed are dependent on the survival of DA neurons within the striatum, since grafting of other tissue produces no behavioral effects [22,23], and removal of transplanted tissue [24] or immune rejection of transplanted neurons [25] reverses transplant-induced behavioral recovery in animal studies. Embryonic day (E) 12–17 fetal rat tissue [26], pig E 27–29 [13] and 6.5–9 weeks old human fetal tissue [5] ventral mesencephalic (VM) donor tissue neuronal exhibit survival and functional effects when transplanted into the adult dopamine depleted striatum. The minimum number of surviving transplanted DA neurons required for behavioral effect in rodent animal models is approximately 100–200 [12]. Using current micro-dissection techniques and cell preparation, only 10% of the transplanted VM cells are phenotypically DA, and only 1–20% of these DA neurons survive implantation depending on trophic and immunological factors [7,17,27–32]. Therefore as many as 10–15 fetal VM per patient may be required for sufficient survival and adequate DA synapse replacement [33].

Factors that are important for maturation and connectivity of DA neurons during normal ontogeny likely also influence development and integration of grafted embryonic tissue when placed in an adult host brain. Current methodology for fetal cell implantation in animal models and patients includes the transfer of numerous types of fetal neuronal and glial progenitor cells. Thus, the implanted neurons are transferred into the host brain with their own contemporaneous glial and angiogenic factor releasing cells, thereby providing a milieu that may contribute to the observed normal cell autonomous development of transplanted fetal VM cells. Adding appropriate trophic factors to fetal cell preparations can enhance survival and growth of implanted DA neurons into animal models of PD [28,34–39]. The ability of fetal neurons to be placed into an ectopic region of an adult brain, survive, and extend neurites within this region is remarkable. The functional effects of VM transplants into DA-depleted striatum is often correlated with degree of striatal reinnervation [26,30]. However there is some limitation in the ability of the transplanted neurons to extend neurites in the adult brain. Even though the graft-induced elevations in tissue DA concentrations are substantial [40], values taken distant from the graft suggest that reinnervation of the whole striatum does not occur. The hypothesis for this sharp decline in density of DA fiber outgrowth is that age-dependent characteristics within the host brain alter outgrowth, since extensive outgrowth can be achieved when transplanted into immature (neonatal) host brain. Expression levels and patterns of adhesion molecules expressed by mature host brain are thought to be the culprits of this reinnervation-inhibitory effect. Allogeneic cell implantation into immature host brain shows robust neuronal and glial migration away from the transplant site and a high degree of integration and target-directed neurite outgrowth [18]. In contrast, fetal neural cells transplanted

into mature brains show neuronal reaggregation around the implant site and less extensive axonal outgrowth into host brain, suggesting an age-dependent increase in inhibitory or decrease in growth-promoting processes. Clearly, both promoting and repulsive host factor and substrate activities influence axonal guidance and extension of transplanted developing neurons [18].

Data arguing against any absolute outgrowth-inhibitory properties of adult brain come from studies showing long-distance and target-specific axonal growth from human embryonic transplants into adult rat brain [41], as well as from porcine embryonic transplants into adult rat brain [13]. The species-specific markers used in our studies of fetal porcine transplants into adult immunosuppressed rat brain allowed comparison of donor glial fiber and donor axonal growth in different host brain regions, demonstrating their distinct trophic characteristics. Target zones in adult host gray matter were selectively innervated by embryonic donor axons normally destined to form synapses there, whereas donor glial fibers grew irrespective of any target orientation within white matter tracts [13]. Xenogenic pig axons branched profusely in gray matter target region and only rarely penetrated or crossed white matter tracts. DA fibers from transplants placed into the SN were found coursing up toward the striatum through myelinated fiber bundles, then branching into host gray matter. Notably, we found that the non-DA VM cells also grew toward distant gray matter target zones, such as mediodorsal and ventral anterior thalamus. These data suggest that directional cues for axons, whether diffusible or substrate-bound, are provided by adult host target regions. Since porcine neural development continues four to five times longer than mouse, these axons may grow and make synapses for a longer time (with slower maturation) than that seen in rat-to-rat studies. These general differences are borne out in the time-course comparisons of functional recovery in rodent porcine-transplant recipients (8 weeks post-transplantation) as compared with allografts (4 weeks post-transplantation) [18].

3. Anatomical and cell type specification of dopamine neurons

The current understanding of the maturation and phenotypic specializations of DA neurons located in the adult substantia nigra parallels the observations made of the development of committed fetal dopamine neurons placed as grafts into the adult CNS (Fig. 1). The molecular signaling necessary for the final morphological specializations and connectivity of the nigro-striatal DA system must therefore be largely intrinsic to the developing DA neurons; or alternatively, present in significant detail in the adult brain for this process to be completed in a normal way. On the post-synaptic host side; different regions of the striatum are associated with specific behaviors in rat: the dorsal striatum receives primary afferents from the motor areas of

neocortex, and has been shown to be preferentially involved in rotational recovery after DA neuron transplantation [42]. In the intact rat, the subpopulation of nigral DA neurons from A9 SN which co-express AHD project their axons to the dorsal-lateral and rostral regions of the striatum. When transplanted into adult DA-denervated rat striatum, these AHD/TH neurons innervated this region of the DA-depleted striatum [18,43], showing a preferential reinnervation of the dorsolateral striatum corresponding to the normal projection pattern of AHD/TH neurons. Specific innervation by subsets of transplanted DA neurons was also demonstrated by Schultzberg, showing reinnervation of the DA-depleted striatum by the population of grafted A9 VM neurons lacking cholecystokinin (CCK) [44]. The CCK⁺ fibers were found in a narrow zone immediately adjoining the graft. These data suggest the presence of mechanisms, which selectively favor the ingrowth of fibers from the appropriate DA neuronal subset. Thus enrichment of the DA-neuron subpopulation which specifically expresses AHD may allow more appropriate reinnervation of striatum after transplantation, and influence the degree of functional recovery in PD [18] (Fig. 1).

4. Repair of synaptic function and regulated dopamine release after implantation of new dopamine neurons

The most important factor in obtaining complete and sustained functional effects may be the presence of new synapses for biochemically and physiologically appropriate DA release in the host striatum. Embryonic DA neurons produce new connections with the mature host striatal neurons. Synaptic connections between transplanted VM cells and host cells, as well as afferents from host neurons to transplanted cells, have been extensively documented [45,46]. Functional analyses indicate that pharmacological delivery into the striatum may not be as effective in ameliorating the motor symptom of PD, as regulated, synaptic release obtained with transplanted DA neurons [33]; When DA is directly administered into the ventricle of PD patients, serious psychosis can develop [47]. Even from a cell biological standpoint, the rationale for normal range DA release is illustrated by differential display experiments that show abnormal upregulation of over 10 genes within the striatum after abnormal DA exposure *in vivo* [48]. Complications associated with unregulated DA levels are obvious when observing effects of long-term L-dopa administration: as PD progresses, and the DA neuron degeneration continues, the unregulated formation of DA within the striatum and abnormal down-stream activity in the basal ganglia can lead to motor abnormalities such as dyskinesias. Physiologically appropriate DA functions can be achieved by DA neurons or, alternatively, cells which express the complete set of feedback elements required to regulate release and uptake of DA. Several studies have shown normalized metabolic activity throughout the basal ganglia after

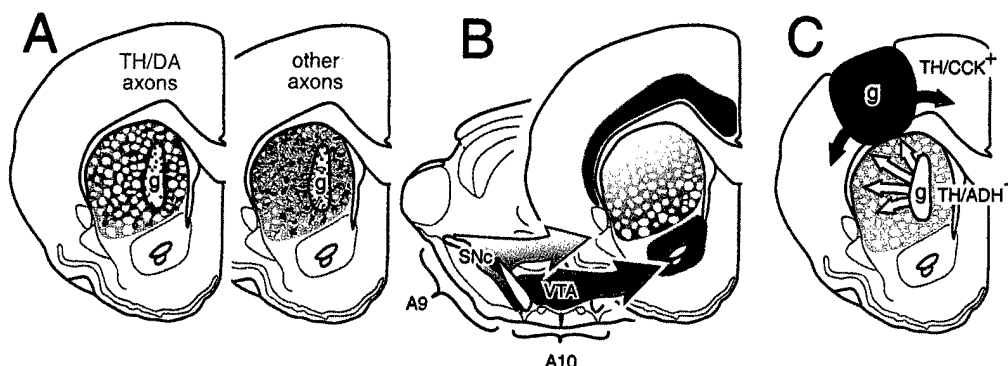


Fig. 1. Target-specific innervation by grafted fetal cells. (A) Target zones in adult host gray matter are selectively innervated by embryonic pig donor DA axons normally destined to form synapses there, whereas non-DA donor fibers grow into host myelinated bundles. (B) In the intact rat, the subpopulation of nigral DA neurons from A9 SNc, which co-express AHD, project their axons to the gray matter of dorsal-lateral regions of the striatum. The ventral tegmental area (VTA) neurons from A10 co-express CCK, and project to ventromedial striatum, nucleus accumbens, neocortex and limbic regions. (C) When the enriched population of TH/AHD neurons obtained from a medial (versus lateral) VM dissection is transplanted into DA-lesioned adult rat striatum, these neurons preferentially reinnervate their normal dorsolateral striatal target, shown to be involved in rotational recovery after DA neuron transplantation. TH/CCK neurons from VM show different patterns of outgrowth when placed into cortex. (Reprinted with permission from Trends in Neurosciences 1997; 20:477–482. © Elsevier.).

transplantation. Using cytochrome oxidase histochemistry as an indicator of neuronal metabolism in the 6-OHDA-lesioned rat; the lesion-induced increases in activity of the entopeduncular nucleus and SN reticulata were reversed by intrastriatal VM grafts, whereas the lesion-induced increases in globus pallidus and subthalamic nucleus were not affected by grafting [49]. Similarly, in MPTP-treated monkey receiving VM transplants, DA cell implants increased the metabolic activity of the implanted striatum, particularly in the region of grafts containing greater numbers of DA neurons [50]. Positron emission tomography (PET) and carbon-11-labeled 2B-carbomethoxy-3B-(4-fluorophenyl)tropane (11C-CFT) have been utilized as markers for striatal presynaptic DA transporters in a unilateral lesion model in rat. In the lesioned striatum, the binding ratio was reduced by 15 to 35% of the intact side. After DA neuronal transplantation, behavioral recovery occurred only after the 11C-CFT binding ratio had increased from 75 to 85% of the intact side, revealing a threshold for functional recovery in the lesioned nigro-striatal system after neural transplantation [23]. Auto-regulation of DA release and metabolism by intrastriatal grafts has been shown by *in vivo* microdialysis. Infusion of a non-selective DA agonist (apomorphine) reduced DA concentrations in the grafted striatum [12,51], indicating auto-regulation of DA levels by transplanted cells. Evidence for the formation of functional synapses and appropriate DA regulation by transplanted fetal DA neurons comes from the observation that dyskinesias, expressed either as contraversive circling after repeated L-dopa injections in rodents [52] or L-dopa-induced dyskinesias in non-human primates are reduced after transplantation. These data indicate that DA levels within the transplanted striatum will be regulated in a functional manner by the transplanted DA neurons.

5. Potential use of stem cells for obtaining donor cell for transplantation

Most living systems undergo continuous growth. There are many examples of cell division and differentiation for maintaining cell populations in adult human bodies; for example, the bone marrow that recruits stem cells capable of dividing into most of the cells necessary for blood and immune systems throughout life. Part of entire adult organs can be regenerated, such as the liver. Cells in the lining of the gut are shed on a daily basis with replacements growing in from layers below. In the skin, the basal cell layers of the dermis provide a plentiful source of growth; that also signifies a continuous growth process. These specialized cells can divide to maintain or increase growth of organ systems in the adult body. The recent fascination with the most pluripotent of such cell; the so-called stem cells, illustrate a renewed interest and deepening molecular understanding of developmental biology. While for the last 60 years most text-books of embryology has detailed most biological sequences in the development of mammals, it is not until recently that a molecular and mechanistic data of cellular signaling pathways involved in cell fate and development of organ systems has been obtained. In addition, recent cloning experiments have illustrated that even mammalian adult cell nuclei (containing DNA) has the material for establishing all cells of a whole organism after transfer to a fertilized egg-cell. The fertilized oocyte goes through a few rounds of cell division and then the resulting cluster of cells (in the range of 250 cells; see Fig. 2) is capable of imbedding itself in the wall of the uterus in mammals. At this stage, each of the cells in the inner cell mass cluster is usually capable of forming any part, or the whole of the entire body plan. This type of cell is therefore

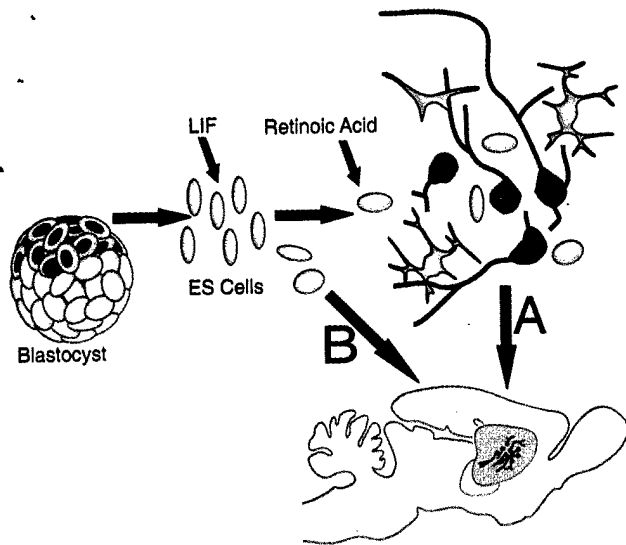


Fig. 2. Basic steps for ES cell procedures including in vitro expansion, chemical or spontaneous induction into neurons after implantation into the adult brain. Totipotent embryonic stem cells derived from the inner cell mass of blastocyst are propagated in culture in the presence of leukemia inhibitory factor (LIF). Prior to transplantation, LIF is removed. The cells are treated with retinoic acid (A) or are transplanted directly (B) into adult brain. Regardless of pre-treatment with retinoic acid, the transplanted ES cells differentiate to form cells with neuron-like morphology and phenotypic expression of neuronal markers.

denoted stem cell, or in this case, embryonic stem cells. From this initial group of stem cells, all other cells that form the living body are generated. The developmental sequential orchestration of the growth of the body into its specialized parts and unique form and function follows a strict pattern and sequence in the embryo and neonate. Nevertheless, as previously mentioned, in the adult organism, many cells with the body remain capable of division and growth into specialized cell systems. Recently, such divisible (yet non-malignant or carcinogenic) cells have gained increased attention. The idea that such multipotent cells present in the blood stream, or even in the brain, are still capable of multiple cellular fates has intrigued biologists and the public. In particular in the brain, in addition to the well-known fact that olfactory epithelium and a few other brain regions (including the dentate gyrus of the hippocampus) there may also be dividing cells capable of other types of growth or repair. Such continuous cell division may be necessary for maintenance and adaptive function of many cellular systems.

In experimentation, stem cell-like behavior has been observed from embryonic stem cells, growth factor-expanded neural progenitors, immortalized cell lines and embryonal carcinoma cells. Growth factor-expanded cells have been implanted into the adult brain, with survival of small cell clusters [53–55]. Immortalized cell lines have shown capacity to differentiate into several neuronal cell types when transplanted (for review, see Ref. [56]). The implantation of immortalized cells into neonatal brain

resulted in differentiation into neurons and glia with apparent region-specific morphology [57–60]. Notably, when transplanted into adult brain such immortalized cells (generated from embryonic striatum or hippocampus) are usually fated to form glia [61]. Brain implants of embryonic carcinoma cell lines have been shown to survive and grow as neurons when treated with retinoic acid [62–65]. We transplanted mouse D3 and E3 normal ES cells into adult mouse striatum and adult 6-OHDA-lesioned striatum, which spontaneously developed into neurons and other cells (Fig. 2). Many TH⁺ neurons were found, while dopamine- β -hydroxylase⁺ cells were infrequent. Non-neuronal regions sometimes were immunoreactive for glial fibrillary acidic protein. Many neurons, including DA and 5-HT catecholaminergic cells, grew axons into the host brain. The axonal growth into gray matter was not abnormal, but did not resemble the five caliber fiber innervation seen in normal DA growth in the striatum [13,66]. ES derived serotonergic neurons grew in a less restricted pattern than TH⁺ neurons. Mouse D3 and E3 ES cells placed into mouse kidney capsule grew into similar neuronal phenotypes as those placed in the adult brain. These data suggest that neuralization is a possible default pathway, and occurs spontaneously if pre-gastrula cells are prevented from getting patterned signals from other embryonic cell layers [67]. This is not entirely surprising, given that the early gastrula ectodermal animal cap, normally destined to become epidermal tissue, will form neural tissue if disrupted [68]. There are known inducing factors discovered for epidermal differentiation during gastrulation, such as BMP4 [69]. Homozygous knock-out mice lacking functional BMP receptor (BMPRI) will not survive past gastrulation [70], a time when epidermis would normally form. Inhibitors of BMP4 or activin, such as noggin, follistatin, and chordin, from the Spemann organizer region, can cause ectopic formation of neural tissue. Taken together, these findings indicate that disruption of these epidermis-inducing signals causes neural differentiation. Given that our experiment involved dissociated and expanded ES cells, this may be equivalent of such disruption. Nonetheless, it remains to be determined if other growth factors present in brain and kidney capsule can induce TH⁺ neurons. The absence of kidney formation in GDNF-knockout mice suggests that GDNF may play a role in both kidney and brain development [71]. While these ES cells form neurons of TH⁺ (putative DA) phenotypes that extend axons, into the adult host striatum, such neurons may also be able to create the kind of behavioral recovery seen with implantation of normal phenotypic fetal DA neurons.

In conclusion, there is a large unmet need for obtaining a donor cell source for clinical cell implantation to PD patients. While human fetal DA donor cells work in principle, as shown in human pilot studies, this cell source is not available or workable in a standard clinical environment. Analogous fetal DA donor cells from other animal species are potential alternatives to human fetal tissue. For example, the pig or rodent meso-striatal DA system also contains cell

groups A8, A9, and A10 that differentiate into the homologous cell groups seen in humans and function after transplantation to the mature brain. Alternatively, functionally appropriate DA neurons could be derived from progenitor or stem cell populations. Moreover, genetic engineering and immortalization technology could be applied to progenitor and stem cells, in order to obtain sufficient numbers of DA neurons of appropriate design for cell transplantation to a large number of PD patients.

Statement on animal experiments: All animal experiments have been carried out in accordance with the National Institutes of Health Guide for the care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978). All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques, if available.

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References

- [1] Madrazo I, Leon V, Torres C. Transplantation of fetal substantia nigra and adrenal medulla to the caudate putamen in two patients with Parkinson's disease. *New Engl J Med* 1988;318:51.
- [2] Backlund E, Granberg P, Hamberger B. Transplantation of adrenal medullary tissue to striatum in parkinsonism. *J Neurosurg* 1985;62:113–69.
- [3] Widner H, Tetrad J, Rehnrona S, Snow B, Brundin P, Gustavii B. Bilateral fetal mesencephalic grafting in two patients with severe parkinsonism induced by MPTP. *New Engl J Med* 1992;327:1556–63.
- [4] Piccini P, Brooks DJ, Bjorklund A, Gunn RN, Grasby PM, Rimoldi O, Brundin P, Hagell P, Rehnrona S, Widner H, et al. Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. *Nat Neurosci* 1999;2:1137–40.
- [5] Freeman TB, Olanow CW, Hauser RA, Nauert M, Smith DA, Borlongan CV, Sanberg PR, Holt DA, Kordower JH, Vingerhoets F, J G, et al. Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. *Ann Neurol* 1995;38:379–88.
- [6] Freed CR, Breeze RE, Rosenberg NL, Schneck SA, Kriek E, Qi JX, Lone T, Zhang YB, Snyder JA, Wells TH, et al. Survival of implanted fetal dopamine cells and neurologic improvement 12 and 46 months after transplantation for Parkinson's disease. *New Engl J Med* 1992;327:1549–55.
- [7] Kordower J, Rosenstein J, Collier T, Burke M, Chen E, Li J, Martel L, Levey A, Mufson E, Freeman T, et al. Functional fetal nigral grafts in a patient with Parkinson's disease: chemoanatomic, ultrastructural, and metabolic studies. *J Comp Neurol* 1996;370:203–30.
- [8] Kordower J, Freeman T, Chen E, Mufson E, Sanberg P, Hauser R, Snow B, Olanow C. Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. *Mov Disord* 1998;13:383–93.
- [9] Isacson O, Riche D, Hantraye P, Sofroniew MV, Maziere M. A primate model of Huntington's disease: cross-species implantation of striatal precursor cells to the excitotoxically lesioned baboon caudate-putamen. *Exp Brain Res* 1989;75:213–20.
- [10] Hantraye P, Riche DMM, Isacson O. Intrastriatal grafting of cross-species fetal striatal cells reduces abnormal movements in a primate model of Huntington's disease. *Proc Natl Acad Sci USA* 1992;89:4187–91.
- [11] Pedersen E, Poulsen F, Zimmer J, Finsen B. Prevention of mouse–rat brain xenograft rejection by a combination therapy of cyclosporin A, prednisolone and azathioprine. *Exp Brain Res* 1995;106:181–6.
- [12] Galpern WR, Burns LH, Deacon TW, Dinsmore J, Isacson O. Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. *Exp Neurol* 1996;140:1–13.
- [13] Isacson O, Deacon TW, Pakzaban P, Galpern WR, Dinsmore J, Burns LH. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. *Nat Med* 1995;1:1189–94.
- [14] Pakzaban P, Isacson O. Neural xenotransplantation: reconstruction of neuronal circuitry across species barriers. *Neuroscience* 1994;62:989–1001.
- [15] Isacson O, Breakefield XO. Benefits and risks of hosting animal cells in the human brain. *Nat Med* 1997;3:964–9.
- [16] Schumacher JM, Ellias SA, Palmer EP, Kott HS, Dinsmore J, Dempsey PK, Fischman AJ, Thomas C, Feldman RG, Kassissieh S, et al. Transplantation of embryonic porcine mesencephalic tissue in patients with PD. *Neurology* 2000;14:1042–50.
- [17] Deacon T, Schumacher J, Dinsmore J, Thomas C, Palmer P, Kott S, Edge A, Penney D, Kassissieh S, Dempsey P, et al. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nat Med* 1997;130:350–3.
- [18] Isacson O, Deacon TW. Neural transplantation studies reveal the brain's capacity for continuous reconstruction. *Trends Neurosci* 1997;20:477–82.
- [19] Brundin P, Duan W, Saur H. Functional effects of mesencephalic dopamine neurons and adrenal chromaffin cells grafted to the rodent striatum. In: Dunnett S, Bjorklund A, editors. *Functional Neural Transplantation*, vol. 2. New York: Raven Press, 1994. p. 9–46.
- [20] Annett L. Functional studies of neural grafts in parkinsonian primates. In: Dunnett S, Bjorklund A, editors. *Functional Neural Transplantation*, vol. 2. New York: Raven Press, 1994. p. 71–102.
- [21] Olsson M, Nikkiah G, Bentlage C, Bjorklund A. Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *J Neurosci* 1995;15:3863–75.
- [22] Dunnett S, Hernandez T, Summerfield, Jones G, Arbutnot G. Graft-derived recovery from 6-OHDA lesions: specificity of ventral mesencephalic graft tissue. *Expl Brain Res* 1988;71:411–24.
- [23] Brownell AL, Livni E, Galpern W, Isacson O. In vivo PET imaging in rat of dopamine terminals reveals functional neural transplants. *Ann Neurol* 1998;43:387–90.
- [24] Bjorklund A, Dunnett S, Stenevi U, Lewis M, Iversen S. Reinnervation of the denervated striatum by substantia nigra transplants: functional consequences as revealed by pharmacological and sensorimotor testing. *Brain Res* 1980;199:307–33.
- [25] Carder R, Snyder-Keller A, Lund R. Behavioral and anatomical correlates of immunologically induced rejection of nigral xenografts. *J Comp Neurol* 1988;277:391–402.
- [26] Bjorklund A, Lindvall O, Isacson O, Brundin P, Victorin K, Strecker R, Clarke D, Dunnett S. Mechanisms of action of intracerebral neural implants: Studies on nigral and striatal grafts to the lesioned striatum. *Trends Neurosci* 1987;10:509–16.
- [27] Olanow CW, Kordower JH, Freeman T, B.M. Fetal nigral transplantation as a therapy for Parkinson's disease. *Trends Neurosci* 1996;19:102–9.
- [28] Yurek D, Lu W, Hipkens S, Wiegandm S. BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons. *Exp Neurol* 1996;137:105–18.
- [29] Nakao N, Frodl E, Duan WM, Widner H, Brundin P. Lazaroids improve the survival of grafted embryonic dopamine neurons. *Proc Natl Acad Sci* 1994;91:12 408–12.

- [30] Costantini LC, Snyder-Keller A. Co-transplantation of fetal lateral ganglionic eminence and ventral mesencephalon can augment function and development of intrastriatal transplants. *Exp Neurol* 1997;145:214–27.
- [31] Brundin P, Isacson O, Gage F, Bjorklund A. Intrastriatal grafting of dopamine-containing neuronal cell suspensions: effects of mixing with target or non-target cells. *Brain Res* 1986;389:77–84.
- [32] Nikkah G, Olsson M, Eberhard J, Bentlage C, Cunningham MG, Bjorklund A. A microtransplantation approach for cell suspension grafting in the rat Parkinson model: a detailed account of the methodology. *Neuroscience* 1994;53:57–72.
- [33] Bjorklund A. Better cells for brain repair. *Nature* 1993;362:414–5.
- [34] Zhou J, Bradford H, Stern G. Influence of BDNF on the expression of the dopaminergic phenotype of tissue used for brain transplants. *Dev Brain Res* 1997;100:43–51.
- [35] Mayer E, Dunnett S, Fawcett J. Mitogenic effect of basic fibroblast growth factor on embryonic ventral mesencephalic dopaminergic Johanson precursors. *Dev Brain Res* 1993;72:253–8.
- [36] Johansson M, Friedemann M, Hoffer B, Stromberg I. Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts in oculo. *Exp Neurol* 1995;134:25–34.
- [37] Wang Y, Tien L, Lapchak P, Hoffer B. GDNF triggers fiber outgrowth of fetal ventral mesencephalic grafts from nigra to striatum in 6-OHDA-lesioned rats. *Cell Tissue Res* 1996;286:225–33.
- [38] Rosenblad C, Martinez-Serrano A, Bjorklund A. Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience* 1996;75:979–85.
- [39] Zawada W, Zastrow D, Clarkson E, Adams F, Bell KCRF. Growth factors improve survival of embryonic dopamine neurons after transplantation into rats. *Brain Res* 1998;786:96–103.
- [40] Rioux L, Gaudin D, Bui L, Gregoire L, DiPaolo T, Bedard P. Correlation of functional recovery after 6-OHDA lesion with survival of grafted fetal neurons and release of dopamine in the striatum of the rat. *Neuroscience* 1991;40:123–31.
- [41] Victorin K, Brundin P, Sauer H, Lindvall OAB. Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. *J Comp Neurol* 1992;323:475–94.
- [42] Dunnett S, Bjorklund A, Schmidt R, Stenevi U, Iverson S. Intracerebral grafting of neuronal cell suspensions. IV Behavioral recovery in rats with unilateral 6-OHDA lesions in different brain sites. *Acta Physiol Scand* 1983;522:29–37.
- [43] Haque N, LeBlanc C, Isacson O. Differential dissection of the rat E16 ventral mesencephalon and survival and reinnervation of the 6-OHDA-lesioned striatum by a subset of aldehyde dehydrogenase-positive TH neurons. *Cell Transplant* 1997;6:239–48.
- [44] Schultzberg M, Dunnett S, Bjorklund A, Stenevi U, Hokfelt T, Dockray G, Goldstein M. Dopamine and cholecystokinin immunoreactive neurons in mesencephalic grafts reinnervating the neostriatum: evidence for selective growth regulation. *Neuroscience* 1984;12:17–32.
- [45] Mahalik T, Finger T, Stromberg I, Olson L. Substantia nigra transplants into denervated striatum of the rat: ultrastructure of graft and host interconnections. *J Comp Neurol* 1985;240:60–70.
- [46] Doucet G, Murata Y, Brundin P, Bosler O, Mons N, Geffard M, Ouimet C, Bjorklund A. Host afferents into intrastriatal transplants of fetal ventral mesencephalon. *Exp Neurol* 1989;106:1–19.
- [47] Venna N, Sabin T, Ordia J, Mark V. Treatment of severe Parkinson's disease by intraventricular injection of dopamine. *Appl Neurophysiol* 1984;47:62–64.
- [48] Gerfen C, Keefe K, Steiner H. Dopamine-mediated gene regulation in the striatum. *Adv Pharmacol* 1998;42:670–3.
- [49] Nakao N, Ogura N, Nakai K, Itakura T. Intrastriatal mesencephalic grafts affect neuronal activity in basal ganglia nuclei and their target structures in a rat model of Parkinson's disease. *J Neurosci* 1998;18:1806–17.
- [50] Collier T, Redmond DJ, Roth R, Elsworth J, Taylor J, Sladek JJ. Metabolic energy capacity of dopaminergic grafts and the implanted striatum in parkinsonian nonhuman primates as visualized with cytochrome oxidase histochemistry. *Cell Transplant* 1997;6:135–40.
- [51] Strecker R, Sharp T, Brundin P, Zetterstrom T, Ungerstedt U, Bjorklund A. Autoregulation of dopamine release and metabolism by intrastriatal nigral grafts as revealed by intracerebral dialysis. *Neuroscience* 1987;22:169–78.
- [52] Gaudin D, Rioux L, Bedard P. Fetal dopamine neuron transplants prevent behavioral supersensitivity induced by repeated administration of L-Dopa in the rat. *Brain Res* 1990;506:166–8.
- [53] Svendsen C, Caldwell M, Shen J, Borg M, Rosser A, Tyers P, Karmio IS, Dunnett S. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. *Exp Neurol* 1997;148:135–46.
- [54] Svendsen C, Clarke D, Rosser A, Dunnett S. Survival and differentiation of rat and human epidermal growth factor-responsive precursor cells following grafting into the lesioned adult central nervous system. *Exp Neurol* 1996;137:376–88.
- [55] Minger S, Fisher L, Ray J, Gage F. Long-term survival of transplanted basal forebrain cells following in vitro propagation with fibroblast growth factor-2. *Exp Neurol* 1996;141:12–24.
- [56] Martinez-Serrano A, Bjorklund A. Immature neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci* 1997;20:530–8.
- [57] Renfranz PJ, Cunningham MG, McKay RDG. Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* 1991;66:713–29.
- [58] Ryder EF, Snyder EY, Cepko CL. Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer. *J Neurobiol* 1990;21:356–75.
- [59] Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwig EA, Hartwig LCC. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* 1992;68:33–51.
- [60] Cattaneo E, Magrassi L, Butti G, Santi L, Giavazzi A, Pezzotta S. A short term analysis of the behavior of conditionally immortalized neuronal progenitors and primary neuroepithelial cells implanted in the fetal rat brain. *Dev Brain Res* 1994;83:197–208.
- [61] Lundberg C, Martinez-Serrano A, Cattaneo E, McKay RDG, Bjorklund A. Survival, integration and differentiation of neural stem cell lines after transplantation to the adult rat striatum. *Exp Neurol* 1997;145:342–60.
- [62] Kleppner SR, Robinson KA, Trojanowski JQ, Lee VM-Y. Transplanted human neurons derived from a teratocarcinoma cell line (NTera-2) mature, integrate, and survive for over 1 year in the nude mouse brain. *J Comp Neurol* 1995;357:618–32.
- [63] Morassutti DJ, Staines WA, Magnuson DSK, Marshall KC, McBurney MW. Murine embryonal carcinoma-derived neurons survive and mature following transplantation into adult rat striatum. *Neuroscience* 1994;58:753–63.
- [64] Wojcik BE, Nothias F, Lazar M, Jouin H, Nicolas J, Peschanski M. Catecholaminergic neurons result from the intracerebral implantation of embryonal carcinoma cells. *Proc Natl Acad Sci USA* 1993;90:1305–9.
- [65] Miyazono M, Lee VM-Y, Trojanowski JQ. Proliferation, cell death, and neuronal differentiation in transplanted human embryonal carcinoma (NTera2) cells depend on the graft site in nude and severe combined immunodeficient mice. *Lab Invest* 1995;73:273–83.
- [66] Isacson O, Deacon TW. Specific axon guidance factors persist in the mature rat brain: evidence from fetal neuronal xenografts. *Neuroscience* 1996;75:827–37.
- [67] Hemmati-Brivanlou A, Melton D. Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell* 1997;88:13–17.
- [68] Grunz H, Tacke L. Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Diff Dev* 1989;28:211–8.

- [69] Wilson PA, Hemmati-Brivanlou A. Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* 1995;376:331–3.
- [70] Hogan B. Bone morphogenic proteins: multifunctional regulators of vertebrate development. *Gene Dev* 1996;10:1580–94.
- [71] Pichel J, Shen L, Sheng H, Granholm A, Drago J, Grinberg A, Lee E, Huang S, Saarma M, Hoffer B, et al. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 1996;382:73–76.

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Improved Surgical Cell Therapy in Parkinson's Disease

Physiological Basis and New Transplantation Methodology

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PHYSIOLOGICAL BASIS FOR TREATMENTS OF PARKINSONISM

In the classical neuropathologic description of Parkinson's Disease (PD) there is abnormal degeneration and dysfunction of the dopaminergic (DA) neurons in the substantia nigra, pars compacta region, with associated DA axonal and synaptic loss in the striatum, subthalamic nucleus, and substantia nigra, pars reticulata. This in effect leaves the patient with signs of resting tremor, bradykinesia, rigidity, and inability to initiate movements unless L-DOPA treated. Other parallel pathologies may be present, but the dopaminergic degeneration probably accounts for the motoric dysfunction.

The ability to observe both physiology and function in small areas within the brain is now possible with high-resolution positron emission tomography (PET) and magnetic resonance (MR) imaging techniques (1–3). The potential use of PET as a research tool in movement disorders has been demonstrated in studies of brain dopamine function (4) and glucose metabolism associated with movement

disorders (5–7). Recently, high-resolution PET imaging has been widely used in studies on animal models of PD (8–15). In addition, advances in receptor studies (8,10,16,17) and magnetic resonance spectroscopy of neurodegeneration (3,15,18–20) provide specific functional neurochemical information that may be of value in determining prognosis and therapy. In PD, the finding of changes in metabolic activity as determined by positron emission tomography (PET) in pre-SMA and PM cortices is intriguing (21). What actually may explain the signs of PD at the physiological circuitry level is the synchronization of neuronal pallidal (GPe and GPi) output signals as a result of the loss of DA tonic input to the putamen together with a reduced thalamic input to the SMA and PM cortices (22) (Figs. 47-1 and 47-2). An apparent recruitment of more cortical regions than normal and the increased and widespread activation of PM and SMA associated cortices suggest that these structures are compensating for the abnormal input to be able to activate the motor cortex for initiation of the movement (see Fig. 47-1). Evidence for this view comes from pallidotomy studies (22) in

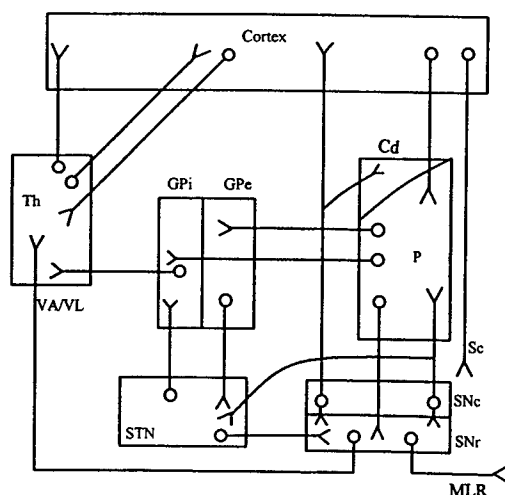


FIG. 47-1. A schematic circuit or network diagram highlighting motor systems interactions. The results of STN lesions (and pallidotomy) may simply provide a release of movement control via the indirect pathway (CP to GPi and GPe via STN regulation of GPi inhibition of thalamic VA/VL). In this way the clinical signs of abnormally reduced or enhanced dopamine release in the CP can be eliminated; the L-DOPA-induced dyskinesias typically seen in advanced PD patients virtually disappear after pallidotomy or STN modulation. Reports indicate that such advanced PD patients can sustain excessive DA activation of CP and the remaining A10 DA neuronal systems (for example, nucleus accumbens) after STN stimulation or pallidotomy. These observations suggest that the indirect basal ganglia loop either blocks cortical motor output (as in dopamine deficiency in PD) or creates an abnormal "oscillation" of cortical output (as in DA/L-DOPA-induced dyskinesias or Huntington's disease).

which the loss of PD signs (tremor, rigidity, and L-DOPA-induced dyskinesias) is reflected in a more localized metabolic activity, as well as appropriate activation in motor association cortex (21; Y. Smith, *personal communication*). Based on all of these data and relevant circuitry of the motor system, we hypothesize that full DA reinnervation, by cell transplantation or regeneration, will rebalance this motor system.

Studies in PD patients and primate PD models have postulated that nigrostriatal DA deficiency leads to decreased inhibitory activity from putamen to the internal segment of

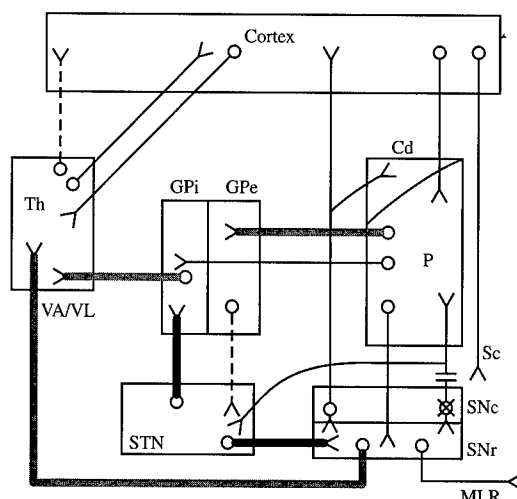


FIG. 47-2. A schematic network of the functional interactions of the motor systems in parkinsonism. The *thick black lines* show enhanced activation. The *thick dark gray lines* are enhanced inhibition. The *broken lines* are reduced inhibitory (GPe) or excitatory (Th) activity.

globus pallidus (23–25). Resulting inhibitory outflow from globus pallidus suppresses the motor thalamus, reducing activation of cerebral motor cortex system and creating the signs of PD. After transplantation therapy, the putamenal inhibitory action of globus pallidus is expected to recover. Longitudinal studies after different transplantation methods with dopamine fetal cells are necessary to determine the underlying biological mechanism of the therapeutic effects on movement initiation.

In the medical history of PD, James Parkinson's description of the disease in 1817 did nothing to relieve the patients of their suffering until the early 1950s, when observations were made about unilateral improvements of PD signs contralateral to a subcortical stroke. Stereotactic thalamotomies and, subsequently, pallidotomies were performed as a rational intervention simulating this therapeutic effect. Surgical trials were fairly extensive and, in many cases, provided long-term improvements and reductions in the patients' signs over several years (see volumes in *Acta Psychiatrica et Neurologica Scandinavica*, 1960). The thermal lesions of this type were, however, superseded

by the discovery that the dopamine system was *prima culpa* in Parkinson's disease. Through findings by Arvid Carlsson's team in Sweden and Oleh Hornykiewicz in Austria, the systemic pharmacologic dosing and delivery of L-DOPA was initiated to patients (26). This became the mainstay of treatment, and only after some time did it become evident that this precursor treatment (overcoming the dopamine rate-limiting enzyme tyrosine hydroxylase) in dopamine neurons would not provide permanent relief for the patients (26–29).

A novel rational idea then emerged from scientists working in neurobiology and cell culture: to replace lost dopamine neurons through neural transplantation (30,31). As a result of animal experiments in the early 1980s and through the beginning of exploratory clinical trials in the late 1980s, the development of a new therapy involving fetal transplantation has achieved some spectacular results (32–37) as well as some evidence of a need for further refinement (38,39). In PD, neural transplantation will potentially replace the missing dopamine neurons and provide an endogenous source (rather than a drug source) of dopamine in the striatum and other dopamine-depleted regions. The last few years have provided clear evidence that dopamine cell transplant therapy additionally improves patients' response to L-DOPA (36,37,40). The L-DOPA can be taken up by transplanted dopamine cells and be appropriately converted by the DOPA decarboxylase into dopamine and released in a physiological way into the anatomic target zones.

COMBINED USE OF DA PHARMACOLOGICAL AND CELL THERAPIES: CAN THE NEURONAL REPLACEMENT ENHANCE L-DOPA RESPONSES AND REDUCE L-DOPA- INDUCED DYSKINESIAS IN PARKINSON'S DISEASE?

After neural transplantation of fetal dopamine cells to PD patients (when appropriate methods are used for transplantation), the vast majority of patients show a reduction in L-DOPA-induced abnormal dyskinesias

and dystonias in the "on" phase as well as percentage time spent "off" (35–37). These findings correlate with the presence of surviving dopaminergic grafts. Between 4 and 12 months after transplantation, up to ten patients worldwide have reduced their L-DOPA usage to zero in association with 50% to 80% reductions in prior symptoms as assessed by clinical rating scales (e.g., UPDRS). Cell replacement therapy is, however, in its infancy. This treatment modality is indeed novel and therefore requires experimentation to become useful and understandable in a neurobiological context (39). The symptomatic recovery in some individual patients from parkinsonism with grafting of fetal neural cells is truly remarkable. However, the six or seven research teams using this methodology around the world are in a sense artisans in that they are using different techniques and clearly have different success rates (35). Moreover, most of these research teams are improving their technology and transplantation techniques every year. Thus, the skepticism levied at some of their results and inconsistencies is understandable.

We have reviewed many active programs in North America and Europe and find that the most reliable finding after transplantation is some reduction of the use of L-DOPA and a dramatic loss of dyskinesias and "on-off" phenomena with full L-DOPA dose. The most likely explanation for this finding is that the biosynthetic machinery provided by the implanted cells will allow L-DOPA, through DOPA decarboxylase and other synaptic vesicular transport mechanisms, to be released and regulated for constancy of concentration at the synaptic sites in the striatum or elsewhere.

A compelling reason for neural transplantation is to provide needed relief for patients from drug side effects, which is a primary source of pain and dysfunction. This interpretation of neural transplantation effects in PD also provides an understanding of the progressive improvements seen. The patients worldwide who have no need for L-DOPA after transplantation all demonstrated a progressive sustained improvement over a 6-month to

6-year period (33,35). Moreover, the degeneration seen of the host dopaminergic systems also continues with no apparent effect on the implanted cells. This becomes apparent when analyzing data from unilaterally transplanted patients (the cohort of Lindvall et al. in Lund, Sweden). In their initial patient series, as the grafted side became functional, the contralateral side continued to degenerate (35). In this way, a form of hemiparkinsonism developed, and one patient was relieved by subsequent bilateral transplantation (35).

This type of clinical case-by-case evidence is complemented by a solid base of research in rodents and monkeys that demonstrates that the CNS dopaminergic system can be repaired (41). Nonetheless, in clinical application the variability in procedures by different transplant teams (on nearly all parameters used in cell preparation, cell number, and sites transplanted) as well as the lack of understanding of the large number of individual forms of parkinsonism among PD patients, make comparisons and conclusions difficult.

In summary, starting with animal experiments in the early 1980s and through the beginning of exploratory clinical trials in the late 1980s, the development of a new therapy involving fetal transplantation has achieved some spectacular results (32–37) as well as much evidence for the need of further refinement (38,39). Essentially, the treatment for PD with neural transplantation will potentially replace all the missing dopamine neurons and thereby provide an endogenous source (rather than a drug source) of dopamine in the striatum and other dopamine-depleted regions. The last few years have provided substantial evidence that dopamine cell transplant therapy also improves drug response (36,37,40). L-DOPA can be taken up by transplanted dopamine cells and be appropriately converted by the DOPA decarboxylase into dopamine and released in a physiological way into the anatomic target zones.

As outlined in the next section, the most recent research for transplantation in PD has shown that more targets than striatum need to be transplanted for optimal anatomic and func-

tional effects (42–48). We feel that triple stereotactic targeting, including the striatum, the subthalamic nucleus, and substantia nigra, pars reticulata may be necessary for optimal recovery in patients. This would achieve reinnervation of frontal (CPU and STN) and caudal output systems (the substantia nigra, pars reticulata and brainstem–spinal systems). Preliminary studies suggest a rapid and complete restoration of function after such procedures (42–48).

TRANSPLANTATION OF DOPAMINE CELLS: WHAT ARE THE APPROPRIATE CELLS AND TRANSPLANTATION SITES?

Experimental work has established protocols for reliable dopamine cell transplant survival, outgrowth, and function as a rational idea for PD intervention derived from neurobiology and cell culture (30,31). Many different strategies to improve the efficacy and survival of fetal ventral mesencephalic grafts have been described, including treatment with growth factors (49,50), antioxidants (51), and variations in cell dissociation techniques (52). The implantation procedure itself can also affect the outcome. Nikkah et al. have used a micrografting paradigm in rodents and shown that several implants of small volumes of cell suspension in the denervated striatum result in better functional recovery than do traditional macrograft implants (53).

Another important question in neural transplantation is the capacity for specific neuronal cell types to reinnervate selectively denervated host target regions (54–57). Transplanted embryonic neurons placed in denervated host targets display a relative specificity of fiber outgrowth into areas typical of their adult phenotype (54–57). The dopaminergic neurons of the midbrain can be divided into various subsets distinguished by different immunoreactivities, for example, dopamine transporter, tyrosine hydroxylase (TH), calbindin, and cholecystokinin (CCK) (54,58). Some of these markers correspond to distinct projection patterns from the substantia nigra and have been

- used to provide evidence for the selective growth regulation of grafted tissue (54).

Fetal cell transplantation experiments generally also make possible discoveries for applications involving stem cells. Simply put, stem cells allow a more abundant generation of transplantable DA cells than sources from fetal primary cells. The current opportunities for PD therapy are limited by access to appropriate cells for transplantation. There are a number of choices beyond the use of human fetal cells, for example, xenogeneic fetal mesencephalic neurons, stem cells, immortalized cells, or genetically modified nonneuronal or neuronal cells. Any of these choices are dependent on the state of research in each particular branch of cell biology. We have found that xenogeneic cells (pig) can be used in both animal models and patients with some success (57,59). Nevertheless, we believe the aim of producing a reliable source of a very large number of dopamine cells will require vigorous research programs for other cell types, such as stem cells. The use of pluripotent cells from the early blastula stage would, if the differentiation steps were known and genetic modification possible, provide a major source for transplantable dopamine cells. We have shown (60,61) that differentiation of such stem cell grafts can lead to the production of nerve cells, including dopaminergic neuronal phenotypes. Nevertheless, the realistic use of a stem cell source can be achieved, in our view, only after considerable experimentation, which will probably be preceded in the clinic by improvements in the standard fetal cell transplantation paradigm. This is important because data from a number of research groups over the last decade demonstrate that fetal cell transplantation can help parkinsonian patients, although there is still considerable room for technical improvements. Many neurosurgeons and neurologists have expressed the view that the neural cell implant treatment could supersede any currently available methodology, such as physiological stimulation or lesioning procedures, pallidotomy, or deep brain stimulation.

Surgical treatments in PD have undergone a renaissance with the advent of electrophysi-

ological interventions using deep brain stimulation such as pallidal or subthalamic high-frequency stimulation. By most accounts, these interventions provide relief from symptoms through depolarization, causing subsequent inhibition or activation of thalamic nuclei and their outflow to the cortex, or through subcortical output systems (22). This type of approach is based on an understanding of the circuitry involved in PD (see Fig. 47-2). Also, neural cell transplantation can be applied to this specific circuitry, but as a form of "repair" in regions involved in the ablative electrical stimulations because these regions are dopaminoceptive. Through dopamine synapse replacement by transplants, improvements to existing patient responses to L-DOPA could be obtained. The DA released in the STN and SNr are of exceptional importance for normal motor function (44-48). The previous focus on transplantation to the dopaminoceptive caudate-putamen, although useful, may have lacked the necessary DA replacement needed for normal function of the STN output to mesencephalic locomotor regions and VA/VL thalamus (Fig. 47-3).

Indeed, research toward transplantation in PD has recently indicated that additional targets to the striatum need to be transplanted for optimal anatomic and functional effects to occur (42-48). Thus, further research is warranted to determine the effects of multiple stereotactic targets including the striatum, subthalamic nucleus, and substantia nigra, pars reticulata. This will allow DA reinnervation of the direct (nigrostriatal) and indirect pathway (including STN) of frontal and caudal output systems (to the substantia nigra, pars reticulata and possibly beyond in the brainstem). The VTA (A10) DA input to layer 6 is relatively intact in PD. Preliminary data, including some from our own laboratories, indicate that fetal (or equivalent) dopaminergic neurons can innervate all DA zones depleted in PD. Experiments in progress investigate the specific contribution of additional targets to functional recovery by addressing whether multiple-target grafting (CPU, SNr, STN) will restore function in a PD primate model.

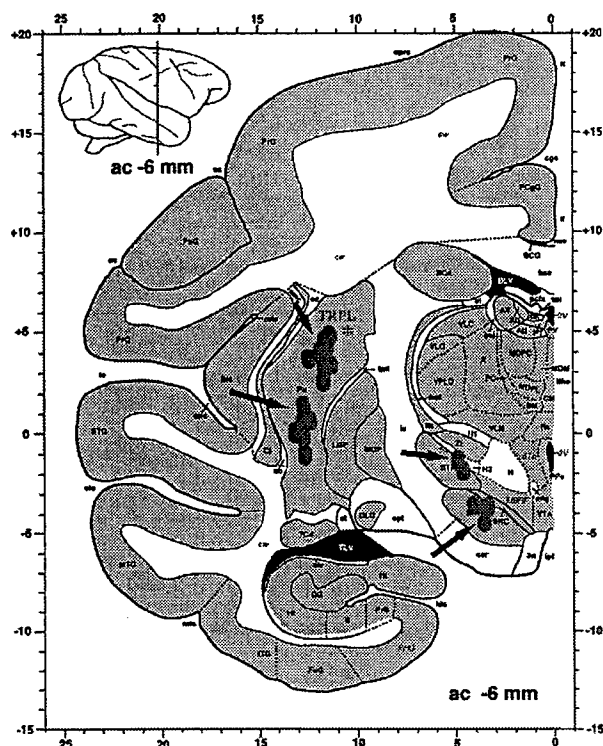


FIG. 47-3. Transplantation sites in putamen, substantia nigra, and subthalamic nucleus. Two sites in the putamen will be transplanted (arrows), and, depending on the paradigm, there will be transplantation to the subthalamic nucleus and the substantia nigra (arrows) (see text).

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REFERENCES

1. Brownell GL, Burnham CA, Stearns CW, et al. Development in high-resolution positron emission tomography at MGH. *Int J Imag Systems Technol* 1989;1:207-217.
2. Derenzo SE, Huecasman RH, Cahoon JL. A positron tomograph with 600 BGO crystals and 2.6 mm resolution. *IEEE Trans Nucl Sci* 1988;35:659-664.
3. Jenkins BG, Brouillet E, Chen YI, et al. Non-invasive neurochemical analysis of focal excitotoxic lesions in models of neurodegenerative illness using spectroscopic imaging. *J Cereb Blood Flow Metab* 1996;16:450-461.
4. Piccini P, Turjanski N, Brooks DJ. PET studies of the striatal dopaminergic system in parkinson's disease (PD). *J Neural Transm [Suppl]* 1995;45:123-131.
5. Hirato M, Ohye C, Takahashi A, et al. Study on the function of basal ganglia and frontal cortex using depth microrecording and PET scan in relation to the outcome of pallidotomy for the treatment of rigid-akinesia-type Parkinson's disease. *Stereotact Funct Neurosurg* 1997; 69:86-92.
6. Antonini A, Leenders KL, Vontobel P, et al. Complementary PET studies of striatal neuronal function in the different diagnosis between multiple system atrophy and Parkinson's disease. *Brain* 1997;120:2187-2195.
7. Moeller JR, Eidelberg D. Divergent expression of regional metabolic topographies in Parkinson's disease and normal aging. *Brain* 1997;120:2197-2206.
8. Hantraye P, Brownell A-L, Elmaleh DR, et al. Dopamine fiber detection by [^{11}C]-CFT and PET in a primate model of parkinsonism. *NeuroReport* 1992;3: 265-268.
9. Wullner U, Pakzaban P, Brownell A-L, et al. Dopamine terminal loss and onset of motor symptoms in MPTP-treated monkeys: A positron emission tomography study with [^{11}C]-CFT. *Exp Neurol* 1994;126:305-309.
10. Brownell A-L, Elmaleh DR, Meltzer P, et al. Cocaine congeners as PET imaging probes for dopamine terminals in normal and MPTP induced parkinsonism in non-human primate brain. *J Nucl Med* 1996;37:1186-1192.
11. Jordan S, Eberling JL, Bankiewicz KS, et al. 6-[^{18}F]Fluoro-L-m-tyrosine: metabolism, positron emission tomography kinetics, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine lesions in primates. *Brain Res* 1997;750: 264-276.
12. Eberling JL, Jagust WJ, Taylor S, et al. A novel MPTP primate model of Parkinson's disease: neurochemical and clinical changes. *Brain Res* 1998;805:259-262.
13. Doudet DJ, Chan GL, Holden JE, et al. 6-[^{18}F]Fluoro-L-

- DOPA PET studies of the turnover of dopamine in MPTP-induced parkinsonism in monkeys. *Synapse* 1998; 29:225-232.
14. Brownell A-L, Livni E, Galpern W, et al. PET imaging of dopamine terminals in a rat transplant model of Parkinson disease. *Ann Neurol* 1998;43:387-390.
 15. Brownell A-L, Jenkins BG, Elmaleh DR, et al. Combined PET/MRS brain studies show dynamic and long-term physiological changes in a primate model of Parkinson disease. *Nature Med* 1998;4:1308-1312.
 16. Frost JJ, Rosier AJ, Reich SG, et al. Positron emission tomographic imaging of the dopamine transporter with ¹¹C-WIN 35,428 reveals marked decline in mild Parkinson's disease. *Ann Neurol* 1993;34:423-431.
 17. Ginovart N, Lundin A, Farde L, et al. PET study of the pre- and post-synaptic dopaminergic markers for the neurodegenerative process in Huntington's disease. *Brain* 1997;120:503-514.
 18. Holshouser BA, Komu M, Moller HE, et al. Localized proton NMR spectroscopy in the striatum of patients with idiopathic Parkinson's disease: a multicenter pilot study. *Magn Reson Med* 1995;33:589-594.
 19. Guimaraes AR, Schwartz P, Prakash MR, et al. Quantitative nuclear magnetic resonance spectroscopic imaging of neuronal loss in excitotoxic rat brain model. *Neuroscience* 1995;69:1095-1101.
 20. Federico F, Simone IL, Lucivero V, et al. Proton magnetic resonance spectroscopy in Parkinson's disease and atypical parkinsonian disorders. *Mov Dis* 1997;12:903-909.
 21. Grafton ST, Waters C, Sutton J, et al. Pallidotomy increases activity of motor association cortex in Parkinson's disease: a positron emission tomographic study. *Ann Neurol* 1995;37:776-783.
 22. Lozano AM, Lang AE, Hutchison WD, et al. New developments in understanding the etiology of Parkinson's disease and in its treatment. *Curr Opin Neurobiol* 1998; 8:783-790.
 23. Alexander GE, Crutcher MD, DeLong MR. Basal ganglia thalamocortical circuits: parallel substrates for motor, oculomotor, "prefrontal" and "limbic" functions. *Prog Brain Res* 1990;85:119-146.
 24. Filion M, Tremblay L. Abnormal spontaneous activity of globus pallidus neurons in monkeys with MPTP-induced parkinsonism. *Brain Res* 1991;547:142-151.
 25. Bergman H, Wichmann T, Karmon B, et al. The primate subthalamic nucleus. II. Neuronal activity in the MPTP model of parkinsonism. *J Neurophysiol* 1994;72: 507-520.
 26. Tolosa E, Marti MJ, Valldeoriola F, et al. History of levodopa and dopamine agonists in Parkinson's disease treatment. *Neurology* 1998;50:S2-S10.
 27. Marsden CD. Parkinson disease. *Postgrad Med J* 1992; 68:538-543.
 28. Lange KW. Clinical pharmacology of dopamine agonists in Parkinson's disease. *Drugs Aging* 1998;13:381-389.
 29. Poewe W. Should treatment of Parkinson's disease be started with a dopamine agonist? *Neurology* 1998;51: S21-S24.
 30. Bjorklund A, Stenevi U. Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. *Brain Res* 1979;177:555-560.
 31. Perlow M, Freed W, Hoffer B, et al. Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. *Science* 1979;204: 643-647.
 32. Hong M, Mendez I. Double grafting of human fetal dopamine cells restores function and anatomy of the nigrostriatal pathway in the rodent model of Parkinson's disease. *Exp Neurol [Suppl]* (in press).
 33. Mendez I, Dagher A, Hong M. Transplantation of fetal dopamine cells hibernated in GDNF: From animal studies to clinical trials. *Exp Neurol [Suppl]* (in press).
 34. Lindvall O, Sawle G, Widner H, et al. Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. *Ann Neurol* 1994;2:172-180.
 35. Lindvall O. Update on fetal transplantation: the Swedish experience. *Mov Dis* 1998;13:83-87.
 36. Freeman TB, Olanow CW, Hauser RA, et al. Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. *Ann Neurol* 1995;38: 379-388.
 37. Wenning G, Odin P, Morrish P, et al. Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. *Ann Neurol* 1997;42:95-107.
 38. Freed CR, Breeze RE, Rosenberg NL, et al. Survival of implanted fetal dopamine cells and neurologic improvement 12 and 46 months after transplantation for Parkinson's disease. *N Engl J Med* 1992;327:1549-1555.
 39. Itakura T, Nakai E, Nakao N, et al. Transplantation of neural tissue into the brain—a new therapeutic modality for the 21st century. *Neurol Med Chir (Tokyo)* 1998; 38:756-762.
 40. Kordower JH, Freeman TB, Snow BJ, et al. Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. *N Engl J Med* 1995;332:1118-1124.
 41. Isacson O, Deacon TW. Neural transplantation studies reveal the brain's capacity for continuous reconstruction. *Trends Neurosci* 1997;20:477-482.
 42. Mendez I, Sadi D, Hong M. Reconstruction of the nigrostriatal pathway by simultaneous intrastriatal and intranigral dopaminergic transplants. *J Neurosci* 1996;15: 7216-7227.
 43. Winkler C, Bentlage C, Nikkhah G, et al. 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* 1999;155:165-186.
 44. van Horne CG, Mahalik T, Hoffer B, et al. Behavioral and electrophysiological correlation of human mesencephalic dopaminergic xenograft function in the rat striatum. *Brain Res Bull* 1990;25:325-334.
 45. Stromberg I, van Horne C, Bygdeman M, et al. Function of intraventricular human mesencephalic xenografts in immunosuppressed rats: electrophysiological and neurochemical analysis. *Exp Neurol* 1991;112:140-152.
 46. van Horne CG, Hoffer BJ, Stromberg I, et al. Clearance and diffusion of locally applied dopamine in normal and 6-hydroxydopamine-lesioned rat striatum. *J Pharmacol Exp Ther* 1992;263:1285-1292.
 47. van Horne CG, Eken S, Henry S, et al. Nigral and striatal grafts into the subthalamic nucleus of 6-OHDA lesioned rats. Paper presented at the Sixth International Neural Transplant Meeting, 1997;2.
 48. van Horne CG, Eken S, Henry S, et al. Nigral and striatal grafts to the subthalamic nucleus in an animal model of Parkinson's disease. *World Soc Stereotact Funct Neurosurg Abstr* 1997;98A-03:75.

49. Haque NSK, Hlavin M-L, Fawcett JW, et al. The neurotrophin NT4/5, but not NT3, enhances the efficacy of nigral grafts in a rat model of Parkinson's disease. *Brain Res* 1996;712:45-52.
50. Mott JL, Eken S, Bowenkamp K, et al. Effects of glial cell line-derived neurotrophic factor on dopaminergic transplants to the 6-OHDA denervated striatum. *Soc Neurosci Abstr* 1996;22:1492.
51. Grasbon-Frodl EM, Nakao N, Brundin P. The Lazaroid U-83836E improves the survival of rat embryonic mesencephalic tissue stored at 4°C and subsequently used for cultures or intracerebral transplantation. *Brain Res Bull* 1996;39:341-347.
52. Barker RA, Fricker RA, Abrous DN, et al. 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* 1995;4:173-200.
53. Nikkah G, Cunningham MG, Jodicke A, et al. Improved graft survival and striatal reinnervation by microtransplantation of fetal nigral cell suspensions in the rat Parkinson model. *Brain Res* 1994;633:133-143.
54. Schultzberg M, Dunnett SB, Bjorklund A, et al. Dopamine and cholecystokinin immunoreactive neurons in mesencephalic grafts reinnervating the neostriatum: evidence for selective growth regulation. *Neuroscience* 1984;12:17-32.
55. Nilsson OG, Clarke DJ, Brundin P, et al. Comparison of growth and reinnervation properties of cholinergic neurons from different brain regions grafted to the hippocampus. *J Comp Neurol* 1988;268:204-222.
56. Isacson O, Deacon TW. Specific axon guidance factors persist in the mature rat brain: evidence from fetal neuronal xenografts. *Neuroscience* 1996;75:825-837.
57. Isacson O, Deacon TW, Pakzaban P, et al. Transplanted xenogeneic neural cells for Parkinson's and Huntington's diseases exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibers. *Nature Med* 1995;1:1189-1194.
58. Haber SN, Ryoo H, Cox C, et al. Subsets of midbrain dopaminergic neurons in monkeys are distinguished by different levels of mRNA for the dopamine transporter: comparison with the mRNA for the D₂ receptor, tyrosine hydroxylase and calbindin immunoreactivity. *J Comp Neurol* 1995;362:400-410.
59. Deacon T, Schumacher J, Dinsmore J, et al. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nature Med* 1997;130:350-353.
60. Deacon T, Dinsmore J, Costantini LC, et al. Blastula-derived stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. *Exp Neurol* 1998;149:28-41.
61. Isacson O. The neurobiology and neurogenetics of stem cells. *Brain Pathol* 1999;9:495-498.

The Potentials of Gene Therapy for Treatment of Parkinson's Disease

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Development of molecular biological techniques has resulted in discovery of genetic mutations that are responsible for various neurodegenerative disorders including Parkinson's disease (PD) and these discoveries are leading to better understanding of the pathogenesis of these disorders ^{1,2,3}. Such technologies also lead to development of genetic engineering methods as potential therapeutic modalities for these disorders ⁴⁻⁶. This combination of development anticipates new treatments for these disorders that have not been amenable to traditional therapies. The idea of gene therapy has been proposed decades ago, but a successful clinical application has not yet been implemented ⁴. As with most of the scientific developments, the initial implementation of ideas reveals unanticipated problems and further improvements and modifications follow. This chapter will discuss the basic concepts and methodology of gene therapy including recent promising advances and limitations of the current techniques. The potential of the gene therapy for PD will then be illustrated through specific examples in animal models of PD.

I. Basic concepts of gene therapy

In its simplest concept, gene therapy delivers DNA materials into host instead of the final desired product, utilizing the internal machinery to transcribe the DNA information into RNA, then protein, which in turn, may produce a desired product. Delivery of DNA provides more efficient, sustained, and localized supply of the product than delivering the product itself. The defective function of abnormal genes in genetic disorders could be complemented by normal genetic information. In cases where the genetic abnormality leads to gain of toxic function, a good understanding of the mechanism of toxicity is necessary in order to intervene and neutralize the toxic function. In many cases, gene therapy does not directly target the genetic mutations, but rather provides a biological minipump that delivers pharmacological compounds directly into the localized sites of the body ⁷.

II. Gene therapy methods for the nervous system

Two general approaches have been employed for delivering therapeutic genes into target tissues: the *ex vivo* gene therapy by which cells are genetically modified *in vitro*, then transplanted into the host, and the *in vivo* gene therapy by which therapeutic gene is introduced directly into the host somatic cells *in situ*, using viral vectors (Fig. 1).

1) *Ex vivo* modality

For *ex vivo* gene transfer, the cells to be transduced with the genes should be easily obtainable, readily cultured, able to express the transgenes, and undergo the selection process that is used to enrich the population of cells transduced. The cells should be non-oncogenic, immunologically compatible with the recipient, and survive well in the brain. The cells then could be genetically modified to secrete the neuroactive substances such as neurotransmitters and neurotrophic factors and serve as a biological pump at a localized site in the brain. Primary skin fibroblast cells taken from adult animals satisfy most of the criteria outline above. Fibroblasts could be easily obtained from patients: their own skin biopsy could be modified into customized immunocompatible donor cells. The astrocyte is also an attractive cell type for grafting studies in the CNS due to its intrinsic supportive role in the CNS. These cells are, however, not as easy to transduce with retroviruses as fibroblasts ⁸.

The ideal cells for CNS somatic gene therapy would be cells of CNS origin with neuronal features such as storage mechanism, secretory pathways, and regulatory signal transduction pathways for the final product. Primary neurons could be genetically modified to boost their survival or alter their phenotypes. To avoid obtaining primary neurons each time, while reducing the risk of tumor formation, techniques of conditional immortalization using non-transforming oncogenes have been employed^{9,10}. More recently, CNS progenitor cells that are capable of cell division have been isolated from fetal and adult brains, particularly from regions that undergo neurogenesis beyond the developmental ages such as subventricular zone, olfactory system and hippocampus ¹¹, but also from other areas of adult brain ¹². These were propagated in the presence of epidermal growth factor ¹³ or fibroblast growth factor ¹¹. These cells can then differentiate into glial or neural phenotypes in culture and in the brain after transplantation ^{11,14}. Understanding proper differentiation signals for the desired neuronal phenotypes would provide a means of generating unlimited supply of transplant donor cells. For example, Nurr1 is a critical transcriptional factor that is important in development of dopaminergic neurons ^{15,16}. Nurr1 has been shown to induce a dopaminergic phenotype, mainly the expression of the first and rate-limiting step of dopamine synthesis, tyrosine hydroxylase (TH) in neuronal precursor cells ¹⁷. However, whether Nurr1 alone is sufficient to induce full-fledged dopaminergic

phenotypic expression is not clear. Meanwhile, modifying progenitor cells by additional neurotransmitter synthesizing genes, such as TH may be necessary^{18,19}.

The advantages of *ex vivo* gene transfer include the ability to control and monitor the gene transfer process before the cells are placed back into the subjects. The biochemical effect of the transgene can be characterized and potential tumorigenesis can be assessed. Toxicity of the virus can be screened. In addition, grafted cells may provide useful functions beyond what is provided by the transgene, such as serving as a substrate for axonal growth, or restoring synaptic contacts with the host neurons. However, for certain applications, direct *in vivo* gene therapy may be more suitable.

2) *In vivo or direct gene transfer*

Host CNS cells may be better equipped to produce the desired products from the genetic information than foreign donor cells since they may possess the machinery necessary for the post-translational modification of the gene products, the appropriate cofactors, and the ability to secrete the neurotransmitters or other neuroactive compounds. Viral solutions may also be less intrusive than grafts to the normal brain physiology because grafts of genetically engineered cells could disrupt the normal architecture. Moreover, grafting genetically modified cells is more suitable for delivery of secretable products that act on cell surface receptors such as neurotransmitters and neurotrophic factors, but not for delivery of intracellular proteins such as protein kinases, receptors and transporters. On the other hand, the consequence of delivering therapeutic molecules (e.g., dopamine) directly into host cells (e.g., striatal neurons) that does not normally express such molecules is not as predictable as delivering them extracellularly by grafts serving as a biological minipump. Expression of the gene product could lead to untoward alteration of the host cell function. The major disadvantages of *in vivo* gene transfer also include the safety of using viruses directly. Use of neurotrophic viruses such as herpes virus, adenovirus, adeno-associated virus, and lentivirus has recently been shown to be effective in gene transfer into neurons.

3) *Vector constructs*

Although many viruses have been explored as vector systems, the following virus vectors have been most extensively applied to CNS gene transfer. Each has its own advantages and disadvantages and there does not seem to be a clear universal vector system of choice at this point. A simplified general scheme of virus vectors is described in Fig. 2.

a) Neurotrophic virus vectors for in vivo gene therapy

Herpes simplex type I virus (HSV-1) vectors infect a wide range of host cells including post-mitotic neurons and can establish latency indefinitely within the neuron. Two general approaches include 1) use of recombinant HSV with various deletions that render the virus replication-defective ^{20,21} and 2) use of an amplicon based on plasmid vectors containing the transgene plus minimal HSV genes such as origin of DNA replication and packaging site. Because of the large size of HSV, development of safe vectors will require extensive understanding of the genome to be able to reduce the neurovirulence and cytotoxicity ^{22,23}. On the other hand, the large size of HSV viral genome (152 kilobase pairs [kb]) also allows insertion of a large foreign DNA, which may prove very useful for certain *in vivo* CNS gene therapy.

The adenovirus represents an attractive candidate for direct gene transfer into the CNS because of its high titer virus stocks, efficient infection of post-mitotic cells, and the relatively benign nature of the virus infection ²⁴. The adenovirus is a double-stranded, linear DNA virus that, in its wild-type form, causes a variety of mild flu-like ailments. The potential for adenovirus vectors to achieve efficient gene transfer to neurons, microglial cells, and astrocytes *in vivo* have been noted ^{25,26}. Expression of the transgene products is limited in duration, probably due to the immune reaction from the host ^{27,28}. Recent constructs of adenovirus vectors which delete all viral genes show high levels of stable expression with minimal toxicity and allow inserts of up to 37 kb size ²⁹.

Adeno-associated virus (AAV) is a nonpathogenic DNA virus, which requires helper viruses such as adenovirus and herpes virus for productive infection. AAV vectors have minimal viral sequences of their own and therefore have minimal deleterious consequences. Because of the small genome, AAV can accommodate only about 5 kb inserts. AAV integrates into a specific site in the chromosome 19q13.3 ³⁰, and long-term expression of

various neural genes have been noted in the CNS with minimal host reactions³¹⁻³⁴. Despite its limited capacity for transgenes, AAV may be the safest vector capable of long-term *in vivo* expression at this point.

b) Retrovirus vectors for ex vivo therapy

Disabled murine leukemia retroviruses have been most widely used for gene therapy, especially for most *ex vivo* applications. Retroviruses infect a broad range of cells with high efficiency. Many generations of modified vectors have been developed to lower the chance of recombination event that can lead to wild type virus generation³⁵. The integration of provirus into the chromosome is stable and precise, but the location is apparently random, and therefore could produce an insertional mutation by disrupting a normal gene. Homologous recombination of the retroviral vectors with the retroviral genome in the packaging cells could generate the wild type virus, which can lead to the formation of tumors³⁶. However, current generation of packaging cells has minimal overlap of sequences with helper viruses and the helper virus genes are separated into two separate plasmids³⁷ to further minimize the possibility of recombination. Disadvantages of retroviral vectors include relatively low viral titers, usually less than 10^6 infectious units per ml. The size of the gene that can be inserted is limited to 8 to 10 kb. Retroviruses also require dividing cells with active replication and DNA synthesis for the provirus integration to occur³⁸. Therefore, retroviruses are not useful for *in vivo* gene transfer into non-dividing cells, but remains as the mainstay tool for *ex vivo* therapies.

c) Hybrid vectors

Hybrid vectors combine advantageous properties of different virus vectors³⁹. A good example of hybrid vectors is lentiviral vector which combines the ability of the human immuno-deficiency virus (HIV-1) to insert a provirus copy into the genome of nondividing cells with wide host range and high infectivity of stomatitis virus G surface glycoprotein. Unlike other retroviruses, lentiviruses can infect non-dividing cells by using nuclear import machinery to gain entry into the nucleus in quiescent cells and then integrating viral DNA into the host chromosome. Stable transgene expression in neurons was noted *in vivo* for up to 3 months without appreciable pathological changes and immune response⁴⁰. Because the HIV prevents cells from undergoing mitosis, development of packaging cell lines that can facilitate vector generation has been hampered. Further safety of the

vectors must be assured before clinical use of this HIV-based vectors can be contemplated. Another example of hybrid viruses is HSV/AAV vector. AAV rep gene and inverted terminal repeats of AAV are incorporated into HSV amplicon vectors to enable amplification and specific integration of desired gene into chromosome ⁴¹. These new generation of hybrid vectors may provide ideal tools circumventing the safety issues and achieving long-term expression of the transgenes.

d) Further development of gene therapy vectors

Before these gene therapy methods can be applied to human neurodegenerative disorders, several issues need to be resolved. Safety issues of gene therapy vectors have been discussed above and a great deal of progress has been made in this regard. Another safety issue concerns the transgene itself. Expression of the transgenes and secretion of the transgene products into the serum can lead to immune responses to the transgene products. However, it is not clear whether immune system can recognize the transgene products that are expressed intracellularly but are not secreted.

Another problem of gene therapy has been the inability to obtain long-term stable transgene expression *in vivo* ⁴². Long-term expression is easily achieved in cultures throughout many passages of donor cells. Once the cells are implanted in animals, however, transgene expression diminishes rapidly ⁴². Although this remains an unresolved problem, several possibilities appear promising. Vectors that lead to integration of the transgenes into the chromosomes are more likely to be stable than vectors that stay episomal. In addition, integration sites utilized by the vectors play a significant role in the transcriptional activity of the transgene. Retroviruses integrate into the chromosome during active division but the integration site may become dormant in the quiescent state that grafted cells assume *in vivo*. Taking advantage of the ability of lentivirus to enter into the nucleus of non-dividing neurons, and to integrate into sites that are active during quiescence, investigators have been able to achieve long-term expression of transgenes in neurons of the CNS ⁴⁰. AAV also integrates into the genome of host neurons and has shown promise in its ability to sustain long-term gene expression *in vivo* ³¹⁻³³.

Promoter types may also significantly influence long-term expression. Most gene therapy vectors contain viral promoters to express the transgenes, but their transcriptional activity may be suppressed in the somatic cells. Promoters of endogenous cellular genes such as a constitutive housekeeping gene promoters⁴³ or cell type-specific promoters⁴⁴ have had some initial success. However, most tissue-specific promoters have a low basal activity. A more recent approach has shown enhanced transcriptional activity from synthetic promoters engineered from a combination of operator elements⁴⁵. The effect of the promoter may depend on the cell type and vectors used^{46,47}.

Although the current focus is on achievement of high and stable expression of transgenes, excessive levels of products such as L-DOPA are likely to produce adverse side effects such as dyskinesias. Optimal levels are critical for physiological function of many substances, and the ability to regulate the transgene expression is important. Promoters that can be regulated externally or have a built-in feedback mechanism will be an important advance for the future. One such system was developed to express the transgene by promoters whose activities can be either turned on or off by administration of tetracycline both *in vitro*¹⁰ and *in vivo*⁴⁸. Other combination of nontoxic substances and heterologous transcription factors that bind to regulatable promoters have been developed^{49,50}.

III. Strategies of gene therapy for Parkinson's disease

1) Dopamine replacement by delivering neurotransmitter synthesizing genes

The pathogenesis of PD is well understood and the efficacy of replacing the neurotransmitter dopamine is well established. However, long-term treatment with the precursor, L-3,4-dihydroxyphenylalanine (L-DOPA) produces dyskinesia and fluctuations in most patients. These problems can be either prevented or reduced by continuous delivery of dopamine into striatum⁵¹. Various dopamine producing cells including dopaminergic fetal neurons can also provide continuous and site-specific dopamine delivery⁵². However, given the limitations in obtaining proper fetal tissues in sufficient amounts, alternative sources of donor cells such as xenografts and genetically engineered cells have been proposed. To provide L-DOPA into the brain by gene therapy, initial studies have focused on introducing TH gene, which is the first and rate-limiting step of dopamine synthesis, using established cell lines

such as rat fibroblast 208F cells⁵³, NIH3T3 cells, and endocrine cell lines, AtT-20 and RIN cells⁵⁴. Subsequently, to overcome the problems with the tumor cell lines, primary cells such as fibroblast cells⁵⁵, or astrocytes⁸ have been shown to produce long-term graft survival without tumor formation or immunological rejection. More recently, neuronal precursor cells have been genetically modified to express dopaminergic phenotype, but their efficacy in *in vivo* models is not clear yet. In addition, direct *in vivo* TH gene transfer into the brain cells using viral vectors has also been attempted^{56,57}. Most of these studies have shown partial reversal of apomorphine-induced rotation, which is used as a rodent behavioral model of PD.

Further refinement of the L-DOPA delivery has been achieved by using the gene GTP cyclohydrolase I (GCH1), which is the first and rate-limiting step in the biosynthesis of tetrahydrobiopterin, an essential cofactor for TH. Experimental gene therapy studies have shown that that double transduction with TH and GCH1, by either *ex vivo*⁵⁸ or *in vivo*³³ gene transfer, is necessary for sufficient L-DOPA and dopamine production in rat models of PD (Fig. 3A). Microdialysis studies have shown direct biochemical evidence for the efficacy. These studies have also pointed out the limitations of the rotational behavioral model, which have contributed to misleading conclusions of previous studies about the efficacy of using TH gene alone. Importance of GCH1 has been underlined by the finding that mutations in GCH1 in L-DOPA-responsive dystonia (DRD) patients lead to loss of its function to generate cofactor, tetrahydrobiopterin (BH₄)⁵⁹. The absence of cofactor results in the lack of L-DOPA and dopamine production, parkinsonism and dystonia of DRD patients. Interestingly, symptoms of DRD patients can be almost completely ameliorated by L-DOPA therapy without development of long-term complications that is commonly observed in PD. Such an ideal response to L-DOPA in DRD abrogates any need for gene therapy, but provides us with an insight as to the importance of events that occur downstream from production of L-DOPA, in the therapy of PD.

The second step in dopamine synthesis is decarboxylation of L-DOPA to dopamine by aromatic L-amino acid decarboxylase (AADC). Although there seems to be some decarboxylation of L-DOPA to dopamine even with severe dopaminergic neuronal loss in animal models and patients with PD^{60,61}, the exact source and sites of AADC *in vivo* are not clear⁶². When additional AADC was provided within the genetically modified grafts producing L-

DOPA, the final levels of L-DOPA and dopamine decreased, presumably due to the feedback inhibition of dopamine on TH⁶³. Therefore, keeping the cytoplasmic dopamine levels low by sequestering it into the vesicles at high concentrations seems to be a critical strategy of dopamine delivery⁶⁴. Combination of L-DOPA-producing cells with cells that can decarboxylate L-DOPA to dopamine and storage them in vesicles may be more optimal, but this has not been tested directly yet (Fig. 3B).

In the strategies of providing a source of L-DOPA or dopamine as described above, the precise control of the exact amount of L-DOPA or dopamine given by the gene therapy will be critical and may be difficult to achieve. Excessive dopamine will be detrimental to the patients. One could attempt to control the precise levels by using the regulatable promoters described earlier. We proposed another approach of combining the precursor L-DOPA administration with genetic machinery to process L-DOPA more efficiently⁶⁵. Providing additional AADC in the denervated striatum of rat models of PD resulted in higher levels of dopamine produced from L-DOPA³⁴. However, increasing the level of dopamine is not as critical as prolonging the duration of elevation and buffering the fluctuating levels of dopamine following administration of L-DOPA. Combined use of AADC for decarboxylation of L-DOPA and vesicular monoamine transporter (VMAT) for efficient storage of dopamine within cells sustained high levels of dopamine from L-DOPA administration compared to using only AADC⁶⁴ (Fig. 3C). This underlines the importance of storage and gradual release of dopamine in pathogenesis and treatment of fluctuating responses in PD.

Although site-specific and sustained delivery of dopamine would provide a major advance in PD therapy, it is not clear whether this approach will be sufficient to restore the entire symptom complex of PD including some of the L-DOPA-associated complications such as dyskinesia. The role of nondopaminergic system in PD needs to be explored further. In addition, restoration of neuronal connectivity by the graft and complex functions such as feedback interaction of dopaminergic neurons with the striatal neurons may be important. However, it is not clear whether such proper interactions could be restored even with fetal dopaminergic neurons or dopaminergic neuronal cell lines generated by *ex vivo* gene therapy.

2) *Repair and protection strategy by neurotrophic factor delivery*

The peptidergic growth factors have wide-ranging effect on the survival of neuronal populations as well as on regulation of neurotransmitter release and modulation of neural activities in the adult CNS. Although the precise mechanism of the neuronal degeneration of PD is not known, neurotrophic factors or growth factors may prevent or slow cell death cascade regardless of the initial triggering event. Several growth factors that have shown to possess trophic activity in the dopaminergic system include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, basic fibroblast growth factor (bFGF), transforming growth factor β (TGFB), and glial cell line-derived factor (GDNF)⁶⁶⁻⁷¹. All of these factors have been shown to enhance dopamine neuronal cell viability in vitro, although this in some cases appear to be mediated by indirect effects on other cell types^{70,72}. In vivo effects have been mostly noted by neurotrophin and GDNF family members. In vivo delivery of BDNF enhances striatal dopamine turnover and decreases nigral dopamine turnover, as well as causes contralateral rotations and locomotor activity in amphetamine treated rats^{73,74}. Direct infusion of BDNF above the SN increases the firing rate and even the number of electrically active dopamine neurons⁷⁵ in this pivotal brain region. The presence and expression of BDNF and its receptor, TrkB in the adult SN indicates that BDNF may regulate DA function under normal physiological conditions^{76,67,77}. As in the developing CNS, striatal BDNF is retrogradely transported to DA neurons in adult brain⁷⁸. Infusion of NT-4/5 into the ventral mesencephalon also causes increased striatal DA turnover and release and locomotor activation following the amphetamine stimulation⁷⁹. The NT-3 molecule also enhances amphetamine-induced contralateral turning and decreases SN DA turnover⁷⁴. One of the most potent factors for the DA substantia nigra is GDNF. In normal animals, GDNF increases both spontaneous and amphetamine-induced motor behavior. These motor effects occur in parallel with increased DA levels and turnover in the SN, and enhanced DA turnover and consequent reduction in striatal DA levels⁸⁰. Infusion of GDNF into the striatum results in retrograde transport to the SN DA neurons⁸¹.

These results support the concept that even in the adult brain, developmentally dependent growth factors may be able to augment DA neuronal function. The problem with pharmacological delivery of the peptidergic growth factors is access to the brain. The non-oral delivery methods of these substances frequently require methods that circumvent the blood-brain-barrier, for instance by neurosurgical intraparenchymal or intraventricular infusions.

These methods are site-specific but invasive and often unpredictable. Tissue damage at the cannula placement site is frequently seen. Moreover, effective delivery is limited by diffusion properties of the factor within brain parenchyma ⁸². Additionally, intraventricular administration of the TrkB ligand BDNF is ineffective due to binding by truncated TrkB receptors which are present in the ependymal lining of the ventricles.

As an alternative to direct infusion, methods for cell-mediated delivery of therapeutic proteins have been developed including ex vivo as well as in vivo gene transfer. Implants of BDNF-producing fibroblast cells protected against neurotoxins such as 6-hydroxydopamine (6-OHDA) ⁸³ and 1-methyl-4-phenylpyridinium (MPP⁺) ⁸⁴. In addition, sprouting of dopaminergic fiber has been demonstrated in BDNF-transduced fibroblasts implanted into the midbrain ⁸⁵. GDNF has been delivered into rat substantia nigra neurons by adenovirus or AAV vectors and protected these neurons from progressive degeneration induced by 6-OHDA lesion of the dopaminergic terminals in the striatum ^{26,86}. A more practical approach of injecting the viruses into the striatum resulted in cellular and behavioral protection from striatal 6-OHDA lesions ^{87,88}.

3) Other potential targets of gene therapy for Parkinson's disease: intervention of pathogenesis

Understanding the etiology and pathogenesis of PD may allow us to intervene directly at the level of pathogenesis and forestall the clinical manifestations or stop progression of the disease. Although the etiology of PD is still not known for most cases, recent discoveries of mutations in α -synuclein in families with autosomal dominant inheritance of PD ^{1,2} and *parkin* in families with autosomal recessive juvenile parkinsonism ³ may provide us new clues. Knowledge of the precise steps by which these mutations lead to dopaminergic neuronal death could allow us to apply these findings to sporadic form(s) of PD as well.

Although the precise strategy awaits further understanding of the mechanism of the toxicity by the genetic mutations, general therapeutic approaches for a known genetic defect can be outlined. For autosomal recessive genetic disorders which commonly confer a "loss of function", augmentation of the missing genetic information may restore the abnormalities. On the other hand, a dominant disorder may involve a "gain of function" induced by the mutant protein. For these disorders, it is not possible to simply replace the defective gene with a normal one.

Techniques for specifically targeting the abnormal sequence and replacing it with a normal sequence exist and are applied in generation of transgenic animals with "knock-out, knock-in" strategy⁸⁹. However, these are not easily applicable to humans. The intervention of gene expression at the level of messenger RNA could be attempted. It may be possible to integrate viral or non-viral vectors carrying catalytic antisense RNAs or ribozymes which bind to, and irreversibly cleave, abnormal mRNAs⁹⁰. Antisense RNA has been used to hamper transcription, processing, transport and/or translation of mRNAs in a variety of cell types.

Intervention further downstream of the abnormal protein expression will be possible once the normal physiology of the mutant protein is known. Pharmacological blockade of the effects of the mutated protein may be envisaged by introducing genes that will produce inhibitory substances. In addition, even in cases where the exact function of the mutant protein is unknown, understanding the general process of dopaminergic cell death could lead to other approaches in preventing disease progression. For example, the genes preventing apoptosis could be expressed in dopaminergic neurons to save them from their demise⁹¹. Given the possible role of oxidative stress in dopaminergic neuronal degeneration in PD, overexpression of free radical scavenging enzyme, such as superoxide dismutase (SOD) may protect dopaminergic neurons from degeneration. Experimental models show that SOD overexpression protects dopaminergic neurons from the neurotoxicity⁹². In addition, SOD enhances the survival of the grafted neurons⁹³.

In summary, gene therapy has the potential to provide an efficient delivery of various genes and products into a localized site. Along with advances in new genetic discoveries and understanding of the pathogenesis of PD, this may provide the most efficient means of therapeutic intervention at appropriate levels. In addition, gene therapy experiments contribute to further understanding of the biology of the diseases. Gene therapy is an evolving concept that could bridge the new molecular understanding of the diseases and current modalities of PD therapy.

REFERENCES

1. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the α -synuclein gene identified in families with Parkinson's disease. *Science* 1997; 276:2045-2047.

2. Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, et al. The ubiquitin pathway in Parkinson's disease [letter]. *Nature* 1998; 395:451-452.
3. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 1998; 392:605-608.
4. Friedmann T. The road toward human gene therapy--a 25-year perspective. *Ann.Med.* 1997; 29:575-577.
5. Hermens WTJM, Verhaagen J. Viral vectors, tools for gene transfer in the nervous system. *Prog.Neurobiol.* 1998; 55:399-432.
6. Kang UJ. Potential of gene therapy for Parkinson's disease: neurobiologic issues. *Mov.Disord.* 1998; 13 Suppl 1:59-72.
7. Gage FH. Intracerebral grafting of genetically modified cells acting as biological pumps. *Trends Pharmacol.Sci.* 1990; 11:437-439.
8. Lundberg C, Horellou P, Mallet J, Björklund A. Generation of DOPA-producing astrocytes by retroviral transduction of the human tyrosine hydroxylase gene: In vitro characterization and in vivo effects in the rat Parkinson model. *Exp.Neurol.* 1996; 139:39-53.
9. Renfranz PJ, Cunningham MG, McKay DG. Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* 1991; 66:713-729.
10. Hoshimaru M, Ray J, Sah DWY, Gage FH. Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-myc oncogene. *Proc.Natl.Acad.Sci.USA* 1996; 93:1518-1523.
11. Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, et al. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc.Natl.Acad.Sci.USA* 1995; 92:11879-11883.
12. Palmer TD, Ray J, Gage FH. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol.Cell.Neurosci.* 1995; 6:474-486.
13. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; 255:1707-1710.
14. Winkler C, Fricker RA, Gates MA, Olsson M, Hammang JP, Carpenter MK, et al. Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain. *Mol Cell Neurosci* 1998; 11:99-116.
15. Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T. Dopamine neuron agenesis in *Nurr1*-deficient mice [see comments]. *Science* 1997; 276:248-250.
16. Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, et al. *Nurr1* is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc Natl Acad Sci U.S.A.* 1998; 95:4013-4018.
17. Wagner J, Akerud P, Castro DS, Holm PC, Canals JM, Snyder EY, et al. Induction of a midbrain dopaminergic phenotype in *Nurr1*-overexpressing neural stem cells by type 1 astrocytes. *Nat.Biotechnol.* 1999; 17:653-659.

18. Anton R, Kordower JH, Maidment NT, Manaster JS, Kane DJ, Rabizadeh S, et al. Neural-targeted gene therapy for rodent and primate hemiparkinsonism. *Exp.Neurol.* 1994; 127:207-218.
19. Sabate O, Horellou P, Vigne E, Colin P, Perricaudet M, Buc-Caron MH, et al. Transplantation to the rat brain of human neural progenitors that were genetically modified using adenoviruses. *Nat.Genet.* 1995; 9:256-260.
20. Dobson AT, Margolis TP, Sedarati F, Stevens JG, Feldman LT. A latent, nonpathogenic HSV-1-derived vector stably expresses b-galactosidase in mouse neurons. *Neuron* 1990; 5:353-360.
21. Johnson PA, Yoshida K, Gage FH, Friedmann T. Effects of gene transfer into cultured CNS neurons with a replication-defective herpes simplex virus type 1 vector. *Mol.Brain Res.* 1992; 12:95-102.
22. Johnson PA, Miyanohara A, Levine F, Cahill T, Friedmann T. Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J.Virol.* 1992; 66:2952-2965.
23. Kennedy PG. Potential use of herpes simplex virus (HSV) vectors for gene therapy of neurological disorders. *Brain* 1997; 120:1245-1259.
24. Neve RL. Adenovirus vectors enter the brain. *Trends Neurosci.* 1993; 16:251-253.
25. Davidson BL, Allen ED, Kozarsky KF, Wilson JM, Roessler BJ. A model system for in vivo gene transfer into the central nervous system using an adenoviral vector. *Nat.Genet.* 1993; 3:219-223.
26. Choi-Lundberg DL, Lin Q, Chang YN, Chiang YL, Hay CM, Mohajeri H, et al. Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* 1997; 275:838-841.
27. Byrnes AP, MacLaren RE, Charlton HM. Immunological instability of persistent adenovirus vectors in the brain: Peripheral exposure to vector leads to renewed inflammation, reduced gene expression, and demyelination. *J.Neurosci.* 1996; 16:3045-3055.
28. Tripathy SK, Goldwasser E, Lu MM, Barr E, Leiden JM. Stable delivery of physiologic levels of recombinant erythropoietin to the systemic circulation by intramuscular injection of replication-defective adenovirus. *Proc.Natl.Acad.Sci.USA* 1994; 91:11557-11561.
29. Burcin MM, Schiedner G, Kochanek S, Tsai SY, O'Malley BW. Adenovirus-mediated regulable target gene expression in vivo. *Proc Natl Acad Sci U.S.A.* 1999; 96:355-360.
30. Samulski RJ, Zhu X, Xiao X, Brook JD, Housman DE, Epstein N, et al. Targeted integration of adeno-associated virus (AAV) into human chromosome 19 [published erratum appears in *EMBO J* 1992 Mar;11(3):1228]. *EMBO J* 1991; 10:3941-3950.
31. McCown TJ, Xiao X, Li J, Breese GR, Samulski RJ. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res.* 1996; 713:99-107.
32. Samulski RJ, During MJ, Kaplitt MG, Xiao X, Redman G, McCown T. Adeno-associated virus vectors yield long-term expression and delivery of potentially therapeutic genes into non-dividing neuronal cells. *J.Neurovirol.* 1997; 3 Suppl. 1:S72-S72
33. Mandel RJ, Rendahl SK, Spratt SK, Snyder LK, Cohen LK, Leff SE. Characterization of intrastriatal recombinant adeno-associated virus-mediated gene transfer of human tyrosine hydroxylase and human GTP-cyclohydrolase 1 in a rat model of Parkinson's Disease. *J.Neurosci.* 1998; 18:4271-4284.

34. Leff SE, Spratt SK, Snyder RO, Mandel RJ. Long-term restoration of striatal L-aromatic amino acid decarboxylase activity using recombinant adeno-associated viral vector gene transfer in a rodent model of Parkinson's disease [In Process Citation]. *Neuroscience* 1999; 92:185-196.
35. Miller AD, Miller DG, Garcia JV, Lynch CM. Use of retroviral vectors for gene transfer and expression. *Methods Enzymol.* 1993; 217:581-599.
36. Kolberg R. Gene-transfer virus contaminant linked to monkeys' cancer. *J.NIH Res.* 1992; 4:43-44.
37. Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. *BioTechniques* 1989; 7:980-989.
38. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol.Cell.Biol.* 1990; 10:4239-4242.
39. Jacoby DR, Fraefel C, Breakefield XO. Hybrid vectors: a new generation of virus-based vectors designed to control the cellular fate of delivered genes [editorial]. *Gene Ther.* 1997; 4:1281-1283.
40. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; 272:263-267.
41. Johnston KM, Jacoby D, Pechan PA, Fraefel C, Borghesani P, Schuback D, et al. HSV/AAV hybrid amplicon vectors extend transgene expression in human glioma cells. *Hum.Gene Ther.* 1997; 8:359-370.
42. Palmer TD, Rosman GJ, Osborne WRA, Miller AD. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc.Natl.Acad.Sci.USA* 1991; 88:1330-1334.
43. Scharfmann R, Axelrod JH, Verma IM. Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc.Natl.Acad.Sci.USA* 1991; 88:4626-4630.
44. Dai Y, Roman M, Naviaux RK, Verma IM. Gene therapy via primary myoblasts: long-term expression of factor IX protein following transplantation in vivo. *Proc.Natl.Acad.Sci.USA* 1992; 89:10892-10895.
45. Li X, Eastman EM, Schwartz RJ, Draghia-Akli R. Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences [see comments]. *Nat.Biotechnol.* 1999; 17:241-245.
46. Roemer K, Johnson PA, Friedmann T. Activity of the simian virus 40 early promoter-enhancer in herpes simplex virus type 1 vectors is dependent on its position, the infected cell type, and the presence of Vmw175. *J.Virol.* 1991; 65:6900-6912.
47. Blomer U, Naldini L, Verma IM, Trono D, Gage FH. Applications of gene therapy to the CNS. *Human Molecular Genetics* 1996; 5:1397-1404.
48. Dhawan J, Rando TA, Elson SL, Bujard H, Blau HM. Tetracycline-regulated gene expression following direct gene transfer into mouse skeletal muscle. *Somat.Cell Mol.Genet.* 1995; 21:233-240.
49. Suhr ST, Gil EB, Senut MC, Gage FH. High level transactivation by a modified Bombyx ecdysone receptor in. *Proc Natl Acad Sci U.S.A.* 1998; 95:7999-8004.
50. Ye X, Rivera VM, Zoltick P, Cerasoli FJ, Schnell MA, Gao G, et al. Regulated delivery of therapeutic proteins after in vivo somatic cell gene transfer. *Science* 1999; 283:88-91.

51. Mouradian MM, Heuser IJ, Baronti F, Chase TN. Modification of central dopaminergic mechanisms by continuous levodopa therapy for advanced Parkinson's disease. *Ann.Neurol.* 1990; 27:18-23.
52. Olanow CW, Kordower JH, Freeman TB. Fetal nigral transplantation as a therapy for Parkinson's disease. *Trends Neurosci.* 1996; 19:102-109.
53. Wolff JA, Fisher LJ, Jinnah HA, Langlais PJ, Iuvone PM, O'Malley KL, et al. Grafting fibroblasts genetically modified to produce L-dopa in a rat model of Parkinson disease. *Proc.Natl.Acad.Sci.USA* 1989; 86:9011-9014.
54. Horellou P, Brundin P, Kalen P, Mallet J, Björklund A. In vivo release of DOPA and dopamine from genetically engineered cells grafted to the denervated rat striatum. *Neuron* 1990; 5:393-402.
55. Fisher LJ, Jinnah HA, Kale LC, Higgins GA, Gage FH. Survival and function of intrastrially grafted primary fibroblasts genetically modified to produce L-DOPA. *Neuron* 1991; 6:371-380.
56. During MJ, Naegele JR, O'Malley KL, Geller AI. Long-term behavioral recovery in parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. *Science* 1994; 266:1399-1403.
57. Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, et al. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat.Genet.* 1994; 8:148-154.
58. Bencsics C, Wachtel SR, Milstien S, Hatakeyama K, Becker JB, Kang UJ. Double transduction with GTP cyclohydrolase I and tyrosine hydroxylase is necessary for spontaneous synthesis of L-DOPA by primary fibroblasts. *J.Neurosci.* 1996; 16:4449-4456.
59. Ichinose H, Ohye T, Takahashi E, Seki N, Hori T, Segawa M, et al. Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase 1 gene. *Nat.Genet.* 1994; 8:236-242.
60. Abercrombie ED, Bonatz AE, Zigmond MJ. Effects of L-DOPA on extracellular dopamine in striatum of normal and 6-hydroxydopamine-treated rats. *Brain Res.* 1990; 525:36-44.
61. Zhu MY, Juorio AV. Aromatic L-amino acid decarboxylase: biological characterization and functional role. *Gen.Pharmacol.* 1995; 26:681-696.
62. Melamed E, Hefti F, Pettibone DJ, Liebman J, Wurtman RJ. Aromatic L-amino acid decarboxylase in rat corpus striatum: implications for action of L-DOPA in parkinsonism. *Neurology* 1981; 31:651-655.
63. Wachtel SR, Bencsics C, Kang UJ. The role of aromatic L-amino acid decarboxylase for dopamine replacement by genetically modified fibroblasts in a rat model of Parkinson's disease. *J.Neurochem.* 1997; 69:2055-2063.
64. Lee WY, Chang JW, Nemeth NL, Kang UJ. Vesicular monoamine transporter-2 and aromatic L-amino acid decarboxylase enhance dopamine delivery after L-3, 4- dihydroxyphenylalanine administration in Parkinsonian rats. *J.Neurosci.* 99 A.D.; 19:3266-3274.
65. Kang UJ, Fisher LJ, Joh TH, O'Malley KL, Gage FH. Regulation of dopamine production by genetically modified primary fibroblasts. *J.Neurosci.* 1993; 13:5203-5211.
66. Hyman C, Hofer M, Barde YA, Juhasz M, Yancopoulos GD, Squinto SP, et al. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* 1991; 350:230-232.

67. Hyman C, Juhasz M, Jackson C, Wright P, Ip NY, Lindsay RM. Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. *J.Neurosci.* 1994; 14:335-347.
68. Hynes MA, Poulsen K, Armanini M, Berkemeier L, Phillips H, Rosenthal A. Neurotrophin-4/5 is a survival factor for embryonic midbrain dopaminergic neurons in enriched cultures. *J.Neurosci.Res.* 1994; 37:144-154.
69. Lin LFH, Doherty DH, Lile JD, Bektess S, Collins F. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 1993; 260:1130-1132.
70. Knusel B, Michel PP, Schwaber JS, Hefti F. Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *J.Neurosci.* 1990; 10:558-570.
71. Poulsen KT, Armanini MP, Klein RD, Hynes MA, Phillips HS, Rosenthal A. TGFb2 and TGFb3 are potent survival factors for midbrain dopaminergic neurons. *Neuron* 1994; 13:1245-1252.
72. Engele J, Bohn MC. The neurotrophic effects of fibroblast growth factors on dopaminergic neurons in vitro are mediated by mesencephalic glia. *J.Neurosci.* 1991; 11:3070-3078.
73. Altar CA, Boylan CB, Jackson C, Hershenon S, Miller J, Wiegand SJ, et al. Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo. *Proc.Natl.Acad.Sci.USA* 1992; 89:11347-11351.
74. Martin-Iverson MT, Todd KG, Altar CA. Brain-derived neurotrophic factor and neurotrophin-3 activate striatal dopamine and serotonin metabolism and related behaviors: Interactions with amphetamine. *J.Neurosci.* 1994; 14:1262-1270.
75. Shen RY, Altar CA, Chiodo LA. Brain-derived neurotrophic factor increases the electrical activity of pars compacta dopamine neurons in vivo. *Proc.Natl.Acad.Sci.USA* 1994; 91:8920-8924.
76. Gall CM, Gold SJ, Isackson PJ, Seroogy KB. Brain-derived neurotrophic factor and neurotrophin-3 messenger RNAs are expressed in ventral midbrain regions containing dopaminergic neurons. *Mol.Cell Neurosci.* 1992; 3:56-63.
77. Seroogy KB, Lundgren KH, Tran TM, Guthrie KM, Isackson PJ, Gall CM. Dopaminergic neurons in rat ventral midbrain express brain-derived neurotrophic factor and neurotrophin-3 mRNAs. *J.Comp.Neurol.* 1994; 342:321-334.
78. Mufson EJ, Kroin JS, Sobriela T, Burke MA, Kordower JH, Penn RD, et al. Intrastriatal infusions of brain-derived neurotrophic factor: Retrograde transport and colocalization with dopamine containing substantia nigra neurons in rat. *Exp.Neurol.* 1994; 129:15-26.
79. Altar CA, Boylan CB, Fritsche M, Jackson C, Hyman C, Lindsay RM. The neurotrophins NT-4/5 and BDNF augment serotonin, dopamine, and GABAergic systems during behaviorally effective infusions to the substantia nigra. *Exp.Neurol.* 1994; 130:31-40.
80. Hudson J, Granholm AC, Gerhardt GA, Henry MA, Hoffman A, Biddle P, et al. Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits in vivo. *Brain Res Bull.* 1995; 36:425-432.

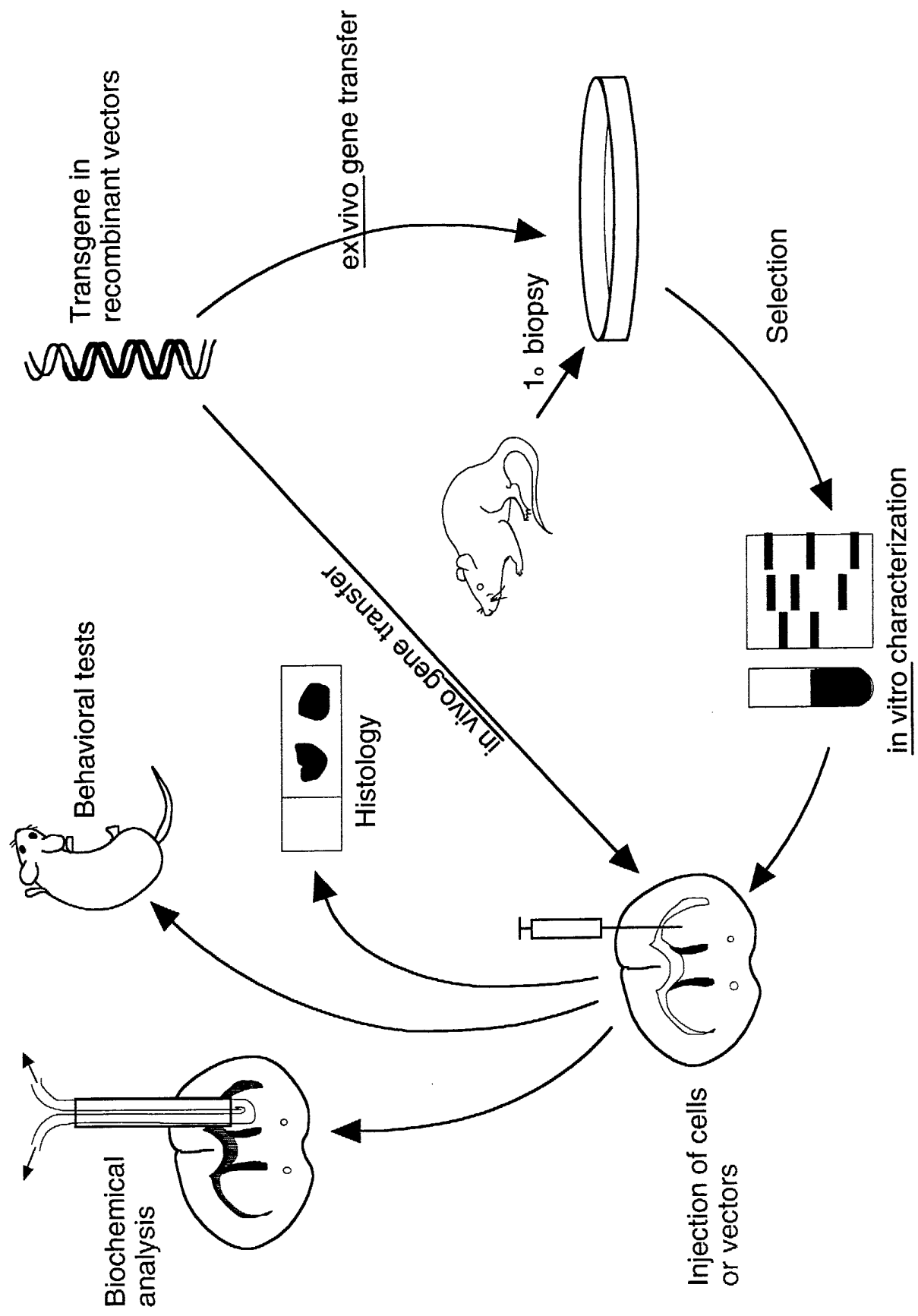
81. Tomac A, Widenfalk J, Lin LFH, Kohno T, Ebendal T, Hoffer BJ, et al. Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proc.Natl.Acad.Sci.USA* 1995; 92:8274-8278.
82. Morse JK, Wiegand SJ, Anderson K, You Y, Cai N, Carnahan J, et al. Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. *J.Neurosci.* 1993; 13:4146-4156.
83. Levivier M, Przedborski S, Bencsics C, Kang UJ. Intrastriatal implantation of fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevents degeneration of dopaminergic neurons in a rat model of Parkinson's disease. *J.Neurosci.* 1995; 15:7810-7820.
84. Frim DM, Uhler TA, Galpern WR, Beal MF, Breakefield XO, Isacson O. Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in the rat. *Proc.Natl.Acad.Sci.USA* 1994; 91:5104-5108.
85. Lucidi-Phillipi CA, Gage FH, Shults CW, Jones KR, Reichardt LF, Kang UJ. BDNF-transduced fibroblasts: production of BDNF and effects of grafting to the adult rat brain. *J.Comp.Neurol.* 1995; 354:361-376.
86. Mandel RJ, Spratt SK, Snyder RO, Leff SE. Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc Natl Acad Sci U.S A.* 1997; 94:14083-14088.
87. Bilang-Bleuel A, Revah F, Colin P, Locquet I, Robert JJ, Mallet J, et al. Intrastriatal injection of an adenoviral vector expressing glial-cell- line-derived neurotrophic factor prevents dopaminergic neuron degeneration and behavioral impairment in a rat model of Parkinson disease. *Proc Natl Acad Sci U.S A.* 1997; 94:8818-8823.
88. Choi-Lundberg DL, Lin Q, Schallert T, Crippens D, Davidson BL, Chang YN, et al. Behavioral and cellular protection of rat dopaminergic neurons by an adenoviral vector encoding glial cell line-derived neurotrophic factor. *Exp.Neurol.* 1998; 154:261-275.
89. Westphal CH, Leder P. Transposon-generated 'knock-out' and 'knock-in' gene-targeting constructs for use in mice. *Curr.Biol.* 1997; 7:530-533.
90. Blaese RM. Gene therapy for cancer. *Scientific American* 1997; June:107-115.
91. Linnik MD, Zahos P, Geschwind MD, Federoff HJ. Expression of bcl-2 from a defective herpes simplex virus-1 vector limits neuronal death in focal cerebral ischemia. *Stroke* 1995; 26:1670-1674.
92. Przedborski S, Kostic V, Jackson-Lewis V, Naini AB, Simonetti S, Fahn S, et al. Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J.Neurosci.* 1992; 12:1658-1667.
93. Nakao N, Frodl EM, Widner H, Carlson E, Eggerding FA, Epstein CJ, et al. Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. *Nature Med.* 1995; 1:226-231.

Figure Legend

Fig. 1 Experimental schemes for transferring genes into the CNS. Two general methods of gene transfer, *in vivo* and *ex vivo*, are schematically depicted. For details, please see the text.

Fig. 2 An idealized scheme for general virus vectors. Wild-type viruses are engineered to produce virus plasmid vector which has minimal viral genes necessary for packaging the vector into the virus particles. Other viral genes are deleted to reduce deleterious effect and make room for insertion of therapeutic genes. Viral components necessary to generate infective particles are introduced into the packaging cells separate from the virus plasmid vector. Packaging cells then produce infective virus particles containing the therapeutic transgene, but minimal or no virus genes.

Fig. 3 Dopamine replacement strategy for Parkinson's disease by gene transfer. A. Cells expressing tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GCH1) are grafted into the striatum. The L-DOPA produced by these cells (dotted line) is converted by endogenous aromatic L-amino acid decarboxylase (AADC) to dopamine (solid line). B. Cells expressing AADC and vesicular monoamine transporter (VMAT) could be cografted with L-DOPA-producing cells to provide additional source of decarboxylation and storage of dopamine. C. An alternative strategy combines the use of AADC/VMAT cells with exogenous administration of L-DOPA. These cells increase the efficacy of L-DOPA by providing additional decarboxylation, and increased storage and sustained release of dopamine.



other viral genes

viral genes necessary for infection

Wild-type virus

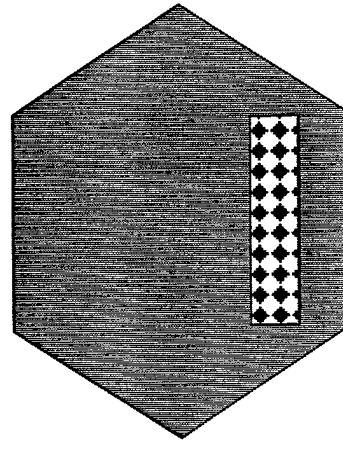
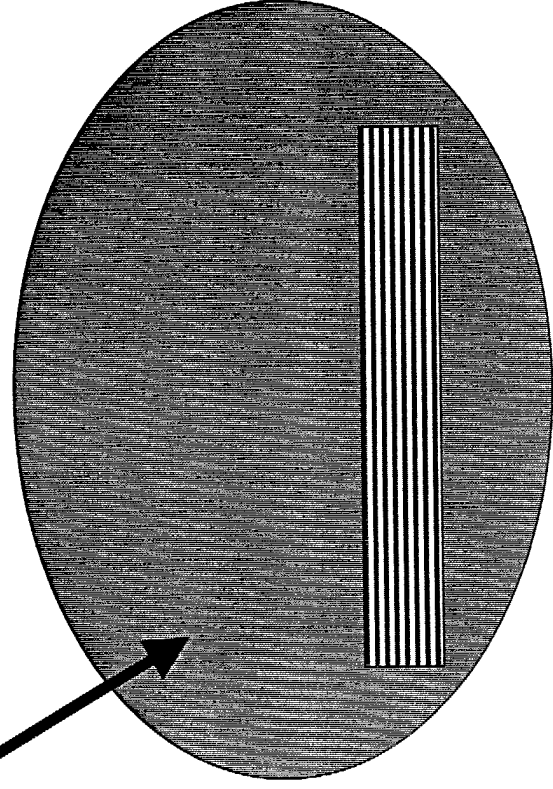
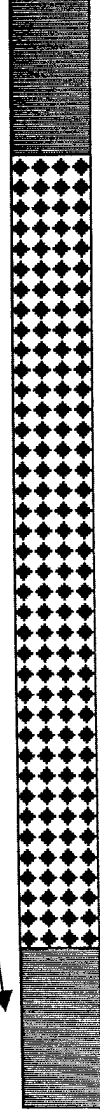
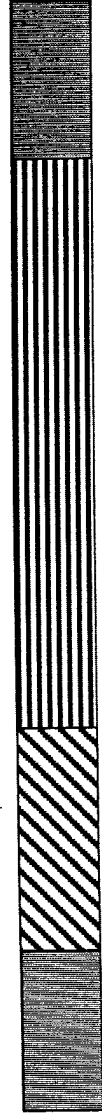
viral genes for
replicaton, packaging

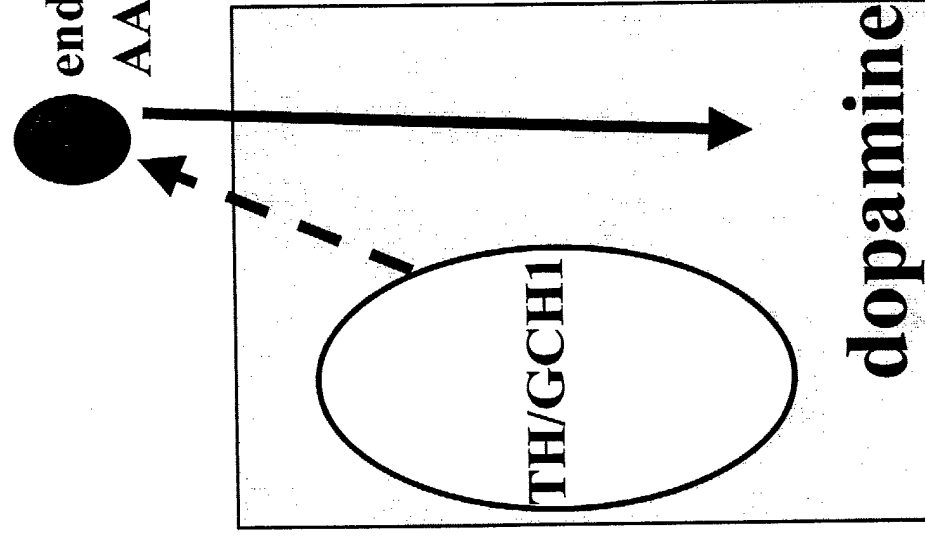
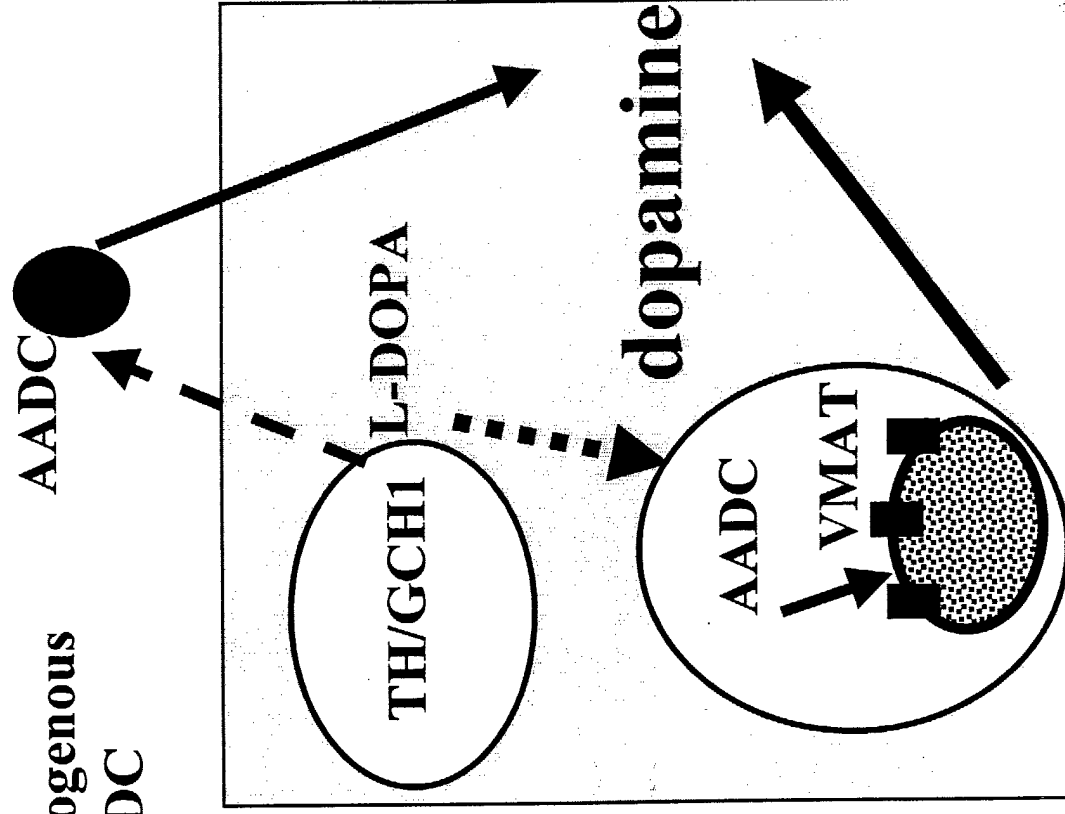
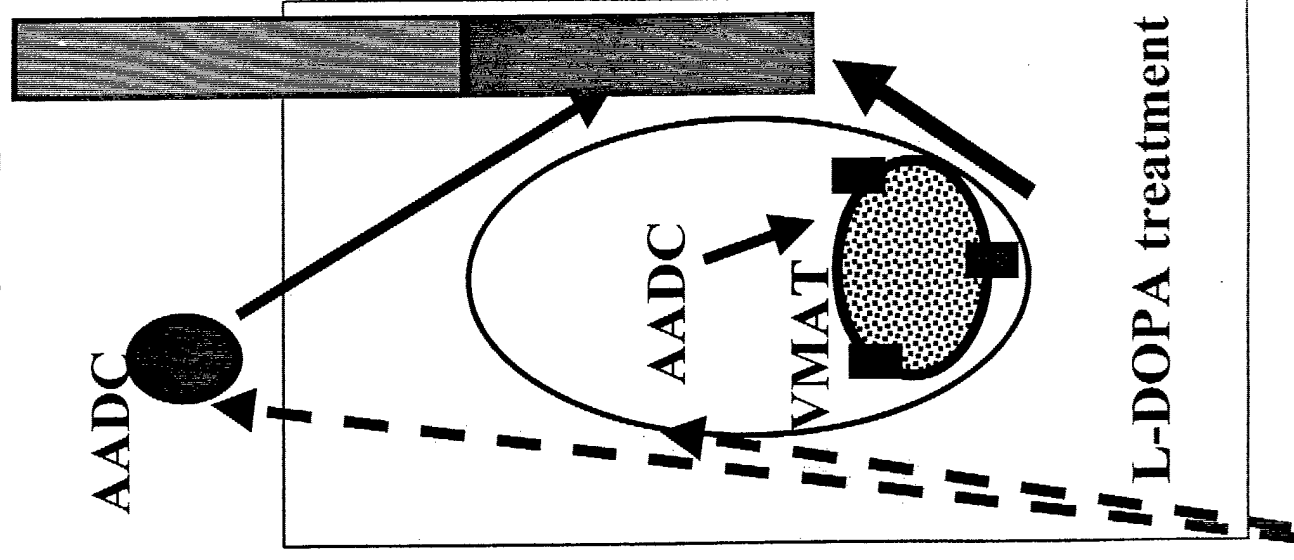
Virus plasmid vector

transgene expression
cassette (promoter +
coding sequences)

Packaging cells

Recombinant Virus vectors



A**B****C** dialysis probe



ENHANCED AXONAL GROWTH FROM FETAL HUMAN BCL-2 TRANSGENIC MOUSE DOPAMINE NEURONS TRANSPLANTED TO THE ADULT RAT STRIATUM

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Abstract—Embryonic neurons transplanted to the adult CNS extend axons only for a developmentally defined period. There are certain intercellular factors that control the axonal extension, one of which may be the expression of the bcl-2 protein. In this study, rats with complete striatal dopamine fiber denervation received embryonic day 14 mouse ventral mesencephalon cells overexpressing human bcl-2 or control wild-type ventral mesencephalon cells. All rats were treated with cyclosporine to prevent rejection and the surviving grafts were analyzed for cell survival and outgrowth of dopaminergic fibers. The results demonstrate that bcl-2 overexpression does not enhance neuronal graft survival. However, the bcl-2 overexpressing neurons had a higher number of dopaminergic fibers that grew longer distances.

These results show that overexpression of bcl-2 can result in longer distance axonal growth of transplanted fetal dopaminergic neurons and that genetic modification of embryonic donor cells may enhance their ability to reinnervate a neuronal target territory. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: xenotransplantation, cell culture, Parkinson's disease, tyrosine hydroxylase.

The adult brain is less permissive for axonal growth and regeneration than the fetal or neonatal brain. However, transplanted fetal neurons can also grow axons in adult brain environments. Typically, fetal dopamine (DA) cells transplanted to the adult striatum will grow and create a new zone of DA axons and synapses associated with behavioral recovery.¹⁴ With current transplantation methods, careful histological analysis has shown that even with a large number of surviving DA neurons, only a part of the target immediately surrounding the graft is fully reinnervated. In contrast, during the host's neonatal period, implanted fetal neurons migrate into the surrounding brain and extend axons for longer distances.^{23,28,35} The host age at which transplanted embryonic ventral mesencephalon (VM) neurons show much restricted axonal elongation occurs between postnatal day 8 and 20.^{20,23}

A number of factors and redundant molecules control

axonal growth. Key factors include growth-associated proteins, activated cytoskeletal proteins, calcium, cyclic AMP and other second-messenger systems.^{22,27,28,36,42} The bcl-2 molecule has been suggested to have the capacity to induce and maintain axonal growth.^{7,20} The bcl-2 gene was discovered as a proto-oncogene of a follicular lymphoma translocation t.^{7,20} In non-pathological roles, bcl-2 is considered an anti-apoptotic factor against ischemia^{29,30}, traumatic brain injury⁸, growth factor deprivation, or generation of free radicals.³²

Bcl-2 is highly expressed in the brain in the middle to late gestational phase. These levels are reduced after birth, except in regions with late differentiation such as the dentate gyrus.^{2,3,33} The highest levels of bcl-2 expression occur during the fetal phase and are seen in the cells of the ventricular zones and in post-mitotic neurons during the period of naturally occurring cell death. Bcl-2 expression is high in the cortical plate, peaking around embryonic day (E) 16.^{32,33} Bcl-2 levels decline in most CNS neurons over the adult period, but remain relatively higher in neurons of the peripheral nervous system (PNS).^{32,33}

The peaks of bcl-2 expression coincide with axonal elongation³³, suggesting a protective function distinct from its anti-apoptotic actions. Chen *et al.*⁷ used mice overexpressing human bcl-2 (hubcl-2), coupled to a neuron specific enolase promoter. Retino-tectal co-cultures prepared from these mice maintained axonal

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Abbreviations: 6-OHDA, 6-hydroxydopamine; AP, antero-posterior; DA, dopamine; E, embryonic day; hubcl-2, human bcl-2; GDNF, glial-derived neurotrophic factor; NGS, normal goat serum; PNS, peripheral nervous system; PBS, phosphate-buffered saline; SN, substantia nigra; SNc, substantia nigra pars compacta; TBS, tris-buffered saline; TH, tyrosine hydroxylase; VM, ventral mesencephalon; VTA, ventral tegmental area.

growth to the tectum after E18, in contrast to wild-type control cultures.^{7,20} Reduced levels of bcl-2 decrease axonal growth of sensory neurons¹⁸ and in co-cultures prepared from retina and tectum of bcl-2 knockout mice. Cultures from E18 homozygote bcl-2 knockout mice showed an 80% decrease of axonal growth compared to wild-type cases. Immunohistochemistry of fetal retinal tissue also indicates an absence of bcl-2 immunoreactivity in normal retinas at E18⁷, the time when axonal extension in this system normally stops. The ability of the PNS to regenerate axons and the persistence of bcl-2 expression in these cells are consistent with the idea of a growth promoting effect of bcl-2. Notably, bcl-2 expression has been described in the superior cervical ganglion and the dorsal root ganglion of 5-month-old mice.³³ Furthermore, axotomized retinal axons in postnatal day 4 bcl-2 overexpressing mice successfully grew across a knife cut lesion to innervate the tectum.⁷ The role of bcl-2 in axonal growth is also demonstrated in a cell line showing that vector expressed hubcl-2 causes increased neurite formation.³⁷ Overexpressing bcl-2 in a neural crest derived cell line by adding sense cDNA results in enhanced axonal outgrowth.⁴⁶

In the present experiment, we hypothesized that transplanted hubcl-2 overexpressing fetal DA neurons would grow axonal terminals over a larger striatal adult rat target territory.

EXPERIMENTAL PROCEDURES

Cell culture

E14 mice were obtained by crossing males overexpressing hubcl-2 (line 73, D.F. Chen, Schepens Eye Research Institute, HMS, Boston, MA) with wild-type female progeny, resulting in 50% bcl-2 transgenic and 50% wild-type embryos. Mice genotypes were determined by polymerase chain reaction from tail samples. Primary VM neuronal cultures (95% neuronal) were obtained, as previously described.^{9,12} Briefly, tissue was dissociated by incubation in 0.025% trypsin solution (37°C, 15 min; Sigma, St. Louis, MO) and triturated in a solution of DNase (0.01%; Sigma) and trypsin inhibitor (0.05%; Sigma). Isolated cells were resuspended in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing heat-inactivated horse serum (10%), glucose (6.0 mg/ml), penicillin (10,000 U/ml), streptomycin (10 mg/ml; Sigma), and glutamine (2 mM; GIBCO). Five hundred microliters of suspension containing 1×10^6 cells/ml were plated onto glass coverslips precoated with poly-L-lysine (Sigma) in each well of 24-well trays (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) containing 500 μ l of serum-containing medium. Unattached cells were aspirated after 1 hr and 1 ml of fresh serum-containing medium was added. After one day of culture, the medium was replaced with defined medium (N2 cocktail GIBCO). After two days, cultures were fixed for 1 h with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS). Fixed cells were preincubated in 10% normal goat serum (NGS), then incubated in rabbit polyclonal antibody against tyrosine hydroxylase (TH, Pel-Freeze, AK; 1:500) and monoclonal antibody against hubcl-2 (Santa Cruz, CA; 1:100) for 48 h at 4°C. After additional rinsing 3×10 min in PBS, cultures were incubated for 1 h in PBS with 2% NGS, biotinylated goat anti-mouse (Sigma, St. Louis, MO; 1:200) and biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA; 1:200) secondary antibodies at room temperature. After rinsing in PBS, cultures were subsequently processed with standard Vectastain (Kit ELITE, Vector Laboratories) with 3,3'-diaminobenzidine substrate resulting in a brown reaction

product (visualization of TH positive cells), and with Vector VIP substrate kit (#SK4600), resulting in a violet reaction product (visualization of hubcl-2 positive cells). Under light microscopy, the two reaction products were clearly distinct by color. Controls with omission of primary antibody were performed to verify the specificity of staining.

6-OHDA lesions

Adult female Sprague-Dawley rats (300 g, Charles River Laboratories, Wilmington, MA) received unilateral 6-hydroxydopamine (6-OHDA) (4 μ l of 3 μ g/ μ l) by stereotaxic injection into the medial forebrain bundle using a 10 μ l Hamilton syringe at the following coordinates: Antero-posterior (AP): -4.4, L: -1.4 and V: -7.8, incisor bar: -2.3 mm. 6-OHDA was infused at a rate of 1 μ l/min, and the needle was left in place for an additional 2 min prior to withdrawal. To verify a complete DA denervation, animals were tested for amphetamine-induced (5 mg/kg) rotational asymmetry using automated rotometers (San Diego Instruments),⁴¹ one to two weeks post-lesion.

Ten rats with a net ipsilateral rotation greater than 800 rotations per 90 min (reflecting an approximate 97% DA depletion) were divided into two experimental groups balanced with respect to pre-transplant rotation scores. The near total striatal DA depletion allowed an analysis of graft-derived (TH-positive) fiber. We did not perform a post-transplantation behavioral study in order to complete a histological analysis as early as five weeks post-transplantation.

All animal experiments were carried out in accordance to the National Institute of Health, USA; Harvard Medical School and McLean Hospital guidelines and all efforts were made to minimize the number of animals used and their suffering.

Transplantation of VM cells

The VM of E14 fetuses from matings of hubcl-2 transgenic males (line 73, D.F. Chen, Schepens Eye Research Institute, HMS, Boston, MA)^{29,30} or wild-type C57BL/6J males with C57BL/6J females, were dissected. Mice genotypes were determined using the polymerase chain reaction method previously described.^{7,29} The dissected pieces from each litter were pooled, incubated in 0.1% trypsin for 20 min, washed with Ca/Mg free Hank's balanced salt solution containing 0.1% DNase and triturated in the same solution through a series of fire polished pipettes of diminishing diameters until a milky suspension with scattered cell clumps was achieved. Cell counts were carried out using acridine orange/ethidium bromide labeling to determine cell concentration. The suspension was diluted to a concentration of 100,000 cells/ μ l.

Two μ l of the cell suspension was injected into the host denervated striatum using a 10 μ l Hamilton syringe and a 45° beveled needle (inner diameter 0.26 mm and outer diameter 0.46 mm) at a rate of 0.5 μ l/min followed by a 2 min pause before retracting the needle (coordinates relative to bregma: AP: +1.0, L: -2.5, V: -4.0 to -5.0, incisor bar: -3.3). One group ($n=5$) received cells from wild-type mice and the other group ($n=5$) was implanted with cells from fetuses of bcl-2 transgenic matings. One day prior to transplantation procedure, animals were given 20 mg/kg of the immunosuppressant cyclosporin A (Sandimmune, diluted in olive oil, sc; Sandoz, East Hanover, NJ). This treatment continued at a lower dose (10 mg/kg/day, s.c.) until the animals were killed. Sacrificed.

Histological fixation and immunostaining of brain sections

Five weeks post-transplantation, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with 100 ml heparinized saline followed by 200 ml 4% paraformaldehyde in 0.1 M PBS, pH ~7.4. The brains were postfixed for 6–8 h in the same fixative before being transferred to 20% sucrose in 0.1 M PBS for cryoprotection. Following equilibration, a series of 40- μ m coronal sections were cut on a freezing microtome and collected in PBS.

Brain sections were immunostained with antibodies to TH

(Pel-Freez, AK; 1:500 and hubcl-2 (Santa Cruz, CA; 1:100). All immunohistochemistry was performed on free-floating sections, using a standard avidin-biotin technique. The sections were incubated in a 0.3% hydrogen peroxide solution containing 50% methanol in PBS (pH 7.4) for 20 min to eliminate endogenous peroxidase. After rinsing three times in PBS, the tissue was incubated in a PBS solution containing 1% bovine serum albumin, 1% NGS, and 0.1% Triton X-100. The following day, sections were rinsed in PBS, then put through two washes of 5% NGS (10 min each) before incubation in secondary antibody solution (biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, CA; or biotinylated goat anti-mouse Sigma; both diluted 1:200 with 2% NGS in PBS). After being rinsed in PBS, the sections were incubated in avidin-biotin-peroxidase complex (Elite ABC kit, Vector Labs) for 90 min. After one wash in PBS, the tissue was transferred to Tris-buffered saline (TBS, 0.05M, pH 7.8) before being developed in 3,3'-diaminobenzidine tetrahydrochloride dissolved in TBS, with 0.03% hydrogen peroxide added. The sections were rinsed in TBS, then PBS before being plated on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA), dehydrated through a series of graded ethanols and xylenes, followed by coverslipping with Permount (Fisher Scientific).

Determination of cell survival and outgrowth from grafted DA neurons

Surviving TH cells were counted over the extent of the grafts with Abercrombie corrections.^{1,10,12} Since all grafts in the adult brain survived as a single placement in isolated aggregates and were located in different striatal regions due to surgical variation, we established a semi-quantitative measure of fiber outgrowth for such grafts. For each rat, sections from AP +1.6 to AP -1.4, where the center of the grafts was observable were digitally captured at low power magnification on a Zeiss Axioplan microscope and imported into Adobe Photoshop 5.0 software. The graft was outlined and concentric circles (Fig. 1; L1, L2, L3) at fixed distances were drawn from the center of the graft going to the periphery (Fig. 1) and the number of TH positive cells were counted. We also measured the distance from the edge of the grafts to the circles to control for variance in graft size. Given that some grafts were smaller than others and that the average TH positive neurons in these grafts had to grow longer distances to reach each concentric circle, axonal growth distances were calculated from a point set between the middle of the graft and the edge of the graft. As shown in Fig. 1, the counting grid was divided into eight sectors. The number of fibers in each sector crossing the concentric circles was counted. The distances from the center of the graft to the edge of the graft (graft area), and to L1, L2 and L3 were measured. All measurements were done using an image analysis system based in Image 1.41 (NIH, Bethesda, MD).

Statistical analysis

The primary hypotheses examined in this study were that cell survival would not differ between the groups (bcl-2 vs. wild-type), and that bcl-2 group would not exceed the control (wild-type) in branching and in axonal outgrowth distances of TH fibers.

We used a single factor analysis of variance (ANOVA) method to compare TH-positive neuron survival between groups. We used a two-factor model to assess between group (bcl-2 vs. wild-type) differences in fiber count and axonal outgrowth distances, with the second (blocking) factor being concentric circles (with levels 1, 2, 3; see Fig. 1). This required a repeated measure analytic method because each experimental animal contributed data at each of the three concentric circles. For this purpose, we used panel-data-based random effects regression modeling methods,¹⁷ with adjustment for clustering within experimental animals. This modeling method permits adjustment for clustering within subjects. We checked the best fit of this modeling using added variable and partial residual plotting methods. We checked for interactions between the groups and concentric circles factors, and included an interaction factor in the modeling analyses where indicated. Because interaction effects

were not predicted in advance, tests of significance of interactions were adjusted using a multiple comparison adjustment method.¹⁹

Using a method of fiber counting (Fig. 1), prior to statistical regression modeling, we examined the distributions of the outcomes data (fibers and distances) for possible disturbances from normality. For both fiber count outcomes, we logarithmically transformed the data to reduce positive skewness and achieve more normal-like distributions tractable for analysis. For the distance data at concentric circles, the distance distributions appeared to approximate normality so that a normalizing transformation was not necessary.

The data management and statistical analysis work was carried out using two software packages: JMP (version 3.1.6; SAS Institute, Cary, NC); and Stata® (version 6; Stata Corporation, College Station, TX). Differences between groups were considered statistically significant at an alpha level of $P < 0.05$.

RESULTS

Expression of human bcl-2 in fetal VM dopaminergic neurons in vivo and in vitro

To confirm the hubcl-2 expression in the adult substantia nigra (SN) of the transgenic mice, we stained the post-mortem tissue of 2-month-old genotyped heterozygous mice. The hubcl-2 was highly expressed in neurons of the substantia nigra compacta (SNc) and the ventral tegmental area (VTA) (Fig. 2a) while lower levels and more variable neuronal expression were observed in other brain regions (data not shown). To address expression and survival of dissociated VM, we performed *in vitro* studies. We found that in E14 hubcl-2 cultured VM neurons, approximately 50% of the neurons co-expressed TH and hubcl-2 (Fig. 2b). This proportion of co-expression was expected since the cultures were prepared from tissue dissected and pooled from crossing of bcl-2 transgenic males with wild-type females, which according to Mendelian genetics would result in 50% transgenic and 50% wild-type embryos.

Five weeks following transplantation of dissociated embryonic E14 VM into the adult rat brain, the ratio of hubcl-2 and non hubcl-2 expressing neurons remained at 50% (Fig. 2c), indicating that overexpression of hubcl-2 does not increase the survival of these neurons. Generally, the surviving grafts were small in both transplanted groups (0.5–1.0 mm³) but all contained high proportion of TH-positive neurons (Figs 1 and 2d). The implanted neurons innervated the completely denervated host striatum and axons could be followed from the center of the graft aggregate into the striatal neuropil.

Fiber outgrowth from wild type and hubcl-2 overexpressing dopaminergic neurons grafted to adult striatum

When the total number of TH-positive neurons within the hubcl-2 grafts was compared with wild-type controls, no significant difference was detected ($P > 0.05$; data not shown). To determine the extent of axonal outgrowth from the grafted neurons, we performed image analysis and semi-quantitative measurements of TH-positive fiber outgrowth from the center of the graft (Fig. 1; see Experimental Procedures for details). The number of TH-positive fibers crossing three circles at set distances from the center of the graft was counted and compared between grafts containing hubcl-2 overexpressing neurons

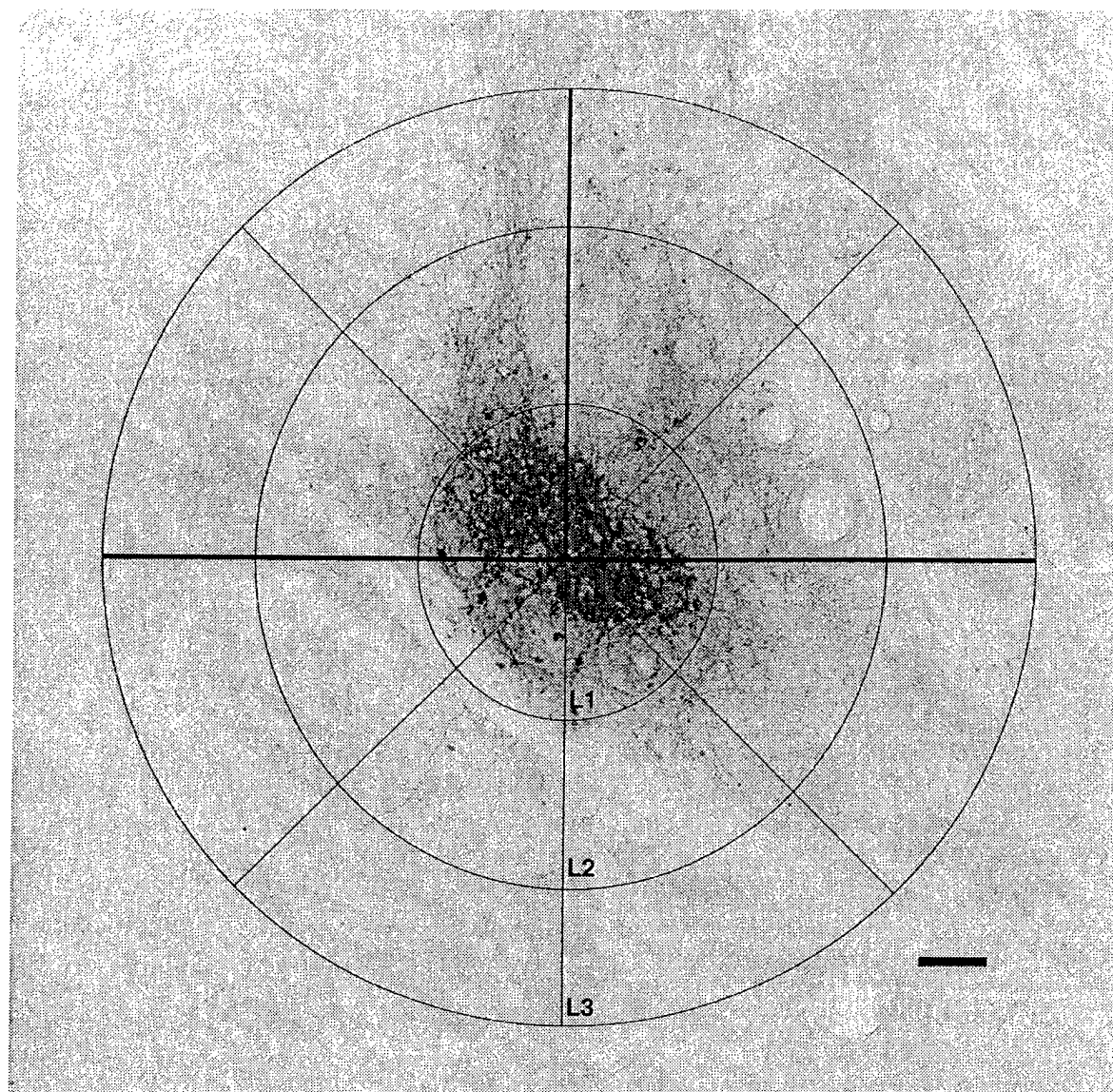


Fig. 1. The method used for measuring approximate size and fiber outgrowth from the graft. Concentric circles (L1, L2, L3) were drawn from the center of the graft and divided into eight sectors. The graft was outlined and the edge of the graft in the section was determined. The number of TH positive neurons in the section through the graft were counted, the distance from the center to the edge of the graft was measured. Segments were selected depending on graft size and shape, and the number of TH positive fibers crossing L1, L2, L3 in the selected segments was noted. The distance from L1, L2 and L3 was constant from the center of the graft in each case. Scale bar = 100 μ m

or grafts with only wild-type neurons (Fig. 3a). To control for graft shape and size, the distance of the graft's edge in a counted sector was determined. A tendency for smaller grafts was seen in hubcl-2 compared to wild-type grafts. This difference did not reach significance ($P > 0.05$). The total number of TH-positive fibers crossing the innermost circle (L1) was not different between hubcl-2 and control grafts (Table 1 and Fig. 3a). Similar results were obtained from TH fibers counts of crossings at the middle circle (L2; Table 1 and Fig. 3a), but there was a significant increase in the number of fibers from hubcl-2 grafts crossing the outermost circle (L3) compared to control grafts. The differences in fiber counts at L3 vs. L1/L2 is shown in Fig. 3a, in which

this statistical interaction effect is very prominent. At L1, the hubcl-2 and wild-type fiber numbers, on average, are equal. At L2, due to increased fibers for hubcl-2 but decreased number of wild-type fibers, there are on average more hubcl-2 fibers than wild-type fibers, but not to any significance. At circle L3, the hubcl-2 vs. wild-type fiber count difference is pronounced. This interaction effect is strongly statistically significant by random effects regression modeling ($z = 3.17$, $P = 0.002$). After adjustment for multiple comparisons (because three group $\times$ concentric circle interaction effects were examined post hoc), the hubcl-2 versus wild-type fiber count at L3 finding remained statistically significant ($P = 0.006$).

The hubcl-2 axonal outgrowth extended for longer

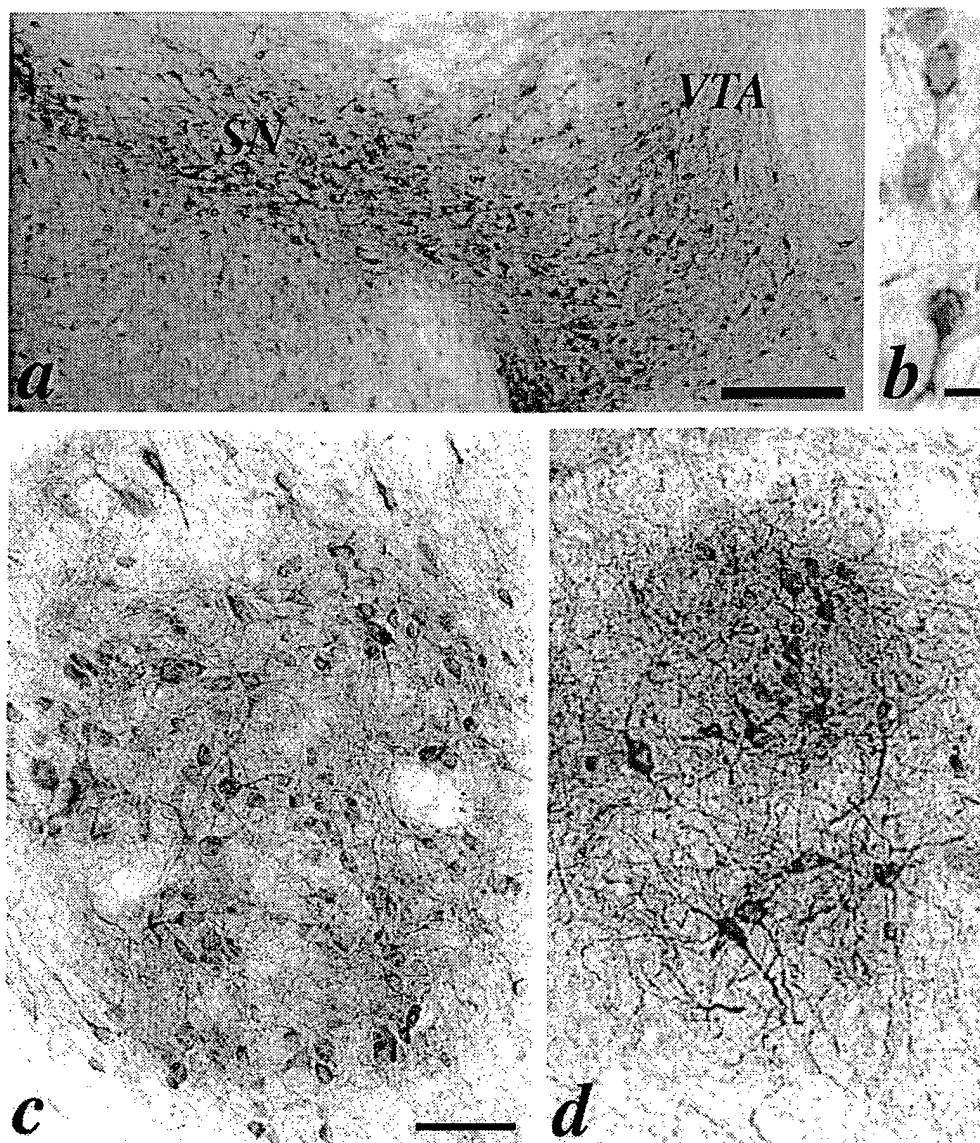


Fig. 2. Low power photomicrographs of hubcl-2 staining of the adult transgenic mouse midbrain area illustrating high expression of hubcl-2 in neuron of the SN and VTA (a). (b) Double staining (immunohistochemistry) for TH and hubcl-2 of E14 VM cell culture depicting TH neuronal expression (grey cells) and TH/hubcl-2 double labeled neurons (cells of red appearance). Appearance of a typical xenograft of E14 VM cell suspension from hubcl-2 transgenic mice transplanted into the adult rat brain (c-d). Hubcl-2 positive (c) and TH positive (d) neurons can easily be identified in the grafts of adjacent sections. Scale bars = 200 μ m (a); 25 μ m (b); 50 μ m (c, d).

distances than the wild-type. The mean outgrowth distance, measured from the center of the graft (Fig. 1) for hubcl-2 vs. wild-type grafts at the three concentric circles, L1, L2, L3 (shown graphically in Fig. 3b). Longer axonal growth mean distances from the center were seen for hubcl-2 compared to wild-type grafts at each circle, with the magnitude of these mean differences being about two standard errors, on average. The random effects regression modeling indicated the hubcl-2 vs. wild-type effect to be highly statistically significant ($z = 2.63$, $P = 0.009$). At each concentric circle, hubcl-2 axonal outgrowth distances, on average, substantially exceed wild-type distances (Fig. 3b).

DISCUSSION

In this study, we grafted VM neurons from mice over-expressing hubcl-2^{29,30} into the striatum of immunosuppressed 6-OHDA lesioned rats. TH-positive cell survival was not significantly different between hubcl-2 grafts and controls, supporting previous observations.^{21,39} In contrast, hubcl-2 appeared to influence the neurons' capacity to successfully extend axons into the adult striatum. Our measurements show that dopamine axons from hubcl-2 expressing neurons grew for significantly longer distances into the neuropil compared to wild-type control grafts.

Table 1. Summary of measurements for dopamine fiber outgrowth

Groups	# of TH cells in graft section	Edge of graft from center of graft (μm)	Distance of L1 from edge (μm)	Number of fibers crossing L1	Distance of L2 from edge (μm)	Number of fibers crossing L2	Distance of L3 from edge (μm)	Number of fibers crossing L3
Bcl2 +/-								
1	38	95.03	82.97	43	262.97	28	436.97	9
2	80	115.12	59.28	33	234.88	48	417.88	65
3	12	86.91	91.09	16	270.09	8	451.09	4
4	11	82.10	94.40	24	270.30	43	420.30	69
5	13	78.80	97.70	15	280.00	13	450.60	12
Mean	30.80	91.59	85.09	26.20	263.65	28.00	435.37	31.80
SEM	13.29	6.48	6.90	5.37	7.68	7.91	7.12	14.44
Wildtype								
1	29	118.50	54.40	30	229.74	6.00	412.48	3.00
2	22	125.01	53.09	29	227.99	14.00	400.99	4.00
3	22	115.33	63.47	11	236.67	23.00	410.57	25.00
4	43	205.61	27.76	17	150.89	21.00	324.99	11.00
5	74	134.70	48.70	43	223.60	49.00	402.20	7.00
Mean	38.00	139.83	38.38	26.00	213.78	22.60	390.25	10.00
SEM	9.78	16.92	16.71	5.57	15.86	7.24	16.47	4.00

Enhanced axonal growth in *hubcl-2* expressing mesencephalic grafts

The grafts of fetal DA neurons always aggregate in SN-like small structures in the adult host. Since the range of placements is always variable (1–2 mm AP/LM) the most appropriate method for axonal counts is concentric to the actual graft.^{9,11,21} Since all rats were fully lesioned and grafts outlined by *hubcl-2* and TH immunostained cell body, the radial TH fiber extent of neurites (from the graft center) allowed for fiber counts between groups of animals.¹¹ The immunohistochemical detection of *hubcl-2* was restricted to cell bodies of grafted neurons, preventing tracing of individual axons from *hubcl-2* expressing neurons. The rat hosts were selected for transplantation on the basis of drug-induced measures for complete denervation, which was confirmed by the behavioral tests and *post mortem* analysis, using loss of TH SN neurons as a criterion. Thus, although an alternative interpretation for the graft-derived increase of TH positive fibers from *hubcl-2* grafts could be a regeneration of rat host TH positive fibers, this is highly unlikely. Moreover, the typical concentric outgrowth of DA fiber from the transplants characteristically formed a halo on the background of a denervated host striatum, and no fibers were seen in the interface between grafts derived DA fibers and denervated host striatum. Our observations of increased axonal growth by transplanted *hubcl2* dopamine neurons is also consistent with the by work Schierle *et al.*³⁹ where grafts of *hubcl-2* overexpressing fetal VM grafts were placed into the denervated rat striatum, and densitometry measurements indicated increased volume of TH-innervation compared to wild-type control grafts.

Ability of embryonic neurons to extend axons in the adult brain

Normal axonal growth appears to proceed by growth cone extension and collapse.^{22,26,28} Concentration gradients

of attractive and inhibitory molecules can provide axonal orientation by inducing asymmetric growth cone collapse and extension.^{13,26} In studies of axonal growth from transplanted fetal neurons, new axons can grow extensively in neonatal, but less so in adult host brain.^{23,35} It is reasonable to assume that the adult CNS environment has less stimulatory trophic molecules and more frequent growth cone collapse, thereby increasing the time needed for the axon to reach its target.²³ Typically, CNS neuronal phenotypes persist in a growing state for a limited time. In fetal allotransplantation to the adult host brain, axonal extension is usually limited to adjacent axonal target zones.²³ Xenotransplantation experiments using donor species with slower embryonic development than the rat host, e.g. pig²⁴ or human⁴⁴ indicate that a longer cell autonomous "time window" for axonal extension provides an opportunity for axons to reach long distance targets. An alternative explanation is that axons from the xenotransplants could be less responsive to growth inhibitory molecules expressed in the host brain. However, some observations indicate that growth from fetal xenotransplants in the adult is as impeded as allotransplants.^{23,24} Moreover, mouse xenogenic fetal tissue placed in rat striatum does not show the increase in axonal growth relative to rat donor tissue, while xenotransplanted human tissue does.^{23,24} Thus the long distance axonal growth from fetal pig or human neurons could be the result of an extended genetically controlled time period of active growth as well as a delay until inhibitory receptors are expressed.^{24,34,45} Given that PNS neurons and some CNS neuronal phenotypes^{20,23} retain regenerative capacity in adulthood, there are many molecules (including *bcl-2*) and intrinsic processes that would allow for such continued axonal growth.

Stimulation of dopaminergic axonal growth from fetal neuronal transplants

High levels of growth factors are present in the brain

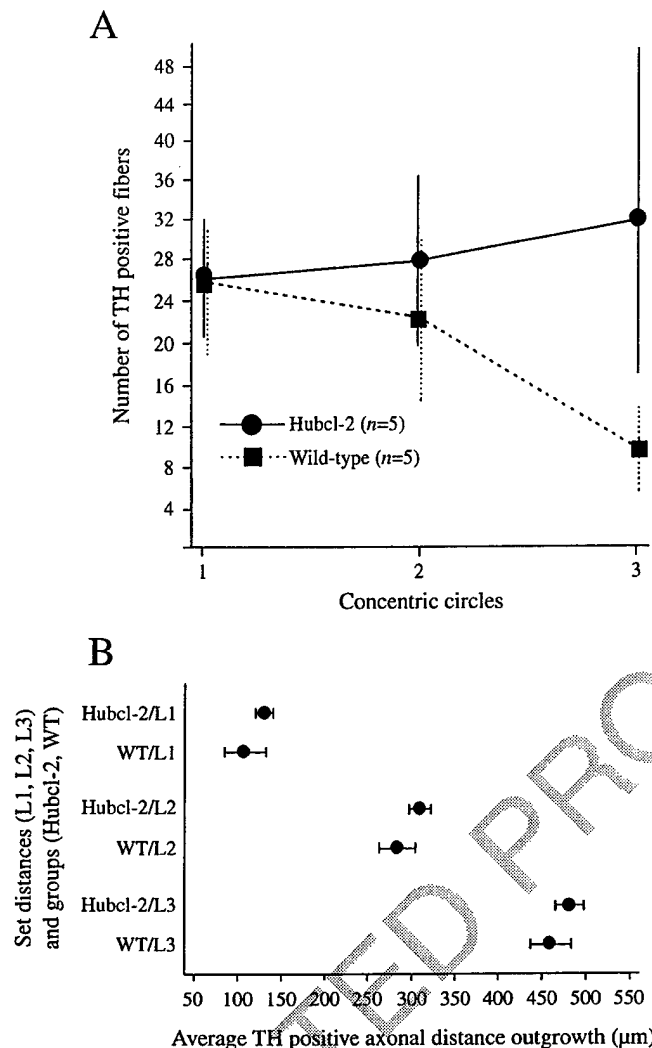


Fig. 3. (a) Statistical analysis (using random effects regression modeling) of the number of TH positive fibers crossing at L1, L2 and L3 (concentric circles). The group differences in the number of TH positive fibers crossings L3 are highly statistically significant ($Z = 3.17$; $P = 0.002$; after adjustment for multiple, post hoc comparisons, $P = 0.006$). (b) Statistical analysis (using random effects regression modeling) of the TH positive axonal outgrowth distance measurements made between the middle of the graft to the edge of the graft (correction for graft size differences) to level L1, L2 and L3. These observations are statistically significant between groups at every level ($Z = 2.63$; $P = 0.009$).

during development and correlate in time and location with enhanced growth of specific groups of neurons. For example, nerve growth factor is highly expressed in the hippocampus and cortex during development²⁵ and likewise glial-derived neurotrophic factor (GDNF) is expressed in the developing striatum.⁴⁰ There are several studies showing improved fetal graft-derived innervation of tissue when the fetal cells are exposed to growth factors. Granholm and colleagues showed more extensive innervation from dopaminergic cells transplanted to the striatum after pretreatment of the fetal tissue with GDNF.⁴⁶ In addition to improved innervation of nearby target tissue, long distance growth has also been achieved using growth factors. For example, Wang *et al.*⁴³ created a track of GDNF along which axons from grafted dopaminergic neurons placed in the SN could grow to the target striatum. Furthermore, expression of

growth factors may be the cause of more extensive or long distance axonal outgrowth from grafted neurons in a number of experiments using co-transplantation of embryonic tissue. Transplantation of a mixture of fetal VM and striatum into adult 6-OHDA lesioned striatum results in a larger area of dense innervation surrounding the graft.⁴ When transplanting embryonic VM to the lesioned SN and creating a bridge of embryonic cortical tissue along an oblique needle track from the SN to the striatum, Dunnett and colleagues¹⁵ showed that dopaminergic fibers from the graft could grow along the bridge-graft all the way to the striatum. Mendez and colleagues³¹ transplanted fetal VM cells simultaneously to the SN and the striatum of adult rats. Surprisingly, growth of dopaminergic axons from the graft in the SN to the VM graft in the striatum was observed, using retrograde labeling with fluorogold. As a control, fetal cortical neurons

transplanted to the striatum did not cause the homotopically placed dopaminergic neurons to grow to the striatum. A possible explanation for this long distance axonal growth could be the release of appropriate trophic factors by fetal cells, thereby stimulating long distance directional growth of axons, since axon-orienting (tropic) factors are still present in the adult CNS.²⁴ Several of these trophic manipulations of fetal grafts are likely to induce axons into an extended growth phase.

These results support the notion that maintained bcl-2 expression can enhance a fetal or neonatal axon's ability to grow.^{3,7,18,20,33,37,42} The down-stream effector molecules of such a process are not determined, but could involve suppression factors involved in cell differentiation, such as p21. Other metabolic intracellular signals, such as cyclic AMP, guanylate cyclase, protein kinase A, growth-associated proteins, along with phosphorylated cytoskeletal proteins and associated growth-cone cell

machinery all dynamically interact to achieve the axonal growth.^{5,6,22,28,34,37,38,42} These results imply that transgenic modification of donor neurons used for transplantation may accomplish desirable changes relevant to therapeutic interventions in neurodegenerative disorders such as Parkinson's disease.

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REFERENCES

1. Abercrombie M. (1946) Estimation of nuclear population from microtome sections. *Anat. Rec.* **94**, 239–247.
2. Bernier P. J. and Parent A. (1998) The anti-apoptosis bcl-2 proto-oncogene is preferentially expressed in limbic structures of the primate brain. *Neuroscience* **82**, 635–640.
3. Bernier P. J. and Parent A. (1998) Bcl-2 protein as a marker of neuronal immaturity in postnatal primate brain. *J. Neurosci.* **18**, 2486–2497.
4. Brundin P., Isacson O., Gage F. H. and Björklund A. (1986) Intrastriatal grafting of dopamine-containing neuronal cell suspensions: effects of mixing with target or non-target cells. *Brain Res.* **389**, 77–84.
5. Cai D., Shen Y., De Bellard M., Tang S. and Filbin M. T. (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* **22**, 89–101.
6. Caroni P. (1997) Intrinsic neuronal determinants that promote axonal sprouting and elongation. *Bioessays* **19**, 767–775.
7. Chen D. F., Schneider G. E., Martinou J. C. and Tonegawa S. (1997) Bcl-2 promotes regeneration of severed axons in mammalian CNS. *Nature* **385**, 434–439.
8. Clark R., Chen J., Watkins S. C., Kochanek P. M., Chen M., Stetler R. A., Loeffert J. E. and Graham S. H. (1997) Apoptosis-suppressor gene bcl-2 expression after traumatic brain injury in rats. *J. Neurosci.* **17**, 9172–9182.
9. Costantini L. C., Chaturvedi P., Armistead D. M., McCaffrey P. G., Deacon T. W. and Isacson O. (1998) A novel immunophilin ligand: distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. *Neurobiol. Disease* **5**, 97–106.
10. Costantini L. C., Cole D., Chaturvedi P. and Isacson O. (1999) Neurophilin ligands: protective and trophic effects in animal models of Parkinson's disease. *Soc. Neurosci. Abstr.* **25**, 293.
11. Costantini L. C. and Isacson O. (2000) Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons. *Expl. Neurol.* **164**, 60–70. *Exp.*
12. Costantini L. C. and Snyder-Keller A. (1997) Co-transplantation of fetal lateral ganglionic eminence and ventral mesencephalon can augment function and development of intrastriatal transplants. *Expl. Neurol.* **145**, 214–227. *Exp.*
13. de la Torre J. R., Hopker V. H., Ming G. L., Poo M. M., Tessier-Lavigne M., Hemmati-Brivanlou A. and Holt C. E. (1997) Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. *Neuron* **19**, 1211–1224.
14. Dunnett S. B. and Björklund A. (1994) Functional Neurotransplantation, **2**. Raven Press, New York.
15. Dunnett S. B., Rogers D. C. and Richards S. J. (1989) Nigrostriatal reconstruction after 6-OHDA lesions in rats: combination of dopamine-rich nigral grafts and nigrostriatal "bridge" grafts. *Expl. Brain Res.* **75**, 523–535. *Exp.*
16. Granholm A. C., Mott J. L., Bowenkamp K., Eken S., Henry S., Hoffer B. J., Lapchak P. A., Palmer M. R., van Horne C. and Gerhardt G. A. (1997) Glial cell line-derived neurotrophic factor improves survival of ventral mesencephalic grafts to the 6-hydroxydopamine lesioned striatum. *Expl. Brain Res.* **116**, 29–38. *Exp.*
17. Greene W. (2000) *Econometric Analysis*, 4th Edition. Upper Saddle River, Prentice-Hall, NJ.
18. Hilton M., Middleton G. and Davies A. M. (1997) Bcl-2 influences axonal growth rate in embryonic sensory neurons. *Curr. Biol.* **7**, 798–800.
19. Holland B. and Copenhaver M. (1987) An improved sequentially rejective Bonferroni test procedure. *Biometrics* **43**, 417–423.
20. Holm K. H. and Isacson O. (1999) Factors intrinsic to the neuron can induce and maintain the ability for neurite outgrowth: a role for bcl-2?. *Trends Neurosci.* **22**, 269–273.
21. Holm K. H., Boonman Z., Tandon P., Costantini L. C., Deacon T. W., Chen D. F. and Isacson O. (1998) Specific graft host synaptic connections in long distance axonal growth and fetal neurotransplantation to adult CNS. *Soc. Neurosci. Abstr.* **24**, 557.
22. Igarashi M., Li W. W., Sudo Y. and Fishman M. C. (1995) Ligand-induced growth cone collapse: amplification and blockade by variant GAP-43 peptides. *J. Neurosci.* **15**, 5660–5667.
23. Isacson O. and Deacon T. (1997) Neural transplantation studies reveal the brain's capacity for continuous reconstruction. *Trends Neurosci.* **20**, 477–482.
24. Isacson O., Deacon T. W., Pakzaban P., Galpern W. R., Dinsmore J. and 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.
25. Kaisho Y., Shintani A., Ono Y., Kato K. and Igarashi K. (1991) Regional expression of the nerve growth factor gene family in rat brain during development. *Biochem. biophys. Res. Commun.* **174**, 379–385.
26. Keynes R. J. and Cook G. M. (1995) Repulsive and inhibitory signals. *Curr. Opin. Neurobiol.* **5**, 75–82.
27. Llenicka G. A., Arcaro K. F. and Calabro J. M. (1998) Activity-dependent development of calcium regulation in growing motor axons. *J. Neurosci.* **18**, 4966–4972.

28. Loschinger J., Bandtlow C. E., Jung J., Klostermann S., Schwab M. E., Bonhoeffer F. and Kater S. B. (1997) Retinal axon growth cone responses to different environmental cues are mediated by different second-messenger systems. *J. Neurobiol.* **33**, 825–834.
29. Martinou J. C., Dubois-Dauphin M., Staple J. K., Rodriguez I., Frankowski H., Missotten M., Albertini P., Talabot D., Catsicas S. and Pietra C., *et al.* (1994) Overexpression of Bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* **13**, 1017–1030.
30. Martinou J. C., Frankowski H., Missotten M., Martinou I., Potier L. and Dubois-Dauphin M. (1994) Bcl-2 and neuronal selection during development of the nervous system. *J. Physiol., Paris* **88**, 209–211.
31. Mendez I., Sadi D. and Hong M. (1996) Reconstruction of the nigrostriatal pathway by simultaneous intrastriatal and intranigral dopaminergic transplants. *J. Neurosci.* **16**, 7216–7227.
32. Merry D. E. and Korsmeyer S. J. (1997) Bcl-2 gene family in the nervous system. *A. Rev. Neurosci.* **20**, 245–267.
33. Merry D. E., Veis D. J., Hickey W. F. and Korsmeyer S. J. (1994) Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* **120**, 301–311.
34. Ming G. L., Song H. J., Berninger B., Holt C. E., Tessier-Lavigne M. and Poo M. M. (1997) cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225–1235.
35. Nikkah G., Cunningham M. G., Cenci M. A., McKay R. D. and Bjorklund A. (1995) Dopaminergic microtransplants into the substantia nigra of neonatal rats with bilateral 6-OHDA lesions. I. Evidence for anatomical reconstruction of the nigrostriatal pathway. *J. Neurosci.* **15**, 3548–3561.
36. Nikolic M., Chou M. M., Lu W., Mayer B. J. and Tsai L. H. (1998) The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. *Nature* **395**, 194–198.
37. Oh Y. J., Swarzenski B. C. and O'Malley K. L. (1996) Overexpression of Bcl-2 in a murine dopaminergic neuronal cell line leads to neurite outgrowth. *Neurosci. Lett.* **202**, 161–164.
38. Polleux F., Morrow T. and Ghosh A. (2000) Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* **404**, 567–573.
39. Schierle G. S., Leist M., Martinou J. C., Widner H., Nicotera P. and Brundin P. (1999) Differential effects of Bcl-2 overexpression on fibre outgrowth and survival of embryonic dopaminergic neurons in intracerebral transplants. *Eur. J. Neurosci.* **11**, 3073–3081.
40. Strömberg I., Bjorklund L., Johansson M., Tomac A., Collins F., Olson L., Hoffer B. and 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. *exp.*
41. Ungerstedt U. and Arbuthnott G. W. (1970) Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res.* **24**, 485–493.
42. Wang Q. and Zheng J. Q. (1998) cAMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. *J. Neurosci.* **18**, 4973–4984.
43. Wang Y., Tien L. T., Lapchak P. A. and Hoffer B. J. (1996) GDNF triggers fiber outgrowth of fetal ventral mesencephalic grafts from nigra to striatum in 6-OHDA-lesioned rats. *Cell Tiss. Res.* **286**, 225–233.
44. Victorin K., Brundin P., Gustavii B., Lindvall O. and Bjorklund A. (1990) Reformation of long axon pathways in adult rat central nervous system by human forebrain neuroblasts. *Nature* **347**, 556–558.
45. Yue Y., Widmer D. A. J., Halladay A. K., Cerretti D. P., Wagner G. C., Dreyer J. -L. and Zhou R. (1999) Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand Ephrin-B2. *J. Neurosci.* **19**, 2090–2101.
46. Zhang K. Z., Westberg J. A., Holtta E. and Andersson L. C. (1996) Bcl2 regulates neural differentiation. *Proc. natn. Acad. Sci. USA* **93**, 4504–4508.

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CHECK AREA FOR YOUR ABSTRACT:

- ☐ Human Trials
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☐ Huntington's Disease Models
☐ Memory Systems
☐ Cortex/Cerebellum
☐ Spinal Cord
☐ Gene Transfer
☐ Stem Cells & Progenitors
☒ Immunology/Xenografts
☐ Trophic Factors
☐ Novel Technology
☐ Other: _____

COMBINED APOPTOSIS AND COMPLEMENT INHIBITORS IMPROVE PORCINE NEURAL XENOTRANSPLANT SURVIVAL IN THE RAT BRAIN.

F. Cicchetti*¹, L. Costantini¹, A. Moore¹, R. Belizaire¹, W. Burton², W. Fodor² & O. Isacson¹. *¹ Neuroregeneration Laboratory, McLean Hospital/MGH Hospital, Harvard Medical School, Belmont, MA, 02478, ² Alexion Pharmaceuticals Inc., New Haven, CT.

We have undertaken a xenotransplantation study in rodents aimed at increasing cell survival of intrastriatal ventral mesencephalic (VM) grafts utilizing 1) inhibitors of cysteine-proteases, 2) the complement membrane attack complex (MAC) and 3) donor cells derived from transgenic pigs expressing human CD59 (a cell-surface molecule which blocks formation of the MAC). The experimental paradigm comprised 4 groups of 10 naïve rats. The groups received cell suspensions of pig E28 VM treated with either a) cysteine-protease inhibitors, b) MAC inhibitors, c) a combination of both or d) untreated VM. Animals also received systemic cyclosporine (CsA) one day before transplantation (30mg/kg) and then daily until sacrifice (15mg/kg). Five weeks post-transplantation, rats were perfused and their brains processed for tyrosine hydroxylase (TH) and pig neurofilament 70 kd protein (NF70) immunohistochemistry. Transplant cell survival (counts of TH-positive cells) and transplant volume (measures of NF70 graft area) were analyzed. There was a significant difference between the number of TH-positive cells in the group of combined inhibitors of cysteine-proteases/MAC versus the control group. This group also had grafts of significantly larger volumes when compared to controls. In summary, our preliminary observations in pig to rat transplants using a combination of inhibitors of cyteine-proteases and the MAC suggest better VM cell survival.

HARVARD UNIVERSITY CURRICULUM VITAE

DEMOGRAPHIC INFORMATION:

Updated 4/25/01

Name: Ole Isacson

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Harvard Medical School
McLean Hospital, MRC 119
115 Mill St.
Belmont, MA 02478
(617) 855-3283 (office)
(617) 855-3284 (fax)

Home Address: 14 Ellery Square
Cambridge, MA 02138

Email: isacson@helix.mgh.harvard.edu

Place of Birth: Kristianstad, Sweden

EDUCATION AND TRAINING:

Education:

1979	Biochemistry, University-College of Kalmar, Sweden
1980	Medical School, University of Lund, Sweden
1981	Research appointment, University of Lund, in the laboratory of Prof. A. Björklund.
1983 M.B.	1 st medical degree, Medical Bachelor, Medical School, University of Lund
1987 M.D.(-Ph.D.)	Doctor of Medicine (Dr. Med. Sc.), University of Lund Thesis: "Neural grafting in an animal model of Huntington's disease" (Medical Neurobiology)

Postdoctoral Training:

1987-1989	Research Fellow, Department of Anatomy (Neuroanatomy Division), University of Cambridge, England
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Licensure and Certification: Not applicable.

PROFESSIONAL APPOINTMENTS:

Academic Appointments:

1981-83	Teaching Assistant Dept. of Histology, University of Lund, Sweden
1983-85	Research Assistant (Demonstrator) Dept. of Histology, University of Lund, Sweden
1986-87	Lecturer & Research Associate Dept. of Medical Cell Research, University of Lund, Sweden
1987-89	Research Fellow Dept. of Anatomy (Neuroanatomy), University of Cambridge, England

PROFESSIONAL APPOINTMENTS: (continued)

Academic Appointments: (continued)

- | | |
|-----------|--|
| 1989- | <i>Docent</i> (Academic Title of Assoc. Professor) of Medical Neurobiology
University of Lund, Sweden |
| 1989-1992 | Assistant Professor of Neurology (Neuroscience)
Harvard Medical School, Boston, MA |
| 1992- | Associate Professor of Neurology (Neuroscience)
Harvard Medical School, Boston, MA |

Hospital or Affiliated Institution Appointments:

- | | |
|-----------|--|
| 1989-1996 | Director of Neuroregeneration Laboratory
Mailman Research Center, McLean Hospital, Belmont, MA |
| 1989-1993 | Assistant in Neuroanatomy
Department of Neurology, Massachusetts General Hospital, Boston, MA |
| 1989- | Investigator
Harvard University/New England Primate Research Center, Southborough, MA |
| 1993- | Associate in Neuroanatomy
Department of Neurology, Massachusetts General Hospital, Boston, MA |
| 1996- | Director of Neuroregeneration Laboratories (a full hospital department) Mailman Research
Center, McLean Hospital, Belmont, MA |

Other Professional Positions and Major Visiting Appointments:

- | | |
|-----------|--|
| 1988-1989 | CNRS and CEA Visiting Research Professor
Hospital de Orsay, Paris, France |
| 1990- | Senior Thesis Advisor, Departments of Biochemistry, Biology and Psychology
Harvard University, Cambridge, MA |
| 1990- | Postdoctoral Research Advisor for Residents in Neurology and Neurosurgery
Massachusetts General Hospital, Boston, MA |
| 1990- | Postdoctoral Research Advisor for Fellows in Neurobiology and Neurology
Harvard Medical School, Boston, MA |
| 1993- | Adjunct Associate Professor of Neuroscience
University of Massachusetts Medical School, Worcester, MA |
| 1996 | Visiting Professorship Lecture Series
The Miami Project of Cure Paralysis, The University of Miami School of Medicine |

AWARDS AND HONORS:

- | | |
|---------|--|
| 1976-78 | Nathorst's Scientific Foundation. Full Scholarship Award at Atlantic College, U.K.
(personal award) |
| 1983 | The Medical Faculty Award for graduate thesis work in medicine, University of Lund,
Sweden (personal award) |

AWARDS AND HONORS: (continued)

1987	The Fernstrom Foundation Scholarship Award 1987 for medical scientists (personal prize/award)
1987	The Swedish Physican's Society Award for studies on neurodegenerative diseases (personal prize/award)
1987	The Royal Swedish Academy of Sciences. Lindahl's Award (personal award)
1989-90	NATO Grant, for studies on neurodegenerative disease. Research grant # CRG 890583
1990-91	NIH Program Project Award: Huntington's Disease Center, (P.I. of sub-contract) Massachusetts General Hospital and McLean Hospital
1991-96	NIH R29 Award: Neurological Science. Research Grant # R29 NS29178
1992-95	NIH RO1 Award: Neurological Science. Research Grant # RO1 NS30064
1995	Milton Fund Award, Harvard University
1995-	NIH RO1 Award: Neurological Science. Research Grant # RO1 NS30064-04
1998-02	USAMRAA Research Grant Award DAMD17-98-1-8618 (R01 level)
1999-02	USAMRAA Research Grant Award DAMD17-99-1-9482 (R01 level)
1999-	NIH: NINDS Parkinson's Research Center of Excellence. (Center Director) P50 NS39793
1999-	The Century Foundation Research Award
1999-	NIH RO1 Award: Neurological Science. Research Grant # RO1 NS30064-07
2000-	NIH RO1 Award: Neurological Science. Research Grant # RO1 NS41263-01
2001-04	USAMRAA Research Grant Award (R01 level)
2001-03	The Kinetics Foundation Research Award

SERVICE ASSIGNMENTS: Not applicable

MAJOR COMMITTEE ASSIGNMENTS:

Harvard Medical School/Affiliated Institutions

1990-1994	Graduate Admission Committee, Program of Neuroscience, Harvard Med. Sch.
1990-	Research Committee, McLean Hospital
1990-	Scientific Advisory Board, New England Regional Primate Research Center, Southborough, MA
1990-	Institutional Animal Care and Use Committee, McLean Hospital
1991-	Space Allocation Committee, McLean Hospital
1991-	Mailman Research Center Steering Committee, McLean Hospital
1993-1996	Business/Accounting Task Force, Research Subcommittee, McLean Hospital
1993-94	Search Committee for Director of Mailman Research Center, McLean Hospital
1997-	Industrial Relations Study Group, McLean Hospital
1997-	Chair of Core Imaging Facility Advisory Committee, McLean Hospital/Shriver Ctr.
1998-	Search Committee, Faculty for Mailman Research Center
2001-	Governance Committee, Harvard Center for Neurodegeneration and Repair

Regional/National/International

1992-	Veterans Administration Merit Review Board
1992-	Internal Review Committee NIH Program Project grants
1993-	Special Review Committes; National Institutes of Health (NINDS): Program Projects (Site-visit teams) and Clinical Research Centers (NIH) (Ad hoc member)
1994-	Neurological Disorders Program Project Review B Committee (NINDS) (Ad hoc member)
1994-	Human Frontier Science Program (review committee)
1994-	Council, American Society for Neural Transplantation
1995-	Natural Sciences and Engineering Research Council of Canada (review committee)
1995-1996	Program Committee, American Society for Neural Transplantation (Chair)
1997-1998	President Elect, American Society for Neural Transplantation
1997	Advisory Group on Parkinson's, presentation to Veterans' Admin, US Congress (Chair)
1998	The Wellcome Trust (review group)

MAJOR COMMITTEE ASSIGNMENTS: (continued)

Regional/National/International (continued)

1998-1999	President, American Society for Neural Transplantation and Repair
1998-	MDCN-2 Study Section (NIH), member
1998-	Council, International Cell Transplant Society
1999-	Program Committee, International Neural Transplantation and Repair

MAJOR ADMINISTRATIVE RESPONSIBILITIES:

1989-1996	Director	Neuroregeneration Laboratory, Mailman Research Center, McLean Hospital
1996-	Director	Neuroregeneration Laboratories (a full hospital department and research program), Mailman Research Center, McLean Hospital
1996-	Dept. Head	Mailman Research Center (formerly Ralph Lowell Laboratories) Research Department
1999-	Center Director	NIH Udall Parkinson's Disease Research Center

PROFESSIONAL SOCIETY INVOLVEMENT:

1989-	International Brain Research Organization (IBRO) (member)
1989-	American Association for the Advancement of Science (AAAS) (member)
1989-	Society for Neuroscience (member)
1989-	European Neuroscience Association (ENA) (member)
1989-	Boston Society of Neurology and Psychiatry (member)
1989-	World Federation of Neurology Research Group on Huntington's Disease (elected member)
1993-	Huntington's Disease Society of America, Massachusetts Chapter
1994-	American Society for Neural Transplantation (ASNT) (founding member)
1994-	American Society for Neural Transplantation (Council member)
1994-	Program Committee, American Society for Neural Transplantation (Co-Chairman)
1995-	International Society of Neuropathology (member)
1995-96	American Society for Neural Transplantation (Secretary)
1995-96	Program Committee, American Society for Neural Transplantation (Chairman)
1996-	New York Academy of Science (member)
1997-	American Academy of Neurology (member)
1997	American Society for Neural Transplantation (President-elect)
1998	American Society for Neural Transplantation and Repair (President)
1998-	International Society for Cell Transplantation (Council member)
1999-	American Society of Transplantation (elected member)

COMMUNITY SERVICE (related to professional work):

Professional Consultation (Other than Patient Care)

a) Scientific and Technical Consultation

1995-1997	Scientific advisor at clinical trials for Parkinson's and Huntington's disease at HMS/Brigham and Women's Hospital, Boston, MA and Lahey Clinic, Burlington, MA
-----------	---

b) Public Presentations as an Expert in Discipline

1990	Huntington Disease Society of America, basic research presentation for patients
1994	Belmont Rotary Chapter, science presentation
1994	American Parkinson Disease Association, Local chapter for patients, Presentation on basic research towards new treatments for PD, Boston

COMMUNITY SERVICE (related to professional work): (continued)

Professional Consultation (Other than Patient Care) (continued)

- | | |
|------|--|
| 1995 | National Youth Leadership Forum, presentation to high school students interested in pursuing research and medical studies, Simmons College, Boston |
| 1995 | Advisory presentation before the US Senate Special Committee on Aging, Capitol Hill, Washington, D.C. |
| 1995 | Advisory presentation before the US House Commerce Committee, Health and Environment Subcommittee, Capitol Hill, Washington, D.C. |
| 1996 | Presentation on current PD research to patient group, Boston, MA |
| 1996 | Research Update, Parkinson's Action Network Third Annual Public Policy Forum, Washington, D.C. |
| 1997 | Advisor to members of Labor, Health and Education Committee on the progress of basic neuroscience research, Washington, D.C. |
| 1999 | Advisory presentation on neurological research to science advisors to the President at the White House, Washington, D.C. |

Other Professionally Related Service

- | | |
|------|---|
| 1996 | National Parkinson Foundation "On the Causes and Treatments of Parkinson's Disease" |
| 1997 | ABC "Turning Point", Television interview on Neural Xenotransplantation |
| 1997 | NPR "Science Friday", Radio interview on Risks and Benefits of Xenotransplantation |
| 1998 | CBS "60 Minutes", Television interview on xenotransplantation. |

EDITORIAL BOARDS:

Member of Editorial Board

- | | |
|-------|--|
| 1989- | Journal of Neural Transplantation and Plasticity |
| 1991- | Cell Transplantation |
| 1997- | Restorative Neurology and Neuroscience |
| 1997- | Experimental Neurology |
| 1998- | inSight |

Guest Editorships

- | | |
|------|--|
| 1995 | Cell Transplantation, ASNT Special Issue |
| 1996 | Cell Transplantation, ASNT Special Issue |
| 1999 | Brain Pathology, Special Issue |
| 1999 | Experimental Neurology, Special Issue |
| 2000 | Neuroscience News, Special Issue |

Current Editorial Review Assignments

Science, Nature, Nature Medicine, Nature Genetics, Journal of Neuroscience, Journal of Comparative Neurology, Neuroscience, Brain Research, Proceedings of the National Academy of Science USA, Journal of Neurochemistry

PART II:

REPORT OF RESEARCH:

Major Research Interests:

- (1) Methods for neuronal repair, regeneration and protection using animal models of Parkinson's, Huntington's and Alzheimer's diseases.
- (2) The pathophysiology and therapy of CNS degeneration in Huntington's, Parkinson's, and Alzheimer's diseases.

Narrative Description of Research:

The research programs focus on mechanisms of neuronal degeneration and innovative methods for cellular repair, with emphasis on the neurodegenerative disorders like Parkinson's (PD), Alzheimer's (AD), and Huntington's (HD). This work has already led to applications of cell therapy for PD and HD. This laboratory is involved in several pharmacological, gene, molecular and cellular studies to obtain neuroprotection or cell and gene delivery in the CNS.

1. Neural Transplantation:

The understanding of regeneration and plasticity of the mammalian nervous system has developed over the last decade. We have participated in research that shows that the brain is a regenerative system which can integrate implanted primary neurons, progenitor or stem cells into the adult brain. These implanted neurons grow physiologically and functionally to repair previously damaged or degenerated neuronal pathways. The specific experiments performed in this laboratory have significantly influenced neural transplantation in clinical trials using porcine and human cells for Parkinson's and Huntington's disease. Encouraging results suggest that cell therapy may become, when fully developed, a useful treatment for neurodegenerative disease. At the very fundamental level, this laboratory also investigates the specific axon guidance factors that persist in the adult brain. In animal models, by transplanting neuroblasts into various locations in the brain, we are determining if reconnection and tropic interactions are possible. The goal of this work is a better understanding of the structural and functional plasticity of the central nervous system that can lead to improved therapies for neurological disease.

2. Neuroprotection:

Starting in 1989, this laboratory has investigated a number of paradigms in which neurotrophic factors can prevent degenerative events. Based on the theory that the neuron at any given moment is in a defined state of vulnerability (dependent on genetic and phenotypic characteristics), we have devised methods to improve the resilience of neurons affected in Huntington's, Parkinson's and Alzheimer's diseases. We are currently investigating several novel molecular (e.g. neurophilins) treatments for Parkinson's disease and Alzheimer's disease models. In addition, we are performing basic research on molecular modifications of striatal neurons to enhance their capacity to withstand insults or modify their genetically induced vulnerability in Huntington's disease.

3. Gene Delivery and Gene Therapy:

Since 1989, this laboratory has developed a number of model systems for gene delivery, ranging from delivery in the peripheral nervous system to specific delivery in anatomical systems involved in Parkinson's and Huntington's disease. We have used modified and transfected cell lines to establish new paradigms for neuroprotection and recently have made inroads into efficient, selective and stable delivery and expression of genes by modified viral vectors into the CNS.

(Please refer to Bibliography to see the development and progress of these research paths.)

Specific Research Funding Information:

1989-90	NATO Grant# CRG 890583, (PI) "Neural transplantation in primate models of neurodegenerative disease".
1990-91	NIH Program Project Award: Huntington's Disease Center, (PI of sub-contract) MGH and McLean Hospital.
1991-96	NIH: Neurological Science. Research Grant NS29178, (PI), "Excitotoxic Cortex Lesions-Degeneration and Remodeling"
1992-93	Biotechnology sponsored research program, (PI) "Cell Based Therapy"
1992-95	NIH: Neurological Science. Research Grant # RO1 NS30064, "Neuronal Replacement in a Model of Huntington's Disease"
1993-94	Biotechnology sponsored research program, (PI) "Cell Based Therapy"
1994-	NIH: MH19905 (Benes, PI) "Clinical Neuroscience Training Program Grant"
1994-95	Biotechnology sponsored research program, (PI) "Immunological masking techniques in intracerebral fetal nerve cell transplantation"
1995	Milton Fund, Harvard University, (PI) "Novel neurotrophic molecules for neuroprotection in Parkinson's disease"
1995-96	Wills Foundation, (Sponsor (OI), Haque, fellowship),
1995-96	Biotechnology sponsored research program, (PI) "New Immunological Methods in Intracerebral Fetal Nerve Cell Transplantation"
1995-98	NIH: Neurological Science. Research Grant # RO1 NS30064, Competing Renewal, (PI) "Neuronal Replacement in a Model of Huntington's Disease"
1996-	NIH: 5-T32-AG00222 (Yankner, PI) "Molecular Biology of Neurodegeneration Training Grant"
1996-97	National Parkinson Foundation, (Sponsor (OI), Costantini, fellowship) "Ex vivo and in vivo gene transfer of CuZn SOD-1: protection from oxidative stress and neurodegeneration"
1996-97	Biotechnology sponsored research program, (PI) "Cholinergic neuronal replacement in animal models"
1996-	Pharmaceutical industry sponsored research program, (PI) "Documentation of neuroprotective and therapeutic effects of compounds against MPTP/MPP+ induced neurotoxicity in mice and rat paradigms of Parkinson's disease"
1997-	Biotechnology sponsored research program, (PI) "Functional pharmacological and therapeutic models relevant to neuroprotection or amyloid formation"
1997-	Biotechnology sponsored research program, (PI) "Identification of the human immune response to porcine fetal mesencephalic cells"
1997-99	Clinical Neuroscience Training Program, (Sponsor (OI), Costantini, fellowship) "Ex vivo and in vivo gene transfer of CuZn SOD-1: protection from oxidative stress and neurodegeneration"
1998-	Federal, USAMRAA, DAMD17-98-1-8618, (PI) "Structural and functional brain repair studies of PD models by novel neurosurgical, PET and MRI/MRS methods"
1999-	Federal, USAMRAA, DAMD17-99-1-9482, New (PI) "Knock-out and transgenic strategies to improve neural transplantation therapy for Parkinson's disease"
1999-	Century Foundation, Sarasota Memorial Hospital, sponsored research program (PI) "Electrophysiology of PD"
1999-	Federal: NINDS/NIH P50 NS39793, Parkinson's disease Research Center of Excellence (Center Director and PI) "Novel Therapeutic Approaches to Parkinson's disease"
1999-	NIH: Neurological Science. Research Grant # RO1 NS30064, Competing Renewal, (PI) "Transgenic Xenotransplants for Huntington's Disease"
2000-	NIH Neurological Science. Research Grant # RO1 NS41263-01, "Novel Anti-inflammatory Therapies for Neurotoxicity Induced PD"
2001-	Federal, USAMRAA, (PI) "Gene Regulation and Stem Cell Transplantation Therapies"
2001-	Kinetics Foundation, "Stem Cell Derived Dopaminergic Cells and DA Gene Regulation"

REPORT OF TEACHING:

Local Contributions:

Graduate Medical Courses/Seminars/Invited Teaching Presentations:

1988	Boston, Dept. of Neurology, Harvard Medical School, Massachusetts General Hospital "Neuronal transplantation and strategies for CNS regeneration" (seminar)
1990-	Faculty, Program of Neuroscience, Harvard Medical School. Faculty, Neurobiology of Behavior course/program, McLean Hospital, Harvard Medical School
1990	Boston, MA "Excitotoxic lesions of the cerebral cortex model degeneration and plasticity seen in neurodegenerative diseases" (lecture)
1990	Cold Spring Harbor, N.Y., "The use of genetically engineered cells as donor tissue in models of intracerebral transplantation" (lecture)
1990	Woods Hole Marine Biology Laboratory, RUNN course lecture: "Studies of neuronal cell death and regeneration in transplantation models" (faculty)
1992	Course organizer: HMS Program of Neuroscience Course; "Paradigms to investigate neuronal health: what happens to neurons in neurodegenerative diseases"
1992	Faculty, Lecturer, Dept. of Neurology, HMS, MGH course: "Intensive Clinical and Basic Neuroscience Update"
1993	Neurobiology 209, Harvard Medical School (lecture)
1994	Massachusetts General Hospital, Scientific Integrity Course (faculty)
1995	McLean Hospital, Clinical Neuroscience Training Program, "Neural transplantation: site-specific CNS delivery of neuroprotectants and neurotransmitters" (lecture)
1996	Gene Therapy: Principles and Practice (Genetics 208), Harvard Medical School "Strategies of gene therapy for dominant and recessive genetic, as well as non-hereditary, diseases" (lecture)
1996	Neurobiology of Disease Course, Harvard Medical School
1997	McLean Hospital, Clinical Neuroscience Training Program, "Neural transplantation: site-specific CNS delivery of neuroprotectants and neurotransmitters" (lecture)
1997	Boston, Brigham and Women's Hospital, "Hosting foreign cells in the brain: will xenogeneic neurons serve as treatments for Parkinson's and Huntington's disease?" (lecture)
1997	Gene Therapy Group, Harvard Medical School "Gene Therapy for Huntington's Disease" (lecture)
1997	Boston, Harvard-Mahoney Neuroscience Institute Forum, "Neural Transplantation in Parkinson's and Huntington's Disease"
1998	Boston, MGH, Current Approaches to Understanding Neurodegenerative Disease Symposium, "Neural transplantation therapy for neurodegenerative disease". (lecture)
1999	Southborough, MA, Harvard Primate Research Center, "How neural transplantation can work in patients with neurodegenerative disease" (lecture)
1999	Boston, Partners Neurology Residency, Basic Neuroscience Course, "Neuronal Death, Regeneration and Transplantation" (lecture)
1999	McLean Hospital, Clinical Neuroscience Training Program, "Neural transplantation: site-specific CNS delivery of neuroprotectants and neurotransmitters" (lecture)
2000	Cambridge, MIT, Modulation of APP and memory by the cholinergic system (lecture)
2000	Boston, HMS, Harvard Medicine at the Millenium, "Parkinson's and Other Neurodegenerative Diseases: Bench to Bedside" (lecture and moderator)
2001	Boston, Brigham and Women's Hospital, Neurosurgery Rounds

Continuing Medical Education Courses:

1992	Dept. of Neurology, HMS, MGH course, Boston, MA "Intensive Clinical and Basic Neuroscience Update" (faculty)
------	--

TEACHING: Local Contributions: (continued)Continuing Medical Education Courses: (continued)

1996	State University of New York, Syracuse, NY, 8th Annual Neuroscience Symposium (Neurofest '96), "Specificity of axonal growth from porcine embryonic neural xenografts in host brain" (lecture)
1998	Co-director, "Anatomy and Physiology of Basal Ganglia Surgery" CME Course and Scientific Conference, Sarasota, FL
1998	Organizer, Second "Cellular and Molecular Treatments of Neurological Diseases" Scientific Conference, Cambridge, MA
1999	Co-director, "Anatomy and Physiology of Basal Ganglia Surgery" CME Course and Scientific Conference, Sarasota, FL
2001	Organizer, Third "Cellular and Molecular Treatments of Neurological Diseases" Scientific Conference, Cambridge, MA

Advisory and Supervisory Responsibilities in Clinical or Laboratory Setting:A. Faculty Mentor, Thesis Advisor or Supervisor in Neuroregeneration Laboratories:A1. Current Predoctoral Students: (Degree)

2001-Present	Peter Sokol-Hessner	BS 2004 Dartmouth University
2001-Present	Robert Fokes Zahra	BS 2003 Harvard University
2001-Present	John Morgan	BS 2003 Harvard University
2001-Present	Michael Brusco	BS 2002 Harvard University
2000-Present	Roger Belizaire	BS 2000 Princeton University
2000-Present	Therese Andersson	MSc 2000 Kalmar University

A2. Past Predoctoral Students: (Degree, current position)

1999-2000	Anna Moore	BSc 2001 Cardiff University
1997-1999	Anna Mattsson	MSc 1998 Kalmar University, PhD Student, Karolinska Institute
1997-1999	Biljana Georgievska	MSc 1998 Kalmar University, PhD Student, Lund University
1997-1998	Karin Holm	MD 1999 Lund University, Resident, Lund University
1997-1998	Zita Boonman	MD 1999 Utrecht University, Resident, Utrecht University
1996-1997	Lina Fine	BS 1997 Harvard University, PhD Studies
1996-1997	Brandi Whatley	BS 1997 Boston University, PhD Student, U. Maryland
1993-1996	Wendy Galpern	BS 1989 Tufts University, PhD 1996, NRL/McLean Hospital & U Mass, MD 1998 U Mass Resident Neurology MGH Instructor, Harvard Medical School
1995-1996	Arif Husain	BS 1996 MIT, Medical School
1995-1996	Paul Borghesani	BS 1994 MIT, MD-PhD 1999, Harvard Medical School
1995	Marcus Ware	BS 1993 Tougaloo College, MD-PhD at Harvard/MIT HST Program
1995	Amy Spiegel	BS 1995 West Chester University, Medical School

TEACHING: Local Contributions: (continued)**A2. Past Predoctoral Students:** (Degree, current position) (continued)

1995-1996	D. Andrina Ngo	BS 1996 Harvard University, PhD Student, Johns Hopkins University
1994	Lisa Genova	Program in Neuroscience, PhD 1998 Harvard Medical School, Consultant, Health Advances, Wellsley
1993-1995	David St. Peter	BS 1995 Harvard University, Director, Biotech. development, New York, NY
1993-1995	Antony Garcia	BS 1995 Harvard University, Director, Computer-software development, Cambridge, MA
1993-1994	Marc Dinkin	BS 1994 Harvard University, Medical School
1992-1993	Tara Uhler	MA 1992 Harvard University, MD, Harvard Medical School
1991-1992	Joseph Simpson	BS 1992 Harvard University, MD, PhD at Washington University, St. Louis
1991-1992	Wendy Yee	BS 1992 MIT, 1999, PhD at Johns Hopkins University
1989-1991	Lisi Fishman	BS 1991 Harvard University, Psychologist, Cambridge, MA

A3. Current Staff and Postdoctoral Fellows:

2001-	Rosario Sanchez-Pernaute	MD 1988 U. Complutense Madrid, 1997 PhD U. Autonoma Madrid
2000-	Kai Sonntag	MD 1993, PhD 1994 U. Heidelberg, Instructor HMS
2000-	Aygun Balcioglu	PhD 1994 Mass College of Pharmacy, Instructor HMS
2000-	Kevin McNaught	PhD 1995 Kings College, London
2000-	Hyemyung Seo	PhD 1999 U. Tennessee, College of Medicine
2000-	Lars Bjorklund	MD 1999, PhD 1998 Karolinska Institute, Sweden
1999-	Sangmi Chung	PhD 1998 Cornell University, New York
1999-	Francesca Cicchetti	PhD 1998 Robert Laval Univ., Quebec
1998-	Anna-Liisa Brownell	PhD 1974 University of Helsinki Asst. Imaging Biologist, McLean Hosp. Assoc. Prof., Harvard Medical School
1998-	Craig van Horne	MD 1992 U. Colorado School of Medicine PhD 1992 U. Colorado School of Medicine Assistant Neurobiologist, McLean Hospital Asst. Prof., Harvard Medical School
1996-	Wendy Galpern	Ph.D. 1996 U Mass/McLean/NRL Instructor, 1997 Harvard Medical School

A4. Past Postdoctoral Fellows: (Degree, current position)

1996-2000	Lauren Costantini	PhD 1996 U. of New York, Albany Assoc. Director of Technol. Dev., Titan, Inc.
1996-1999	Ling Lin	MD 1985 Henan Medical U. Graduate School, Hong Kong
1992-1999	Terry Deacon	PhD 1984 Harvard U., Associate Professor Boston University (formerly Assoc. Prof. at Harvard U.)
1998-1999	Pushpa Tandon	PhD 1985 U. Lucknow, India Research Scientist, Igen, Inc.
1995-1996	Nadia S. K. Haque	PhD 1995 Cambridge U., Research Scientist, Geron, Inc.

TEACHING: Local Contributions: (continued)

A4. Past Postdoctoral Fellows: (Degree, current position)(continued)

1992-1996	Lindsay Burns	PhD 1991 Cambridge U., Research Scientist, Neurex, Inc.
1993-1995	Stephen Tatter	MD/PhD 1990, 1989 Rockefeller U. /Cornell Med. School, Asst. Prof. Neurosurgery, North Carolina Baptist Hospital
1992-1994	Peyman Pakzaban	MD 1984 MGH, Neurosurgery, U. Texas Med. Center
1991-1993	David Frim	PhD/MD 1988 Harvard Medical School , Neurosurgery, Assoc. Prof., U. Chicago Medical Center
1991-1992	Ullrich Wullner	MD-PhD 1989 U. Gottingen, Asst. Prof., Lab Director, Neurology, University of Tübingen
1991-1992	Philippe Hantraye	PhD 1987 U. of Paris Assoc. Prof., Director, Research Unit, Centre National de Recherche Scientifique (France)
1990-1992	William Rosenberg	MD 1987 Harvard Medical School, Asst. Prof., Neurosurgery, U. Cincinnati
1989-1990	James Schumacher	MD 1986 U. Washington, Neurosurgery, Sarasota Memorial Hospital Instructor, Harvard Medical School 2000

B. Recent Theses/Dissertations Directed:

B1. Graduate

Therese Andersson, MSc Thesis, 2001
 Anna Mattsson, MSc Thesis, 1998
 Biljana Gjorgijevska, MSc Thesis, 1998
 Karin Holm, MSc, MB Thesis, 1998
 Wendy R. Galpern, PhD "Neuroprotection and Neurotransplantation Strategies in Models of Parkinson's Disease", Awarded 1996

B2. Undergraduate (Primary research supervisor and mentor roles)

Anna Moore, BSc (Hons) Neuroscience Thesis, 2000 Cardiff University
 Brandi Whatley, BS, Senior Honors Thesis, 1997 Boston University
 Amy Spiegel, BS, Senior Honors Thesis, 1995 West Chester University
 David St. Peter, BS, Senior Honors Thesis, Biology, 1995 Harvard University
 Antony Garcia, BS, Senior Honors Thesis, Biology, 1995 Harvard University
 Marc Dinkin, BS, Senior Honors Thesis, Biology, 1994 Harvard University
 Joseph Simpson, BS, Senior Honors Thesis, Biology, 1992 Harvard University
 Wendy Yee, BS, Senior Honors Thesis, Biology, 1992 MIT
 Lisi Fishman, BS, Senior Honors Thesis, Biology, 1991 Harvard University

C. PhD Dissertation or Examining Committees:

1993-1995	Macrene Alexiades, Thesis Advisory Committee, HMS
1994	Wendy R. Galpern, Oral Qualifying Examination Committee, U Mass Medical School
1994-1996	Paul Borghesani, Thesis Advisor, MD-PhD Program, HMS
1999	Richard Christie, PhD Thesis Examination Committee, HMS

Leadership Roles:

1992-1993	"On Neuronal Health", Graduate course, HMS (organizer)
1994	HMS Neuroscience Student Visit to McLean Hospital (organizer)

TEACHING: Local Contributions: (continued)

Leadership Roles: (continued)

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| 1995 | National Youth Leadership Forum, presentation to high school students interested in pursuing research and medical studies, Simmons College, Boston |
| 1995 | Scientific Program Organizer, "Cellular and Molecular Treatments of Neurological Diseases" Conference, Three day international conference of 50 invited participants including students from HMS Program in Neuroscience and residents of Neurology and Neurosurgery Programs at HMS hospitals. |
| 1998 | Scientific Program Organizer, Second "Cellular and Molecular Treatments of Neurological Diseases" Conference, American Academy of Arts and Sciences, Cambridge, MA. Three day international conference of 150 invited participants including students from HMS Program in Neuroscience and residents of Neurology and Neurosurgery Programs at HMS hospitals |
| 2001 | Scientific Program Organizer, Third "Cellular and Molecular Treatments of Neurological Diseases" Conference, American Academy of Arts and Sciences, Cambridge, MA. Three day international conference of 150 invited participants including students from HMS Program in Neuroscience and residents of Neurology and Neurosurgery Programs at HMS hospitals |

TEACHING: National or International Contributions:

Medical/Graduate School Courses/Seminars/Invited Teaching Presentations:

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| 1981-83 | Seminars and tutorials in Cell Biology, Histology and Neurobiology at the Medical Faculty, University of Lund, Sweden |
| 1983-85 | Lecturer in Neurobiology and Histology at the Medical Faculty, University of Lund. |
| 1985-87 | Lecturer and Assistant Director of Medical Neurobiology Course, Lecturer in Histology. Supervisor for research students in Medicine, Co-supervisor for 2 PhD students, University of Lund. |
| 1987-89 | V. Fellow, Jesus College, Cambridge, University of Cambridge, England. Supervisor for Medical Part II students, University of Cambridge, England. |
| 1990 | Faculty, Lecturer, RUNN Course (Review and Update in Neurobiology for Neurosurgeons and Neurologists) Woods Hole, MA |
| 1990 | Faculty, Lecturer, Cold Spring Harbor Course: "Molecular Genetic Analysis of Diseases of the Nervous System", N.Y. |
| 1994 | Woods Hole, MA, RUNN Course "Affecting Neural Function by Transplantation" (faculty) |
| 1995 | Chicago, IL, for Rush University Research Week (Keynote speaker) |
| 1996 | Miami, FL, The University of Miami, The Miami Project to Cure Paralysis "Specificity of connections formed by transplanted fetal neurons to the mature CNS" (Visiting Professor) |
| 1998 | Karolinska Institute, Stockholm, Introductory lecture for PhD thesis examination for Lars Bjorklund on Intraocular Cellular Transplants |
| 2001 | France, INSERM Workshop "Neural stem cells" (faculty) |
| 2001 | Amsterdam, 22 nd Intl. Summer School, "Plasticity in the Adult Brain" (faculty) |

Invited Presentations:

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| 1983 | Hamburg, European Neuroscience Association "Monitoring of neuronal survival in suspensions of embryonic CNS tissue" (paper) |
| 1984 | University of Cambridge, Downing Site "Functional neuronal replacement in the ibotenic acid lesioned neostriatum by neostriatal neural grafts" (lecture) |
| 1984 | Lund, Nordic Meeting in Neuropsychiatry "Functional neuronal replacement in an animal model of Huntington's disease" (paper) |

TEACHING: National or International Contributions: (continued)

Invited Presentations: (continued)

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| 1984 | Oxford, Dept. of Pharmacology, University of Oxford "Striatal neural transplant in the excitotoxically lesioned neostriatum" (lecture) |
| 1985 | Uppsala, Nordic Physiology Meeting "Neuronal replacement in an animal model of Huntington's disease" (paper) |
| 1985 | Munchen, Glial-neuronal communication symposia "The use of neural transplants in the study of lesion models of the adult CNS" (lecture) |
| 1985 | Oxford, European Neuroscience Association "Morphological and behavioural changes following neural grafting in rats with lesions of the anteromedial neostriatum" (paper) |
| 1986 | Avoriaz, Symposium at European Winter Congress on Brain Research "Neural replacement by intracerebral grafts in animal models of Parkinson's and Huntington's disease" (chairman and lecture) |
| 1986 | New York, New York Academy of Sciences "Morphology and function of striatal neural grafts" (lecture) |
| 1986 | Dusseldorf, Dept. of Neurology "The use of neural grafting in studies of CNS development and regeneration" (lecture) |
| 1986 | Spetses-ETP, Research program at European Training Program "Autumn School" "The use of neural grafting in experimental studies of CNS regeneration and development" (lecturer) |
| 1987 | London, Royal Free Hospital, Dept. of Psychiatry "Aspects of degeneration and regeneration in the adult CNS using intracerebral transplants" (lecture) |
| 1987 | London, Maudsley Hospital, Inst. of Psychiatry "Neural grafting in animal models of neurodegenerative disease" (lecture) |
| 1987 | Venice, 2nd Symposium on Restorative Neurology "The use of fetal neurons to replace neurons in the CNS" (lecture) |
| 1987 | Rochester, New York, at Neural transplantation into the mammalian CNS meeting, "Fetal cortical grafts into the excitotoxically lesioned neocortex: a model for trophic interactions in Alzheimer's disease?" (paper) |
| 1987 | Pécs, Hungary, Satellite Symposium on Neural Regeneration and Transplantation "Striatal cell suspension grafts in an animal model of Huntington's disease" (paper) |
| 1987 | Paris, Dept of Neurology, Frédéric Joliot Hospital, Orsay "A primate model of Huntington's disease" (lecture) |
| 1988 | Paris, Dept. of Neurology, Frederic Joliot Hospital, Orsay "Excitotoxic lesions models of CNS degeneration" (lecture) |
| 1988 | Paris, Dept. of Neurology, Frederic Joliot Hospital, Orsay "The use of neural transplantation in patients with neurodegenerative disease: basic research and recent clinical experiments" (lecture) |
| 1988 | Lyon, Conference: Trends in Neurobiology "Neuron-target interaction in the CNS: neuronal degeneration and regeneration theories" (paper) |
| 1989 | Cambridge, England, Neural transplantation meeting: molecular bases to clinical application "Neural transplantation in a primate model of Huntington's disease" (paper) |
| 1990 | Lund, Sweden "From pharmacological to neuronal replacement in Huntington's disease" (paper) |
| 1991 | St. Louis, Missouri, CNS Transplants in Adult Damaged Sensory Thalamus and Neocortex (lecture) |
| 1991 | Washington, D.C., Georgetown University, Neural Transplantation in Animal Models of Huntington's Disease (lecture) |
| 1991 | Paris, La Salpetriere Hospital, "Animal Models of Neuronal Protection, Degeneration and Regeneration: Concepts of Neuronal Health" (lecture) |

TEACHING: National or International Contributions: (continued)

Invited Presentations: (continued)

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| 1991 | Stockholm, Karolinska Institute, "CNS degeneration and regeneration models: new concepts of neuronal damage and protection" (lecture) |
| 1992 | Nagoya, Japan, "International Conference on Biochemistry of Disease" (lecture) |
| 1992 | Washington, D.C. "IV International Symposium on Neural Transplantation" (lecture) |
| 1992 | Brussels, "25th International Congress of Psychology" (lecture) |
| 1993 | Frankfurt, Symposium on anti-excitotoxic therapy: "Neuronal protection, gene-transfer and circuitry repair in the basal ganglia" (lecture) |
| 1994 | Hancock, MA, Third Berkshire Neuroscience Symposium (lecture) |
| 1994 | Chatenay-Malabry (Paris), 5th International Symposium on Neural Transplantation (lecture) |
| 1995 | Winter Conference on Brain Research, "Primate models of caudate-putamen motor functions" (lecture) |
| 1995 | Paris, ANPP Meeting "Novel Therapeutics in the Nervous System: Gene Transfers and Trophic Factors" (lecture) |
| 1995 | Philadelphia, PA, Intl. Conf. on Gene Therapy for CNS Disorders, "Gene Therapy for Huntington's Disease" (lecture) |
| 1995 | National Press Club, Washington D.C. "New therapies for Parkinson's disease" (lecture) |
| 1995 | U.S. Senate Special Committee on Aging, Washington D.C. Advisory presentation on Parkinson's disease |
| 1995 | House Subcommittee on Health and Environment, Washington, D.C., Advisory presentation on Parkinson's disease |
| 1995 | Maastricht, Holland, Annual Meeting of NECTAR (lecture) |
| 1996 | Washington, DC, National Foundation for Brain Research, Gene Therapy for Parkinson's Disease Consortium |
| 1996 | San Francisco, CA, Annual Meeting of American Diabetes Association "Neural xenotransplants in degenerative disease" (lecture) |
| 1996 | Miami, FL, The University of Miami, The Miami Project to Cure Paralysis "Specificity of connections formed by transplanted fetal neurons to the mature CNS" (Visiting Professor) |
| 1996 | New York, NY, New York Academy of Sciences "Cellular protection and repair of the brain using cell transplantation" (lecture) |
| 1996 | New Haven, CT, Yale University, Ninth Gene Therapy User Group Meeting, "Gene delivery strategies for the treatment of neurodegenerative diseases" (lecture) |
| 1997 | Denver, CO, Univ. of Colorado "Hosting foreign cells in the brain: will xenogeneic neurons serve as treatments for Parkinson's and Huntington's disease?" (lecture) |
| 1997 | U.S. Veterans Administration, Washington, D.C. (1997) Chairman, Advisory Committee on Parkinson's disease research |
| 1998 | Austrian Parkinson Society, Vienna, "Reconnections of neural circuitry in Parkinson's disease patients by xenogeneic dopaminergic neurons." (lecture) |
| 1998 | Karolinska Institute, Stockholm, Introductory lecture for thesis examination on Intraocular Cellular Transplants |
| 1998 | New York, NY, 5th Intl. Congress of Parkinson's Disease and Movement Disorders. "Gene Therapy for Parkinson's Disease", (plenary lecture) |
| 1998 | Tokyo, Japan, The Molecular Medicine Revolution Conference, "Neural cell transplants to physiologically repair circuitry in neurodegenerative disease" (plenary lecture) |
| 1998 | Cardiff, Wales, The Physiological Society, "Cell transplantation as a therapy for Parkinson's disease" (lecture) |
| 1999 | Cornell Medical School/New York Hospital "Developing nerve cells against neurodegeneration" (grand rounds & lecture) |
| 1999 | Montreux, Switzerland, The International Cell Transplant Society, "Primary neuronal cell transplantation for Parkinson's disease (lecture) |

TEACHING: National or International Contributions: (continued)Invited Presentations: (continued)

1999	Keystone Symposia, "Neural xenotransplantation for neurodegenerative disease" (lecture)
1999	Dalhousie University, Halifax, Clinical Neuroscience (rounds) and Dept. of Anatomy and Neurobiology (lecture)
1999	University of Pittsburgh Medical Center, Dept. of Pathology (lecture)
1999	University of Rochester, Experimental Therapeutics Workshop (lecture) and Neurology Grand Rounds
1999	Vancouver, BC, XIIIth Intl. Congress on Parkinson's Disease (lecture)
1999	Odense, Denmark, 7th Intl. Neural Transplantation Meeting (lecture)
1999	Boston, European Behavioral Pharmacology Society and Behavioral Pharmacology Society Conference (lecture)
1999	Austrian Parkinson Society, Vienna (lecture)
1999	Bonn, Intl. Neuroscience Symposium "Molecular Basis of CNS Disorders" (lecture)
1999	London, The Novartis Foundation "Neural Transplantation in Neurodegenerative Disease"
1999	Miami, 6th National Parkinson's Foundation Intl. Symposium on Parkinson's Research (lecture)
2000	Louisville, "The Neuroscience of Developing Cell Therapies for Parkinson's Disease" (lecture)
2000	Zurich, Intl. Study Group on the Pharmacology of Memory, (lecture)
2000	Tokyo, Intl. Workshop: Stem Cell Biology & Cellular Molecular Treatment (lecture)
2000	Il Ciocco, Italy, Gordon Research Conference (lecture)
2000	Rome, Intl. Cong. of the Transplantation Society (lecture)
2000	Turin, Italy, Cellular & Molecular Mechanisms of Brain Repair (lecture)
2000	Stockholm, Karolinska Institute, Neural Donor Cells for Transplantation (lecture)
2001	Colorado, Winter Conf. on Brain Repair, Cell and Gene Therapy for Basal Ganglia Disorders (panel organizer)
2001	San Francisco, AAAS, Stem Cell Biology and Parkinson's Disease (session lecture)
2001	Paris, Association pour la Neuro-Psycho-Pharmacologie, Huntington a Model Disease (lecture)
2001	Valencia, Spain, Fundacion Valenciana de Estudios Avanzados, The Impact of Stem Cell Research on Neural Transplantation (lecture)
2001	Potomac, MD, Workshop on Department of Defense Sponsored Parkinson's Related Research (Session Chair)
2001	Philadelphia, World Parkinson's Day Symposium, Thomas Jefferson University (lecture)
2001	Detroit, MI, Wayne State University Center for Molecular Medicine and Genetics (lecture)
2001	Osaka, Japanese Society for Neural Growth, Regeneration and Transplantation (lecture)
2001	Halifax, NS, Canadian Congress of Neurological Sciences (lecture)
2001	Princeton, NJ, Intl. Neurodegeneration Conference (lecture)
2001	Colorado Springs, CO, Intl. Neurotoxicology Meeting (lecture)

Advisory and Supervisory Responsibilities in Clinical or Laboratory Setting:A. Faculty Mentor, Thesis Advisor or Supervisor:

1988-1989	Boris Lambs	MD 1990 Cambridge U. MD at Cambridge University, England
1986-1988	Walter Fischer	MD 1991 Lund University MD, PhD at Lund University Medical School, Sweden

TEACHING: National or International Contributions: (continued)

Advisory and Supervisory Responsibilities in Clinical or Laboratory Setting: (continued)

1986-1988	Klas Wictorin	MD 1991 Lund University MD, PhD at Lund University Medical School, Sweden
1985-1986	Lars Anderson	MD 1989 Lund University MD at Lund University Medical School, Sweden

B. Theses/Dissertations Directed:

Walter Fischer, PhD at Lund University, (Asst. Supervisor) Awarded 1988

Klas Wictorin, PhD at Lund University, (Asst. Supervisor) Awarded 1988

Boris Lambs, MA at Cambridge U., (Co-advisor) Awarded 1989

C. Ph.D. Dissertation or Examining Committees:

1994 Principal examiner, Serge Marty's Doctoral Thesis, INSERM, Paris, France

1998 Principal examiner, Lars Björklund's Doctoral Thesis, Karolinska Institute, Sweden

Professional Leadership Roles related to Teaching:

1995-1996	Chair, Program Committee, American Society for Neural Transplantation
1995	Organizer, "Cellular and Molecular Treatments of Neurological Diseases" Conference, Three day international conference with invited speakers and participants, Cambridge, MA, (Sept. 7-10, 1995)
1998	Co-director, "Anatomy and Physiology of Basal Ganglia Surgery" CME Course and Scientific Conference, Sarasota, FL (March 13-15 1998)
1998	Organizer, "2nd Cellular and Molecular Treatments of Neurological Diseases" CME Course and Scientific Conference, Cambridge, MA (Oct. 8-11, 1998)
1999	Co-director, "2nd Anatomy and Physiology of Basal Ganglia Surgery" CME Course and Scientific Conference, Sarasota, FL (May 15-16, 1999)
1998	Organizer, "3rd Cellular and Molecular Treatments of Neurological Diseases" CME Course and Scientific Conference, Cambridge, MA (Sept. 20-22, 2001)

CLINICAL ACTIVITIES: (N/A)

(Dr. Isacson does not have a clinical appointment or license.)

1995-1997	Scientific advisor at clinical trials for Parkinson's and Huntington's disease at HMS and Lahey Clinic, Burlington, MA
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PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports

1. Isacson O, Brundin P, Kelly PAT, Gage, FH and Björklund A. Functional neuronal replacement by grafted striatal neurons in the ibotenic acid lesioned rat striatum. *Nature* 1984;311:458-60.
2. Gage FH, Dunnett SB, Brundin P, Isacson O, Björklund A. Intracerebral grafting of embryonic neural cells into the adult host brain: an overview of the cell suspension method and its application. *J Dev Neurosci* 1984;6:137-51.
3. Brundin P, Isacson, O, Björklund A. Monitoring of cell viability in suspensions of embryonic CNS tissue and its use as a criterion for intracerebral graft survival. *Brain Res* 1985;331:251-9.
4. Isacson O, Brundin P, Gage FH, Björklund A. Neural grafting in a rat model of Huntington's disease: Progressive neurochemical changes after neostriatal ibotenate lesions and striatal tissue grafting. *Neuroscience* 1985;16:799-817.
5. Gage FH, Brundin P, Isacson O, Björklund A. Rat fetal brain tissue survive and innervate host brain following five day pregraft tissue storage. *Neuroscience Lett* 1985;60:133-7.
6. Brundin P, Barbin G, Isacson O, Mallat M, Chamak B, Prochiantz A, Gage FH Björklund A. Survival of intracerebrally grafted rat dopamine neurons previously cultured in vitro. *Neuroscience Lett* 1985;61:79-84.
7. Zetterström T, Brundin P, Gage FH, Sharp T, Isacson O, Dunnett SB, Ungerstedt U, Björklund A. In vivo measurement of spontaneous release and metabolism of dopamine from intrastriatal nigral grafts using intracerebral dialysis. *Brain Res* 1986;362:344-9.
8. Isacson O, Dunnett SB, Björklund A. Behavioural recovery in an animal model of Huntington's disease. *Proc Natl Acad Sci USA* 1986;83:2728-32.
9. Brundin P, Isacson O, Gage FH, Björklund A. Intrastriatal grafting of dopamine-containing neuronal cell suspensions: effects of mixing with target or non-target cells. *Dev Brain Res* 1986;24:77-84.
10. Brundin P, Isacson O, Gage FH, Prochiantz A, Björklund A. The rotating 6-hydroxydopamine lesioned mouse as a model for assessing functional effects of neuronal grafting. *Brain Res* 1986;366:346-49.
11. Sofroniew MV, Isacson O, Björklund A. Cortical grafts prevent atrophy of cholinergic basal nucleus neurons induced by excitotoxic cortical damage. *Brain Res* 1986;378:409-15.
12. Sofroniew MV, Pearson RCA, Isacson O, Björklund A. Experimental studies on the induction and prevention of retrograde degeneration of basal forebrain cholinergic neuron. *Prog Brain Res* 1986;70: 363-89.
13. Pritzel M, Isacson O, Brundin P, Wiklund L, Björklund A. Afferent and efferent connections of striatal grafts implanted into the ibotenic acid lesioned neostriatum in adults rats *Exp Brain Res* 1986;65:112-26.
14. Dunnett SB, Whishaw IQ, Jones GH, Isacson O. Effects of dopamine-rich grafts on conditioned rotation in rats with unilateral 6-hydroxydopamine lesions. *Neurosci Lett* 1986;68:127-33.
15. Isacson O, Dawbarn D, Brundin P, Gage FH, Emson PC, Björklund A. Neural grafting in a rat model of Huntington's disease: Striosomal organization as revealed by immunocytochemistry, acetylcholinesterase histochemistry, and receptor autoradiography. *Neuroscience* 1987;22:481-97.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports (continued)

16. Isacson O, Fischer W, Wictorin K, Dawbarn D, Björklund A. Astroglial response in the excitotoxically lesioned neostriatum and its projection areas. *Neuroscience* 1987;20:1043-56.
17. Peschanski M, Isacson O, Fetal homotypic transplants in the excitotoxically neuron depleted thalamus I: Light microscopy. *J Comp Neurol* 1988;274:449-63.
18. Clarke DJ, Dunnett SB, Isacson O, Sirinathsinghji DJS, Björklund A. Striatal grafts in rats with unilateral striatal lesions I: Ultrastructural evidence of afferent synaptic inputs from the host nigrostriatal pathway. *Neuroscience* 1988;24:791-801.
19. Sirinathsinghji DJS, Dunnett SB, Isacson O, Clarke DJ, Björklund A. Striatal grafts in rats with unilateral neostriatal lesions II: In vivo monitoring of GABA release in the globus pallidus and substantia nigra. *Neuroscience* 1988;24:803-11.
20. Dunnett SB, Isacson O, Clarke DJ, Björklund A. Striatal grafts in rats with unilateral striatal lesions III: recovery from dopamine dependent motor asymmetry and deficits in skilled paw reaching. *Neuroscience* 1988;24:813-20.
21. Brundin P, Barbin G, Strecker RE, Isacson O, Prochiantz A, Björklund A. Survival and function of dissociated rat dopamine neurones grafted at different developmental stages or after being cultured in vitro. *Dev Brain Res* 1988;39 233-43.
22. Peschanski M, Rudin M, Isacson O, Delepiere M, Roques B. Magnetic resonance imaging of intracerebral neural grafts. *Prog Brain Res* 1988;78:619-25.
23. Isacson O, Wictorin K, Fischer W, Sofroniew M, Björklund A. Fetal cortical suspension grafts to the excitotoxically lesioned neocortex: anatomical and neurochemical studies of trophic interactions. *Prog Brain Res* 1988;78:13-27.
24. Fischer W, Wictorin K, Isacson O, Björklund A. Trophic effects on cholinergic striatal interneurons by submaxillary gland transplants. *Prog Brain Res* 1988;78:409-13.
25. Wictorin K, Isacson O, Fischer W, Nothias F, Peschanski M, Björklund A. Connectivity of striatal grafts implanted into the ibotenic acid-lesioned striatum I: subcortical afferents. *Neuroscience* 1988;27:547-62.
26. Nothias F, Wictorin K, Isacson O, Björklund A, Peschanski M. Morphological alteration of thalamic afferents in the excitotoxically lesioned striatum. *Brain Res* 1988;461:349-54.
27. Lams BE, Isacson O, Sofroniew MV. Loss of transmitter-associated staining following axotomy does not indicate death of brainstem cholinergic neurons. *Brain Res* 1988;475:401-6.
28. Sofroniew MV, Isacson O. Distribution of degeneration of cholinergic neurons in the septum following axotomy in different portions of the fimbria fornix: a correlation between the degree of cell loss and the proximity of neuronal somata to the lesion. *J Chem Neuroanat* 1988;1:327-37.
29. Sofroniew MV, Isacson O, O'Brien TS. Nerve growth factor receptor immunoreactivity in the rat suprachiasmatic nucleus. *Brain Res* 1989;476: 358-62.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports (continued)

30. Wictorin K, Simerly RB, Isacson O, Swanson LW, Bjorklund A. Connectivity of striatal grafts implanted into the ibotenic acid lesioned striatum II: efferent projecting graft neurons and their relationship to host afferents within the grafts. *Neuroscience* 1989;30:313-30.
31. Isacson O, Riche D, Hantraye P, Sofroniew MV, Maziere M. A primate model of Huntington's disease: cross-species implantation of striatal precursor cells to the excitotoxically lesioned baboon caudate-putamen. *Exp Brain Res* 1989;75:213-20.
32. Dusart I, Isacson O, Nothias F, Gumpel M, Peschanski M. Schwann cells migrate into CNS excitotoxic lesions. *Neurosci Lett* 1989;105:246-50.
33. O'Brien TS, Svendsen CN, Isacson O, Sofroniew M. Loss of true blue labelling from the medial septum following transection of the fimbria-fornix; evidence for the death of cholinergic and non-cholinergic neurons. *Brain Res* 1990;508:249-56.
34. Isacson O, Hantraye P, Maziere M, Sofroniew MV, Riche D. Apomorphine-induced dyskinesias after excitotoxic caudate-putamen lesions and the effects of neural transplantation in non-human primates *Prog Brain Res* 1990;82:523-33.
35. Hantraye P, Riche D, Maziere M, Isacson O. An experimental primate model for Huntington's disease: anatomical and behavioural studies of unilateral excitotoxic lesions of the caudate-putamen in the baboon. *Exp Neurol* 1990;108:91-104.
36. Sofroniew MV, Galletly N.P, Isacson O, Svendsen CN. Adult basal forebrain neurons do not require target neurons for survival. *Science* 1990;247:338-42.
37. Denys A, Leroy-Willig A, Hantraye P, Riche D, Isacson O, Maziere M, Syrota A. In Vivo MRI of neural transplants in a primate model of Huntington's disease. *Amer J of Roent* 1991;158:215-16.
38. Schumacher JM, Short MP, Hyman BT, Breakefield XO, Isacson O. Intracerebral Implantation of Nerve Growth Factor-Producing Fibroblasts Protects Striatum Against Neurotoxic Levels of Excitatory Amino Acids. *Neuroscience* 1991;45:561-70.
39. Levisohn A, Isacson O. Excitotoxic lesions of the rat entorhinal cortex. Effects of selective neuronal damage on acquisition and retention of a non-spatial reference memory task. *Brain Res* 1991;564:230-44.
40. Isacson O, Peschanski M. Is There Capacity for Anatomical and Functional Repair In The Adult Somatosensory Thalamus? *Exp Neurol* 1992;115:173-6.
41. Hantraye P, Loc'h C, Maziere B, Khalili-Varasteh M, Crouzel C, Fournier D, Yorke J-C, Stulz O, Riche D, Isacson O, Maziere M. 6-[¹⁸F] Fluoro-L-Dopa uptake and [⁷⁶Br] bromolisuride binding in the excitotoxically lesioned caudate-putamen of nonhuman primates studied using positron emission tomography. *Exp Neurol* 1992;115:218-27.
42. Hantraye P, Riche D, Maziere M, Isacson O. Intra-striatal Grafting of Cross-Species Fetal Striatal Cells Reduces Abnormal Movements in a Primate Model of Huntington's Disease. *Proc Natl Acad Sci USA* 1992;89:4187-91.
43. Isacson O, Sofroniew MV. Neuronal loss or replacement in the injured adult cerebral neocortex induce extensive remodeling of intrinsic and afferent neural systems. *Exp Neurol* 1992;117:151-75.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports (continued)

44. Hantraye P, Brownell A-L, Elmaleh D, Spealman RD, Wullner U, Brownell GL, Madras BK, Isacson O. Dopamine fiber detection by 11C-CFT and PET in a primate model of Parkinsonism. *NeuroReport* 1992;3:265-8.
45. Schumacher JM, Hantraye P, Brownell A-L, Riche D, Madras BK, Davenport PD, Maziere M, Elmaleh DR, Brownell GL, Isacson O. Stereotactic CT-guided lesion method and CNS transplantation in a primate model of Huntington's disease. *Cell Transplant* 1992;1:313-22.
46. Rosenberg WS, Breakefield XO, DeAntonio C, Isacson O. Detection of the E. coli lacZ gene product in the rat brain by histochemical methods. *Mol Brain Res* 1992;16:311-5
47. Beal MF, Swartz KJ, Isacson O. Developmental changes in brain kynurenic acid concentrations. *Dev Brain Res* 1992;68:136-9.
48. Hantraye P, Leroy-Willig A, Denys A, Riche D, Isacson O, Maziere M, Syrota A. Magnetic resonance imaging to monitor pathology of caudate-putamen after excitotoxin-induced neuronal loss in the non-human primate brain. *Exp Neurol* 1992;118:18-23.
49. Frim DM, Short MP, Rosenberg WS, Simpson J, Breakefield XO, Isacson O. Local protective effects of nerve growth factor-secreting fibroblasts against excitotoxic lesions in the rat striatum. *J Neurosurg* 1992;78:267-73.
50. Yee WM, Frim DM, Isacson O. Relationships between stress protein induction and NMDA-mediated neuronal death in the entorhinal cortex. *Exp Brain Res* 1993;94:193-202.
51. Simpson JR, Isacson O. Mitochondrial impairment reduces the threshold for in vivo NMDA-mediated neuronal death in the striatum. *Exp Neurol* 1993;121:57-64.
52. Frim DM, Simpson J, Uhler T, Short MP, Bossi SR, Breakefield XO, Isacson O. Striatal degeneration induced by mitochondrial blockade is prevented by biologically delivered NGF. *J Neurosci Res* 1993;35:452-8.
53. Bossi SR, Simpson JR, Isacson O. Age dependence of striatal neuronal death caused by mitochondrial dysfunction. *NeuroReport* 1993;4:73-6.
54. Frim DM, Short MP, Breakefield XO, Isacson O. Biological gene-product delivery to the brain: a protocol for retroviral gene transfer into cultured cells and intracerebral transplantation. *NeuroProtocol* 1993;3:63-8.
55. Frim DM, Uhler TA, Short MP, Ezzedine ZD, Klagsbrun M, Breakefield XO, Isacson O. Effects of biologically delivered NGF, BDNF, and bFGF on striatal excitotoxic lesions. *NeuroReport* 1993;4:367-70.
56. Frim DM, Yee WM, Isacson O. NGF reduces striatal excitotoxic neuronal loss without affecting concurrent neuronal stress. *NeuroReport* 1993;4:655-8.
57. Wullner U, Brouillet E, Isacson O, Young AB, Penney JB. Glutamate receptor binding sites change in MPTP-treated mice. *Exp Neurol* 1993;121:284-7.
58. Burns LH, Sato K, Wullner U, Isacson O. Intra-nigra infusion of AMPA attenuates dopamine-dependent rotation in the rat. *NeuroReport* 1993;4:1075-8.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports (continued)

59. Pakzaban P, Deacon T, Burns L, Isacson O. Increased proportion of AChE-rich zones and improved morphologic integration in host striatum of fetal grafts derived from the lateral but not the medial ganglionic eminence. *Exp Brain Res* 1993;97:13-22.
60. Brownell AL, Hantraye P, Wullner U, Hamberg L, Shoup T, Elmaleh DR, Madras B, Frim DM, Brownell GL, Rosen BR, Isacson O. PET- and MRI-based assessment of glucose utilization, dopamine receptor binding, and hemodynamic changes after lesions to the caudate-putamen in primates. *Exp Neurol* 1994;125:41-51.
61. Davar G, Kramer MF, Garber D, Roca AL, Andersen JK, Bebrin W, Coen DM, Kosz-Vnenchak M, Knipe DM, Breakefield XO, Isacson O. Comparative efficacy of gene delivery to mouse sensory neurons using herpes virus vectors. *J Comp Neurol* 1994;339:3-11.
62. Andersen JK, Frim DM, Isacson O, Breakefield XO. Herpes-virus mediated gene delivery into the rat brain: specificity and efficiency of the neuron-specific enolase promoter. *Cell Mol Neurobiol* 1994;13:503-15.
63. Uhler TA, Frim DM, Pakzaban P, Isacson O. The effects of mega-dose methylprednisolone and U-78517F on glutamate-receptor mediated toxicity in the rat neostriatum. *Neurosurgery* 1994;34:122-8.
64. Wullner U, Hantraye P, Brownell A-L, Pakzaban P, Burns L, Shoup T, Elmaleh D, Petto A, Speelman RD, Brownell GL, Isacson O. Dopamine terminal loss and onset of motor symptoms in MPTP-treated monkeys: a positron emission tomography study with 11C-CFT. *Exp Neuro*. 1994;126:305-9.
65. Frim DM, Uhler TA, Galpern W, Beal MF, Breakefield XO, Isacson O. Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in the rat. *Proc Natl Acad Sci USA* 1994;91:5104-8.
66. Andersen JK, Frim DM, Isacson O, Breakefield XO. Catecholaminergic cell atrophy in a transgenic mouse aberrantly overexpressing MAO-B in neurons. *Neurodegeneration* 1994;3:97-109.
67. Frim DM, Wullner U, Beal MF, Isacson O. Implanted NGF-producing fibroblasts induce catalase and modify ATP levels but do not affect glutamate receptor binding or NMDA receptor expression in the rat striatum. *Exp Neurol* 1994;128:172-80.
68. Pakzaban P, Geller A, Isacson O. Effect of exogenous nerve growth factor on neurotoxicity of and neuronal gene delivery by a herpes simplex amplicon in the rat brain. *Human Gene Therapy* 1994;5:987-95.
69. Andersen JK, Frim DM, Isacson O, Beal MF, Breakefield XO. Elevation of neuronal MAO-B activity in a transgenic mouse model does not increase sensitivity to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Brain Res* 1994;656:108-14.
70. Deacon TW, Pakzaban P, Burns LH, Dinsmore J, Isacson O. Cytoarchitectonic development, axon-glia relationships and long distance axon growth of porcine striatal xenografts in rats. *Exp Neurol* 1994;130:151-67.
71. Deacon TW, Pakzaban P, Isacson O. The lateral ganglionic eminence is the source of striatal phenotypes: neural transplantation and developmental evidence. *Brain Res* 1994;668:211-9.
72. Burns LH, Pakzaban P, Deacon TW, Brownell A-L, Tatter SB, Jenkins BG, Isacson O. Selective putaminal excitotoxic lesions in non-human primates model the movement disorder of Huntington disease. *Neuroscience* 1995;64:1007-17.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports (continued)

73. Pakzaban P, Deacon TW, Burns LH, Dinsmore J, Isacson O. A novel mode of immunoprotection of neural xenotransplants: masking of donor major histocompatibility complex class I enhances transplant survival in the CNS. *Neuroscience* 1995;64:983-96.
74. Tatter SB, Galpern WR, Hoogeveen AT, Isacson O. Effects of striatal excitotoxicity on Huntingtin-like immunoreactivity. *NeuroReport* 1995;6:1125-9.
75. Garcia AR, Deacon TW, Dinsmore J, Isacson O. Long-distance axonal growth from fetal porcine neural grafts in the excitotoxically lesioned adult rat cortex. *Cell Transplant* 1995;4:515-27.
76. Isacson O, Frim DM, Galpern, WR, Tatter SB, Breakefield XO, Schumacher JM. Cell-mediated delivery of neurotrophic factors and neuroprotection in the neostriatum and substantia nigra. *Rest Neurol Neurosci* 1995;8:59-61.
77. Isacson O, Deacon TW, Pakzaban P, Galpern WR, Dinsmore J, Burns LH. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. *Nature Med* 1995;1:1189-94.
78. Dinsmore J, Deacon T, Ratliff J, Pakzaban, P, Jacoby, D, Isacson O. Embryonic stem cells differentiated in vitro as a novel source of cells for transplantation. *Cell Transplant* 1996;5:131-43.
79. Galpern WR, Frim DM, Tatter SB, Altar CA, Beal MF, Isacson O. Cell-mediated delivery of brain-derived neurotrophic factor enhances dopamine levels in a MPP+ rat model of substantia nigra degeneration. *Cell Transplant* 1996;5:225-32.
80. Galpern WR, Burns LH, Deacon TW, Dinsmore J, Isacson O. Xenotransplantation of porcine fetal ventral mesencephalon in a rat model of Parkinson's disease: functional recovery and graft morphology. *Exp Neurol* 1996;140:1-13.
81. Isacson O, Deacon TW. Specific axon guidance factors persist in the adult rat brain as demonstrated by pig neuroblasts transplanted to the rat. *Neuroscience* 1996;75:827-37.
82. Galpern WR, Matthews RT, Beal MF, Isacson O. NGF attenuates 3-nitrotyrosine formation in a 3-NP model of Huntington's disease. *NeuroReport* 1996;7:2639-42.
83. Deacon T, Schumacher J, Dinsmore J, Thomas C, Palmer P, Kott S, Edge A, Penney D, Kassissieh S, Dempsey P, Isacson O. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. *Nature Med* 1997;3:350-3.
84. Haque N, Isacson O. Antisense gene therapy for neurodegenerative disease? *Exp Neurol* 1997;144:139-46.
85. Haque NSK, LeBlanc CJ, Isacson O. Differential dissection of the rat E16 ventral mesencephalon and survival and reinnervation of the 6-OHDA-lesioned striatum by a subset of aldehyde dehydrogenase-positive TH neurons. *Cell Transplant* 1997;6:239-48.
86. Costantini LC, Lin L, Isacson O. Medial fetal ventral mesencephalon: a preferred source for dopamine neuron grafts. *NeuroReport* 1997;8:2253-7.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports (continued)

87. Chen YCI, Brownell A-L, Galpern W, Keltner JR, Matthews RT, Isacson O, Rosen BR, Jenkins BG. Detection of dopaminergic neurotransmitter activity using pharmacologic MRI (phMRI): correlation with PET, microdialysis and behavioral data. *Magn Res Med* 1997;38:389-98.
88. Deacon T, Dinsmore J, Costantini LC, Ratliff J, Isacson O. Blastula-stage stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. *Exp Neurol* 1997;149:28-41.
89. Lin L, Leblanc C, Deacon T, Isacson O. Chronic cognitive deficits and amyloid precursor protein elevation after selective immunotoxic lesions of the basal forebrain cholinergic system. *NeuroReport* 1997;9:547-52.
90. Brownell A-L, Livni E, Galpern W, Isacson O. In vivo PET imaging in rat of dopamine terminals reveals functional neural transplants. *Ann Neurol* 1997;43:387-90.
91. Costantini LC, Chaturvedi P, Armistead DM, McCaffrey PG, Deacon TW, Isacson O. Distinct branching effects of a novel immunophilin ligand on dopaminergic neurons in culture, and neurotrophic actions after oral administration in an animal model of Parkinson's disease. *Neurobiol Disease* 1998;5:97-106.
92. Brownell A-L, Jenkins BG, Elmaleh DR, Deacon TW, Spealman, RD, Isacson O. Combined PET/MRS studies of the brain reveal dynamic and long-term physiological changes in a Parkinson's disease primate model. *Nature Medicine* 1998;4:1308-12.
93. LeBlanc CJ, Deacon TW, Whatley BR, Dinsmore J, Lin L, Isacson O. Morris water maze analysis of 192-IgG-saporin lesioned rats and porcine cholinergic transplants to the hippocampus. *Cell Transplant* 1999;8:131-42.
94. Deacon TW, Whatley B, LeBlanc C, Lin L, Isacson O. Pig fetal septal neurons implanted into the hippocampus of aged or cholinergic deafferented rats grow axons and form cross-species synapses in appropriate target regions. *Cell Transplant* 1999;8:111-49.
95. Brownell, A-L, Jenkins, B. and Isacson, O. Dopamine Imaging Markers and Predictive Mathematical Models for Progressive Degeneration in Parkinson's Disease. *Biomed & Pharmacother* 1999;53:131-40.
96. Kordower JH, Isacson O, Emerich, DF. Cellular delivery of trophic factors for the treatment of Huntington's disease: is neuroprotection possible? *Exp Neurol* 1999;159:4-20.
97. Lin L, Georgievska B, Mattsson A, Isacson O. Cognitive changes and modified processing of amyloid precursor protein in the cortical and hippocampal system after cholinergic synapse loss and muscarinic receptor activation. *Proc Natl Acad Sci USA* 1999;96:12108-13.
98. Costantini LC, Jacoby DR, Wang S, Fraefel C, Breakefield XO Isacson O. Gene transfer to the nigrostriatal system by hybrid HSV/AAV amplicon vectors. *Human Gene Therapy* 1999;10:2481-94.
99. Chen YI, Brownell A-L, Galpern W, Isacson O, Bogdanov M, Beal MF, Livni E, Rosen BR, Jenkins BG. Detection of dopaminergic cell loss and neural transplantation using pharmacological MRI, PET and behavioral assessment. *Neuroreport* 1999;10:2881-6.
100. Haque NSK, Isacson O. Neurotrophic factors NGF and FGF-2 alter levels of huntingtin in striatal neuronal cell cultures. *Cell Transplant* 2000;in press.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Original Reports (continued)

101. Costantini LC, Isacson O. Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons. *Exp Neurol* 2000;164:60-70.
102. Schumacher JM, Elias SA, Palmer EP, Kott HS, Dinsmore J, Dempsey PK, Fischman AJ, Thomas C, Feldman RG, Kassissieh S, Raineri R, Manhart C, Fink JS, Isacson O. Transplantation of embryonic porcine mesencephalic tissue in patients with Parkinson's disease. *Neurology* 2000;54:1042-50.
103. Fink JS, Schumacher JM, Elias SL, Palmer EP, Saint-Hilaire M, Shannon K, Penn R, Starr P, van Horne C, Kott HS, Dempsey PK, Fischman AJ, Raineri R, Manhart C, Dinsmore J, Isacson O. Porcine xenografts in Parkinson's disease and Huntington's disease patients: tentative outcomes. *Cell Transplant* 2000;9:273-8.
104. Costantini LC, Isacson O. Neurophilin ligand enhances reinnervation of host striatum from fetal dopamine transplants. *Neuroscience*, 2000;105:515-20.
105. Freeman TB, Cicchetti F, Hauser RA, Deacon TW, Randall TS, Masiello I, Li X-J, Hersch SM, Nauert GM, Sanberg PR, Kordower JH, Saporta S, Isacson O. Survival of fetal striatal transplants in a patient with Huntington's disease, *Proc Natl Acad Sci* 2000;97:13877-82.
106. Costantini LC, Cole D, Isacson O. Immunophilin ligands can prevent progressive dopaminergic degeneration in animal models of Parkinson's disease. *Eur. J. Neurosci.* 2001;13:1085-92.
107. Holm KH, Cicchetti F, Bjorklund L, Boonman Z, Tandon P, Costantini LC, Deacon TW, Huang X, Chen DF, Isacson O. Enhanced axonal growth from fetal human Bcl-2 transgenic mouse dopamine neurons transplanted to the adult rat striatum. *Neuroscience* 2001;104:397-405.
108. Browne SE, Lin L, Mattsson A, Georgievska B, Isacson O. Selective antibody-induced cholinergic cell and synapse loss produce sustained hippocampal and cortical hypometabolism with correlated cognitive deficits. *Exp. Neurol.* 2001, in press.
109. Moore AE, Cicchetti F, Hennen J, Isacson O. Parkinsonian motor deficits are reflected by proportional A9/A10 dopamine neuron degeneration in the rat. *Exp. Neurol.* 2001, in press.

Reviews/Book Chapters

1. Isacson O, Brundin P, Dawbarn D, Kelly PAT, Gage FH, Emson PC, Björklund A. Striatal grafts in the ibotenic acid lesioned striatum. In: Bjorklund A, Stenevi U, eds. *Neural grafting in the mammalian CNS*. Amsterdam: Elsevier Science Publishers, B.V., 1985:539-49.
2. Brundin P, Isacson O, Gage FH, Stenevi U, Björklund A. Intracerebral grafts of neuronal cell suspensions. In: Bjorklund A, Stenevi U, eds. *Neural grafting in the mammalian CNS*. Amsterdam: Elsevier Science Publishers, B.V., 1985:51-9.
3. Emson PC, Dawbarn D, Rossor MN, Rehfeldt JF, Brundin P, Isacson O, Björklund A. Cholecystokinin content in the basal ganglia in Huntington's disease and expression of cholecystokinin-immunoreactivity in striatal grafts to the ibotenic acid lesioned striatum of the rat. In: Vanderhaegen JJ, Crawley JN, eds. *Ann NY Acad Sci USA* 1985;448: 489-94.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Reviews/Book Chapters (continued)

4. Isacson O, Brundin P, Gage FH, Björklund A. Compensation for lesions induced changes in cerebral metabolism and behaviour by striatal neural implants in a rat model of Huntington's disease. In: Will, Schmitt, Dalrymple-Alford, eds. Brain plasticity, learning and memory, Plenum Press, 1985:519-35.
5. Gage FH, Björklund A, Isacson O, Brundin A. Uses of neuronal transplantation in models of neurodegenerative diseases. In: Das GP, Wallace RB, eds. Neural transplantation and regeneration. Springer-Verlag, 1986:103-24.
6. Isacson O, Björklund A, Dunnett SB. Conditions for neuronal survival and growth as assessed by the intracerebral transplantation technique in lesion models of the adult CNS. In: Althausen A, Seifert W, eds. Glial-neuronal communication in development and regeneration. Heidelberg: Springer Verlag, 1986:529-44.
7. Brundin P, Strecker RE, Lindvall O, Isacson O, et al. Intracerebral grafting of dopamine neurons: experimental basis for clinical trials in patients with Parkinson's disease. In: Cell and Tissue Transplantation into the Adult Brain. Ann NY Acad Sci USA 1987;495:473-97.
8. Isacson O, Pritzel M, Dawbarn D, Brundin P, et al. Striatal neural transplants in the ibotenic acid lesioned rat neostriatum: cellular and functional aspects. In: Cell and Tissue Transplantation into the Adult Brain. Ann NY Acad Sci USA, 198; 495:537-56.
9. Björklund A, Lindvall O, Isacson O, Brundin P, Wictorin K, Strecker RE, Clarke DJ, Dunnett SB. Mechanism of action of intracerebral neural implants: studies on nigral and striatal grafts to the lesioned striatum. Trends Neurosci 1987;10:509-16.
10. Björklund A, Brundin P, Isacson O. Neuronal replacement by intracerebral neural implants in animal models of neurodegenerative disease. In: Waxman SG, ed. Physiological basis for functional recovery in neurological disease. Advances in Neurology, NY: Raven Press, 1988;47:455-92.
11. Gage FH, Brundin P, Strecker R, Dunnett SB, Isacson O, Björklund A. Intracerebral neuronal grafting in experimental models of age-related motor dysfunction. In: Central Determinants of Age-Related Declines in Motor Function. Ann NY Acad Sci, 1988;575:383-93.
12. Dunnett SB, Isacson O, Sirinathsinghji DJS, Clarke DJ, Björklund A. Striatal grafts in the ibotenic acid lesioned neostriatum: functional studies. In: Gash DM, Sladek JR, eds. Neural Transplantation in the Mammalian CNS. Prog in Brain Res, Amsterdam:Elsevier 1988;78:39-47.
13. Wictorin K, Isacson O, Fischer W, Nothias F, Peschanski M, Björklund A. Studies on host afferent inputs to fetal striatal transplants in the excitotoxically lesioned striatum. In: Gash DM, Sladek JR, eds. Neural Transplantation in the Mammalian CNS, Prog in Brain Res, Amsterdam:Elsevier 1988;78:55-61.
14. Peschanski M, Nothias F, Dusart I, Isacson O, Roudier F. Reconstruction of the cytoarchitecture, synaptology and vascularization of the neuron-depleted thalamus using homotypic fetal neurons In: Bentivoglio M, Spreafico R, eds. Cellular Thalamic Mechanisms. Elsevier Science, 1988:543-54.
15. Clarke DJ, Dunnett SB, Isacson O, Björklund A. Striatal grafts in the ibotenic acid-lesioned neostriatum: ultrastructural and immunohistochemical studies. In: Gash DM, and Sladek JR, eds. Neural Transplantation in the Mammalian CNS Prog in Brain Res, Amsterdam:Elsevier, 1988;78:47-55.
16. Dunnett S, Isacson O. Trophic mechanisms are not enough. Trends Neurosci 1989;12:257.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Reviews/Book Chapters (continued)

17. Hantraye PH, Riche D, Maziere M, Maziere B, Loc'h C, Isacson O. Anatomical, behavioural and positron emission tomography studies of unilateral excitotoxic lesions of the baboon caudate-putamen as a primate model of Huntington's disease. In: Crossman AR, and Sambrook MA, eds. Neural mechanisms in disorders of movement. London: J Libbey & Co LTD, 1989:183-93.
18. Bjorklund A, Isacson O, Brundin P, Dunnett SB. Nigral and striatal grafts to the lesioned striatum: models of graft function. In: Gage FH, Privat A, Christen Y, eds. Intracerebral grafts and Alzheimer's disease. Springer-Verlag, 1989;4-20.
19. Peschanski M, Nothias F, Dusart I, Ontoniente B, Geffard M, Isacson O. Differential neural plasticity of diffuse monoaminergic and point-to-point systems as demonstrated by responses to target deprivation and fetal neural transplants. In: Gage FH, Privat A, Christen Y, eds. Intracerebral grafts and Alzheimer's disease. Springer-Verlag, 1989:177-88.
20. Dunnett SB, Bjorklund A, Brundin P, Isacson O, Gage FH. Transplantation of dopamine cell suspensions to the dopamine-depleted neostriatum. In: Stem G, ed. Parkinson's disease. London: Chapman Hall, 1990;239-65.
21. Sofroniew MV, Dunnett SB, Isacson O. Remodelling of intrinsic and afferent systems in neocortex with cortical transplants. In: Dunnett SB, Richards SJ, eds. Neural transplanation: from molecular bases to clinical application. Amsterdam: Elsevier 1990:313-320.
22. Isacson O, Arriagada PV, Hyman BT. Presence of morphologically distinct amyloid protein positive structures in non-demented elderly and Alzheimer's disease. *Neurobiol Aging* 1990;11:286.
23. Isacson O. Rules governing specificity and plasticity of neurons as demonstrated studies of neuronal transplants into the mature brain. *Medecine/Sciences* 1990;6:863-69.
24. Sofroniew MV, Svendsen CN, Isacson O. Changes in Basal Forebrain Cholinergic Systems Following Excitotoxic Cell Death In: Yoshida, ed. *The Hippocampus and Cerebral Neocortex*. 1991.
25. Isacson O, Hantraye P, Riche D, Schumacher JM, Maziere M. The Relationship Between Symptoms and Functional Anatomy in Chronic Neurodegenerative Diseases: From Pharmacological to Biological Replacement Therapy in Huntington's Disease. In: *Intracerebral Transplantation in Movement Disorders*. Restorative Neurology, Elsevier, 1991;4:245-58.
26. Riche D, Hantraye P, Isacson O, Maziere M. A Primate Model of Huntington's Disease: Unilateral Striatal Lesions and Neural Grafting in the Baboon. In: Bernardi O, et al., eds. *Advances in Behavioral Biology*. New York: Plenum Press, *The Basal Ganglia III* 1991;39:561-72.
27. Isacson O. *Neurology and Neurobiology*, Vol 60. *Advances in Neural Regeneration Research*, Seil F.J., ed. J. Neurosurg, 1991;75:491-92.
28. Frim DM, Andersen JK, Schumacher JM, Short MP, Isacson O, Breakefield X. Gene transfer into the central nervous system: neurotrophis factors. In: Moody TW, ed. *Growth Factors, Peptides & Receptors*. New York: Plenum Press, 1993;83-91.
29. Isacson O. On Neuronal Health. *Trends in Neuroscience*, 1993;16:306-308.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Reviews/Book Chapters (continued)

30. Isacson O. Clinical and Preclinical PET Correlates of Parkinsonism with 11C-WIN 35,428. *Ann Neurol*, 1994;35:377-378.
31. Isacson O. Fetal nerve cell transplantation: Advances in the treatment of Parkinson's disease. *On the Brain, The Harvard Mahoney Neuroscience Institute Letter*. 1994;3:4-5.
32. Pakzaban P, Isacson O. Neural xenotransplantation: reconstruction of neuronal circuitry across species barriers. *Neuroscience*, 1994;62:989-1001.
33. Tatter SB, Galpern WR, Isacson O. Mechanisms of neurotrophic factor protection against excitotoxic neuronal death. *The Neuroscientist*, 1995;1:286-97.
34. Breakefield XO, Sena-Esteves M, Pechan P, Kramm C, Yoshimoto Y, Lin Q, Davar G, Livermore J, Isacson O, Chiocca EA, Bohn M, Kaye E. Gene Therapy for the Nervous System-Status 1994, In: *Genetic Therapy Fundacion BBV*, 1995:503-25.
35. Isacson O. On behavioral effects and gene delivery in Parkinson's rat model. *Science*, 1995;269:856-57.
36. Isacson O. Review of "Neuroregeneration." *J Chem Neuroanat* 1995;9:149-52.
37. Freeman TB, Sanberg PR, Isacson O. Development of the human striatum: implications for fetal striatal transplantation. *Cell Transplantation* 1995;4:539-45.
38. Isacson O. Testimony for Hearing before the Special Committee on Aging, United States Senate: Breakthroughs in Brain Research: A National Strategy to Save Billions in Health Care Costs. June 27, 1995, Senate Hearing. 1995; Serial No. 104-230, 88-99.
39. Isacson O. Testimony for Hearing before the Subcommittee on Health and Environment of the Committee on Commerce US House of Representatives: Research Efforts with Respect to Combating Parkinson's Disease and other Neurological Disorders July 21, 1995. Serial No. 104-68, 1996;52-61
40. Isacson O, Kordower J. The neurobiological and clinical potential of neural cell transplantation: special issue based on the meeting of the American Society for Neural Transplantation, 1995 *Cell Transplantation* 1996;5:123-25.
41. Dinsmore JH, Pakzaban P, Deacon TW, Burns L, Isacson O. Survival of transplanted porcine neural cells treated with F(AB')₂ antibody fragments directed against donor MHC class-I in a rodent model *Transplant Proc* 1996; 28:817-18.
42. Pakzaban P, Burns LH, Isacson O. Xenotransplantation, brain. In: *Encyclopedia of Neuroscience*. 1996;273:(CD Rom).
43. Isacson O. On the Causes and Treatments of Parkinson's Disease. In: *Parkinson NPF Report*, National Parkinson Foundation, Inc., 1996;Vol. XVII, Issue 1: 8-11.
44. Galpern WR, Tatter S, Isacson O. Neurotrophic factor protection in models of neurodegeneration: implications for the treatment of neurodegenerative disorders In: Beal MF, Howell N, Bodis-Wollner I, eds., *Mitochondria and Free Radicals in Neurodegenerative Diseases*, New York: John Wiley & Sons Inc, 1997:557-83.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Reviews/Book Chapters (continued)

45. Isacson O, Pakzaban P, Galpern WR. Transplanting fetal neural xenogeneic cells in Parkinson's and Huntington's disease models. In: Freeman TB, Widner H, eds., *Fetal Transplantation in Neurological Diseases*. New Jersey: The Humana Press, 1997
46. Haque NSK, Borghesani P, Isacson O. Therapeutic strategies for Huntington's disease based on a molecular understanding of the disorder. *Molec. Med Today*. 1997;3:175-83.
47. Schumacher JM, Isacson O. Neuronal xenotransplantation in Parkinson's disease. *Nature Med*, 1997;3:474-75.
48. Isacson O, Haque N. Gene Therapy of Huntington's Disease. In: Chiocca AE, Breakefield X, eds., *Gene Transfer and Therapy for Neurological Disorders*. New Jersey: The Humana Press, 1997:423-40.
49. Isacson O, Breakefield XO. Benefits and risks of hosting animal cells in the human brain. *Nature Med*, 1997;3:964-69
50. Isacson O, Deacon T. Neural transplantation studies reveal the brain's capacity for continuous reconstruction. *Trends Neurosci*, 1997;20:477-82.
51. Sanberg P, Borlongan CV, Victorin K, Isacson O. Fetal-tissue transplantation for Huntington's disease: Preclinical studies. In: Freeman TB, Widner H, eds., *Cell Transplantation for Neurological Disorders*. New Jersey: The Humana Press, 1998:77-93.
52. Isacson O, Pakzaban P, Galpern WR. Transplanting fetal neural xenogeneic cells in Parkinson's and Huntington's disease models. In: Freeman TB, Widner H, eds., *Cell Transplantation for Neurological Disorders*. New Jersey: The Humana Press, 1998:189-210.
53. Isacson O, Deacon T, Schumacher J. Immunobiology and neuroscience of xenotransplantation in neurological disease. In: Tuszynski M, Kordower JH, eds., *CNS Regeneration, Basic Science and Clinical Applications*, San Diego: Academic Press, 1998:365-87.
54. Emerich DF, Kordower JH, Isacson O. Cellular delivery of neurotrophic factors as a potential treatment for Huntington's disease. In: Tuszynski M, Kordower JH, eds., *CNS Regeneration, Basic Science and Clinical Applications*, San Diego: Academic Press, 1998:477-502.
55. Holm K, Isacson O. Factors intrinsic to the neuron can induce and maintain the ability for neurite outgrowth: a role for bcl-2? *Trends Neurosci*, 1999;22:269-73.
56. Boonman Z, Isacson O. Caspases in neuronal development and transplantation. *Exp Neurol*, 1999;156:1-15.
57. Costantini LC, Isacson O. Dopamine neuron grafts: development and molecular biology. In: di Porzio U, Pernas-Alonso R, Perone-Capano C, eds., *Dopamine Neuron Development*, Landes Company, Georgetown, 1999:123-37.
58. Isacson O, Costantini LC, Galpern WR. Molecules for neuroprotection and regeneration in animal models of Parkinson's disease. In: R Dean, D Emerich, eds., *Innovative animal models of CNS diseases: From molecule to therapy*, Humana Press, Totowa, NJ, 1999:187-207.
59. Isacson O. The Neurobiology and Neurogenetics of Stem Cells. *Brain Pathol*. 1999;9:495-8.

PART III: BIBLIOGRAPHY (183 Total: 109 Original, 72 Reviews/Chapters, 2 Books)

Reviews/Book Chapters (continued)

60. Isacson O, Sladek J. Cellular and Molecular Treatments of Neurological Diseases. *Exp. Neurol.* 1999;159:1-3.
61. Isacson O, Kang, UJ. The Potential of Gene Therapy for Treatment of Parkinson's Disease. In: *Principles of Surgery for Parkinson's Disease and Movement Disorders*, Krauss, K., Jankovic, J., Grossman, R. eds. Lippincott-Raven, 1999;in press.
62. Isacson O. (Discussant) Novartis Foundation Symposium, J. Goode, ed., 1999;in press.
63. Costantini LC, Bakowska JC, Breakfield XO, Isacson O. Gene Therapy in the CNS. *Gene Therapy* 2000;7:93-109.
64. Isacson O. Book Review: "Neural Transplantation" by William J. Freed *Nature Medicine* 2000;6:621.
65. Kordower JH, Isacson O, Leventhal L, Emerich DF. Cellular delivery of trophic factors for the treatment of Huntington's disease: is neuroprotection possible? *Progress in Brain Research* 2000;127:413-430.
66. Isacson O, Costantini L, Schumacher JM, Cicchetti F, Chung S, Kim K-S. Cell Implantation Therapies for Parkinson's Disease Using Neural Stem, Transgenic or Xenogenic Donor Cells. *Parkinson's Disease and Related Disorders*, Elsevier Science Ltd. 2000, in press.
67. Isacson O, van Horne C, Schumacher JM, Brownell A-L. Improved surgical cell therapy in Parkinson's disease: physiological basis and new transplantation methodology. In: *Parkinson's Disease, Advances in Neurology*, D. Calne, ed. Lippincott Williams Wilkins, Philadelphia, PA, 2000, in press.
68. Emilien G, Ponchon M, Caldas C, Isacson O, Maloteaux M. Impact of genomics on drug discovery and clinical medicine. *Quarterly Journal of Medicine (UK)*. 2000;93:391-423.
69. Isacson O, Lin L. Cholinergic modulation of amyloid processing and dementia in animal models of Alzheimer's disease. *Annals of the New York Acad. Sci.* 2000;920:309-14.
70. Björklund L, Herlihy D, Isacson O. Cell and synaptic replacement therapy for Parkinson's disease: current status and future directions. *Neuroscience News* 2000;6:6-12.
71. Isacson O. Making regeneration and cell therapy possible for neurological disease. *Neuroscience News* 2000;6:4-5.
72. Isacson O, Björklund L, Sanchez Pernaute R. The need for more research on "neural transplantation" for Parkinson's disease. *Nature Neurosci.*, in press.

Books

1. Isacson O. *Neural grafting in an animal model of Huntington's disease*. Lund, Sweden: Lund University Library, 1987:1-188 (ISBN 91-7900-258-7).
2. Sanberg P, Victorin K, Isacson O. *Cell Transplantation for Huntington's Disease*. Austin: R.G. Landes & Co, 1994 (ISBN 1-57059-079-6).