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CHAPTER 12

# Discovering novel phenotype-selective neurotrophic factors to treat neurodegenerative diseases

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**Abstract:** Astrocytes and neurons in the central nervous system (CNS) interact functionally to mediate processes as diverse as neuroprotection, neurogenesis and synaptogenesis. Moreover, the interaction can be homotypic, implying that astrocyte-derived secreted molecules affect their adjacent neurons optimally vs remote neurons. Astrocytes produce neurotrophic and extracellular matrix molecules that affect neuronal growth, development and survival, synaptic development, stabilization and functioning, and neurogenesis. This new knowledge offers the opportunity of developing astrocyte-derived, secreted proteins as a new class of therapeutics specifically to treat diseases of the CNS. However, primary astrocytes proliferate slowly in vitro, and when induced to immortalize by genetic manipulation, tend to lose their phenotype. These problems have limited the development of astrocytes as sources of potential drug candidates. We have successfully developed a method to induce spontaneous immortalization of astrocytes. Gene expression analysis, karyotyping and activity profiling data show that these spontaneously immortalized type-1 astrocyte cell lines retain the properties of their primary parents. The method is generic, such that cell lines can be prepared from any region of the CNS. To date, a library of 70 cell lines from four regions of the CNS: ventral mesencephalon, striatum, cerebral cortex and hippocampus, has been created. A phenotype-selective neurotrophic factor for dopaminergic neurons has been discovered from one of the cell lines (VMCL1). This mesencephalic astrocyte-derived neurotrophic factor (MANF) is a 20 kD, glycosylated, human secreted protein. Homologs of this protein have been identified in 16 other species including *C. elegans*. These new developments offer the opportunity of creating a library of astrocyte-derived molecules, and developing the ones with the best therapeutic indices for clinical use.

**Keywords:** Cell culture; cell lines; neuroprotection; Parkinson's disease; spontaneous immortalization; type-1 astrocytes

## Introduction

The selective death of specific neuronal phenotypes that underlies the etiology of each of the four major neurodegenerative diseases: Parkinson's disease (PD), Alzheimer's disease (AD), Lou Gehrig's disease or amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD), lead to debilitating clinical consequences, but is of profound scientific interest. Dopaminergic neurons in the zona compacta of the substantia nigra (SNc) die in PD (Fig. 1), while GABAergic neurons in the substantia nigra zona

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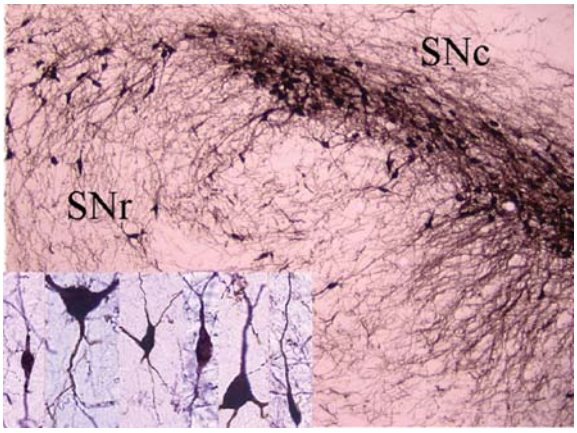



Fig. 1. Subphenotypes in the zona compacta of rat substantia nigra. Illustration of a normal zona compacta of the substantia nigra (SNc) of rat, immunostained to identify dopaminergic (TH+) neurons. Six morphological dopaminergic subphenotypes (inset, lower left) were identified in the SNc. Do different neurotrophic factors exert differing effects on these different subphenotypes?

reticulata (SNr) and dopaminergic neurons in the medial ventral tegmental region (VTA) survive (Hardman et al., 1996; McRitchie et al., 1997). These observations suggest that SNc dopaminergic neurons are more susceptible to death, compared with the adjacent SNr GABAergic or VTA dopaminergic neurons. All three types of neurons occupy the same small, ventral mesencephalic space.

The above results imply that the death of a dopaminergic neuron in the SNc in PD is a discrete, specific event. One possible mechanism is activity-dependent production of excessive reactive oxygen species (ROS) secondary to a high level of oxidative deamination (Gotz et al., 1990; Kristal et al., 2001; Li et al., 2001). It is unclear whether the metabolic profiles of the SNc and VTA dopaminergic neurons are so different to cause such opposing effects. Toxic, target-derived molecules could also be involved, given that the SNc and VTA dopaminergic neurons project to different targets, the neostriatum and nucleus accumbens respectively (Morgan et al., 1986; Anglade et al., 1995). However, no dopaminergic toxins have been identified in the striatum, although functional, injury-induced, dopaminergic neuroprotective activity has been reported in the striatum (Niijima et al., 1990; Maggio et al., 1997). Dopaminergic neurons in the Parkinsonian brain that express calbindin-$D_{28K}$ appear to be less susceptible to neurodegeneration (Yamada et al., 1990). The significance of this observation to the general pathology in PD has been difficult to assess. A variety of other etiologies have been suggested for dopaminergic neuronal death in PD, including a complement-dependent toxin of serum origin, defects in cytochrome C oxidase in the SNc, functional defects in mitochondrial P450 enzymes (Defazio et al., 1994; Itoh et al., 1997; Riedl et al., 1998) and other less well-defined causes (Masalha et al., 1997; Plante-Bordeneuve et al., 1997; Yoritaka et al., 1997). None have been proven to date.

We are therefore faced with the task of treating and possibly curing a disease whose fundamental cause we do not understand. There are two essential requirements. The first is to arrest further neuronal death after diagnosis, and then protect the remaining dopaminergic neurons over the long-term. The second is to replace, at least in part, the neurons that died prior to diagnosis. It follows that neuroprotection combined with cell therapy is likely to be the most realistic and effective therapeutic approach to the treatment of PD. In recent years, we have acquired powerful tools to protect neurons in vitro. Indeed, neuroprotection, the ability to protect neurons from death in a consistent and robust way (still mainly in the laboratory), is one of the most powerful phenomena in modern neurobiology (Takeshima et al., 1996; Gozes, 2001). The challenge is to understand the cellular and molecular mechanisms that are the basis of this neuroprotection, and transfer the knowledge to the in vivo, clinical setting to treat the neurodegenerative diseases.

Three sources of neurotrophic factors are recognized: autocrine, in which the neuron elaborates self-protective molecules (Davies, 1996; Aliaga et al., 1998); target-derived, which is particularly important in developmental neurobiology (Hashino et al., 2001; Lotto et al., 2001; Von Bartheld and Johnson, 2001); and paracrine, in which the trophic molecules are derived from adjacent, mainly nonneuronal cells or type-1 astrocytes (Fig. 2A), (Takeshima et al., 1994; Albrecht et al., 2002).

The focus of this review will be on paracrine, astrocyte-derived, neuroprotective protein molecules. As therapeutics they tend to be less toxic, nonteratogenic and specific (Henderson, 1995; Jain, 1998).

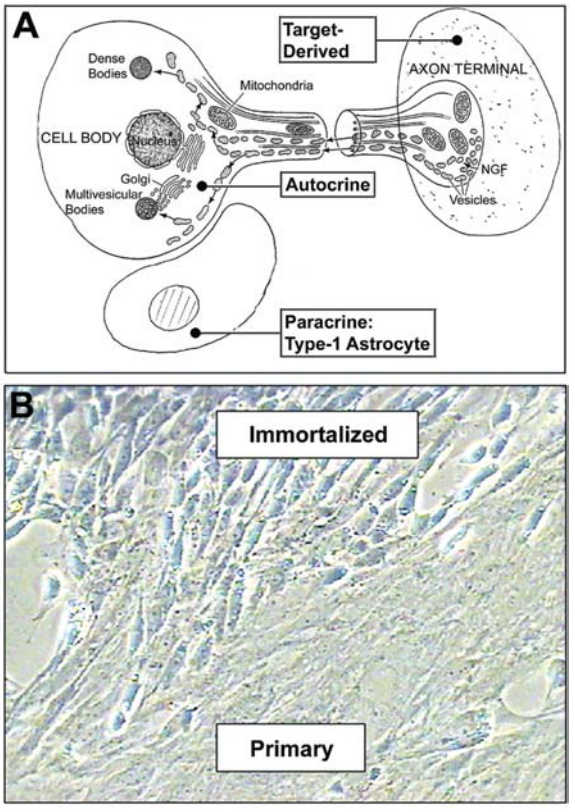



Fig. 2. Types of neurotrophic factors and spontaneous immortalization of astrocytes. (A) Neurotrophic factors may be autocrine, target-derived, or paracrine (modified from Purves and Litchman, 1985. Fig. 11, Chapter 7). The main source of paracrine neurotrophic factors in the CNS is type-1 astrocytes. (B) Spontaneous immortalization of type-1 astrocytes is signaled by a dramatic change in the morphology of the cells from broad and flat (primary) to small and spindle shaped (immortalized). Immortalized cells were marked, picked and subcloned to produce pure cell lines. The duration of the immortalization process is 45–65 days.

Increasing knowledge in the development peptide mimetics from neurotrophic factors will increase the value of these molecules as therapeutics to treat neurodegenerative diseases (Skaper and Walsh, 1998; Beglova et al., 2000; Xie and Longo, 2000).

In terms of a demonstrated, causal link between loss of neuroprotection in nigral dopaminergic neurons and degeneration of dopaminergic neurons leading to PD, the evidence is still indirect. Tooyama et al. (1994) have observed that the expression of basic fibroblast growth factor (bFGF) is diminished in dopaminergic neurons in Parkinsonian patients, but persists in the nigra of age-matched controls. bFGF acting independently, or indirectly as a mitogen for astrocytes which then secrete the neuroprotective molecules, is potently neuroprotective for dopaminergic neurons in vitro (Mena et al., 1995; Hou and Mytilineou, 1996). A reduction of hippocampal brain-derived neurotrophic factor (BDNF) has been shown to precede neuronal degeneration in the CA1 region (Yamasaki et al., 1998). Our current understanding is that the actions of neurotrophic factors are translated via complex molecular cascades (Rozengurt, 1995; Kahn et al., 1997), which therefore offer the opportunity of developing small molecules to promote those reactions that mediate neuroprotection, and inhibit cell death-promoting interactions. In terms of cell therapy, we have also acquired the capacity, although still limited, to expand dopaminergic neurons in vitro. Moreover, new methods of packaging cells optimally for use in cell therapy, and maintaining them at $>90\%$ viability for 24 h have recently been developed (Peaire et al., 2003). Within a few years, these new methods should allow us to produce enough dopaminergic neurons of high viability, for transfer to collaborative neurosurgical centers, to conduct a pilot clinical trial. The work presented in this chapter will explore the scientific basis and technologies associated with these new approaches to the treatment of PD. Our objective is to implement a workable strategy for the effective clinical treatment of PD.

## Astrocytes and neuroprotection

There is a solid body of evidence demonstrating that secreted proteins present in conditioned medium (CM) prepared from ventral mesencephalic type-1 astrocytes are selectively neurotroprotective for dopaminergic neurons (O'Malley et al., 1992; Takeshima et al., 1994; Panchision et al., 1998; Petrova et al., 2003). There is also suggestive evidence that the astrocyte-neuron interaction can be homotypic. The term homotypic implies that molecules derived from ventral mesencephalic astrocytes are more effective in protecting ventral mesencephalic neurons compared with striatal or cerebral cortical neurons (O'Malley

et al., 1994; Takeshima et al., 1994). It follows that neurotrophic factors to treat PD and HD should be derived from ventral mesencephalic and striatal astrocytes, respectively.

Most of the evidence in support of the above contention is derived from experiments utilizing primary astrocytes (Takeshima et al., 1994). However, primary astrocytes are an impractical source of molecules when used over an extended period of time. They are likely to be heterogeneous. They are difficult to maintain, and tend to expand slowly in culture. Quality control for inter-batch preparations is challenging. Errors, requiring an experiment to be repeated, pose practical problems. To be of value as a drug development tool, astrocytes must be immortalized, but with retention of their phenotype. Most methods of immortalization rely on the insertion of foreign DNA into the genome, generally resulting in a changed phenotype. The large tumor antigen from simian virus-40 (SV40-LTA) is the instrument of choice in many immortalization protocols (Azuma et al., 1996; Engele et al., 1996), although other methods have been used as well (Louis et al., 1992; Goletz et al., 1994; Seidman et al., 1997). We have successfully developed an alternative immortalization protocol that is based on the serial passage of proliferating astrocytes (Panchision et al., 1998). Gene expression analysis, karyotyping and biological activity profiling data suggest that cells that were immortalized by this new protocol retain their phenotype (Panchision et al., 1998). Immortalization is signaled by an abrupt change in the morphology of the cell from broad and flat (Fig. 2B, bottom), to small and spindle-shaped (Fig. 2B, top), and by localized expansion at the sites of immortalization in the culture dish. The putative, immortalized cells are then marked, picked and subcloned, and pure cell lines prepared. The method is generic. It can be used to develop cell lines from any region of the CNS. To date, we have prepared a library of seventy such rodent cell lines from four regions of the CNS: ventral mesencephalon (55), striatum (5), cerebral cortex (5) and hippocampus (5). Conditioned medium prepared from one of these cell lines, ventral mesencephalic cell line 1 (VMCL1), contained glial cell line-derived neurotrophic factor (GDNF) and transforming growth factor beta-2 (TGF-$\beta$2), both of which had been previously identified in the primary parent cells by RT-PCR (unreported observation). An unidentified peak of activity was also present that was indicative of an unknown neuroprotective molecule. Standard chromatographic methods were used to separate the different biological activities. GDNF and TGF-$\beta$2 were identified by ELISA according to the manufacturer's instructions (Promega, Madison, WI).

One of the crucial elements in this search for novel neurotrophic factors is a robust, functional bioassay that is capable of identifying neurotrophic activity reliably during sequential purification of a factor. The assay utilizes a ventral mesencephalic cell culture that contains 20% of dopaminergic neurons (Fig. 3) (Takeshima et al., 1996). This high content of dopaminergic neurons provides a high, absolute number of dopaminergic neurons for analysis, permitting test compounds of similar neurotrophic potencies to be resolved reliably. As shown in Fig. 4B, in the absence of neuroprotection, almost all of the plated cells died after seven days (DIV7) in culture, in response to a serum-deprivation insult. However, when treated with astrocyte CM, neuronal survival was pronounced (Fig. 4C). We also know the neuronal phenotypic composition of the culture system as determined 24 h after plating (dopaminergic 20%, GABAergic 50%, serotonergic 5%, cholinergic 8%, bi-potential vimentin+ cells 5%, astrocytes (GFAP+) 0%, with 12% remaining unknown to date. Therefore, the relative specificity of a test CM or pure neurotrophic molecule for a given neuronal phenotype can be determined using this bioassay (Takeshima et al., 1996). It is evident that without a working knowledge of the phenotypic composition of a bioassay, it cannot be used to identify phenotype-selective neurotrophic factors.

In summary, we had decided on a combined strategy of neuroprotection and cell therapy; identified type-1 astrocytes as a source of neuroprotective molecules; built the concept of homotypic astrocyte-neuron interaction into our thinking and used it as a guide in selecting the regions of the CNS from which to develop cell lines; developed a method of preparing astrocyte cell lines that retain their phenotype; developed a novel bioassay of known phenotypic composition with a high content of dopaminergic neurons for use in identifying dopaminergic-selective neurotrophic factors. These initiatives are complementary

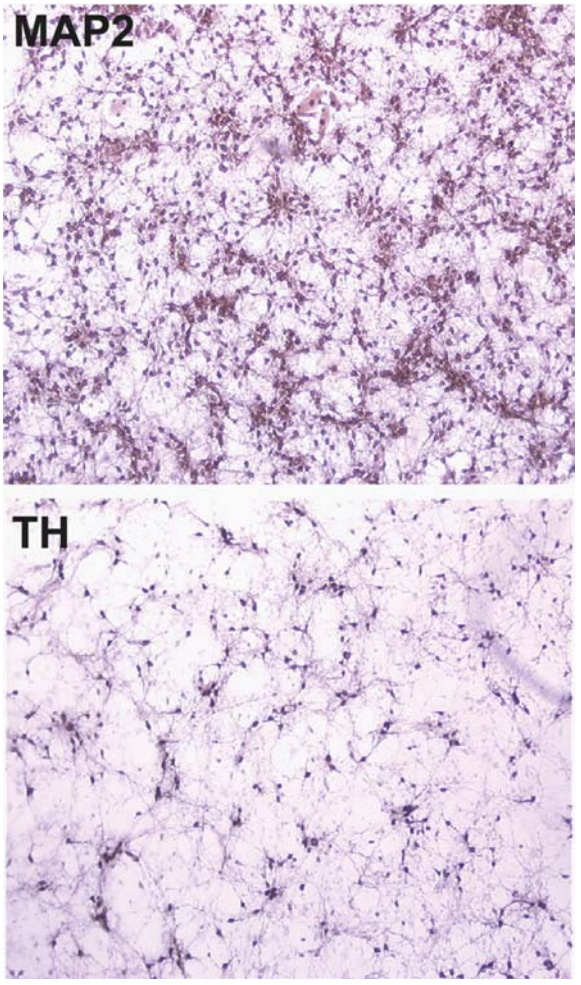



Fig. 3. Ventral mesencephalic cell culture at DIV1. Rat, E14 ventral mesencephalon (1.0 $mm^3$) was microdissected, dispersed at a density of $1.0 \times 10^6$ cells/ml, and plated as 25 μl microisland droplets, in an area of 12.5 $mm^2$. This plating technique results in cells that are homogeneously distributed, at high density, at the center of the microisland (MAP2). The microdissection technique is used routinely, and results in cultures containing 20% of dopaminergic neurons (TH). The cells were immunostained using the Vector ABC method. MAP2+, ×10 Objective; TH+, ×10 Objective. Column factor: 1.0.

to other developments that were taking place in the field. Others have described progress in neurogenesis from adult stem cells and deriving dopaminergic neurons from progenitors (Studer et al., 1998; Song et al., 2002). Methods for the effective delivery of proteins to discrete sites in the brain are being developed (Kordower et al., 2000). The genes that are

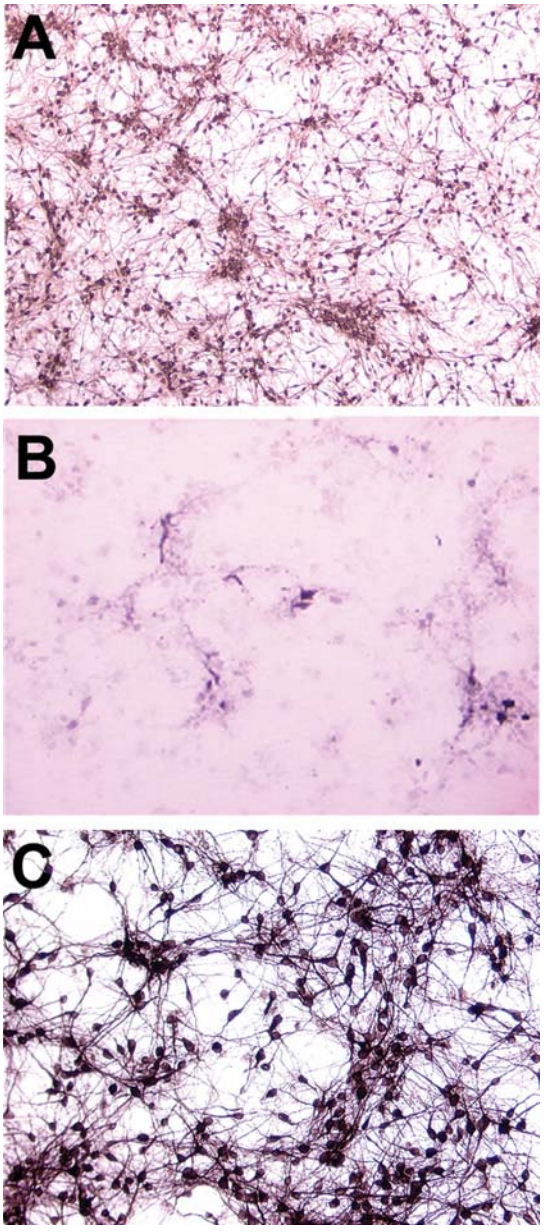



Fig. 4. Neuroprotection. The cultures were prepared and plated using the same methods explained in the legend of Fig. 3. The experiment was designed to illustrate the neuroprotective power of conditioned medium prepared from certain ventral mesencephalic type-1 astrocyte cell lines. (A) The center of the microisland culture (DIV1). At DIV7, and in the absence of neuroprotection, nearly all of the cells had died (B) When treated with 25% v/v of type-1 astrocyte conditioned medium, there was a dramatic rescue of the cells (C). (A), (B) and (C): MAP2 staining using Vector ABC method. A: ×10. B and C: ×20.

implicated in the small familial component of the Parkinsonian population have been cloned (Farrer et al., 1998; Shimura et al., 2000; Lee et al., 2002).

To date, it has been difficult to develop neurotrophic molecules deliberately as potential drug candidates targeted at specific diseases. Discovery has been mainly by serendipity. The sources have been diverse, and a wide variety of methods of testing biological activities have yielded conflicting and inconclusive results. The methods and principles being outlined in this report will likely change the field significantly, at least with regard to paracrine-derived neurotrophic factors targeted at the CNS.

## Protein purification, sequencing, expression and testing

It took several decades (1948–1992) to discover the four members of the neurotrophin family: nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and NT-4/5 (Barde, 1994), but just five years (1993–1998) to discover the four members of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs): GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) (Airaksinen and Saarma, 2002). Factors that are neuroprotective for dopaminergic neurons are illustrated in Table 1. Only deprenyl (selegiline) in this list is actually used clinically to treat PD. The clear implication is that factors to treat neurodegenerative diseases must be developed in a targeted way. We have attempted to pursue such a discovery strategy with MANF.

MANF is derived from a spontaneously immortalized VMCL1. The activity profile of VMCL1-CM demonstrated significant protection of dopaminergic neurons (TH+) in a dose-dependent manner, but not of the general neuronal population (MAP2+) (Panchision et al., 1998). Using classical protein purification and analysis, MANF was purified from 5 L VMCL1-CM, in five sequential chromatographic steps: affinity, gel filtration, weak interactions, ion exchange and high resolution gel filtration (Petrova et al., 2003). Our efforts were concentrated on a peak of activity lacking GDNF and TGF-$\beta$2, which were identified by ELISA. At each stage in the analysis, the chromatographic fractions were tested using the bioassay, usually over a 5-day period. The cultures were treated at DIV0, 2 and 4, scored daily, fixed and stained on DIV5 to identify TH+ and MAP2+ cells. The live cultures ($n=4$) were scored daily on a scale of 0–10, based on visual inspection.

At the last, high resolution gel filtration step, there were 40 fractions (Fig. 5). Biological activity was localized in fractions #18–24. The active fractions and inactive side fractions were pooled separately, and retested to verify the presence and absence respectively of biological activity. Further analysis of the samples is described in detail elsewhere (Petrova et al., 2003). The last analytical step was done using a

TABLE 1
Dopaminergic neurotrophic factors

| Family | Examples | References |
|---|---|---|
| Neurotrophins | BDNF | Hyman et al., 1991 |
| | NT-4/5 | Altar et al., 1994 |
| GDNF-Family ligands (GFLs) | GDNF | Lin et al., 1993 |
| | NRTN | Kotzbauer et al., 1996 |
| | ARTN | Baloh et al., 1998 |
| | PSPN | Baloh et al., 1998 |
| (New factor) | MANF | Petrova et al., 2003 |
| TGF family | TGF-$\beta$1 | Henrich-Noack et al., 1994 |
| | TGF-$\beta$2 | Krieglstein and Unsicker, 1994 |
| | TGF-$\beta$3 | Poulsen et al., 1994 |
| | TGF-$\alpha$ | Alexi and Hefti, 1993 |
| Cytokines | IL-2 | Alonso et al., 1993 |
| | IL-6 | von Coelln et al., 1995 |
| | IGF-II | Liu and Lauder, 1992 |
| Mitogens | bFGF | Engele and Bohn, 1991 |
| | EGF | Casper et al., 1991 |
| | PDGF-AA | Giacobini et al., 1993 |
| | PDGF-BB | Nikkhah et al., 1993 |
| MAO B-I | Deprenyl | Roy and Bédard, 1993 |
| | Rasagiline | Finberg et al., 1998 |
| Miscellaneous | CNTF | Hagg and Varon, 1993 |
| | S-100-$\beta$ | Liu and Lauder, 1992 |
| | VEGF | Silverman et al., 1999 |
| | cAMP | Hartikka et al., 1992 |

cAMP: cyclic adenosine mono phosphate; ARNT: artemin; BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; EGF: epidermal growth factor; FGF: fibroblast growth factor; GDNF: glial cell line-derived neurotrophic factor; IGF: insulin-like growth factor; IL: interleukin; MANF: mesencephalic astrocyte-derived neurotrophic factor; MAO B-I: monoamine oxidase B inhibitors; NRTN: neurturin; NT-3: neurotrophin 3; PDGF: platelet-derived growth factor; PSPN: persephin; TGF: transforming growth factor; VEGF: vascular endothelial growth factor.

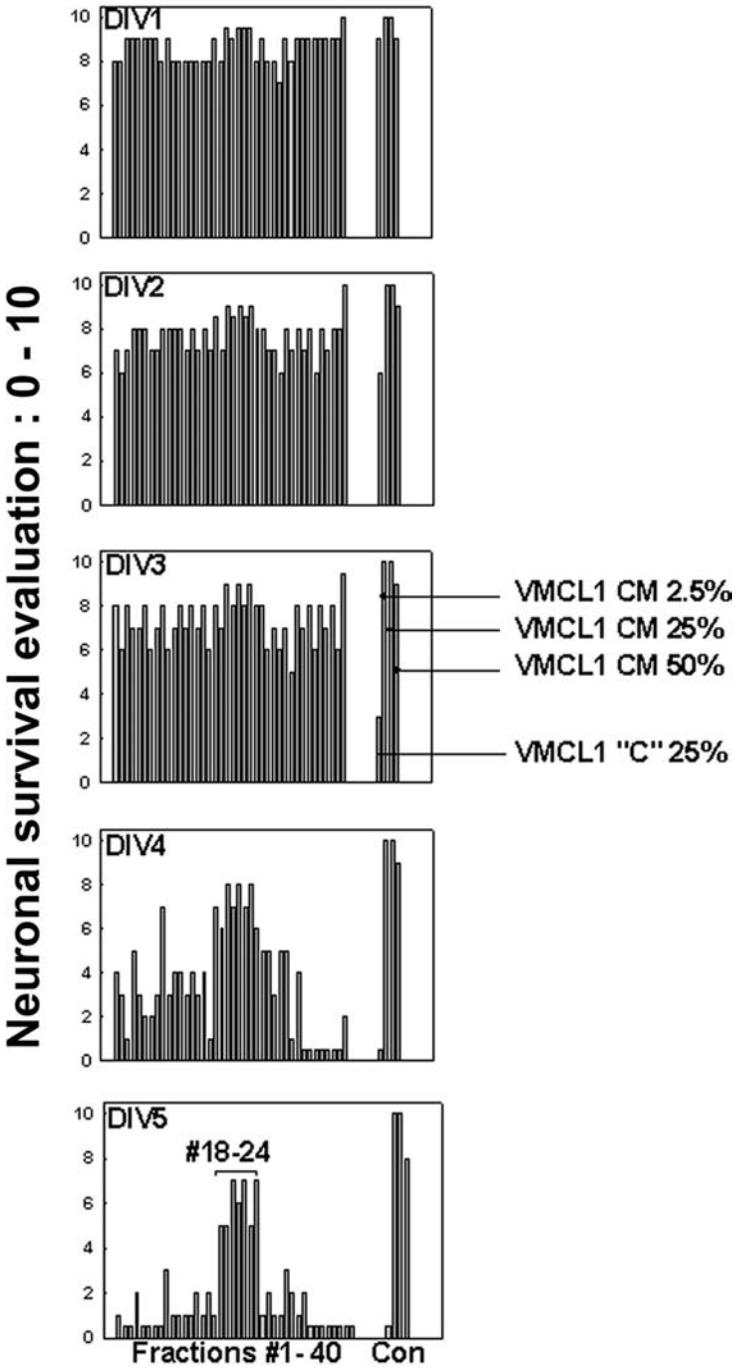



Fig. 5. Use of the bioassay to identify active fractions during purification of MANF. Neuroprotective activity was scored on a scale of 0–10, based on visualization of the live cells in culture, at 24 h intervals. Negative and positive controls were the unconditioned growth medium ('C') and VMCL1-CM (2.5, 25 and 50% v/v respectively). As shown, by DIV2, it was possible to begin to localize the active fractions. By DIV4/5 localization of the active fractions was readily apparent, as illustrated. Biological activity was localized in fractions #18–24 in the last purification step, using a BioSil 125 high resolution gel filtration column.

12% SDS–PAGE gel which yielded a single band in the expected 20 kD range. The gel was fixed (methanol/acetic acid/water in the ratio 50:10:40), washed (20, 10 and 10 min), stained (overnight, using GelCode Blue Stain Reagent at room temperature) and washed. The 20 kD band was excised and placed in a 1.5 ml microcentrifuge tube and stored on at −86°C until analyzed.

Prior to analysis, the gel band was destained (100 mM ammonium bicarbonate/30% acetonitrile, 30 to 60 min), washed (deionized water, 10, 10, 10 min), dehydrated (acetonitrile), rehydrated (50 mM ammonium bicarbonate) and digested (200 ng of modified trypsin, Promega, Madison, WI) overnight at 37°C. The digest was then transferred to an eppendorf tube, evaporated to dryness (Savant centrifugal evaporator), redissolved (5 μl 5% acetonitrile/0.1% trifluoroacetic acid (desalted, ZipTipTM C18 tips; Millipore, Bedford, MA), and reconstituted in 5 μl 75% methanol/0.5% acetic acid. Peptide analysis was done by nanoelectrospray ionization-tandem mass spectrometry (nESI-MS/MS) (Blackburn and Anderegg, 1997; Li et al., 2000). Two novel peptides were identified from the 20 kD band, ... DVTFSPATIE... and ...QIDLSTVDL. A search of the NCBI protein sequence database for matching proteins was done, as well as BLAST searches. The 20 kD protein was tentatively identified as a human arginine-rich protein or ARP, that contained translated sequences that were 100% homologous with the two peptides identified from nESI-MS/MS analysis. This human ARP cDNA had previously been cloned (Shridhar et al., 1996), but never expressed. The name arginine-rich protein is derived from the fact that 22 of the first 55 N-terminal residues in this transcript are arginines, a property not compatible with a secreted protein. A more detailed search of the public databases identified seventeen homologs of ARP, including those of rat, mouse, *C. elegans* and a second human transcript. All of these, including the second human transcript, lacked the Arg-rich, N-terminal sequence identified in the first human sequence. Additional analysis revealed that AA56 in ARP was methionine. It therefore seemed evident that the start methionine in the secreted rat protein (and the other homologs as well) responsible for the neuroprotective activity observed in the bioassay was the equivalent of the first ARP identified, minus the first 55 AAs. Further, the Signal-P program indicated two possible signal sequences: (1) The 15-AA sequence: M-56 to V-70 (MWATQGLAVRVALSV...), and (2) the 21-AA sequence: M-56 to A-76 (MWATQGLAVRV ALSVLPGSRA...). Edman microsequencing analysis of the secreted recombinant protein revealed a 5-AA N-terminal sequence of ...LRPGD, that is identical to the equivalent 5-AAs in ARP. This result confirmed the 21-AA signal sequence, and assigned a definitive sequence to the biologically active, secreted protein.

The original human ARP cDNA was therefore subcolned to delete the first 55-AAs, making it equivalent to the other homologs identified in the data base. The re-engineered cDNA was tagged for further purification (His-tag) and identification (HSV-tag) and ligated into the Novagen pTriEx vector. Proof reading PCR and the Novagen pTriEx vector system were used according to the Supplier's recommendations. The protein expressed lacked the R-rich, 55-AA N-terminal region of the original ARP molecule. It was therefore renamed mesencephalic astrocyte-derived neurotrophic factor (MANF) to reflect its origin from a mesencephalic astrocyte, and its neurotrophic activity. Tag-free MANF was expressed in *E. coli*, and tagged MANF in VMCL1, COS and HEK293 using standard expression protocols (Durocher et al., 2002). A larger scale expression of recombinant MANF (25 mg) in HEK293-EBNA cells was done as previously described (Durocher et al., 2002). MANF is encoded by a small 4.3 Kb gene with 4 exons, located on the short arm of human chromosome 3. Two domains in MANF: 39-AA and 109-AA respectively, and the 8 cysteines are conserved from *C. elegans* to man. The secondary structure is dominated by $\alpha$-helices (47%) and random coils (37%). Studies to determine the localization of MANF in the brains of rat, monkey and man, as well as the receptor, signaling pathways and biologically active peptide mimetics are in progress.

Figure 6 demonstrates an SDS-PAGE gel of tag-free hrMANF expressed in *E. coli*. Unpurified and purified MANF are shown in lanes #2 and #3, respectively. The band in lane #3 was excised and analyzed by nESI-MS/MS. Eight peptides (box inset) were identified, all of which are derived from MANF.

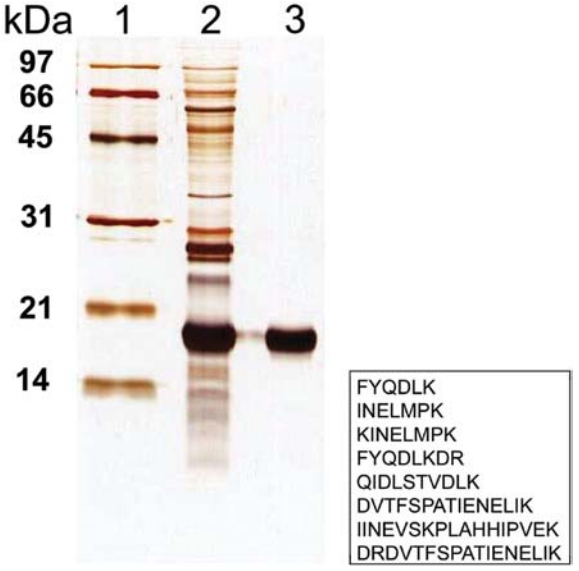
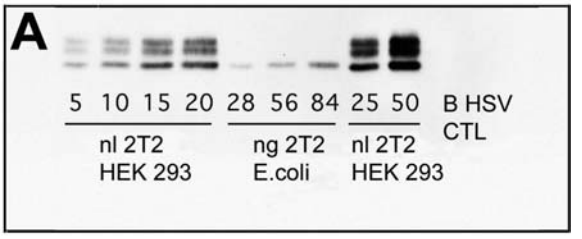
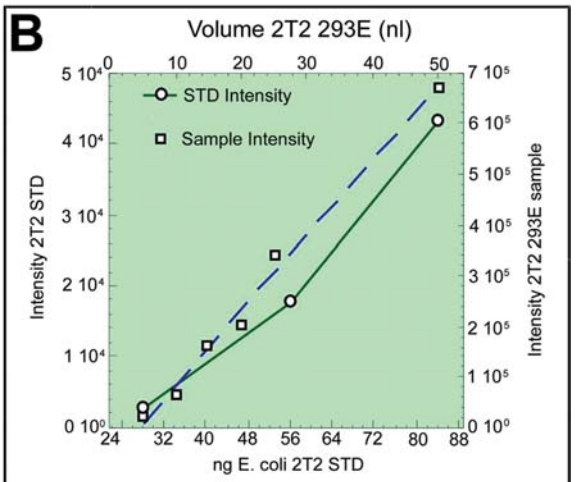



Fig. 6. Highly purified tag-free MANF expressed in *E. coli*. Tag-free hrMANF expressed in *E. coli* (lane 2) was purified (lane 3) by high resolution gel filtration (BioSil 125), analyzed by SDS-PAGE and stained with coomassie blue. The single band indicated in lane 3 was destained, digested using papain, and the peptide digest analyzed by nESI-MS/MS. Eight peptides were identified, all of which were derived from hrMANF. No bacterial protein was identified. Lane 1: MW Standard.

There were no contaminating bacterial proteins. Tag-free MANF expressed in *E. coli* was shown to be biologically active (Petrova et al., 2003). It was used to quantitate MANF expressed in HEK293, as illustrated in Fig. 7. It is evident (Fig. 7A) that MANF expressed in HEK293 is glycosylated. Independent verification of glycosylation was obtained by the action of neuraminidase (Petrova et al., 2003). Polyclonal antibodies to MANF have been prepared (Fig. 8). At a dilution of 1:5000, 15.6 ng of MANF was easily identified (Fig. 8, lane #9). Experiments to identify MANF by immunocytochemistry in the human brain are in progress.

The first test of hrMANF expressed in VMCL1 was in the determination of the viability of ventral mesencephalic cells at DIV5, using the Live Cell/Dead Cell Assay (Molecular Probes). Cell viability in the cultures treated with VMCL1-CM containing hrMANF was significantly increased (Fig. 9, #c, $p < 0.05$) vs positive controls (#b and #d). This was the first indication that hrMANF was biologically




| ng 2T2 | Intensity | nl 2T2 | Intensity |
|---|---|---|---|
| 28 | 2662 | 5 | 23109 |
| 56 | 17666 | 10 | 63861 |
| 84 | 43506 | 15 | 162220 |
| | | 20 | 202220 |
| | | 25 | 342220 |
| | | 50 | 672220 |

Fig. 7. Quantitation of hrMANF expressed in HEK293 cells. hrMANF expressed in HEK293 cells was quantitated using Western blot combined with densitometry. A standard curve was constructed (o) with 28, 56 and 84 ng of purified hrMANF expressed in *E. coli* (A: Center values, and B: [o]). Subsequently, secreted hrMANF in 5, 10, 15, 20, 25 and 50 nl of the HEK293 medium (A) was plotted as illustrated (B: [□]), and the equivalent quantities of hrMANF determined. Note the difference in units of density on the Y axis for pure hrMANF (left: ng 2T2) and unknown hrMANF (right: nl 2T2). 2T2 is the name assigned to hrMANF in this experiment.

active. The presence of hrMANF in the CM used in these experiments (Fig. 9) was independently verified by Western blot (Petrova et al., 2003). Glycosylated hrMANF expressed in HEK293 cells was tested in the bioassay, and the cultures stained by indirect, double immunofluorescence to identify TH and

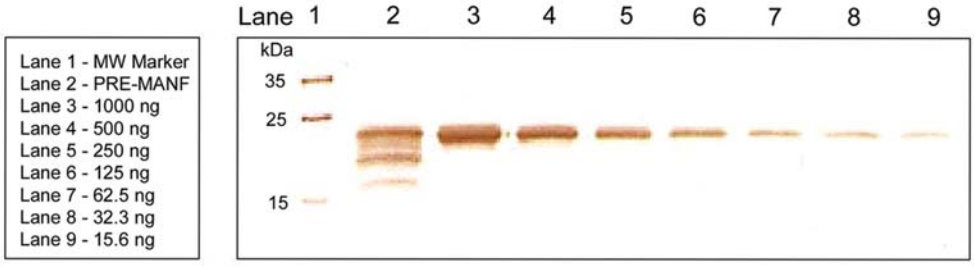



Fig. 8. Sensitivity of anti-MANF polyclonal antibodies. Affinity-purified anti-MANF polyclonal antibodies diluted 1:5000 recognized 15.6 ng of hrMANF. Seven quantities of MANF (15.6–1000 ng, lanes #3–9) were all identified by the antibody. Lane #2 contains MANF prior to purification. Lane #1: MW Standard. The analysis was done by Western blot.

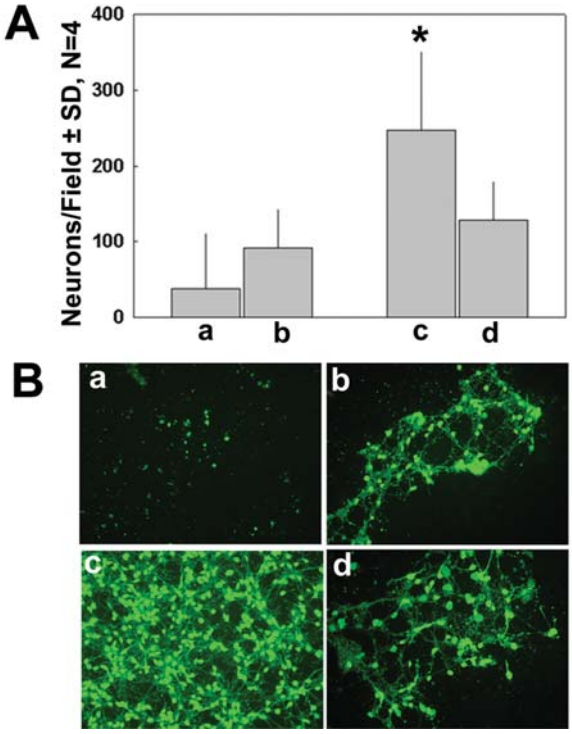



Fig. 9. hrMANF-induced increased viability of ventral mesencephalic neurons. (A) 25% v/v of the VMCL1-CM containing secreted hrMANF (verified independently by Western blot) caused a significant increase at DIV5 ($p < 0.05$ by SNK) in the viability of ventral mesencephalic cells (c) compared to the controls (a, b and d). Conditions a, b, and d are negative control (unconditioned growth medium), positive control (astrocyte conditioned medium) and CM from mock transfected VMCL1 cells respectively. * c is significantly different from a, b and d. ANOVA: $p < 0.01$. (B) Representative images from the four conditions illustrated in (A) above. There is a close correspondence between the number of cells/field indicated in the images (B), and the mean number of cells/field in the histograms (A).

MAP2 at DIV4 (Fig. 10). The data demonstrate that MANF at 0.05 and 50 ng/ml (Fig. 10A, #3 and #4, respectively), caused significant protection ($P < 0.05$) of dopaminergic neurons versus negative control. The MAP2 data suggest that MANF also increased general neuronal protection. However, we have suggested elsewhere that GABAergic and serotonergic neurons appear not to maintain the expression of GAD and tryptophan hydroxylase respectively in the presence of MANF, while the expression of TH is maintained in dopaminergic neurons (Petrova et al., 2003). This result represents a variation in mechanism of neuroprotection. The GABAergic and serotonergic neurons appear not to die. Instead, they appear to downregulate the enzymes that are unique to these neurons.

## Understanding the problem

We are attempting to treat and possibly cure PD, but without a firm understanding of the etiology of the death of dopaminergic neurons that causes the disease (Ruberg et al., 1995; Plante-Bordeneuve et al., 1997; Hubble et al., 1998). As shown (Fig. 1, inset), six morphological subphenotypes of dopaminergic neurons can be identified in the SNc. Other evidence suggests that the expression of tyrosine hydroxilase (TH) mRNA in SNc dopaminergic neurons can also be binned into several levels of expression (Weiss-Wunder and Chesselet, 1991). The electrophysiological firing patterns of dopaminergic neurons are also heterogeneous (Grace and Bunney, 1984a, b). Heterogeneity of dopaminergic neurons has been identified even at the level of the dopamine

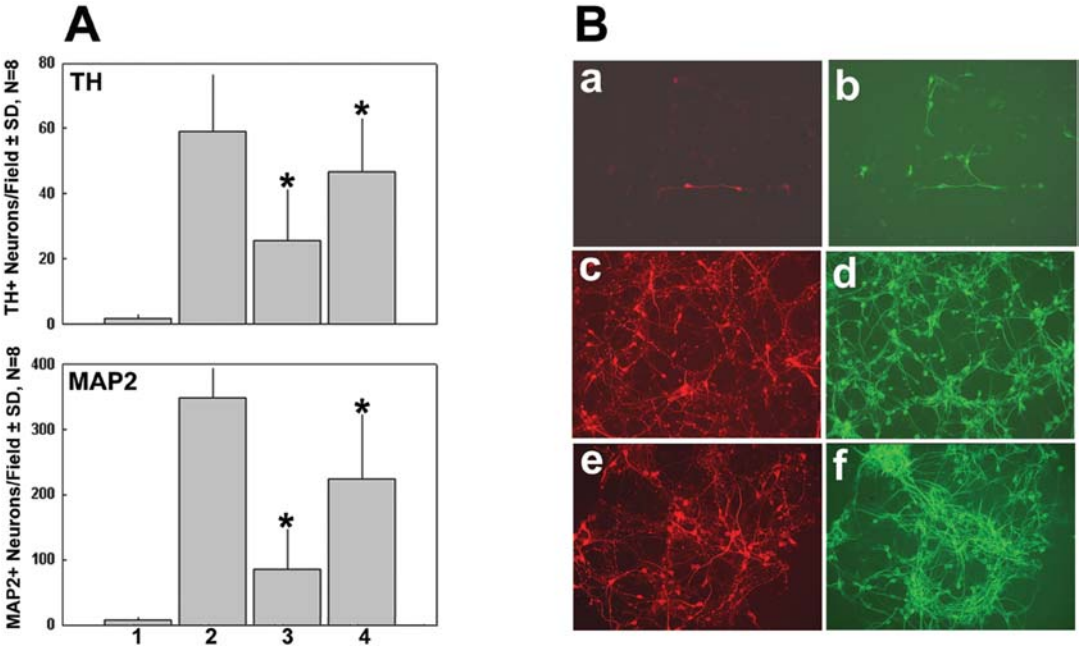



Fig. 10. hrMANF increases the survival of dopaminergic neurons in vitro. (A) Purified hrMANF expressed in HEK 293 was tested in the bioassay. At DIV4, hrMANF at 0.05 (#3) and 50 (#4) ng/ml increased the survival of dopaminergic neurons significantly ($p < 0.05$ by SNK) vs the negative control (#1: unconditioned growth medium). The positive control (#2) is 25% v/v astrocyte conditioned medium. See text for an explanation of the MAP2 data, with regard to the selectivity of MANF for the dopaminergic phenotype. * Significantly different, $p < 0.05$ vs negative control (#1). (B) Representative fluorescence images TH (left panels) and MAP2 (right panels) from the negative control (a and b, same field), positive control (**c** and **d**, same field) and hrMANF, 50 ng/ml (**e** and **f**, same field). Indirect immunofluorescence, TH (CY3): **a**, **c** and **e**; MAP2 (FITC): **b**, **d** and **f**. All fields × 20.

transporter (Burchett and Bannon, 1997; Katz et al., 1997). Dopaminergic neurons are also heterogeneous with regard to the expression of the calcium-binding protein calbindin-$D_{28K}$ (Sanghera et al., 1994; Sanghera et al., 1995; Betarbet et al., 1997). We have usually assumed, but really do not know whether these neuronal sub-groups are homogeneous in terms of their physiological, neuroprotective requirements. Some of our recent evidence (unreported observation) suggests that CM from different astrocyte cell lines exert differential neuroprotective effects on different dopaminergic morphological subphenotypes.

These diverse results detailed above strongly suggest that dopaminergic neurons in the SNc may be a functionally heterogeneous population. If they are, especially in terms of their neuroprotective requirements, then the effective treatment of PD with a single neurotrophic factor might not be feasible. In that case, we will need to discover factors that are specific not to generic dopaminergic neurons, but to subsets of dopaminergic neurons. If there is an equivalent functional heterogeneity in the ventral mesencephalic astrocyte population that matches the morphological heterogeneity of the nigral dopaminergic neurons, then the actual in vivo situation may be complex. At one extreme, and assuming a one to one match between astrocytes and dopaminergic neurons, at least six subphenotypes of ventral mesencephalic astrocytes may be implicated in the support of the six dopaminergic subtypes. Functional overlap may reduce this number to three. In that case a cocktail of at least two, and possibly three neurotrophic factors derived from ventral mesencephalic astrocytes would be needed to treat PD effectively. Although the scenario detailed above is somewhat speculative, and may seem unduly complex, it really is not, in terms of what is known about organizational complexity in other parts of the CNS, particularly the cerebellum (Eccles et al., 1967; Ito, 1997). Given the enormous

effort, and lack of substantive progress over the last two decades in understanding and treating PD, we ought to begin to explore the difficult issues from a new perspective. The large number of molecules that are neuroprotective for dopaminergic neurons (Table 1), albeit in widely differing experimental paradigms, indirectly support the idea of multiple physiological neuroprotective molecules in vivo.

Patients with long-standing PD that have lost nearly 100% of their dopaminergic neurons will likely not benefit from neuroprotective drug therapy. Cell therapy will likely be the treatment of choice for this group of patients. Fortunately, substantial progress is being made in this area. The days of using minced pieces of nigral tissue in PD cell therapy should now be at end. We are transitioning into an era in which cells for cell therapy to treat PD will be prepared from stem cells or progenitors, packaged optimally, and prepared to retain viability of $>90\%$ over a 24 h period. The phenotypic composition of the cell preparation should also be known, with dopaminergic neurons accounting for 25–50%, and astrocytes $<1.0\%$. These new ideas are presented in a recent publication (Peaire et al., 2003). When cell therapy is used to treat PD, it will be prudent to combine it with a neuroprotective strategy. The working assumption should be that exogenous dopaminergic neurons transplanted into the brain of a PD patient will die, as did their endogenous predecessors, if not protected. Significant progress has been made in delivering neuroprotective factors to localized regions of the primate CNS using lentiviral vectors, and the expression of the protein maintained over many months (Kordower et al., 2000; Galimi and Verma, 2002). The new Medtronics pump will likely prove to be clinically useful (Gardner et al., 1995). The issue of delivery remains an important topic in the application of protein therapeutics to treat neurodegenerative diseases. However, if there is a molecule with the right clinical indices that is strongly indicated for the treatment of PD, it is difficult to believe that an effective delivery mechanism will not be found. The effective movement of BDNF across the blood–brain barrier of rat has been described (Pardridge et al., 1994). Recent successes in the preparation of peptide mimetics that are more effective than their parent precursors is also encouraging (Brenneman and Gozes, 1996).

The idea of astrocyte-derived secreted proteins as homotypic neuroprotective molecules may be an instance of a more general glia–neuron interaction in the nervous system. In a model system of tectal astrocytes and retinal ganglion cells (RGCs), astrocyte-derived molecules regulated the development, functioning and stabilization of synapses in vitro (Nagler et al., 2001; Ullian et al., 2001). In yet another paradigm, hippocampal astrocyctes were six-fold more effective in promoting neurogenesis from hippocampal adult stem cells, vs spinal cord astrocytes (Song et al., 2002). In two recent paradigms of differentiation of dopaminergic neurons, ventral mesencephalic glia were also involved (Wagner et al., 1999; Matsuura et al., 2001). Astrocyte-derived tumor necrosis factor alpha (TNF$\alpha$) strengthened synaptic transmission in both dispersed and slice cultures, specifically by mediating increased expression of AMPA receptors in the postsynaptic neurons by an activity-dependent process (Beattie et al., 2002). Astrocytes can also modulate synaptic transmission at both inhibitory and excitatiory synapses (Kang et al., 1998; Newman and Zahs, 1998; Haydon, 2000). Similar results have been reported for the action of Schwann cells at the frog neuromuscular junction in the peripheral nervous system (PNS) (Robitaille, 1998). These diverse results of glia–neuron interactions indicate that we are beginning to acquire a clearer understanding of the functional and molecular interplay between glia and neurons in the CNS and PNS. These developments bode well for the development of drug candidates in the difficult search to find effective therapies to treat peripheral neuropathies and the major neurodegenerative diseases.

There has not yet been a dramatic breakthrough in the treatment of PD, but there are significant, modest accomplishments. The concept of homotypic, astrocyte-derived neurotrophic factors from the mesencephalon will likely prove to be important (Le Roux and Reh, 1995; Araque et al., 2001; Petrova et al., 2003). The use of lentiviral vectors to deliver factors to nondividing cells in the CNS over extended periods of several months is also a significant advance (Kordower et al., 2000). The preparation of dopaminergic neurons from progenitors initially,

and eventually from stem cells for use in PD cell therapy, combined with methods to package the cells optimally and keep them viable over extended periods represents solid progress (Hynes and Rosenthal, 2000; Rodriguez-Pallares et al., 2001; Bjorklund et al., 2002; Peaire et al., 2003). There is an optimistic mood in the Parkinsonian research community. The challenge is to translate optimism into actual, effective therapy.

## Abbreviations

| | |
|---|---|
| cAMP | cyclic adenosine mono phosphate |
| ARTN | artemin |
| BDNF | brain-derived neurotrophic factor |
| CNS | central nervous system |
| CNTF | ciliary neurotrophic factor |
| EGF | epidermal growth factor |
| FGF | fibroblast growth factor |
| GDNF | glial cell line-derived neurotrophic factor |
| GFLs | GDNF family ligands |
| IGF | insulin-like growth factor |
| IL | interleukin |
| MANF | mesencephalic astrocyte-derived neurotrophic factor |
| MAO B-I | monoamine oxidase B inhibitors |
| NRTN | neurturin |
| NT-3 | neurotrophin-3 |
| PD | Parkinson's disease |
| PDGF | platelet-derived growth factor |
| PSPN | persephin |
| RGCs | retinal ganglion cells |
| SNc | substantia nigra zona compacta |
| SNr | substantia nigra zona reticulata |
| TGF | transforming growth factor |
| VEGF | vascular endothelial growth factor |

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