NOVEL CDNF/MANF PROTEIN FAMILY: MOLECULAR STRUCTURE, EXPRESSION AND NEUROTROPHIC ACTIVITY

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SELECTED ABBREVIATIONS

aa	amino acid
ARTN	artemin
ATF	activating transcription factor
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
CDNF	conserved dopamine neurotrophic factor
CNS	central nervous system
CXXC	cysteine-X-X-cysteine
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
ERAD	ER associated protein degradation
GABA	γ-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF family receptor alpha
GSL	glycosphingolipid
ICV	intracerebroventricular
IHC	immunohistochemistry
ISH	*in situ* hybridization
kDa	kilodalton
KDEL	lysine-aspartic acid-glutamic acid-leucine
LC	locus coeruleus
MANF	mesencephalic astrocyte-derived neurotrophic factor
MCAO	middle cerebral artery occlusion
mDA	mesencephalic dopaminergic neuron
MFB	medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MW	molecular weigth
NCAM	neural cell adhesion molecule
NRTN	neurturin
NTF	neurotrophic factor
6-OHDA	6-hydroxydopamine
PCD	programmed cell death
PD	Parkinson's disease
PDI	protein disulphide isomerase
pI	pH value of isoelectric point
PNS	peripheral nervous system
PSPN	persephin
RET	rearranged during transfection
ROS	reactive oxygen species
SapA-B	saposin A-B
SAPLIP	saposin-like protein
SE	status epilepticus
SNpc	substantia nigra pars compacta
TH	tyrosine hydroxylase
TRX	thioredoxin
UPR	unfolded protein response
XBP1	X-box binding protein 1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles that will be referred to in the text by their Roman numerals:

I. **Päivi Lindholm**, Merja H. Voutilainen, Juha Laurén, Johan Peränen, Veli-Matti Leppänen, Jaan-Olle Andressoo, Maria Lindahl, Sanna Janhunen, Nisse Kalkkinen, Tõnis Timmusk, Raimo K. Tuominen, and Mart Saarma (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. *Nature* 448:73-77. Supplementary material is linked for the online version of the article at www.nature.com/nature.

II. **Päivi Lindholm**, Johan Peränen, Jaan-Olle Andressoo, Nisse Kalkkinen, Zaal Kokaia, Olle Lindvall, Tõnis Timmusk, and Mart Saarma (2008). MANF is widely expressed in mammalian tissues and differently regulated after ischemic and epileptic insults in rodent brain. *Molecular and Cellular Neuroscience* 39:356-371.

III. Vimal Parkash*, **Päivi Lindholm***, Johan Peränen, Nisse Kalkkinen, Esko Oksanen, Mart Saarma, Veli-Matti Leppänen, and Adrian Goldman (2009). The structure of the conserved neurotrophic factors MANF and CDNF explains why they are bifunctional. *Protein Engineering, Design & Selection* 22:233-241. *Equal contribution to the publication. Supplementary material is linked for the online version of the article at www. peds.oxfordjournals.org.

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Cover image

Illustrative three-dimensional (3D) structure of full-length mature human CDNF. The crystal structure of CDNF amino-terminal saposin-like domain (green) was combined by image processing with the carboxy-terminal domain (grey) of human MANF. The four cysteine bridges are indicated in yellow. Below the 3D structure is a schematic presentation of the primary structure of mature CDNF. The formation of cysteine bridges is indicated. Original 3D structural images of CDNF and MANF were provided by Veli-Matti Leppänen and Vimal Parkash.

ABSTRACT

Neurotrophic factors (NTFs) are secreted proteins which promote the survival of neurons, formation and maintenance of neuronal contacts, and regulate synaptic plasticity. NTFs are also potential drug candidates for the treatment of neurodegenerative diseases. Parkinson's disease (PD) is mainly caused by the degeneration of midbrain dopaminergic (mDA) neurons. Current therapies for PD do not stop the neurodegeneration or repair the affected neurons. Thus, search of novel neurotrophic factors for midbrain dopaminergic neurons, which could also be used as therapeutic proteins, is highly warranted.

In the present study, we identified and characterized a novel protein named conserved dopamine neurotrophic factor (CDNF), a homologous protein to mesencephalic astrocyte-derived neurotrophic factor (MANF). Others have shown that MANF supports the survival of embryonic midbrain dopaminergic neurons *in vitro*, and protects cultured cells against endoplasmic reticulum (ER) stress. CDNF and MANF form a novel evolutionary conserved protein family with characteristic eight conserved cysteine residues in their primary structure. The vertebrates have CDNF and MANF encoding genes, whereas the invertebrates, including *Drosophila* and *Caenorhabditis* have a single homologous CDNF/MANF gene.

In this study we show that CDNF and MANF are secreted proteins. They are widely expressed in the mammalian brain, including the midbrain and striatum, and in several non-neuronal tissues. We expressed and purified recombinant human CDNF and MANF proteins, and tested the neurotrophic activity of CDNF on midbrain dopaminergic neurons using a 6-hydroxydopamine (6-OHDA) rat model of PD. In this model, a single intrastriatal injection of CDNF protected midbrain dopaminergic neurons and striatal dopaminergic fibers from the 6-OHDA toxicity. Importantly, an intrastriatal injection of CDNF also restored the functional activity of the nigrostriatal dopaminergic system when given after the striatal 6-OHDA lesion. Thus, our study shows that CDNF is a potential novel therapeutic protein for the treatment of PD.

In order to elucidate the molecular mechanisms of CDNF and MANF activity, we resolved their crystal structure. CDNF and MANF proteins have two domains; an amino (N)-terminal saposin-like domain and a presumably unfolded carboxy (C)-terminal domain. The saposin-like domain, which is formed by five α-helices and stabilized by three intradomain disulphide bridges, may bind to lipids or membranes. The C-terminal domain contains an internal cysteine bridge in a CXXC motif similar to that of thiol/disulphide oxidoreductases and isomerases, and may thus facilitate protein folding in the ER.

Our studies suggest that CDNF and MANF are novel potential therapeutic proteins for the treatment of neurodegenerative diseases. Future studies will reveal the neurotrophic and cytoprotective mechanisms of CDNF and MANF in more detail.

1. REVIEW OF THE LITERATURE

1.1. Neurotrophic factors: The concept

Correct formation of neuronal contacts is essential for the development of vertebrate nervous system. Different cell-intrinsic and cell-extrinsic factors regulate this process, including intrinsic transcriptional programs, and extrinsic, secreted or cell-surface bound molecules provided by neighbouring cells, tissues and extracellular matrix. Neurotrophic factors are an important group of cell-extrinsic, secreted proteins, which regulate the neuronal survival and death at the time of synapse formation with the target tissue or with other neurons. As the name implies, neurotrophic factors provide “nourishment” for postmitotic neurons which they need for the survival.

During embryogenesis neurons are produced in excess. The overproduced neurons are removed during the developmental period of programmed cell death (PCD), which is to ensure that the target tissue receives optimal number of neuronal contacts. It has been estimated that 20-80% of neurons in a given population die by apoptosis, a cell-intrinsic default program of death (Oppenheim 1991). NTFs have a crucial role in preventing the apoptotic death and in regulating the number of target-innervating neurons. The concept of neurotrophic factors is largely based on the studies on nerve growth factor (NGF), the first growth factor and neurotrophic factor identified in the 1950’s (Levi-Montalcini 1987). According to the original neurotrophic factor hypothesis, the target tissue secretes NTFs in limited amounts, which leads to neuronal competition of the trophic support. As a result, only neurons receiving a sufficient trophic support survive and synapse with the target, whereas other neurons die apoptotically. The target-derived NTF binds to a receptor at axon terminals, the ligand-receptor complex is endocytosed and retrogradely transported into the cell soma, where survival promoting signal pathways are activated, and apoptotic death is inhibited. NGF functions as a target–derived factor for sensory and sympathetic neurons in the peripheral nervous system (PNS) (Huang and Reichardt 2001; Glebova and Ginty 2005). The neurotrophic factor concept is well-established in the development of the PNS; however, the role of NTFs in the development and maintenance of the central nervous system (CNS) is less clear, although intensively studied.

The classification of “neurotrophic factor” includes a wide range of proteins with different functional specificities. Traditionally, neurotrophic factors consist of three main protein families: Neurotrophins (Lu et al. 2005; Huang and Reichardt 2001), glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) (Airaksinen and Saarma 2002; Bespalov and Saarma 2007), and neuropoietic cytokines (also referred as interleukin-6 (IL-6) family) (Bauer et al. 2007; Heinrich et al. 2003). All these factors signal via transmembrane receptor tyrosine kinases, or have receptors associated with kinases (Bespalov and Saarma 2007). In addition to these three families, various other growth factors also have neurotrophic activities. Examples of these include the members of transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and fibroblast growth factor (FGF) families (Zacchigna et al. 2008; Grothe and Timmer 2007).

Different NTFs affect on specific sets of neuronal subpopulations, which are genetically competent to respond, i.e. express cognate receptors on their surface, and have appropriate transcriptional programs. In addition to the survival promoting activity, NTFs affect on neuronal migration, differentiation and maturation. They regulate neuritogenesis, formation and maintenance of synaptic contacts, and synaptic plasticity. They also promote the regeneration of neurons after injury (Airaksinen and Saarma 2002; Huang and Reichardt 2001). It is well established that neurons may receive neurotrophic support from various sources; not only from the the target (Fig. 1) (Davies 2003; Krieglstein 2004). Neurotrophic factors also affect on non-neuronal cells and tissues, and have important roles, for example, in angiogenesis (Zacchigna et al. 2008).

Evidence suggests that NTFs function also in the development of invertebrate nervous system. In the fly *Drosophila*, glial-derived neurotrophic support has been reported (Booth et al. 2000). As in the vertebrates, fly neurons are originally overproduced, and the excess of neurons is removed by PCD during development (White et al. 1994). However, the identification of NTFs in arthropods and nematodes was for long unsuccessful (Jaaro et al. 2001; Bothwell 2006). Only recently Zhu and colleagues (Zhu et al. 2008) described *Drosophila* Neurotrophin 1 (DNT1; spätzle 2) part of which is structurally homologous to all known neurotrophins. Furthermore, the first ligand-receptor pair homologous to neurotrophins and Trk receptors has been described in the mollusc *Aplysia* snail (Kassabov 2008). Although GFL homologs have not been found in insect genomes (Airaksinen et al. 2006), an orphan homolog for GDNF receptor RET (rearranged during transfection) is present in *Drosophila* (Sugaya et al. 1994). Several

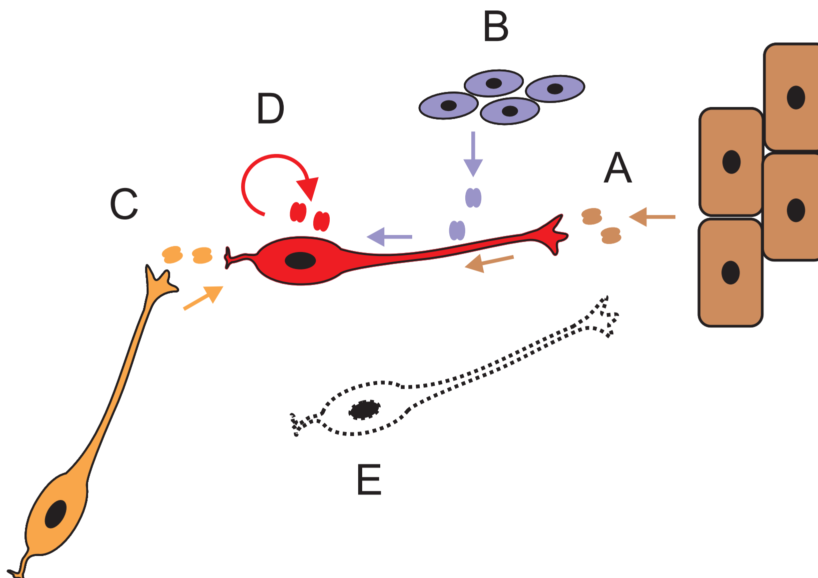


Figure 1. Neurons may receive NTFs from different sources, including the target (**A**); intermediate target cells or glial cells (**B**); or presynaptic neurons (**C**). **D**) Autocrine trophic support. **E**) Neurons without a sufficient trophic support die by apoptosis.

insect genomes also contain a GDNF family receptor (GFR)-like gene, which is found in *C. elegans* as well (Airaksinen et al. 2006). Thus, NTFs seem necessary not only for the development and maintenance of the "complex" vertebrate nervous system with high number of neurons, but also for the more "simple" nervous system of invertebrates.

1.2. Neurotrophic factors for midbrain dopaminergic neurons

In this work our interest has been on neurotrophic factors supporting the survival of dopaminergic neurons located in the substantia nigra (SN) in the midbrain. These neurons form the major neuronal population degenerating in Parkinson's disease, a progressive neurodegenerative movement disorder. The role of target-derived NTFs in the development of midbrain dopaminergic neurons has remained unclear (Andressoo and Saarma 2008). Potential target-derived factors for these neurons include GDNF, neurturin (NRTN), brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4) and FGF-2 (Krieglstein 2004).

1.2.1. Neurotrophins

The family of neurotrophins include four members in mammals: NGF, BDNF (Barde et al. 1982; Leibrock et al. 1989), neurotrophin-3 (NT-3) (Ernfors et al. 1990; Hohn et al. 1990; Maisonpierre et al. 1990; Rosenthal et al. 1990) and NT-4 (Berkemeier et al. 1991; Hallböök et al. 1991). Neurotrophins signal via two transmembrane receptor systems; tropomyosin-related kinase (Trk) receptor or p75 neurotrophin receptor ($p75^{NTR}$), a member of tumour necrosis factor (TNF) receptor family (Chao et al. 2006). Ligand binding is selective to the different Trk receptors (TrkA, B, C), whereas all neurotrophins bind to $p75^{NTR}$. NGF binds specifically to TrkA, BDNF and NT-4 bind to TrkB, and NT-3 binds to TrkC. Signalling mediated by Trk receptor preferentially promotes the cell survival, whereas signalling mediated by $p75^{NTR}$ can either facilitate Trk-mediated survival or, in the absence of Trk receptors, mediate apoptotic cell death (Roux and Barker 2002).

Structurally neurotrophins are classified to the cystine-knot cytokines (Structural Classification of Proteins; SCOP) (Murzin et al. 1995), and they function as homodimers. They are synthetized as precursors, pro-neurotrophins of 30-35 kDa. The pro-part is proteolytically cleaved resulting to a mature protein of 12-13 kDa (Lu et al. 2005). Both the pro- and mature forms of neurotrophins are active in signalling, although their biological effects can be opposite: proNGF and proBDNF signalling via $p75^{NTR}$ mediates apoptotic cell death whereas mature NGF and BDNF bind to Trk receptors and promote cell survival (Chao et al. 2006; Lee and Chao 2001; Lu et al. 2005). Thus, the biological activity of neurotrophins can be regulated by the cleavage of pro-region (Chao and Bothwell 2002).

Neurotrophins support various neuronal populations in the PNS and CNS (Huang and Reichardt 2001). As mentioned earlier, NGF promotes the survival of sympathetic and sensory neurons in the PNS. It also promotes the survival of cholinergic neurons in the basal forebrain, which presumably participate in memory functions that are affected in Alzheimer's disease (AD) (Chao et al. 2006). In adult, NGF has an essential role in pain mechanisms (Hefti et al. 2006).

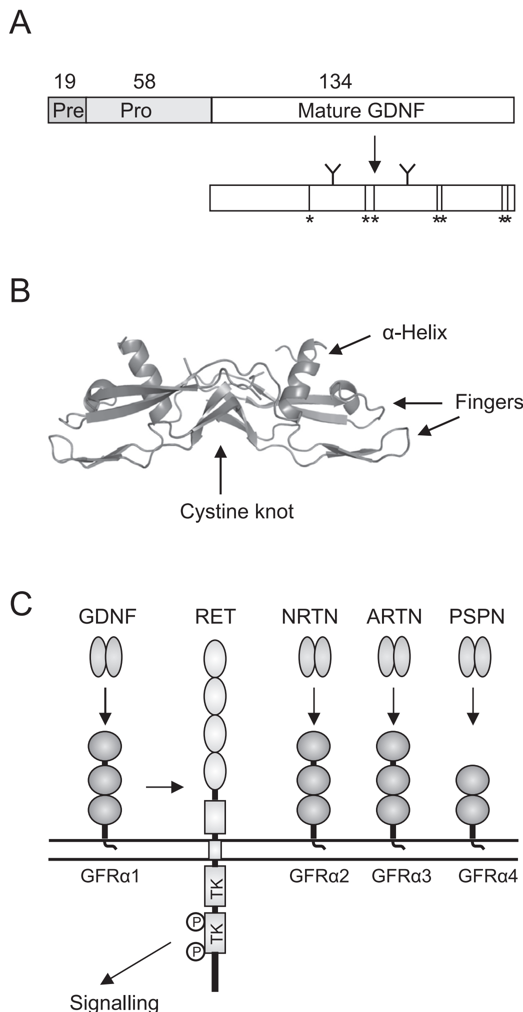
BDNF promotes the survival of peripheral sensory neurons (Huang and Reichardt 2001), and modulates synaptic plasticity of various brain neurons (Arancio and Chao 2007). Outside the nervous system, BDNF has a role in vascular development and angiogenesis after injury (Kermani and Hempstead 2007). NT-3 promotes the survival of sympathetic and sensory neurons and promotes the growth of sympathetic axons towards their targets (Glebova and Ginty 2005; Huang and Reichardt 2001).

BDNF was the first NTF described to promote the survival and dopamine uptake of embryonic midbrain dopaminergic neurons *in vitro* (Hyman et al. 1991; Knusel et al. 1991). Also NT-3 and NT-4 promote the survival of embryonic midbrain dopaminergic neurons in culture (Hyman et al. 1994; Hynes et al. 1994). BDNF and its receptor TrkB are expressed in the dopaminergic neurons in the SN (Numan and Seroogy 1999; Venero et al. 2000), and BDNF mRNA has been detected in the striatum, although at low level (Hofer et al. 1990). BDNF can be retrogradely transported by dopaminergic neurons from the striatum to SN (Mufson et al. 1994), which suggests a role for BDNF as a target-derived factor for nigral dopaminergic neurons. However, gene ablation studies in mice have not revealed an essential role for endogenous BDNF or TrkB on the survival and maturation of midbrain dopaminergic system. BDNF deficient mice, which die at age of ~21 days, do not have significant alterations in the nigrostriatal dopaminergic system (Conover et al. 1995; Ernfors et al. 1994; Jones et al. 1994). However, Baker and colleagues (Baker et al. 2005) reported that the number of dopaminergic dendrites was reduced postnatally in the SN of BDNF-deficient mice, although the number of dopaminergic neurons in the SN was not affected, suggesting that BDNF has a role in the phenotypic maturation of dopaminergic neurons. Conditional removal TrkB from the dopaminergic system in embryo did not affect the numbers of nigral dopaminergic neurons even at 2 years of age (Kramer et al. 2007), indicating a minor role for TrkB signalling in target innervation or maintenance of dopaminergic neurons in the SN. Differently from this, conditional removal of BDNF expression from the developing midbrain-hindbrain (MHB) region in embryonic mice resulted in early postnatal reduction of a subpopulation of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the SN, although the total number of nigral neurons was not significantly reduced (Baquet et al. 2005), suggesting that BDNF is required for the differentiation of dopaminergic phenotype. Although it now seems clear that BDNF is not an essential survival factor for midbrain dopaminergic neurons *in vivo*, its role in the dopaminergic system is not fully understood; thus, further studies with conditional mouse models are needed.

1.2.2. GDNF family ligands

GDNF is a potent neurotrophic factor for midbrain dopaminergic neurons. It was originally isolated from the culture medium of a rat B49 glial cell line based on its ability to promote dopamine uptake, survival and morphological differentiation of embryonic midbrain dopaminergic neurons in culture (Lin et al. 1993). Other members of GFLs include NRTN (Kotzbauer et al. 1996), artemin (ARTN) (Baloh et al. 1998) and persephin (PSPN) (Milbrandt et al. 1998). The homology of GFL amino acid sequences is 40-50% (Saarma 2000).

Structurally GFLs are related to TGFβ protein superfamily which belongs to cystine-knot cytokines (Murzin et al. 1995). Although the overall amino acid sequence homology between GDNF and the members of TGFβ superfamily is less than 20%, seven cysteine residues are conserved in their primary structure and determine the protein fold (Lin et al. 1993). The cysteines form three intramolecular and one intermolecular disulphide bonds (Eigenbrot and Gerber 1997; Ibáñez 1998). Similarly to neurotrophins, all members of the TGFβ family are functional as dimers (Sun and Davies 1995; Vitt et al. 2001).



GDNF family ligands are synthesized as prepro-proteins, from which the signal peptide (pre) is cleaved upon secretion (Fig. 2A). An enzymatic cleavage of the proGFL results in an active, mature protein, which is functional as a disulfide-bonded homodimer (Fig. 2B). Mature GDNF consists of 134 amino acids and contains two N (asparagine)-linked glycosylation sites. The molecular mass of an unglycosylated GDNF monomer is 16 kDa (Lin et al. 1993; Lin et al. 1994). GDNF, NRTN and ARTN are basic proteins which bind extracellular matrix heparan-sulphate proteoglycans; thus, their diffusion is limited in tissue (Airaksinen and Saarma 2002). The extracellular matrix may also serve as a depot for storage and slow release of GDNF (Lin et al. 1994) and other GFLs.

GFLs signal using transmembrane receptor tyrosine kinase RET and glycosyl-phosphatidylinositol (GPI) linked GDNF family alpha (GFRα) coreceptors, which determine the ligand specificity (Fig. 2C). Dimeric GFL binds first to a GFRα coreceptor on the cell membrane, and the complex induces subsequent dimerization and tyrosine phosphorylation of RET.

Figure 2. A) Schematic presentation of the primary structure of prepro-GDNF and mature GDNF. Seven conserved cyteines (*) and two glycosylation sites (Y) of mature GDNF are indicated. According to Saarma and Sariola (1999). **B**) GDNF homodimer (Eigenbrot and Gerber 1997). Finger-regions are formed by antiparallel β-strands connected by loop regions. **C**) GFL receptor system. Dimeric GFL binds to coreceptor GFRα, the GFL-GFRα complex dimerizes and activates RET. Phosphorylation (P) of tyrosine kinase domain (TK) of RET leads to the activation of intracellular signalling cascades. Preferred ligand-coreceptor pairs are indicated. According to Airaksinen and Saarma (2002).

This leads to the activation of intracellular signalling cascades (Airaksinen and Saarma 2002; Santoro et al. 2004) which induce subsequent changes in gene expression, protein synthesis and cellular response. Four coreceptors (GFRα1-4) have been identified, and each GFL binds preferentially one of them: GFRα1 is the main co-receptor for GDNF; GFRα2 for NRTN; GFRα3 for ARTN, and GRFα4 for PSPN, respectively (Airaksinen and Saarma 2002; Bespalov and Saarma 2007). The crystal structure of GDNF dimer bound to GFRα1, and ARTN in a complex with GFRα3 coreceptor has been recently determined (Parkash et al. 2008; Wang et al. 2006). An alternative receptor for GFLs, in addition to RET, is a neural cell adhesion molecule (NCAM) (Paratcha et al. 2003). RET receptor was originally identified as an oncogene, and activating mutations of RET are related to the development of cancer (Santoro et al. 2004). Inactivating mutations of RET induce the development of Hirschsprung disease characterized by aganglionic megacolon (Santoro et al. 2004).

In addition to the effects on midbrain dopaminergic neurons, GDNF affects several neuronal populations in the CNS and PNS (Airaksinen and Saarma 2002). GDNF promotes the survival of noradrenergic neurons in locus coeruleus (LC) (Arenas et al. 1995), and seems crucial for their maintenance in adult (Pascual et al. 2008). GDNF also promotes the survival of motoneurons (Henderson et al. 1994; Oppenheim et al. 1995), sensory (Henderson et al. 1994; Oppenheim et al. 1995; Yan et al. 1995), sympathetic (Trupp et al. 1995; Coulpier and Ibáñez 2004), and parasympathetic neurons (Buj-Bello et al. 1995; Hashino et al. 2001). GDNF has also important functions outside the nervous system. GDNF signalling is essential for migration and proliferation of enteric neuronal precursors (Cacalano et al. 1998; Enomoto et al. 1998; Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996), and for kidney morphogenesis, as GDNF knockout mice have severe renal agenesis at the time of birth (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996). GDNF is also important for spermatogonial renewal and differentiation (Meng et al. 2001).

Similarly with GDNF, NRTN is a potent survival factor for midbrain dopaminergic neurons *in vitro* and *in vivo* (Horger et al. 1998). It has protective and restorative effects on mDA neurons in animal models of PD which are comparable to those of GDNF. For example, intrastriatal NRTN given after striatal 6-OHDA lesion showed protective effects on mDA neurons (Rosenblad et al. 1999), and NRTN-secreting intranigral grafts protected against medial forebrain bundle (MFB) lesion induced by 6-OHDA (Åkerud et al. 1999) in rats. NRTN expressing adeno-associated virus serotype 2 (AAV2; CERE-120) induced functional recovery and preservation of mDA neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys (Kordower et al. 2006; Herzog et al. 2007), and has been tested in clinical trials (see 1.4.5). PSPN also promotes the survival of mDA neurons *in vitro* (Milbrandt et al. 1998). *In vivo*, intranigral PSPN protected against intrastriatal 6-OHDA lesion in rats (Milbrandt et al. 1998), and PSPN expressing intrastriatal grafts protected against striatal 6-OHDA lesion in mice (Åkerud et al. 2002). It is likely that NRTN and PSPN signalling in mDA neurons is mediated by GFRα1 instead of GFRα2 and GFRα4, respectively, since mDA neurons derived from GFRα1 knockout mouse embryos do

not respond to NRTN *in vitro* (Cacalano et al. 1998) and most of the *Gfra4* transcripts expressed in the central nervous system are not functional (Lindahl et al. 2000; Lindfors et al. 2006).

1.2.3. Effects of GDNF on midbrain dopaminergic neurons

Since GDNF was originally discovered as a trophic factor for midbrain dopaminergic neurons, its function on the midbrain dopaminergic system *in vivo* has been extensively studied. The administration of exogenous GDNF protein into adult rodent brain clearly affects the midbrain dopaminergic system by increasing locomotor activity (Hebert et al. 1996; Hoane et al. 1999; Hudson et al. 1995), TH expression (Hoffer et al. 1994; Hudson et al. 1995), dopamine levels and dopamine metabolism (Lapchak et al. 1997), and by inducing sprouting of dopaminergic neurons (Hudson et al. 1995). Negative effects related to the administration of exogenous GDNF have also been reported in rats, including decrease in the food consumption (Hudson et al. 1995), loss of body weight and allodynia (pain) (Hoane et al. 1999). Downregulation of TH expression and aberrant sprouting of dopaminergic fibers were seen after sustained lentiviral expression of GDNF in the striatum (Georgievska et al. 2002).

Whether GDNF is a target-derived neurotrophic factor for midbrain dopaminergic neurons is not clear (Andressoo and Saarma 2008; Krieglstein 2004). PCD of midbrain dopaminergic neurons occurs postnatally in two phases, peaking at the postnatal day 2 (P2) and P14 (Oo and Burke 1997). GDNF mRNA is expressed at higher levels in the early postnatal striatum as compared to that of adult (Choi-Lundberg and Bohn 1995; Schaar et al. 1993; Strömberg et al. 1993), which supports the potential role of GDNF as a target derived NTF. RET and GFRα1 are also expressed in the SN, where the dopaminergic cell bodies are located (Choi-Lundberg and Bohn 1995; Golden et al. 1998; Trupp et al. 1997; Widenfalk et al. 1997). Furthermore, intrastriatally injected GDNF is transported retrogradely from the adult rat striatum to SN (Tomac et al. 1995b), in consistent with the role of GDNF as a target-derived NTF.

GDNF knockout mice die at birth (Airaksinen and Saarma 2002); thus, only the prenatal development of these mice can be studied. At the time of birth, the survival of dopaminergic neurons in the SN and noradrenergic neurons in the LC is not affected in the GDNF deficient mice (Cacalano et al. 1998; Enomoto et al. 1998; Moore et al. 1996; Pichel et al. 1996; Treanor et al. 1996). Similarly with the GDNF deficient mice, RET or GFRα1 knockout mice, which also die at birth, do not have alterations in nigral dopaminergic neurons as compared to the wild type (Airaksinen and Saarma 2002). GDNF heterozygous mice are viable and fertile (Granholm et al. 2000). When aged, they show decline in motor performance and TH expression in nigral dopaminergic neurons, suggesting that GDNF has a role in the maintenance of midbrain dopaminergic system (Boger et al. 2006).

Recent studies with conditional mouse models of GDNF (Pascual et al. 2008) and RET (Kramer et al. 2007) indicate that GDNF is necessary for the maintenance of adult mouse dopaminergic neurons. When GDNF expression was suppressed ubiquitously in adult mice, a significant reduction in the number of dopaminergic neurons in the SN and ventral tegmental area (VTA) was observed at seven months later, and the noradrenergic neurons of LC

were almost absent (Pascual et al. 2008). The mice also showed a progressive hypokinetic syndrome. These results suggest that continuous supply of GDNF is essential for the survival of adult cathecolaminergic neurons (Pascual et al. 2008). In contrast, conditional embryonic inactivation of *Ret* in cathecolaminergic neurons did not cause significant defects in midbrain dopaminergic neurons in mice less than one year of age, as analyzed by two research groups (Jain et al. 2006, Kramer et al. 2007), suggesting that RET signalling is not crucial for the survival of mDA neurons during the early postnatal PCD periods. However, conditional removal of *Ret* caused progressive loss of nigral dopaminergic neurons in adult mice starting around one year of age, which was accompanied with the degeneration of striatal nerve terminals. In addition, significant gliosis and inflammation were deteced in the striatum and SN, respectively, in the aged mice (Kramer et al. 2007). Differently from the conditional ablation of GDNF in adult (Pascual et al. 2008), conditional inactivation of *Ret* did not cause marked defects in the dopaminergic neurons of the VTA or in the noradrenergic neurons of LC (Kramer et al. 2007). The milder phenotype of RET deficient mice (Kramer et al. 2007) as compared to the GDNF deficient mice (Pascual et al. 2008) suggests that the survival promoting signals of GDNF on dopaminergic neurons and noradrenergic neurons are mediated not only by RET but also by another receptor. An alternative possibility that could explain the differences between the above phenotypes is that the lack of RET signalling in embryonic dopaminergic neurons is compensated with other survival promoting signals independent of GDNF and RET. The difference may also be due to the different genetic background of the mice used. Finally, it is not completely excluded that the phenotype loss of GDNF deficient mice (Pascual et al. 2008) is unspecific due to high concentrations of tamoxifen used. It would be important to create comparable animal models for the current conditional GDNF and RET models (i.e. conditionally inactivate *Gdnf* in embryonic cathecolaminergic neurons, and ubiquitously inactivate *Ret* in adult mice) in order to clarify the significance of GDNF-RET signalling in the development and maintenance of midbrain dopaminergic system.

1.2.4. Other trophic factors for midbrain dopaminergic neurons

In addition to the GFLs and neurotrophins, several growth and trophic factors have neurotrophic effects on midbrain dopaminergic neurons, including the members of TGFβ family, FGF-2 and cytokines (Table 1). Cytokines are a large group of pleiotropic molecules, which include interleukins, tumour necrosis factor (TNF) family and hematopoietic cytokines (e.g. erythropoietin; EPO). Neuropoietic cytokines, one of the three main groups of neurotrophic factors, affect both neurons and hematopoietic cells. They function in inflammation and immune response, regulate neurogenesis and stem cell fate, and modulate synaptic plasticity and various behaviours (Bauer et al. 2007; Heinrich et al. 2003). Members of this group include interleukin-6 (IL-6), IL-11, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), oncostatin M (OSM), cardiotrophin-like cytokine (CLC) and B cell stimulating factor 3 (BSF3) (Heinrich et al. 2003). They have a characteristic four-helix bundle three-

dimensional structure, and they signal using glycoprotein (gp)130, LIF and OSM receptors. IL-6 and CNTF show trophic effects on midbrain dopaminergic neurons (Engele 1998; Hagg and Varon 1993) and CNTF on motoneurons in particular (Sendtner et al. 2000).

Some of the survival factors, including FGF-2 and bone morphogenetic proteins (BMPs), affect midbrain dopaminergic neurons indirectly by stimulating glial cells which likely synthetize and release trophic factors thus promoting neuronal survival (Knusel et al. 1990; Jordan et al. 1997). Different trophic factors may also function in a co-operative manner. TGFβ potentiates the survival promoting effects of GDNF on midbrain dopaminergic neurons *in vitro* (Krieglstein et al. 1998). However, TGFβ2 and GDNF double knockout mice dying at birth do not show defects in the midbrain dopaminergic system (Rahhal et al. 2009). Further studies are needed to elucidate the effects of different growth factors on the development and maintenance of midbrain dopaminergic neurons.

1.3. Midbrain dopaminergic system

Dopamine, a catecholamine, is a neurotransmitter of anatomically and functionally distinct neuronal groups in the CNS. The largest group of dopaminergic neurons is located in the ventral midbrain (mesencephalon) and is referred as midbrain (mesencephalic) dopaminergic neurons. These neurons form two major nuclei, the substantia nigra pars compacta (SNpc, A9) and ventral tegmental area (VTA, A10; Fig. 3A) (Prakash and Wurst 2006). Cell bodies in the SNpc project axons into dorsal striatum (caudate nucleus and putamen) forming the nigrostriatal pathway, which is important in controlling voluntary movements. Degeneration of this pathway is the major cause of PD (Dauer and Przedborski 2003). Neurons in the VTA send projections to the limbic areas (nucleus accumbens, amygdala, and olfactory tubercle) and prefrontal cortex forming the mesolimbic and mesocortical pathways, respectively. These pathways participate in the regulation of emotion-related behaviour such as motivation and reward. A third, smaller group of mDA neurons is located in the retrorubral field (A8), and is functionally related to the A10 group (Abeliovich and Hammond 2007; Prakash and Wurst 2006). Dysfunction of the dopaminergic limbic system is related to schizophrenia, depression and addiction (Van den Heuvel and Pasterkamp 2008). In humans, mDA cell groups consist of 400.000-600.000 neurons, of which over 70% is located in the SNpc. In rats, mDA neuronal groups consist of 40.000-45.000 cells, half of which is located in the SNpc (Björklund and Dunnett 2007).

The postmitotic precursors of mDA neurons are born in the ventricular-subventricular zone in the midbrain-hindbrain region around embryonic day 10.5 (E10.5) of mouse development (Fig. 3B) (Prakash and Wurst 2006). They migrate from the ventricular zone along radial glia to the ventral midbrain where SNpc and VTA are subsequently formed (Smits et al. 2006). The dopaminergic fate is determined by interplay between extracellular inductive signals and transcriptional programs of the cell. FGF8 secreted from the midbrain-hindbrain border (isthmus) and sonic hedgehog (SHH) secreted from the notochord are inductive signals which determine the permissive region where mDA progenitors are born (Smidt and Burbach 2007). Extracellular signalling of TGFβ is essential for the induction, and Wnt signalling is important in establishing

Table 1. Other growth factors with neurotrophic effects on midbrain dopaminergic neurons.

Factor	Symbol	Survival promoting effect *in vitro* on mDA neurons (Ref.)	Neuroprotective effects in PD models *in vivo*	Neurorestorative effects in PD models *in vivo*
Erythropoietin	EPO	Promotes differentiataion of mDA progenitors under hypoxia (Studer et al. 2000)	Intrastriatal EPO protected midbrain dopaminergic neurons against striatal 6-OHDA lesion in rats (Xue et al. 2007) and mice (Signore et al. 2006). EPO protected against MPTP toxicity in mice (Genc et al. 2001).	EPO delivered by HSV vector into striatum 2 weeks after the start of continual MPTP intoxication restored dopaminergic function (Puskovic et al. 2006). Intraventricular infusion of EPO started 30 min after striatal 6-OHDA lesion induced neuroprotective/rescue effects in rats (Kadota et al. 2009).
Vascular endothelial growth factor	VEGF*	(Silverman et al. 1999)	Encapsulated VEGF secreting cells in striatum protected against striatal 6-OHDA in rats (Yasuhara et al. 2004).	Encapsulated VEGF secreting cells in striatum implanted 1 or 2 weeks after striatal 6-OHDA lesion induced rescue effects in rats (Yasuhara et al. 2005).
Transforming growth factor beta	TGFβ	(Poulsen et al. 1994; Krieglstein and Unsicker 1994)	GDNF protective effect in mouse MPTP model requires TGFβ (Schober et al. 2007)	ND
Bone morphogenetic protein	BMP*	(Jordan et al. 1997)	Intranigral BMP7 protected against 6-OHDA MFB lesion in rats (Harvey et al. 2004).	ND
Growth and differentiation factor 5	GDF5*	(Krieglstein et al. 1995)	GDF5 injected into lateral ventricle and into SN immediately before 6-OHDA injection into MFB protected against lesion in rats (Sullivan et al. 1997).	GDF5 injected 1 week after 6-OHDA induced recovery in behaviour, but not GDF5 injected 2 weeks after 6-OHDA in rats (Hurley et al. 2004).
Macrophage-inhibitory cytokine-1	MIC-1 (GDF 15)	(Strelau et al. 2000)	GDF15 injected into ventricle and above SN protected against 6-OHDA injected at the same time into the MFB in rats (Strelau et al. 2000).	ND
Fibroblast growth factor 2	bFGF/ FGF-2*	(Engele and Bohn 1991)	FGF2 secreting fibroblast grafts in striatum protected against striatal 6-OHDA in rats (Shults et al. 2000).	Striatal bFGF protein treatment starting 6 months after administration of MPTP, and given every second week for 6 months, induced functional recovery in monkeys (Fontan et al. 2002)
Epidermal growth factor	EGF*	(Casper et al. 1991)	Continuous striatal infusion of EGF simultaneously with nigral 6-OHDA lesion, and continuing after lesion, induced protection in the striatum but not in nigra in rats (Iwakura et al. 2005).	ICV infusion of EGF started 35 days after unilateral mechanical transection of MFB induced functional recovery in rats (Pezzoli G. et al 1991)

Factor	Symbol	Survival promoting effect *in vitro* on mDA neurons (Ref.)	Neuroprotective effects in PD models *in vivo*	Neurorestorative effects in PD models *in vivo*
Heparin-binding EGF-like growth factor	HB-EGF*	(Farkas and Krieglstein 2002)	HB-EGF protein injected into the SN and lateral ventricle, protected from 6-OHDA lesion in MFB in adult rats (Hanke et al. 2004).	ND
Transforming growth factor alpha	TGFα*	(Alexi and Hefti 1993)	ND	Continuous infusion of TGFα into the ipsilateral striatum starting 2 weeks after 6-OHDA injection into the SN-VTA induced functional recovery with cell proliferation in rats (Fallon et al. 2000).
Platelet-derived growth factor, BB isoform	PDGF-BB*	(Nikkhah et al. 1993)	ND	Continuous ICV infusion of PDGF-BB starting 3 weeks after 6-OHDA MFB lesion increased cell proliferation in the striatum and SN (Mohapel et al. 2005). Continuous ICV administration of PDGF-BB starting 3 weeks after MPTP treatment induced recovery of dopaminergic function in monkeys (Zachrisson et al. 2008).
Insulin-like growth factor	IGF1* IGF2*	(Knusel et al. 1990)	ICV or peripheral IGF1 protected against 6-OHDA induced MFB lesion in rats (Quesada and Micevych 2004).	Lentiviral IGF1-expressing human neurospheres transplanted into striatum 7 days after intrastriatal 6-OHDA lesion induced functional recovery in rats (Ebert et al. 2008).
Ciliary neurotrophic factor	CNTF	(Magal et al. 1993)	CNTF infusion starting immediately after transection of nigrostriatal pathway prevented neuronal loss in rats (Hagg and Varon 1993).	ND
Interleukins	IL*-1β, -6, -7, -10	(von Coelln et al. 1995)	IL-1β infusion into SN and subsequent astrocytic activation protected nigral cell bodies but not striatal terminals from striatal 6-OHDA lesion in rats (Saura et al. 2003). AAV2-delivered intrastriatal IL-10 protected nigral neurons against striatal 6-OHDA (Johnston et al. 2008).	Polymer pellets containing slow-released IL-1 implanted into striatum 4 weeks after 6-OHDA lesion in SN induced behavioral recovery in rats (Wang et al. 1994).

*Glial mitogen; trophic effect is most probably mediated by glial cells. HSV, herpes simplex virus; ICV, intracerebroventricular; ND, not described.

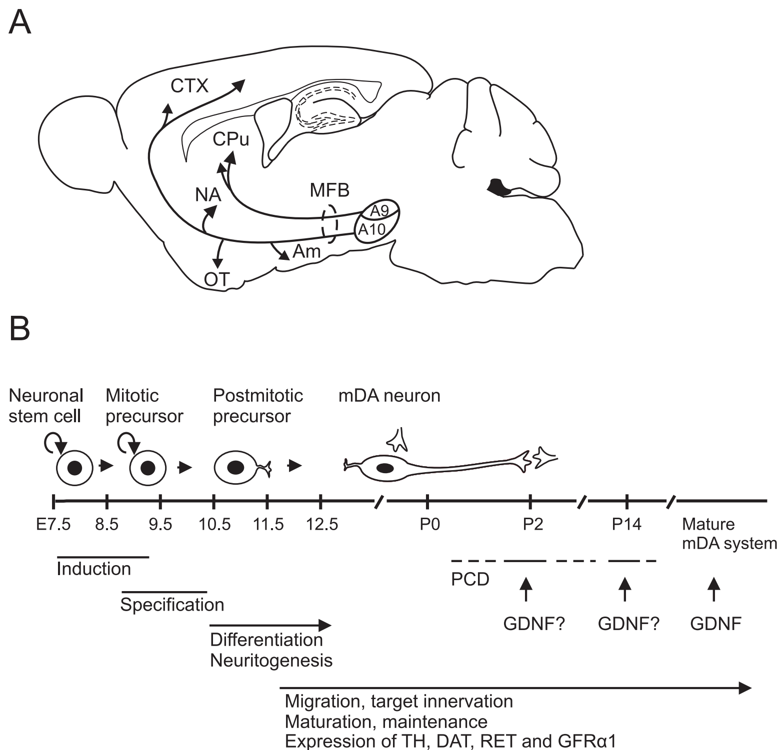


Figure 3. A) Schematic presentation of mesencephalic dopaminergic pathways in mouse brain. A9, substantia nigra; A10, ventral tegmental area. Am, amygdala; CTX prefrontal cortex; NA, nucleus accumbens; OT, olfactory tubercle. Figure modified from "The Mouse Brain in Stereotaxic Coordinates" by George Paxinos and Keith B. Franklin (2nd edition), copyright by Academic Press (2001). **B**) Development of mDA neurons (Abeliovich and Hammond 2007; Prakash and Wurst 2006). GDNF is a potential target-derived factor for mDA neurons, which may counteract early postnatal PCD. GDNF maintains mDA system in adult (Pascual et al. 2008).

midbrain-hindbrain region (Smidt and Burbach 2007). During differentiation of the postmitotic mDA neuron precursors, the transcription factors expressed include nuclear orphan receptor Nr4a2 (Nurr1), engrailed 1 and 2 (En1/2), LIM homeobox transcription factor Lmx1b and paired-like homeodomain transcription factor 3 (Pitx3) (Abeliovich and Hammond 2007; Smits et al. 2006). Nurr1 regulates the expression of proteins required for dopamine synthesis and transport, including TH, vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT) (Burbach and Smidt 2006). TH is the rate-limiting enzyme of dopamine biosynthesis, and used commonly as a marker for dopaminergic neurons. Expression of TH is induced at E11.5 (Burbach and Smidt 2006). The first mDA axons can be detected at E11 in mouse, and at E14.5 many of them have reached their targets (Abeliovich and Hammond 2007). At E18 striatal dopaminergic innervation is still partial, but completed by P4 in rodents (Burke 2004). Axon guidance molecules including ephrins, netrins and slits instruct the growth of dopaminergic axons

towards their targets (Van den Heuvel and Pasterkamp 2008).

As described earlier, developing neuronal populations undergo a regulated natural cell death which determines the final neuronal number in adults. The purpose of this developmental cell death is to remove excess of neurons and adjust neuronal numbers with size of the target, and to eliminate neurons with incorrect target connections (Oppenheim 1991). Whether the number of midbrain dopaminergic neurons is regulated by PCD during development is still uncertain. Burke and colleagues (Burke 2004; Jackson-Lewis et al. 2000; Oo and Burke 1997) have shown that mDA neurons in the SN undergo developmental apoptotic cell death. According to these studies, a natural cell death in the SNpc occurs postnatally in rats and mice in a biphasic manner. The first, major peak of death is readily after birth at P2, and the second, minor peak at P14 (Oo and Burke 1997; Jackson-Lewis et al. 2000). In contrast to these results, some evidence indicates that the number of mDA neurons is not regulated by apoptotic cell death in mice (Lieb et al. 1996). The rodent mDA system is mature around the third postnatal week (Prakash and Wurst 2006).

1.4. Parkinson's disease

PD is a gradually progressive neuro-degenerative disorder, which affects about 1-2% of population over 50 years of age (Thomas and Beal 2007). The cardinal motor symptoms of PD and other parkinsonian sydromes include resting tremor, rigidity, slowness of voluntary movement (hypokinesia) and postural instability (Dauer and Przedborski 2003). The main cause of motor symptoms is the degeneration of dopaminergic neurons in the SNpc. These neurons project axons to the putamen and nucleus caudatus (collectively known as striatum) forming the nigrostriatal pathway, which is degenerated in the disease. However, the neurodegeneration in PD is not restricted to the SNpc. Non-motor symptoms of PD include anosmia (inability to perceive smells) and dysfunction of the autonomic nervous system. Characteristic pathology at cellular level is the accumulation of intracytoplasmic protein aggregates called Lewy bodies, which contain misfolded protein α-synuclein and other proteins, including ubiquitin, proteasome subunits, heat-shock proteins and neurofilaments (Lotharius and Brundin 2002). As clinical symptoms appear, the disease is already highly progressed: about 60% of the dopaminergic cells in the SNpc are degenerated, and dopamine levels in the putamen are reduced by ~80% (Dauer and Przedborski 2003). The dopaminergic neurons of VTA are much less affected than those of SNpc (Dauer and Przedborski 2003). The majority of the PD cases are sporadic (idiopathic) whereas familial etiology is found in about 5-10% of patients.

Although the main pathological feature of PD is the degeneration of nigrostriatal dopaminergic pathway, other regions of the CNS are also affected, including LC and raphe nuclei. Locus coeruleus contains noradrenergic neurons and is located in the brainstem. Raphe nuclei are also located in the brainstem, and contain neurons using 5-hydroxytryptamine (5-HT; serotonin) as a neurotransmitter. According to some authors the disease may originate from nondopaminergic neurons (Braak et al. 2004). Braak and colleagues (Braak et al. 2003; Braak et al. 2004) suggest a scheme of six stages for the progression of PD pathology in the human brain. According

to this scheme, the sporadic PD starts from the nondopaminergic structures of lower brain stem or olfactory bulb, and progresses upward to the cerebral cortex. Lewy bodies or Lewy neurites are first detected in the dorsal motor nucleus of vagal nerve, and in the anterior olfactory structures. The first pathological changes are found in asymptomatic persons. As disease progresses, the Lewy pathology spreads gradually into the SN and amygdala, and the clinical symptomps become evident. At final stages of PD, the pathology progresses to the neocortex (Braak et al. 2004). Recently, Braak's scheme has been critically discussed (Burke et al. 2008); and evidently the relation between abnormal α-synuclein immunostaining in the human brain and the progression of PD needs further studies.

1.4.1. Mechanisms of dopaminergic neurodegeneration in PD

Why are the nigral dopaminergic neurons especially vulnerable? Current knowledge of the molecular mechanisms leading to the degeneration and death of midbrain dopaminergic neurons is relatively limited. Evidently aging is a major risk factor for neurodegenerative diseases, including PD. Molecular alterations which are seen in normal aging, including increased oxidative stress, damaged proteins and nucleic acids are amplified in the affected neuronal populations in neurodegenerative diseases (Mattson and Magnus 2006).

Oxidative stress resulting from the oxidation of dopamine is the major hypothesis explaining the endogenous vulnerability of mDA neurons (Ahlskog 2007). Dopamine metabolism creates reactive oxygen species (ROS), including superoxide anions, dopamine-quinones and hydroxyl radicals which create oxidative stress in the cell, by damaging proteins, lipids and DNA (Lotharius and Brundin 2002). Nigral dopaminergic neurons contain neuromelanin, which binds iron and may thus facilitate oxidation reactions (Ahlskog 2007). Subsequently, dopaminergic neurons need high antioxidant capacity for the protection against oxidative stress and cell death. Reduced levels of glutathione, an antioxidant, and high levels of oxyradical products have been found in the brains of PD patients (Dauer and Przedborski 2003).

Mitochondrial dysfunction may have a major role in the development of PD pathology (Mattson et al. 2008). Significantly reduced activity of mitochondrial respiratory chain complex I has been detected in the SN of PD patients (Schapira et al. 1989). Furthermore, deletions in the mitochondrial DNA (mtDNA) leading to functional deficiences of the respiratory chain are significantly increased in substantia nigra neurons of aged PD patients (Bender et al. 2006). MPTP toxin, which causes degeneration of nigrostriatal system and PD-like symptoms (see 1.4.2.) (Langston et al. 1984) selectively inhibits complex I function suggesting that complex I impairment has a major role in the disease. The inhibition of complex I leads to ROS production and oxidative stress, and induces further alterations in the mitochondria (Schapira 2008). Functional impairment of the ubiquitin proteasome system is also related to the PD pathology (Dauer and Przedborski 2003). Decreased activity of ubiquitin proteasome complex has been detected in the SN of PD patients (McNaught et al. 2003).

Apoptotic cell death and activation of caspases are found in PD pathology

(Vila and Przedborski 2003). Another typical feature of PD pathology is inflammation. Even in the normal brain, the density of microglial cells is high in the SN (Teismann et al. 2003). Activated microglia and astrocytes (Hirsch et al. 2005; Teismann et al. 2003) and increased levels of proinflammatory cytokines have been detected in the brain of PD patients (Hirsch et al. 2005). However, the role of inflammation in the disease process is unclear. Although usually considered as detrimental, gliosis may also have protective effects on mDA neurons. Glial cells may secrete trophic factors and protect against ROS (Teismann et al. 2003).

One possible cause for the development of PD and other neurodegenerative diseases is the shortage of neurotrophic factors in the brain. Decreased GDNF levels have been detected in the SN of PD patients (Chauhan et al. 2001) and also in the striatum and SN of aged rats (Yurek and Fletcher-Turner 2001). Also the levels of BDNF and NGF are reduced in the SN of PD patients (Howells et al. 2000; Mogi et al. 1999; Parain et al. 1999). However, direct link between the lack of neurotrophic factors or their receptors and the development of neurodegenerative diseases has not been found.

Since the nigrostriatal dopaminergic pathway is degenerated in sporadic as well as familial forms of PD, it is assumed that common molecular mechanisms function behind the both etiologies. Several (currently 13) genetic loci have been linked to PD, and mutations in α-synuclein, parkin, PINK1 (PTEN-induced putative kinase 1), DJ-1 and LRRK2 (leucine-rich repeat kinase 2) are clear genetic causes of PD (Thomas and Beal 2007). Alpha-synuclein is enriched in presynaptic nerve terminals, where it apparently has a role in vesicle recycling and dopamine neurotransmission (Moore et al. 2005). The protein is natively unstructured and prone to fibril formation and subsequent aggregation. However, the mechanism connecting fibrillar α-synuclein to the dysfunction and death of mDA neurons have not been resolved (Moore et al. 2005; von Bohlen und Halbach 2004). Parkin is an ER-associated E3 ubiquitin ligase (Imai et al. 2000), which targets misfolded proteins to the ubiquitin-proteasome pathway for degradation. The expression of Parkin is induced by ER stress, suggesting that Parkin has a role in the adaptation to ER stress (Kim et al. 2008). Parkin is also translocated to mitochondria where it seems to affect mitochondrial fission/fusion together with PINK1 (Van Laar and Berman 2009). DJ-1 is a mitochondrial protein which presumably functions as an antioxidant (Thomas and Beal 2007). LRRK2 is multidomain protein which has a role in synaptic vesicle recycling and in the functions of mitochondria, Golgi and lysosomes (Thomas and Beal 2007). In summary, the known genetic causes of PD suggest two main pathways for PD pathogenesis: 1) Mitochondrial dysfunction and oxidative stress, in which the antioxidant capacity of the cell is unable to neutralize ROS; 2) dysfunction of the ubiquitin proteasome system and impaired degradation of misfolded proteins. These pathways are presumably inter-related, which affects disease progression. Whether ER stress contributes to the PD progression or has a protective role against disease development is currently unclear (Kim et al. 2008).

1.4.2. Modelling of PD

Animal and cell culture models mimicking the PD pathology seen in humans are valuable tools to study molecular mechanisms behind the disease. Toxin-induced models are used to study the pathogenesis of PD at cellular and molecular level, *in vitro* and *in vivo*. The most commonly used toxins are 6-OHDA and MPTP (Table 2). They are used especially in preclinical studies of novel therapeutic agents. MPTP was discovered at early 1980's as a contaminant of a

Table 2. PD models in rodents.

Toxin models	**Reference**	**Cellular effects of the toxin**	**Toxin-induced phenotype**
6-OHDA (rat)	Jenner 2008; Ungerstedt and Arbuthnott 1970	ROS production, oxidative stress	Nigrostriatal DA neuron degeneration, motor dysfunction, no inclusion bodies
MPTP/MPP+ (mouse; used also in non-human primates)	Jenner 2008; Langston et al. 1983	Mitochondrial respiratory chain complex I inhibition	Nigrostriatal DA neuron degeneration, motor dysfunction, intracellular inclusions
Genetic mouse models	**Reference**	**Known function of the protein (cellular effects of mutation)**	**Mutation phenotype**
α-synuclein (human mutation A53T or A30P transgene)	Giasson et al. 2002; Lee et al. 2002	Associates with synaptic terminals, vesicle recycling	Motor dysfunction, *no clear degeneration of nigrostriatal DA neurons*, intracellular inclusions
DJ-1 knockout	Chen et al. 2005; Goldberg et al. 2005	Mitochondrial protein, antioxidant activity	Motor dysfunction, *no clear degeneration of nigrostriatal DA neurons*, elevated striatal DA and DA reuptake
Parkin knockout	Goldberg et al. 2003	E3 ubiquitin ligase	Motor dysfunction, *no clear degeneration of nigrostriatal DA neurons*, increased striatal DA content, reduced striatal neuron excitability
PINK1 knockout	Gautier et al. 2008; Kitada et al. 2007	Serine/threonine kinase, fission/fusion of mitochondria	*No clear degeneration of nigrostriatal DA neurons*, reduced striatal DA release, striatal synaptic plasticity impairments, impaired mitochondrial respiration in striatum
Pitx3 knockout (Aphakia)	Hwang et al. 2005	Homeodomain transcription factor, maturation of mDA neuron phenotype	Nigrostriatal DA neuron degeneration, motor dysfunction
Tfam conditional knockout in mDA neurons (MitoPark)	Ekstrand et al. 2007	Mitochondrial transcription factor (Reduced mtDNA expression, respiratory chain deficiency)	Nigrostriatal DA neuron degeneration, progressive motor dysfunction, intraneuronal inclusions

synthetic narcotic, and caused severe PD-like symptoms in drug users (Langston et al. 1983). MPTP damages the nigrostriatal dopaminergic pathway in a manner similar to that of seen in the PD patients, but without classical Lewy body formation (Dauer and Przedborski 2003). MPTP readily crosses the blood-brain barrier (BBB) and it is metabolized in astrocytes to toxic 1-methyl-4-phenylpyridinium (MPP+) which is subsequently released and taken up by dopaminergic neurons. MPP+ inhibits the complex I in the mitochondrial electron transport chain, which results in ROS production, oxidative stress, energy depletion, and apoptotic cell death. MPTP is used commonly in mice (since rats are less sensitive to it) and non-human primates to test novel treatment strategies for PD.

Exposure to some environmental toxins, including pesticide rotenone and herbicide paraquat, increases the risk of PD in humans. These toxins have also been used in modelling of PD *in vitro* and *in vivo* (Bove et al. 2005). Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is also used in a rodent model of PD (Gao et al. 2002; Herrera et al. 2000). Discovery of familial PD mutations has opened a way to develop genetic models of PD (Table 2). Deletion of mitochondrial transcription factor A (Tfam) in mDA neurons resulted in progressive PD symptomps in mice (Ekstrand et al. 2007). Interestingly, transgenic expression of mutant human LRRK2 caused Parkinsonism-like phenotype in *Drosophila* (Liu et al. 2008). Studies on the overexpression of mutated human α-synuclein in mice have resulted only mild, if any, effects on mDA neurons (Schneider, Zufferey et al. 2008).

1.4.3. 6-OHDA model of PD

6-OHDA is a structural analog of dopamine and noradrenaline, and it selectively destroys cathecolaminergic neurons. It is taken up by dopaminergic or noradrenaline transporters (DAT or NAT, respectively) into the cell. 6-OHDA can affect all cathecolamine neurons in the body, and systemic injection of 6-OHDA induces degeneration of sympathetic nerve endings (Blum et al. 2001). Since 6-OHDA does not cross BBB, it has to be injected directly into the brain to target cathecolaminergic pathways. The 6-OHDA lesion model of PD is commonly applied on rats. In order to target the nigrostriatal dopaminergic pathway, 6-OHDA can be injected either into the striatum, SN, or MFB (Fig. 3A). The specificity of 6-OHDA for dopaminergic neurons is increased with noradrenaline transport blockers, which inhibit 6-OHDA uptake by noradrenergic neurons (Deumens et al. 2002). The injection site affects severity of the lesion; injection into the MFB or SN induces pronounced destruction of dopaminergic neurons, whereas 6-OHDA injection into the striatum leads to more protracted degeneration (Deumens et al. 2002). After striatal injection, the axon terminals of mDA neurons are destroyed first, and the axonal degeneration gradually proceeds towards cell bodies in the SNpc. This retrograde degeneration may continue up to 8-16 weeks (Sauer and Oertel 1994). Usually 6-OHDA toxin is injected into the brain unilaterally, which creates dopamine imbalance between the hemispheres. The severity of the lesion can be estimated by measuring the rotational behaviour of rats after administration of drugs which stimulate either dopamine release (e.g. amphetamine; rotation towards the lesion), or dopamine receptor activity

(e.g. apomorphine; rotation away from the lesion) (Ungerstedt and Arbuthnott 1970; Deumens et al. 2002). Characteristic feature of PD, the formation of Lewy bodies, has not been reliably detected in the 6-OHDA model of PD (Bove et al. 2005).

The mechanism of 6-OHDA toxicity is not completely known. However, it is largely based on oxidative stress: ROS and quinones derived from 6-OHDA diminish the antioxidant capacity of the cell, resulting oxidative damage on proteins, lipids and DNA (Schober 2004). 6-OHDA injection also increases iron levels in the SN, which further induces generation of ROS (Blum et al. 2001). 6-OHDA has been shown to inhibit mitochondrial respiratory chain complex I suggesting that it impairs mitochondrial function in cells (Glinka and Youdim 1995), which creates ROS, reduces ATP levels and leads to cell death. Apoptosis markers including cleaved caspase-3 and condensed chromatin, are detected in cells treated with 6-OHDA. It has been shown that 6-OHDA induced cell death depends on *de novo* protein synthesis. Transcription factor CHOP (C/EBP homologous protein) has been shown to mediate 6-OHDA induced apoptosis *in vivo* (Silva et al. 2005). CHOP is upregulated in ER stress induced unfolded protein response (UPR; see 1.5.2.) and regulates expression of proapoptotic genes (Szegezdi et al. 2006b). 6-OHDA treatment upregulated genes involved in the UPR and ER stress, including CHOP, in PC12 cells (Ryu et al. 2002) and in MN9D cells (Holtz and O'Malley 2003). However, the role of UPR and ER stress in the 6-OHDA toxicity *in vivo* is still unclear (Silva et al. 2005). 6-OHDA injection also induces gliosis and astrocytic activation (Bove et al. 2005; Henning et al. 2008).

6-OHDA model is commonly used in preclinical studies to test the potency of novel molecules for treatment of PD. When designing an experimental setup for 6-OHDA rat model of PD, several aspects have to be considered, including the site of 6-OHDA injection (striatum, SN or MFB), the site of protein delivery (striatum, SN or ventricle), and the method of protein delivery (single/multiple injection(s), continuous infusion by pumps, protein secreting cell grafts or viral vectors). Neuroprotective effects of a trophic factor can be studied by injecting the factor before the toxin. More challenging is the experimental setup of "neurorestoration" in which the lesion is induced first and the trophic factor injected afterwards. The latter model resembles more closely to PD, in which the neurodegeneration is severe before the clinical symptoms appear and treatment is started. Several trophic factors have neuroprotective effects in PD models (Table 1), whereas only GDNF and NRTN have well-established neurorestorative effects on mDA neurons in PD models shown by several independent research groups.

1.4.4. GDNF is protective and restorative in PD models

Neurotrophic activity of GDNF has been intensively studied in rodent and primate models of PD. These studies have proved the potency of GDNF as a neurotrophic factor for midbrain dopaminergic neurons. One of the first studies showed the neuroprotective and neurorestorative effects of GDNF on adult nigrostriatal dopaminergic system in mouse MPTP model of PD (Tomac et al. 1995a). GDNF injected into the striatum or SN before MPTP or into the striatum after MPTP was able to increase dopamine levels, TH-positive fibre densities and improve motor behaviour (Tomac et al. 1995a). GDNF injected into the SN was unable

to induce sprouting of dopaminergic fibers in the striatum when given after MPTP suggesting a target-derived role for GDNF (Tomac et al. 1995a). GDNF injections into the SN also protected midbrain dopaminergic neurons from MFB axotomy-induced degeneration in rats (Beck et al. 1995).

GDNF has significant neuroprotective and neurorestorative effects against striatal 6-OHDA lesion in rats, as shown by several studies. Striatally injected GDNF induced functional recovery of the midbrain dopaminergic system and sprouting of dopaminergic fibers after striatally injected 6-OHDA (Aoi et al. 2000; Rosenblad et al. 1998). Combination of intraventricular and intrastriatal GDNF, or intraventricular GDNF alone induced protection and restoration of the mDA system when given after striatal 6-OHDA lesion in rats (Kirik et al. 2001; Rosenblad et al. 1999). Continuous infusion of GDNF over the SN prevented nigral death and atrophy induced by intrastriatal 6-OHDA (Rosenblad et al. 2000; Sauer et al. 1995). However, GDNF injected close to the SN did not preserve dopaminergic innervation in the striatum, nor induced functional recovery after intrastriatal 6-OHDA (Sauer et al. 1995; Winkler et al. 1996).

GDNF shows protective and restorative activities on mDA neurons also in PD models in which 6-OHDA is given into the MFB or SN. GDNF was neuroprotective when it was given into the SN before nigral 6-OHDA (Kearns et al. 1997), or when it was given into the SN and lateral ventricle before 6-OHDA injection into the MFB (Sullivan et al. 1998). Consistently, GDNF-secreting intranigral grafts were protective against 6-OHDA injection into MFB (Åkerud et al. 1999). GDNF induced functional recovery of nigrostriatal system when it was delivered into the SN after 6-OHDA lesion in the MFB (Bowenkamp et al. 1995; Hoffer et al. 1994), or after 6-OHDA lesion in the SN (Kearns and Gash 1995).

The potency of GDNF has also been shown in unilateral and bilateral MPTP models of PD in non-human primates (Costa et al. 2001; Gash et al. 2005; Gash et al. 1996; Grondin et al. 2002; Zhang et al. 1997). GDNF delivered either intranigral, intracaudal or intracerebroventricular (ICV) route induced functional recovery of MPTP induced hemiparkinsonism in monkeys (Gash et al. 1996; Zhang et al. 1997; Gash et al. 2005).

1.4.5. Neurotrophic factors as therapeutic proteins for PD

Currently, no PD therapies exist that could stop the degeneration of dopaminergic neurons or induce neuronal recovery. All therapies available are directed to alleviate the disease symptoms, usually by correcting dopamine neurotransmission in the striatum. Levodopa (3,4-dihydroxy-L-phenylalanine), an intermediate of dopamine biosynthesis, is widely used (Lewitt 2008), but it usually causes severe side effects after prolonged use. Thus, novel treatment strategies are needed. Since neurotrophic factors have many beneficial effects on neurons, they may be useful in the treatment of neurodegenerative diseases, including PD, AD, amyotrophic lateral sclerosis (ALS) and spinal cord injury. Although lack of NTFs have not been shown to directly cause neurodegenerative diseases, the levels of NTFs are decreased in neurodegeneration (Chao et al. 2006). Thus, delivery of neurotrophic factors may help to resore the NTF deficiency in disease. Also the synergistic effects of neurotrophic factors may have clinical relevance.

Method of delivery is problematic in the NTF therapy, as proteins do not cross BBB. Different delivery methods, including gene therapy by viral vectors or direct protein infusion are under investigation. Only two NTFs, GDNF and NRTN have been taken to clinical trials on PD patients. However, results from these trials have been somewhat disappointing. Intracerebroventricular infusion of GDNF created side-effects, and did not improve Parkinsonism (Nutt et al. 2003). The infused GDNF did apparently not reach the targets SN and putamen (Kordower et al. 1999; Nutt et al. 2003). In contrast, direct infusion of GDNF into the putamen of PD patients led to significant clinical improvement without serious side-effects (Gill et al. 2003; Patel et al. 2005; Slevin et al. 2005). A post-mortem study of one patient showed sprouting of dopaminergic fibers induced by intraputamenal GDNF (Love et al. 2005). Differently from these encouraging phase I results, a double blind phase II study showed low clinical efficacy of GDNF, and formation of GDNF function blocking antibodies (Lang et al. 2006). The clinical trials with GDNF are currently interrupted.

Although phase I clinical trial of NRTN gene therapy on PD patients using AAV2-vector based delivery into the putamen showed safety, tolerability and potential efficacy (Marks et al. 2008), recently completed phase II study did not show difference between patients treated with CERE-120 and the control group (Ceregene Inc., press release on November 26th, 2008). Thus, it is important to search and study novel neurotrophic factors for mDA neurons that could also be applied to clinic.

1.5. Mesencephalic astrocyte-derived neurotrophic factor (MANF)

1.5.1. MANF is a survival factor for midbrain dopaminergic neurons

The first report of human arginine rich protein (*ARP*) gene described it as an oncogene mutated in renal cell carcinomas (Shridhar et al. 1996b). *ARP* (or *ARMET*; arginine-rich, mutated in early stage tumors) was later shown to encode protein with neurotrophic effects, and the protein was re-named as mesencephalic astrocyte-derived neurotrophic factor (Petrova et al. 2003).

MANF was originally identified from the culture medium of rat type-1 astrocyte ventral mesencephalic cell line 1 (VMCL1) based on its trophic effects on cultured embryonic dopaminergic neurons (Petrova et al. 2003). Previously it was shown that the culture medium of VMCL1 cells supported the survival and promoted the neurite outgrowth of embryonic mesencephalic dopamine neurons in culture (Panchision et al. 1998). After sequential purification steps, the survival promoting, active rat protein was isolated and found homologous to the predicted human ARP protein of 234 amino acids with an amino-terminal arginine rich region (NP_006001) (Shridhar et al. 1996b). ARP protein had never been expressed *in vitro* or purified from tissue. According to the report by Petrova et al. (2003), the amino acid number 56 of human ARP equals to the initiation methionine of human MANF. Thus, human MANF consists of 179 amino acids (amino acids 56-234) and lacks the arginine-rich sequence (amino acids 1-55) of human ARP. The amino-terminal arginine-tract of ARP is apparently not synthesized *in vivo* (Petrova et al. 2003). Thus, ARP or

ARMET name is misleading, and we prefer the name MANF. MANF protein is highly conserved from *C. elegans* and *D. melanogaster* to human with eight conserved cysteine residues of similar spacing (Petrova et al. 2003; Shridhar et al. 1996b).

Human *MANF* (*ARMET, ARP*) gene is located in the short arm of chromosome 3 (3p21.1) (Shridhar et al. 1996b). Several reports associated mutations in the arginine-rich region of *ARP* with different forms of cancer (Shridhar et al. 1996a; Shridhar et al. 1996b; Shridhar et al. 1997). However, these have been contrasted by other studies showing that the variations in the arginine-rich region represent normal polymorphisms (Evron et al. 1997; Piepoli et al. 2006; Tanaka et al. 2000).

According to Petrova et al. (2003) human MANF is a 20 kDa, glycosylated (sialylated) protein. Recombinant human MANF promoted the survival and induced sprouting of E14 rat dopaminergic neurons in culture (Petrova et al. 2003). The authors reported that the survival promoting activity of MANF was specific on dopaminergic neurons versus GABAergic or serotonergic neurons present in the embryonic mesencephalic cultures, although MANF also increased general viability of cultured mesencephalic neurons. The authors suggested that MANF can maintain TH expression in dopaminergic neurons but not the expression of glutamate decarboxylase (GAD) or tryptophan hydroxylase (TPH) in GABAergic or serotonergic neurons, respectively. MANF also increased the survival of cultured dorsal root ganglion (DRG) neurons, but not the survival of sympathetic or cerebellar granule neurons (Petrova et al. 2003). Extracellularly applied MANF was reported to enhance the frequency of $GABA_A$-receptor mediated inhibitory post-synaptic currents in dissociated postnatal rat dopaminergic neurons and in midbrain slices (Zhou et al. 2006). Thus, GABA-mediated inhibitory effect on neuronal firing may contribute the neuroprotective action of MANF on dopaminergic neurons (Zhou et al. 2006).

1.5.2. MANF is induced by ER stress and protects against ischemic cell death

Recent studies indicate that MANF is an endoplasmic reticulum (ER) stress response protein (Apostolou et al. 2008; Lee et al. 2003; Mizobuchi et al. 2007; Tadimalla et al. 2008). Accumulation of unfolded proteins in the ER causes ER stress and the induction unfolded protein response (UPR), which is a signal-transduction pathway that counteracts ER stress (Fig. 4) (Szegezdi et al. 2006b). By UPR, the protein folding capacity and degradation of misfolded proteins is increased in the cell, and protein translocation across the ER membrane is reduced. Molecular chaperones function to refold proteins, and ER associated protein degradation (ERAD) removes misfolded proteins. Three main UPR pathways are mediated by the ER transmembrane receptors inositol-requiring enzyme 1 (IRE1), pancreatic ER kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Szegezdi et al. 2006b). Also the transcription factor X-box binding protein 1 (XBP1) regulates UPR target gene expression (Szegezdi et al. 2006b).

Microarray analysis of mouse embryonic fibroblasts treated with tunicamycin, an inhibitor of N-linked glycosylation and ER stress agent, revealed *Armet* (*Manf*) as an ER stress response gene (Lee et al. 2003). *Armet*

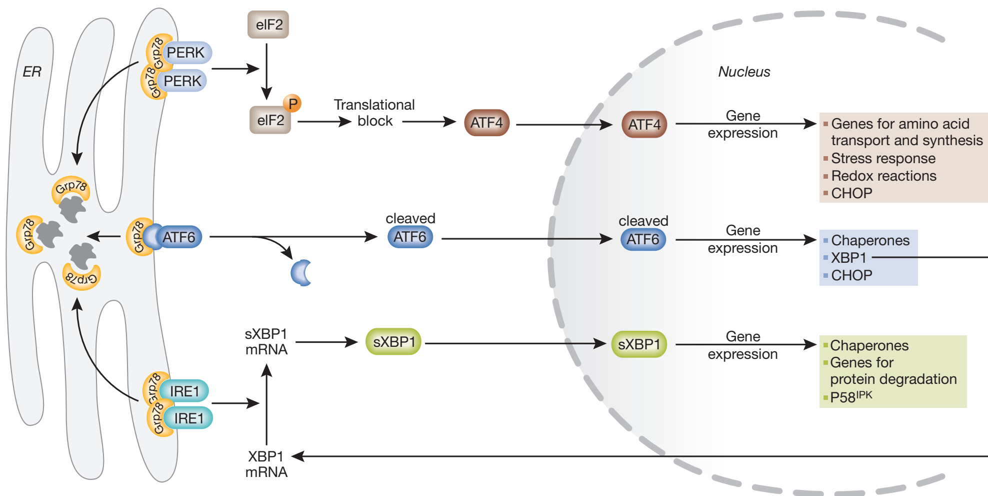


Figure 4. Activation of the UPR. Transmembrane receptors PERK, ATF6 and IRE1 are normally associated with ER chaperone Grp78 (glucose regulated protein 78; BiP). Accumulation of unfolded proteins dissociates Grp78, and triggers UPR. Activated PERK phosphorylates eukaryotic initiation factor 2 (eIF2), which blocks protein translation. Phosphorylation eIF2 enables translation of transcription factor ATF4. ATF4 induces transcription of genes required for the restoration of ER balance. Activated ATF6 regulates expression of ER chaperones and XBP1. XBP1 mRNA is spliced by IRE1. Spliced XBP1 protein (sXBP1) controls transcription of chaperones and genes involved in protein degradation. Reprinted by permission from Macmillan Publishers Ltd: EMBO reports (Szegezdi et al.) copyright (2006).

expression was upregulated in wild-type cells but not in XBP1-/- cells when ATF6 was knocked down by small interfering RNA (siRNA), indicating that *Armet* expression requires either XBP1 or ATF6. Thus, *Armet* may have a role in the quality control of proteins during ER stress (Lee et al. 2003). In consistence, another microarray study reported upregulation of *Armet* by ATF6 expression in the heart of ATF6 transgenic mouse (Belmont et al. 2008). *Armet* was also upregulated in pancreatic β cells of insulin-Akita mouse (Mizobuchi et al. 2007). These cells undergo continuous ER stress caused by the accumulation of unfolded proinsulin. The expression of *Armet* was upregulated by ER stress response element-II (ERSE-II) located in the *Armet* promoter (Mizobuchi et al. 2007). Transcription factors XBP1, ATF6, CBF/NF-Y and luman/CREB3 are known to bind ERSE-II (Liang et al. 2006; Yamamoto et al. 2004) and may thus regulate *Armet* expression (Mizobuchi et al. 2007). *Armet* was also upregulated in human osteosarcoma U2OS and embryonic kidney HEK293 cells by different UPR inducers (Apostolou et al. 2008). Ischemia is known to induce ER stress response (Szegezdi et al. 2006a). In line, hypoxia induced upregulation of *Manf* in HeLa cells (Romero-Ramirez et al. 2004), and *Manf* was also upregulated in the mouse myocardium *in vivo* after myocardial infarction (Harpster et al. 2006; Tadimalla et al. 2008).

Knockdown of *Armet* expression by siRNA increased the susceptibility of HeLa cells to tunicamycin-induced death (Apostolou et al. 2008). It also induced

expression of UPR response factors, which was further aggravated by tunicamycin treatment. In line, overexpression of *Armet* in U2OS osteosarcoma cells made the cells more resistant to tunicamycin-induced death, supporting the role of ARMET as a protective protein against ER stress-induced cell death (Apostolou et al. 2008). Importantly, addition of recombinant MANF protein to the culture medium of cardiac myocytes prevented the simulated ischemia induced cell death *in vitro* (Tadimalla et al. 2008). Furthermore, MANF protein was recently shown to protect against ischemic brain injury induced by middle cerebral artery occlusion (MCAO) in rats *in vivo* (Airavaara et al. 2009). The proposed protective role of MANF in ER stress and in relation to our data is discussed in more detail in the Discussion.

1.6. Saposin-like proteins (SAPLIPs)

1.6.1. SAPLIPs have diverse functions

The family of saposin-like proteins is defined by a characteristic three-dimensional fold, which consists of four to five α-helices connected by three disulphide bridges in a globular conformation (Fig. 5B). The disulphide bridges are formed by six cystine residues, spacing of which is conserved in the SAPLIP primary structure, making the saposin-fold highly stable (Bruhn 2005). SAPLIPs are highly variable in amino acid sequences with identities below 25-30%. The group is named according to the sphingolipid-activator proteins (saposins) A-D which function as co-factors of glycosphingolipid (GSL) degradation by GSL hydrolases in the endosomes and lysosomes. A common feature of SAPLIPs is their ability to interact with membranes or lipids (Bruhn 2005).

SAPLIPs are found from primitive amoebazoans to mammals, and they exert various functions. Mammalian NK-lysin (Andersson et al. 1995) and human granulysin (Jongstra et al. 1987; Stenger et al. 1998) are produced by natural killer (NK) cells and cytotoxic T-lymphocytes, and function as antimicrobial proteins. *Entamoeba histolytica*, a protozoan parasite, synthesizes antimicrobial saposin-like amoebapores (Keller et al. 1989). Amoebapores form ion channels on the membrane of a target cell by oligomerization, which leads to cytolysis and death (Hecht et al. 2004; Zhai and Saier 2000). Surfactant protein B (SP-B), a water insoluble and dimeric SAPLIP, reduces surface tension in the air/liquid interface in alveolar ducts (Cochrane and Revak 1991).

Some of the SAPLIPs, including prosaposin (PSAP) and saposin C (SapC; see 1.6.2. for more details) have also neurotrophic activities. Overexpression of ER-resident MSAP (myosin regulatory light chain (MRLC)-interacting (MIR)-interacting saposin-like protein; Cnpy2) induced neurite outgrowth in neuroblastoma and phaeochromocytoma cell lines (Bornhauser et al. 2003) and migration of glioma cells *in vitro* (Bornhauser and Lindholm 2005). Canopy1, a zebrafish SAPLIP and an ER resident protein, regulates FGF signalling in the midbrain-hindbrain boundary (MHB) during embryonic development and interacts with a fibroblast growth factor receptor 1 (FGFR1) (Hirate and Okamoto 2006).

Although most of the SAPLIPs are single-domain proteins, a saposin-like domain may also belong to a multidomain protein. Leukocytic acyloxyacyl hydrolase

(AOAH), a two-subunit lipase protein, cleaves acyl chains of bacterial LPS, thus reducing LPS toxicity. The amino (N)-terminal saposin-like domain of AOAH contributes to intracellular targeting, LPS recognition and catalytic function of AOAH. Acid sphingomyelinase (ASM) participates in lysosomal sphingolipid degradation (Ponting 1994), and its saposin-like domain stimulates the enzymatic activity and stabilizes protein structure (Kolzer et al. 2004). Saposin-like domain (swaposin) is also found in prophytepsin, a plant aspartic protease (Kervinen et al. 1999).

1.6.2. Sphingolipid activator proteins (saposins)

Saposins A, B, C and D (SapA, B, C and D) are non-enzymatic proteins which function as co-factors of sphingolipid degradation in the lysosomes (Kishimoto et al. 1992). They are small (~80 aa; 9 kDa), heat-stable glycoproteins, and ubiquitously expressed, with high levels in the spleen, liver and kidney (Kolter and Sandhoff 2005). Each of the saposins A-D promotes the degradation of particular GSLs. Functional deficiencies of SapA-D lead to sphingolipid storage diseases characterized by accumulation of lipid aggregates within the lysosomes. Neurological defects are usually related to these diseases (Sun et al. 2008; Kolter and Sandhoff 2005). In the endocytic pathway, saposins facilitate the association of lipid antigens to cluster of differentiation 1 (CD1) molecules, which present antigens to natural killer T cells (Winau et al. 2004; Yuan et al. 2007; Zhou et al. 2004). Sap-B has been shown to interact with coenzyme Q10, a component of mitochondrial respiratory chain and an antioxidant (Jin et al. 2008).

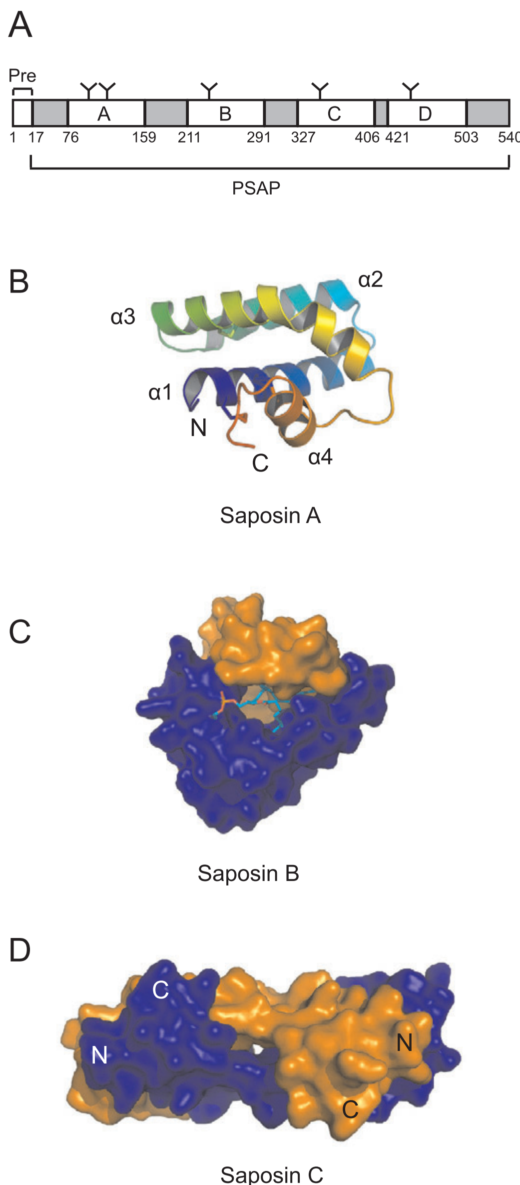


Figure 5. A) Scematic presentation of prosaposin, from which saposins A-D are processed. Signal sequence (pre) and glycosylation sites (Y) are indicated. **B)** Saposin A monomer (Ahn et al. 2006). **C)** Saposin B homodimer with phospholipid (Ahn et al. 2003). **D)** Saposin C homodimer at pH 4 as a domain-swapped dimer (Rossmann et al. 2008).

Saposins A-D are synthetized as a precursor protein, prosaposin (PSAP), from which they are proteolytically cleaved in late endosomes or lysosomes (Fig. 5A) (Kishimoto et al. 1992). PSAP-deficient mice die either shortly after birth or survive for about five weeks (Fujita et al. 1996), accumulate glycosphingolipids in cells and show complex neurodegeneration (Fujita et al. 1996). PSAP is secreted into body fluids, including cerebrospinal fluid and seminal plasma (Kishimoto et al. 1992).

Neurotrophic activity of PSAP and SapC has been mainly studied by O'Brien and colleagues. According to these studies, PSAP and SapC increased neurite outgrowth in human neuroblastoma NS20Y cells *in vitro* (O'Brien et al. 1994). PSAP also promoted the survival of cultured hippocampal neurons and the regeneration of sciatic nerve after transection (Kotani et al. 1996a; Kotani et al. 1996b). The neurotrophic activity of PSAP was located into the amino-terminal domain of SapC (Campana et al. 1996; O'Brien et al. 1994; O'Brien et al. 1995; Qi et al. 1996), and a corresponding peptide derived from SapC was shown to rescue dopaminergic cells in the SN when given subcutaneously after MPTP in a mouse model of PD (Liu et al. 2001). This peptide also prevented ischemia-induced learning disability and hippocampal neuronal loss *in vivo* in gerbils (Sano et al. 1994). More recent study shows anti-apoptotic activity of SapC that is mediated by PI3K (phosphatidylinositol 3'-kinase)/Akt (protein kinase B) pathway in prostate cancer cells *in vitro* (Lee et al. 2004).

The putative receptor and signalling pathways of PSAP and SapA-D still remain unclear. Extracellular PSAP can be endocytosed by low-density lipoprotein receptor-related protein (LRP) or by mannose-6-receptor (M-6-R) in murine embryonic fibroblasts or taken up by LRP or M-6-R from bloodstream to the liver *in vivo* (Hiesberger et al. 1998). O'Brien and colleagues presented evidence that receptor for PSAP is G-protein coupled (Hiraiwa et al. 1997), and PSAP associates with gangliosides on neuroblastoma cell membranes *in vitro* (Misasi et al. 1998). Recently, PSAP signalling was reported to occur through lipid rafts *in vitro* (Sorice et al. 2008).

1.6.3. NK-lysin and granulysin

NK-lysin and granulysin are antimicrobial defence proteins of innate immune system which have a saposin-like three-dimensional fold. NK-lysin was originally isolated from porcine small intestine based on its antibacterial activity (Andersson et al. 1995). NK-lysin is also cytotoxic against fungal cells and tumour cell lines (Andersson et al. 1995). Human granulysin is structurally and functionally related to porcine NK-lysin, although identity between the amino acid sequences of these two proteins is only 35% (Peña and Krensky 1997). Porcine NK-lysin and human granulysin are basic proteins of 78 and 74 amino acids, respectively. Granulysin is synthetized as a precursor of 15 kDa which is secreted and processed into 9 kDa form in the acidic environment of cytolytic granules (Hanson et al. 1999). Mammalian homologues to NK-lysin have been characterized from equine (Davis et al. 2005) and bovine (Endsley et al. 2004), whereas mouse does not have an orthologue (Bruhn 2005).

Expression of the porcine NK-lysin mRNA is induced by interleukin-2 (IL-2) treatment in T lymphocytes and NK cells, and the protein is usually localized in

intracellular granules (Andersson et al. 1995). Also the bone marrow and spleen, which are known to contain high amounts of lymphocytes, express NK-lysin mRNA (Andersson et al. 1995). Similarly with NK-lysin, human granulysin is expressed in NK cells and cytolytic T lymphocytes. Granulysin mRNA expression starts 3-5 d after T cell activation (Jongstra et al. 1987). The protein is located in the cytolytic granules which are exocytosed upon antigenic stimulation of T cells. Granulysin exerts antimicrobial activity against a broad range of Gram-negative and Gram-positive bacteria including *Mycobacterium tuberculosis*, fungi and parasites (Stenger et al. 1998). *In vitro* studies using human T lymphocyte Jurkat cell line have shown that membrane disruption by granulysin results to ion fluxes, ceramide generation and subsequent apoptosis, which is mediated by cytochrome c release and activation of caspases (Krensky and Clayberger 2005). A secreted granulysin precursor is also active: it induces apoptosis of keratinocytes and mediates epidermal necrosis related to adverse drug reactions in humans (Chung et al. 2008).

1.6.4. Membrane interactions of SAPLIPs

SAPLIPs are usually found in a closed conformation with a small hydrophobic core. However, structural studies indicate that conformational changes and oligomerization are important for the membrane interaction and function of SAPLIPs (Bruhn 2005). For example, oligomerization may facilitate pore formation or membrane fusion. Membrane interactions of SAPLIPs are usually pH-dependent.

NMR structure of NK-lysin was the first SAPLIP structure resolved (see Fig. 9E in Results) (Liepinsh et al. 1997). In this structure, the five amphiphatic α-helices form a single globular domain. The authors suggest that the positions of α-helices may change in membrane interactions (Liepinsh et al. 1997). Positively charged side chains of arginine and lysine residues form an equatorial belt around NK-lysin molecule, which may interact with negatively charged membrane lipid head groups (Liepinsh et al. 1997). Similarly with NK-lysin, the crystal structure of human granulysin revealed a saposin-fold (Fig. 9C) (Anderson et al. 2003). Granulysin contains five cysteines, and two disulfide bonds connecting helices 1 and 5, and helices 2 and 3, respectively. Positively charged residues distribute mostly as a ring around the molecule, and the charge distribution steers the orientation of granulysin relative to the membrane. Lytic process induced by granulysin may involve motion of the helical regions as “scissors” which would expose the hydrophobic inner surfaces of granulysin to the membrane (Anderson et al. 2003).

Altough saposins are usually monomeric, saposin B crystallized as a homodimer with a bound phospholipid (Fig. 5C) (Ahn et al. 2003). In the shell-like SapB dimer, the monomers adopted an open V-shaped conformation and a hydrophobic cavity was located between the monomers. The authors suggest that the conformational change in the SapB dimer facilitates lipid access to the inner cavity and is important for the lipid extraction from membranes (Ahn et al. 2003). SapB is a lipid-transport protein (Vogel et al. 1991), that does apparently not bind membranes directly, but extracts

GSLs from membranes and forms a soluble complex, which is subsequently recognized by the lipid degrading enzyme (Vaccaro et al. 1999).

Resembling the SapB dimer structure, significant conformational change in the NMR structure of monomeric SapC was detected in the presence of detergent sodium dodecyl sulfate (SDS) (Hawkins et al. 2005). In the absence of SDS, SapC is in closed conformation (de Alba et al. 2003). When binding to SDS, the SapC monomer opens to a U-shaped tertiary structure, in which the hydrophobic pocket is exposed to solvent. The hydrophobic pocket is likely to contact with membrane lipids (Hawkins et al. 2005).

Saposins function in the acidic environment of pH 4.0-5.5 in lysosomes. It has been shown that pH affects the conformation and activity of saposins. SapC participates in vesicle destabilization and fusion in a pH-dependent manner (Vaccaro et al. 1994). At acidic pH, the hydrophobicity of SapA, SapC, and SapD are increased, which promotes their interaction with membranes (Vaccaro et al. 1995). Saposins A and C are monomeric at neutral pH 7.0, whereas SapA forms dimers and SapC trimers at acidic pH 4.8, in the presence of polyethyleneglycol detergent (Ahn et al. 2006). Lysosomal lipid-rich acidic environment may induce conformational changes in SapA and SapC that are needed for their activities (Ahn et al. 2006). Recently Rossman et al. (2008) crystallized SapC at pH 4.0 in open configuration as domain-swapped dimers, which may function in vesicle fusion (Fig. 5D).

2. AIMS OF THE STUDY

Neurotrophic factors, which by definition have beneficial effects on neurons, have been acknowledged as potential targets for drug development for neurodegenerative diseases. Therefore, it is important to search and study new proteins with neurotrophic effects. The starting point to this project was a publication describing MANF, a novel survival factor for midbrain dopaminergic neurons (Petrova et al. 2003). Soon thereafter we identified conserved dopamine neurotrophic factor (CDNF), a homologous protein for MANF. The aim of this work was to characterize the molecular structure, tissue expression and potential neurotrophic activities of CDNF and MANF proteins.

The specific aims were:

- To characterize the expression of CDNF and MANF in mouse and human tissues.
- To produce and characterize CDNF and MANF proteins *in vitro*.
- To study the effect of CDNF protein on midbrain dopaminergic neurons in a rat 6-OHDA model of Parkinson's disease.
- To determine the crystal structure of CDNF and MANF proteins, and subsequently elucidate their modes of action.

3. MATERIALS AND METHODS

Materials and methods used in this work are described in detail in the original articles. The methods are summarized in Table 3, and a short overview is given below.

Table 3. Methods used in this study.

Method	Article		
DNA and RNA methods	**I**	**II**	**III**
RNA isolation and reverse transcription	I	II	
PCR, cloning and DNA sequencing	I	II	III
Northern hybridization		II	
In situ hybridization	I	II	
Recombinant protein production	**I**	**II**	**III**
Baculovirus production	I		III
SeMet labelling			III
Chromatographic purification	I	II	III
N-terminal sequencing and mass spectrometry	I	II	III
Crystallization and structure determination			III
Immunological methods	**I**	**II**	
Antibody production	I	II	
Tissue protein extracts and western blotting	I	II	
Immunohistochemistry	I	II	
Cell culture methods	**I**	**II**	**III**
Primary neuronal cultures	I		
Transfection	I	II	III
Animal models	**I**	**II**	
Rat 6-OHDA model of PD	I		
Rat model of global forebrain ischemia		II	
Rat model of status epilepticus		II	

3.1. DNA and RNA methods

3.1.1. RNA isolation and reverse transcription

Human RNAs derived from post-mortem brain regions and mouse RNAs were isolated with Ambion's RNAwiz reagent (Applied Biosystems). Human RNAs of total brain and peripheral tissues were purchased from Clontech. First strand cDNA synthesis was performed with Superscript II reverse transcriptase (Invitrogen) as described in (I, II).

3.1.2. PCR, cloning and DNA sequencing

RT-PCR analysis of *CDNF* and *MANF* mRNA expression in human and mouse tissues was performed using primers listed in Table 4. The PCR programs are presented in (I, II). *CDNF* and *MANF* cDNAs were cloned into pCRII-TOPO vector (Invitrogen), verified by sequencing, and subcloned into selected expression vectors (Tables 5 and 6).

Table 4. Primers used for RT-PCR analysis of *CDNF* and *MANF* mRNA tissue expression.

Gene	Primer	Sequence	Article
Mouse *Cdnf*	m-*Cdnf*-ATG	5'-ACCATGCGGTGCATCAGTCCAACTGC-3'	I
Mouse *Cdnf*	m-*Cdnf*-stop-del	5'-GAGCTCCGTTTGGGGGTATATC-3'	I
Human *CDNF*	h-*CDNF*-ATG	5'-ACCATGTGGTGCGCGAGCCCAGTTGC-3'	I
Human *CDNF*	h-*CDNF*-stop-del	5'-GAGCTCTGTTTTGGGGTGTGTC-3'	I
Mouse *Manf*	m*Manf*-ATG	5'-ACCATGTGGGCTACGCGCGGGCT-3'	II
Mouse *Manf*	m*Manf*-stop-del	5'-CAGATCAGTCCGTGCGCTGGCTG-3'	II
Human *MANF*	h*MANF*-ATG2	5'-ACCATGTGGGCCACGCAGGGGCT-3'	II
Human *MANF*	h*MANF*-stop-del	5'-CAAATCGGTCGGTGCACTGGCTG-3'	II

Table 5. CDNF and MANF constructs for protein expression.

Construct	Elements of construct	Vector	Host cell	Article
Flag-His-CDNF (E490)	EGT signal peptide, Flag, 6His, human CDNF aa 1-161 without endogenous signal peptide	pK503.9 (Keinänen et al. 1998)	Sf9	I
Flag-His-T-CDNF (E491)	EGT signal peptide, Flag, 6His, thrombin cleavage site, human CDNF aa 1-161 without endogenous signal peptide	pK503.9	Sf9	III
CDNF-ΔC	EGT signal peptide, Flag, 6His, thrombin cleavage site, human CDNF aa 1-107 without endogenous signal peptide	pK503.9	Sf9	III
mCDNF	Fusion protein*, 6His, thrombin cleavage site, mouse CDNF aa 1-163 without endogenous signal peptide, 6His	T7*lac* based (Peränen et al. 1996)	*E.coli* Origami B	I, II
hMANF	Fusion protein*, 6His, TEV cleavage site, human MANF aa 1-158 without endogenous signal peptide	T7*lac* based	*E.coli* Origami B	I, II
hMANF	Fusion protein*, 6His, thrombin cleavage site, human MANF aa 1-158 without endogenous signal peptide	pHAT based (Peränen et al. 1996)	*E.coli* Origami DE3	III
hMANF-V5-His (E445)	Honeybee melittin signal peptide, human MANF without endogenous signal peptide, V5, 6His	pMIB (Invitrogen)	Sf9	II

EGT, ecdysone-S-glycotransferase of *Autographa californica* nuclear polyhedrosis virus (AcNPV). TEV, Tobacco Etch Virus. Thrombin cleavage site: LVPRGS; cleavage between R-G. *Unpublished (J. Peränen).

Table 6. CDNF and MANF constructs for transient mammalian expression.

Construct	Number	Elements of construct	Vector	Host cell	Article
hCDNF-stop	E511	human CDNF aa 1-187	pCR3.1 (Invitrogen)	HEK293T	I
hCDNF-V5-His	E493	human CDNF aa 1-187, V5, 6His	pcDNA3.1 (Invitrogen)	COS-7	I
mCDNF-V5-His	E494	mouse CDNF aa 1-187, V5, 6His	pcDNA3.1	COS-7	II
hMANF-stop	E742	human MANF aa 1-179	pCR3.1	HEK293T	II
hMANF-V5-His	E443	human MANF aa 1-179, V5, 6His	pcDNA3.1	COS-7	II
mMANF-V5-His	E444	mouse MANF aa 1-179, V5, 6His	pcDNA3.1	COS-7	II

3.1.3. Northern and in situ *hybridization*

For northern analysis, *Manf*-specific DNA probe was generated with Rediprime II system and Redivue dCTP-^{32}P (Amersham). Hybridization was performed on MTN mouse tissue blot (BD Biosciences) as described (II). For *in situ* hybridization, probes (Table 7) were synthesized by *in vitro* transcription using ^{35}S-labelled UTP (Amersham) as described (I, II). Hybridization temperature was 52 ºC. Washing was performed at 55 ºC or at 65 ºC for *Cdnf* or *Manf,* respectively. Hybridized sections were exposed in NTB2 emulsion (Kodak) for 5-6 weeks (*Cdnf*) or 5 days (*Manf*) (I, II).

Table 7. Probes for *in situ* and northern hybridization.

Probe	GenBank Accession No.	Nucleotides	Length	Vector	Construct No.	Article
Mouse *Cdnf**	NM_177647	63-623	561 bp	pCRII	E496	unpublished
Mouse *Cdnf*-P*	NM_177647	409-823	414 bp	pCRII	E753	I
Mouse *Manf*	NM_029103	41-577	536 bp	pCRII	E509	II

*Only for *in situ* hybridization.

3.2. Recombinant protein production

3.2.1. CDNF production, purification and SeMet labelling

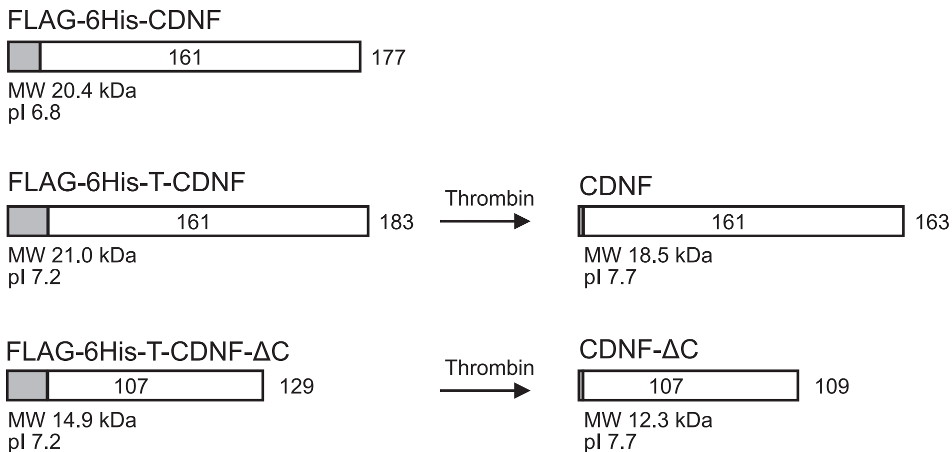
Recombinant baculoviruses encoding human CDNF (I) or the N-terminal domain of CDNF (CDNF-ΔC; III) were produced according to Bac-to-Bac protocol (Gibco-BRL). Protein expression of each baculoviral stock was tested and optimized in Sf9 *Lepidopteran* cells by varying the amount virus stock used for the infection and the infection time.

CDNF was purified from cleared Sf9 culture supernatants at three days post-infection as described in detail (I, III). Briefly, CDNF-containing supernatant (adjusted at pH 7.5) was incubated batchwise with nickel-charged chelating sepharose (Amersham). After washing with 0.5M NaCl, CDNF was eluted with 0.5 M imidazole, pH 8.0, and concentrated. Subsequently, CDNF was applied to

anion exchange chromatography HiTrap Q HP column (Amersham) and eluted with 0-1 M NaCl gradient at pH 8.2. For crystallization and structure determination, CDNF proteolytic fragment (CDNF-ΔC) that co-purified with full-length CDNF, was purified with gel filtration (III). Baculovirally expressed CDNF-ΔC was labelled with selenium-methionine (SeMet), purified and crystallized as described in (III). Constructs used for CDNF production are listed in Table 5, and recombinant proteins are presented schematically in Fig. 6A.

3.2.2. MANF production and purification

Recombinant human MANF was produced in *E.coli* and purified as described (I, II, III). In addition, a stable Sf9 cell line expressing recombinant human MANF was created, and MANF was purified from culture supernatant as described in (II). Constructs used are listed in Table 5, and recombinant MANF proteins are presented schematically in Fig. 6B.



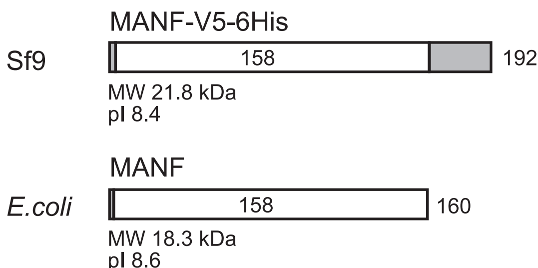


Figure 6. Schematic presentation of recombinant CDNF (**A**) and MANF (**B**) proteins. Mature CDNF or MANF is indicated in white; tags and additional residues in grey. Relative numbers of amino acids are shown. Proteins are presented from N to C terminus.

3.2.3. N-terminal sequencing and mass spectrometry

Amino-terminal sequence analysis was performed with Procise 494A Sequencer (Perkin Elmer), and Q-TOF instrumet (Micromass Ltd.) was used for electrospray mass spectrometry (I, II, III). Peptide mass fingerprints were analyzed with Ultraflex TOF/TOF (Bruker Daltonik GmbH) matrix-assisted laser desorbtion/ionization time-of-flight (MALDI-TOF) mass spectrometer (I, II).

3.2.4. Crystallization and structure determination

Sitting-drop vapour-diffusion technique of Helsinki robot crystallization facility was used to screen crystallization conditions for CDNF-ΔC, SeMet substituted CDNF-ΔC and full-length MANF as described in (III). Data for structural determination was collected at European Synchrotron Radiation Facility (ESRF) as described in (III).

3.3. Immunological methods

3.3.1. Antibody production

Recombinant mouse CDNF or human MANF proteins produced in *E.coli* were used for the immunization of rabbits as described (I, II). Antisera were affinity purified by using CDNF or MANF coupled to cyanogen bromide sepharose (Amersham) beads (I, II). Antibody specificity was tested by preincubating the antibody with recombinant CDNF or MANF before application on western blotting or immunohistochemistry (I, II).

3.3.2. Tissue protein extracts and western blotting

Total post-nuclear proteins from freshly frozen mouse tissues were extracted in high salt-high detergent lysis buffer as described (I, II). Proteins were detected in the western blots using ECL™ detection reagents (Amersham) (I, II). Antibodies used are listed in Table 8.

3.3.3. Immunohistochemistry

Tissue processing and immunohistochemistry was performed as described (I, II). Antibodies used are listed in Table 8.

3.4. Cell culture methods

3.4.1. Primary neuronal cultures

Cultures for mouse P1 superior cervical ganglion (SCG) sympathetic neurons, mouse E14 or E15 dorsal root ganglion (DRG) sensory neurons and rat E14

Table 8. Primary antibodies used for western blotting and IHC.

Antibody	Host	Source/Manufacturer	Dilution	Article	
CDNF	Rabbit	Saarma group	1:750* 1:40**	I	II
MANF	Rabbit	Saarma group	1:1000* 1:100**	I	II
TH	Mouse	MAB318, Chemicon	1:2000**	I	II
Actin	Mouse	AC-40, Sigma	1:1000*	I	II
NeuN	Mouse	MAB377, Chemicon	1:500**	I	II

* Used in Western blotting; ** used in IHC.

motoneurons were established as described (I).

3.4.2. Transfection

Transfection of CDNF and MANF vectors into different mammalian cell lines with Lipofectamine 2000 reagent (Invitrogen) was performed according to the manufacturer's instructions (I, II). Mammalian expression vectors encoding CDNF or MANF are listed in Table 6.

3.5. Animal models

3.5.1. 6-OHDA rat model of Parkinson's disease

Activity of recombinant CDNF was tested in a partial rat 6-OHDA model of PD (I). Experimental setup for the neuroprotection and neurorestoration experiments, respectively, is presented in Fig. 7.

3.5.2. Rat model of global forebrain ischemia or status epilepticus

Ischemia was induced with bilateral occlusion of common carotid arteries combined with hypotension. Circulation was restored after 10 min by blood reinfusion and removal of occlusion clasps (II). Status epilepticus (SE) was induced electrically as described (II). Animals were decapitated at 2h, 24h and 1 week after the reinfusion (ischemia) or the end of SE.

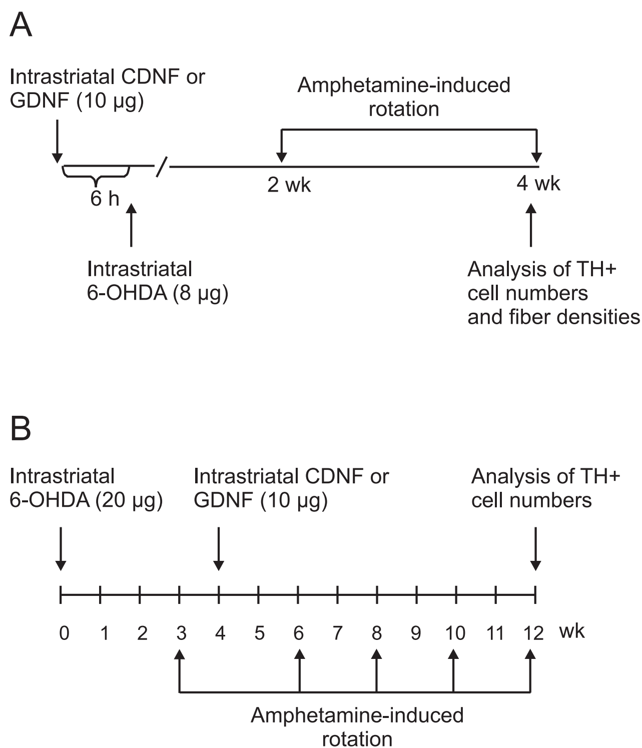


Figure 7. Experimental setup for neuroprotection (**A**) and neurorestoration (**B**) studies in a 6-OHDA model of PD.

4. RESULTS

4.1. CDNF is a vertebrate paralogue of MANF (I)

Bioinformatic search retrieved partial human and mouse expressed sequence tag (EST) sequences homologous to MANF (*ARMET*) cDNA. These cDNAs encoded previously unknown protein of 187 amino acids (Fig. 8A, B), which we named as conserved dopamine neurotrophic factor, CDNF. We amplified *CDNF* cDNA from human and mouse tissues by RT-PCR. In public databases, CDNF is also referred as ARMET-like 1 (ARMETL1). However,

A

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H.s.CDNF   MWCASPVAVVAFCAGLLVSHPVLTQGQEAGGRPGADCEVCKEFLNRFYKS 50
M.m.CDNF   MRCISPTALVTFCAGFCISNPVLAQGLEAGVGPRADCEVCKEFLDRFYNS 50
H.s.MANF   MWATQGLA-VALALSVLPGSRALRPG---------DCEVCISYLGRFYQD 40
M.m.MANF   MWATRGLA-VALALSVLPDSRALRPG---------DCEVCISYLGRFYQD 40
D.m.MANF   MKTWYMVVVIGFLATLAQTSLALKEE---------DCEVCVKTVRRFADS 41
C.e.MANF   MS--RLVLLISLVIVVASAAAP-------------QCEVCKKVLDDVMAK 35
           *        : :   .                   :**** . :  .  .

H.s.CDNF   LIDRGVNFSLDTIEKELISFCLDTKGKENRICYYLGATKDAATKILSEVT 100
M.m.CDNF   LLSRGIDFSADTIEKELLNFCSDAKGKENRICYYLGATTDAATKILGEVT 100
H.s.MANF   LKDRDVTFSPATIENELIKFCREARGKENRICYYIGATDDAATKIINEVS 90
M.m.MANF   LKDRDVTFSPATIEEELIKFCREARGKENRICYYIGATDDAATKIINEVS 90
D.m.MANF   LDDSTKK-DYKQIETAFKKFCKAQKNKEHRFCYYLGGLEESATGILNELS 90
C.e.MANF   VPAGDKS-KPDAIGKVIREHCETTRNKENKFCFYIGALPESATSIMNEVT 84
           :       .   *   : ..*   :.**:::*:*:*.  ::** *:.*::

H.s.CDNF   RPMSVHMPAMKIC-EKLKKLDSQICELKYEKTLDLASVDLRKMRVAELKQ 149
M.m.CDNF   RPMSVHIPAVKIC-EKLKKMDSQICELKYGKKLDLASVDLWKMRVAELKQ 149
H.s.MANF   KPLAHHIPVEKIC-EKLKKKDSQICELKYDKQIDLSTVDLKKLRVKELKK 139
M.m.MANF   KPLAHHIPVEKIC-EKLKKKDSQICELKYDKQIDLSTVDLKKLRVKELKK 139
D.m.MANF   KPLSWSMPAEKIC-EKLKKKDAQICDLRYEKQIDLNSVDLKKLKVRDLKK 139
C.e.MANF   KPLSWSMPTEKVCLEKLKGKDAQICELKYDKPLDWKTIDLKKMRVKELKN 134
           :*::  :*. *:* ****  *:***:*:* * :*  ::** *::* :**:

H.s.CDNF   ILHSWGEECRACAEKTDYVNLIQELAPKYA--ATHPKTEL 187
M.m.CDNF   ILQRWGEECRACAEKSDYVNLIRELAPKYV--EIYPQTEL 187
H.s.MANF   ILDDWGETCKGCAEKSDYIRKINELMPKYAPKAASARTDL 179
M.m.MANF   ILDDWGEMCKGCAEKSDYIRKINELMPKYAPKAASARTDL 179
D.m.MANF   ILNDWDESCDGCLEKGDFIKRIEELKPKYS------RSEL 173
C.e.MANF   ILGEWGEVCKGCTEKAELIKRIEELKPKYV------KEEL 168
           **  *.* * .* ** : :. *.** ***       : :*
```

B

H.s.CDNF Pre 26 161 187

59%

H.s.MANF Pre 21 158 179

49%

D.m.MANF Pre 22 151 173

46%

C.e.MANF Pre 16 152 168

Figure 8. A) Alignment of CDNF and MANF amino acid sequences from selected organisms. Signal peptides are underlined and conserved cyteines boxed. N-linked glycosylation site in human CDNF is indicated in red and putative ER-retention signals in blue. **B**) Schematic presentation of CDNF and MANF proteins from human, fly and worm. Percent of identical amino acids as compared to human CDNF is indicated.

the name *ARMET* is misleading, since only human *MANF* but not genes homologous to human *CDNF* or *MANF* from other species contains an amino-terminal arginine-rich region. Furthermore, the arginine-rich region of human *MANF* is apparently not translated *in vivo* (Petrova et al. 2003).

CDNF and MANF encoding genes are highly conserved in evolution. Analyses of EST and genomic sequences of different organisms suggest that vertebrates have *CDNF* and *MANF* genes, whereas invertebrates, including *C. elegans* and *D. melanogaster*, have a single homologous *CDNF/MANF* gene. Characteristic feature of CDNF and MANF proteins is eight cystine residues, spacing of which is conserved (Fig. 8A, B).

4.2. CDNF and MANF are secreted proteins (I, II)

Transiently overexpressed human CDNF and MANF proteins were efficiently secreted from mammalian cells (I, II). Similar results were obtained with mouse CDNF and MANF proteins (data not shown). The signal peptide of native human CDNF or MANF was cleaved after residue 26 (glycine) or 21 (alanine), respectively, resulting mature protein of 161 (I) and 158 amino acids (II) (Fig. 8B). CDNF and MANF proteins seem not to contain pro-sequence, suggesting that enzymatic cleavage is not necessary for their activity (I, II).

The amino acid sequence of human CDNF contains one potential N-linked glycosylation site, whereas mouse CDNF or MANF, or human MANF sequences do not contain potential N- or O-linked (serine/threonine-linked) glycosylation sites. Based on ESI-MS analysis, recombinant human CDNF-V5-6His and MANF-V5-6His proteins secreted from COS-7 cells were not glycosylated or post-translationally modified (I, II).

4.3. Protein production and purification (I, II, III)

Recombinant Flag-6His-hCDNF produced by baculoviral expression in insect cells was purified to an estimated final purity of ~90% (I). Based on ESI-MS, Flag-6His-hCDNF was not post-translationally modified (I). This CDNF protein was used in the 6-OHDA model of PD (I). Schematic presentation of recombinant CDNF proteins expressed by baculovirus in Sf9 cells, and MANF proteins expressed in a stable Sf9 cell line or in *E.coli* is shown in Materials and Methods (Fig. 6).

Since the crystallization of recombinant mature Flag-6His-hCDNF failed due to its high solubility, and production trials of mature CDNF using *E.coli* expression system gave partially degraded CDNF and low protein yields (data not shown), we decided to crystallize a proteolytic fragment of CDNF (CDNF-ΔC; aa 1-107) which was identified in the baculoviral cultures of Flag-6His-hCDNF (III). SeMet-labelled CDNF-ΔC fragment was produced using baculoviral expression in insect cells, purified and crystallized (III).

Mature human MANF was expressed in *E.coli*, purified (II) and crystallized (III). Also human MANF-V5-6His produced in a stable Sf9 cell line was successfully purified (II). Human CDNF and MANF proteins produced in insect cells were detected exclusively as monomers when analysed in non-denaturating SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown), or in gel filtration chromatography (III).

4.4. Crystal structure of CDNF and MANF (III)

The crystal structure of N-terminal domain of CDNF (CDNF-ΔC) was solved from a crystal of SeMet-substituted protein at resolution of 1.6Å (Fig. 9B). The structure model consists of residues 9-105 of the mature CDNF in two chains. CDNF-ΔC contains five α-helices followed by a turn of 3_{10} helix, which form a globular, saposin-like “closed” conformation. The saposin-like fold is stabilized by three intramolecular disulphide bonds, which connect α1 and the C-terminal 3_{10} helix, α1 and α5 helix, α2 and α3 helix, respectively (Fig. 9A).

The structure of human MANF (residues 1-137 of mature protein) was resolved by molecular replacement using the structure of CDNF-ΔC, and was refined at resolution of 2.8Å (Fig. 9D). Residues 138-158 are not visible in the structure. The N-terminal domain of MANF is saposin-like and essentially similar to that of CDNF. The C-terminus of MANF appears to be natively unfolded. It contains a loop region (residues 95-111) followed by two parallel α-helices α6 and α7, which are connected with a loop formed by residues 123-130. MANF C-terminus contains one CXXC motif (^{127}CKGC^{130}), which forms an internal disulphide bond. Presumably the C-terminal structure of CDNF is similar to that of MANF, and contains a disulphide bridge in the ^{132}CRAC^{135} motif.

The closest structural homologs for CDNF-ΔC and the N-terminal domain of MANF are saposin-like proteins (SAPLIPs) granulysin (Fig. 9C) (Anderson et al. 2003) and NK-lysin (Fig. 9E) (Liepinsh et al. 1997).

4.5. CDNF expression in mammalian CNS (I)

Cdnf transcripts were detected by RT-PCR in the developing embryonic (E12-E18) and postnatal (P1, P4, P7, P14) mouse brain, and in all adult mouse and adult human brain regions analyzed. *CDNF* mRNA was detected in the caudate nucleus and putamen (striatum) and SN of adult human and mouse, and in the mouse embryonic (E13, E18) and postnatal (P1, P10) midbrain (I).

In situ hybridization analysis of *Cdnf* mRNA expression was performed with two *Cdnf* cRNA probes (414 bp and 561 bp; see Materials and Methods). We used low stringency washing for *Cdnf*, since high stringency used in *Manf* detection protocol gave no signal with the *Cdnf* cRNA probe (561 bp) tested. *Cdnf* cRNA probe (414 bp) gave a specific although weak hybridization signal in several brain regions of P1 and P10 mouse brain, in line with the results of CDNF immunohistochemical analysis (I). Relatively high *Cdnf*-specific signal was detected in the hippocampus and thalamus of P1 and P10 mouse brain, and in the olfactory bulb of P1 mouse (I). With the full-length *Cdnf* probe (561 bp), a specific hybridization signal was detected only in the thalamus of P10 mouse brain (data not shown), but not in any other brain regions at P10, or in brain sections from other developmental stages analyzed (P1, P5 or adult; data not shown).

Consistently with the obtained ISH data, immunohistochemical analysis indicated widespread expression of CDNF protein in P1, P10 and adult mouse brain. In the adult cerebral cortex,

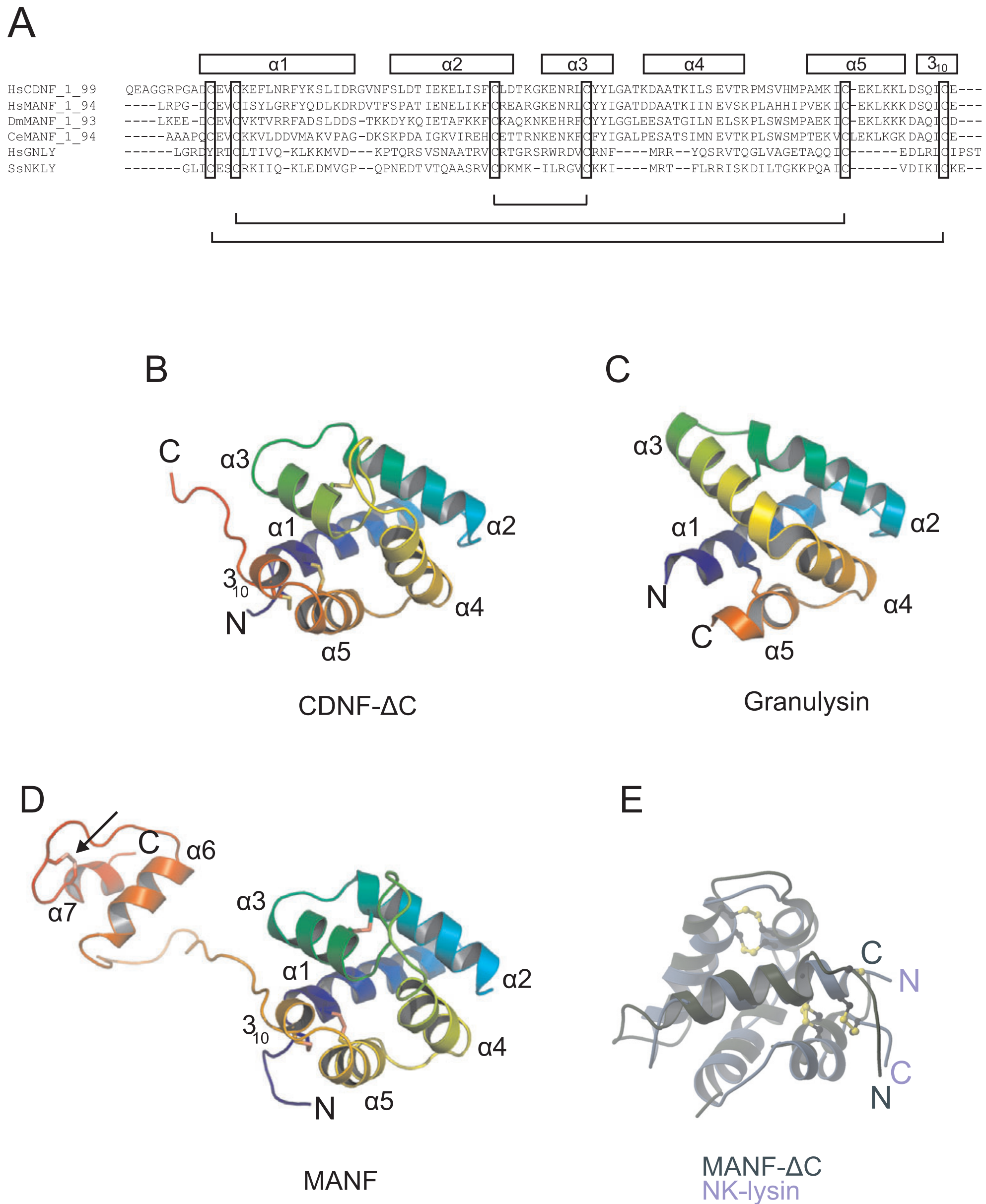

Figure 9. A) Alignment of saposin domains of CDNF and MANF, granulysin (Swiss-Prot P22749; residues 62-142) and NK-lysin (Swiss-Prot Q29075; residues 46-126). Six* conserved cysteines are boxed, and intramolecular cysteine bridges are indicated below the alignment. Helical regions of CDNF and MANF are shown above the sequences. Hs, human; Dm, fruitfly; Ce, roundworm; Ss, pig. *Granulysin contains five cysteines and two intramolecular disulphide bridges. CDNF saposin domain (**B**) and granulysin (**C**) in a closed conformation. **D**) Crystal structure of MANF. The C-terminal cysteine bridge in a CXXC motif indicated with an arrow. **E**) Overlay of MANF saposin-domain and NK-lysin.

CDNF labelling co-localized with NeuN, indicating neuronal expression of CDNF. In the SN, CDNF labelling did not co-localize with TH suggesting that CDNF is not expressed in dopaminergic neurons. Locus coeruleus, which contains noradrenergic neurons, stained positive for CDNF (I). We were unable to detect CDNF signal in embryonic mouse CNS by IHC.

The expression analysis of CDNF on tissue sections by ISH and IHC techniques was difficult since both methods gave only weak specific signals, suggesting that *Cdnf* mRNA and protein were expressed at low levels in mouse tissues. In contrast, the expression of *Manf* mRNA and protein was easy to detect using ISH and IHC methods suggesting higher levels of expression as compared to those of CDNF. To evaluate antibody specificity, we pre-incubated anti-CDNF and anti-MANF antibodies with recombinant CDNF or MANF proteins before applying them on tissue sections (I, II). Although this control suggested specificity for the both antibodies, it is important to test the antiboodies on tissue samples derived from CDNF or MANF knockout mice to further evaluate their specificity, and the accuracy of obtained expression data. This work is currently ongoing.

4.6. MANF expression in mammalian CNS (II)

Based on RT-PCR analysis, *Manf* had a wide expression pattern in the brain, thus resembling that of *Cdnf*. *Manf* transcripts were detected in the embryonic (E13, E15), postnatal (P9, P14) and adult (P60) mouse brain and in all human brain regions analyzed, including the SN, caudate nucleus and putamen (II).

Consistently, *in situ* hybridization analysis indicated widespread *Manf* mRNA expression in several brain regions of P1, P10 and adult mouse brain. *Manf* signal was relatively abundant in the cerebral cortex, hippocampus, and cerebellar Purkinje cells of adult mouse, and it was also detected in the striatum and SN. In the brain, *Manf* signal localized mainly in cells with large nuclei, suggesting neuronal expression. High levels of *Manf* mRNA were also detected in the non-neuronal cells of choroid plexus.

Results from the IHC analysis of MANF protein expression were consistent with the results obtained from *in situ* hybridization analysis. MANF protein was detected in the cerebral cortex, hippocampus and cerebellar Purkinje cells of adult mouse. It was also detected in the striatum and substantia nigra of P1, P10 and adult mouse. In the adult SN, MANF was detected in TH positive neurons.

Also the *in situ* hybridization and immunohistochemical analyses of embryonic tissues gave consistent results. *Manf* mRNA and protein were expressed at high levels in the developing cerebral cortex of E12.5 and E14 mouse embryos, respectively. MANF protein was also detected in the developing midbrain of E16 mouse embryos, although at low level. MANF protein was localized in embryonic PNS, including dorsal root, trigeminal and superior cervical ganglia of E17 mouse embryos.

4.7. MANF expression after epileptic and ischemic insults (II)

Manf mRNA expression was significantly increased 2h after SE in the hippocampal dentate granule cell layer (to 171% of control), in the thalamic reticular nucleus (to 170% of control) and in the

retrosplenial cortex (to 175% of control). After 24h, expression had increased in the piriform and parietal cortical areas (to 175 and 136% of control, respectively). The increase in *Manf* expression was transient, and not detectable 1 week after SE.

After global forebrain ischemia, *Manf* mRNA levels were increased mainly in the hippocampal formation. Transient increase of *Manf* mRNA transcripts was detected in the granule cell layer of dentate gyrus, in hilus and in the CA1 region (to 157, 137, and 151% of control, respectively). Increase of *Manf* mRNA levels were detected also in the retrosplenial cortex (to 136% of control) 2h after the insult. However, no changes were detected in other cortical areas or in thalamic reticular nucleus. The increase in *Manf* expression was transient, and not detected 1 week after ischemia. After both insults, *Manf* expression was mostly neuronal in all examined structures.

4.8. CDNF and MANF expression in non-neuronal tissues (I, II)

Based on RT-PCR analysis, *CDNF* mRNA was expressed in several non-neuronal tissues of adult human and mouse. Relatively high levels of *CDNF* transcripts were detected in the skeletal muscle and testis of human and mouse, and in the mouse heart (I). Consistently, western blot analysis indicated high levels of CDNF protein in the heart, skeletal muscle and testis (I). High CDNF protein levels were detected also in the seminiferous tubules and skeletal muscle of adult mouse by IHC (data not shown).

RT-PCR and northern blot analyses revealed high *Manf* expression in the liver and testis of adult mouse, in contrast to skeletal muscle, in which *Manf* expression was relatively low (II). The results were in line with the western blot analysis of MANF protein expression in adult mouse tissues. Based on the ISH and IHC analyses, MANF expression was abundant in the seminiferous tubules of adult mouse, especially in the early spermatocytes (II). Consistently with the expression data obtained from mouse, *MANF* was widely expressed in non-neuronal tissues of adult human (II).

In situ hybridization analysis of embryonic mouse tissues revealed high *Manf* levels in the E12.5 mouse liver, cartilage primordia of head and vertebra, and umbilical vessels (II). Consistent results were obtained from analysis of E15 mouse embryos (data not shown). MANF expression was high in many secretory tissues. Robust MANF expression was detected in the salivary gland of E17 mouse embryos by ISH and IHC, and in pancreas by IHC (II).

4.9. Neuroprotective effect of CDNF in 6-OHDA rat model of PD (I)

In the neuroprotection studies, CDNF (10 μg) injected into the striatum 6h before intrastriatally injected 6-OHDA significantly reduced amphetamine-induced rotational behaviour at two ($P < 0.01$) and at four weeks ($P < 0.05$) post-lesion. Consistently, the number of dopaminergic TH-positive cells in the SNpc and density of TH-positive fibers in the striatum were significantly higher in CDNF-treated rats (96% of the intact side, $P < 0.001$ for TH-positive cells; 75% of the intact side, $P < 0.001$ for TH-positive fibers) as compared to the vehicle-treated rats (65% for TH-positive cells and 55% for TH-positive fibers). The neuroprotective effect of CDNF was similar to that of GDNF. GDNF (10 μg) injection before 6-OHDA significantly

reduced the rotational behaviour at two (P < 0.01), but not at four weeks post-lesion and increased the number of nigral TH-positive cells (93% of the intact side, P < 0.001) and the density of striatal TH-positive fibers (69%, P < 0.01) at four weeks post-lesion. The neuroprotective effect of CDNF was dose-dependent: at 3 µg CDNF significantly reduced the rotational behaviour and the increased number of TH-positive cells in the SNpc, but less efficiently than at the dose of 10 µg. The protective effect of CDNF at the dose of 1 µg was not statistically significant.

4.10. Neurorestorative effect of CDNF in 6-OHDA rat model of PD (I)

In the neurorestoration studies, CDNF (10 µg) injection into the striatum four weeks after striatal 6-OHDA injection was able to restore the functional activity of nigrostriatal dopaminergic system in adult rats. The amphetamine-induced rotational behaviour of CDNF-treated rats was significantly reduced 8 weeks after the protein injection (i.e. 12 weeks after the 6-OHDA injection), as compared with the vehicle-treated control group. The neurorestorative effect of GDNF (10 µg) was similar to that of CDNF at 12 weeks post-lesion. In line with the behavioural studies, the number of TH-positive cells in the SNpc was higher in the CDNF-treated rats (58% of the intact side, P = 0.0629), and in the GDNF-treated rats (57%, P < 0.05) as compared to the vehicle-treated rats (26% of the intact side).

4.11. Studies on the survival promoting activity of CDNF on primary neuronal cultures (I)

CDNF (100 ng/ml) did not promote the survival of P1 mouse superior cervical ganglion (SCG) sympathetic neurons or E14 and E15 mouse dorsal root ganglion (DRG) sensory neurons in culture. CDNF (0.1-100 ng/ml) had no effect on the survival of E14 rat motoneurons *in vitro* (I).

5. DISCUSSION

5.1. A possible dual role of CDNF and MANF

In the present study we identified and characterized CDNF (I, III), a novel vertebrate protein homologous to MANF (Petrova et al. 2003). CDNF and MANF form a novel protein family conserved from humans to invertebrates, including *C. elegans* and *D. melanogaster*. Recent evidence by us and others suggest a dual role for these proteins as extracellular neurotrophic factors and as ER resident cytoprotective proteins. CDNF (I) and MANF (Petrova et al. 2003; Voutilainen et al. submitted) promote the survival of midbrain dopaminergic neurons, and MANF is also cytoprotective against ER stress induced cell death (Apostolou et al. 2008; Tadimalla et al. 2008). CDNF and MANF have widespread expression in mammalian tissues (I, II) suggesting that they affect many cell types. We have resolved the crystal structure of MANF and CDNF, which will help to reveal the action mechanisms of these new proteins at molecular level (III).

5.1.1. Secretion and ER localization of CDNF and MANF

In line with the observed neurotrophic activities, CDNF and MANF are secreted proteins. We showed that CDNF and MANF are secreted from transiently transfected cells (I, II), and a recent study presented consistent results (Apostolou et al. 2008). Importantly, the authors also show that endogenous MANF is secreted from human cervical cancer HeLa cells (Apostolou et al. 2008). CDNF and MANF are highly soluble and monomeric at neutral pH 7 (data not shown) (Mizobuchi et al. 2007). Based on mass analysis, human CDNF secreted from transiently transfected mammalian cell lines was not glycosylated (I). Human CDNF contains a potential N-linked glycosylation site in the saposin-like domain (Fig. 8A), and others have shown that a glycosylated form of human CDNF is also secreted from transiently overexpressing cells (Apostolou et al. 2008). However, we have shown that unglycosylated CDNF is biologically active (I), indicating that glycosylation is not crucial for its activity. Mouse CDNF, or human and mouse MANF do not have potential N- or O-linked glycosylation sites. MANF secreted from transiently transfected cells was not glycosylated (II), in line with the study by Apostolou et al. (2008), and in contrast to the original report (Petrova et al. 2003) which introduced MANF as a silalylated protein. The reason for this discrepancy is unclear. Many SAPLIPs are glycoslated, but the glycosylation has not been related to their activity, suggesting that it regulates protein stability or lifetime (Bruhn 2005). Neurotrophic factor GDNF has two glycosylation sites, but unglycosylated recombinant GDNF is biologically active (Lin et al. 1994), although the glycosylated forms are more stable and thus more potent in inducing tyrosine phosphorylation of RET *in vitro* (Mart Saarma, personal communication).

In line with the role of MANF in ER stress, MANF also localizes in the ER (Apostolou et al. 2008; Mizobuchi et al. 2007). MANF and CDNF have a C-terminal sequence (RTDL and KTEL, respectively) closely resembling the classical ER retention signal (KDEL) (Fig. 8A). This sequence may function as

a partial ER retention signal (Apostolou et al. 2008; Raykhel et al. 2007).

Whether the secretion of endogenous CDNF or MANF is regulated by physiological stimuli or injury is unclear. ER stress had only a minor effect on the secretion of MANF and CDNF from transiently transfected cells (Apostolou et al. 2008), suggesting a cell-autonomous role in the ER stress.

5.1.2. CDNF and MANF: Lipid binding proteins?

Analysis of the crystal structure shows that CDNF and MANF consist of two domains: the saposin-like N-terminal domain with five α-helices stabilized by three disulphide bridges, and presumably unstructured C-terminal domain with a disulphide bridge in a CXXC motif (III) (Fig. 9B, D).

Characteristic feature of saposin-like proteins is their ability to interact with membranes or lipids (Bruhn 2005). The closest structural homologues of human CDNF and MANF N-terminal domain are SAPLIPs granulysin (Anderson et al. 2003) and NK-lysin (Liepinsh et al. 1997) which function as defence proteins against bacterial cells (Andersson et al. 1995; Peña et al. 1997; Jongstra et al. 1987). They are able to disrupt a target cell membrane and increase its permeability. Granulysin and NK-lysin are proteolytically processed in cytolytic granules from pro-polypeptides. Activity of granulysin is decreased in the acidic environment of cytolytic granules, which is to protect the cell from damage (Hanson et al. 1999). In contrast, CDNF and MANF are likely functional upon synthesis and secretion; they do not seem to contain pro-sequences for enzymatic activation (I, II). However, whether they are proteolytically cleaved remains open.

The first obvious question based on the structural data is whether CDNF and MANF interact with lipids or membranes. Our recent studies indicate that they do (Hongxia Zhao and Mart Saarma, personal communication). Indeed, lipid interaction may be crucial for the activity of CDNF and MANF proteins.

5.1.3. The putative membrane interaction of CDNF and MANF: Dimerization, conformational changes and pH effect?

Molecular mechanisms of lipid and membrane interactions of saposins, NK-lysin and granulysin have only partially been revealed. Characteristic features of the membrane interactions of SAPLIPs are oligomerization and conformational changes in the saposin fold. Composition of membrane lipids and pH are known to affect membrane binding of SAPLIPs.

Charge distribution on the surface of SAPLIPs may have an important role in the membrane targeting. NK-lysin (Liepinsh et al. 1997) and granulysin (Anderson et al. 2003) have a ring of positively charged residues around the molecule, which may direct their interaction towards the negatively charged lipid head groups in the membrane. The saposin-like domain of CDNF and MANF has conserved positively charged residues on the surface in two patches; in MANF, one patch is formed by residues R44, K46, R49 and K96, the other by residues K70, K80, K84, K86 and K87, respectively. These residues may contribute to the membrane interactions (III).

Oligomerization facilitates the membrane interaction of many SAPLIPs. Amoebapores form oligomers on a target cell membrane that are necessary for pore formation and subsequent lysis (Zhai and

Saier 2000). Consistently, cooperation between granulysin monomers may induce membrane disruption (Anderson et al. 2003). Interestingly, the saposin-like domain of CDNF crystallized as a dimer at pH 4.6, whereas the full-length, mature MANF crystallized as a monomer at neutral pH (III). Whether the dimerization of CDNF (or MANF) at low pH is biologically relevant is currently unknown. Structurally unrelated neurotrophic factors, GFLs and neurotrophins, function as homodimers showing that dimerization is a common theme in the function of neurotrophic factors.

Conformational changes in the saposin-like domain seem to regulate the membrane/lipid interactions and biological activities of SAPLIPs. Hydrophobic residues located at conserved positions are buried in the domain interior, forming a hydrophobic core, which may have a role in the lipid interactions of SAPLIPs (Bruhn 2005). In the crystals, the saposin-like domain of CDNF and MANF was in a globular, compact conformation, with a relatively small hydrophobic core (III). In this conformation, two "leafs" (Bruhn 2005), one formed by helices α1, α2, and α3; and the other formed by helices α4 and α5, are closed together (III). Conformational changes of SAPLIPs have been reported in the presence of detergents and in response to pH changes. Whether conformational changes in the SAPLIP domain facilitate the lipid interactions of CDNF and MANF will be revealed by future studies.

SapB, a protein with lipid transfer activity (Vogel et al. 1991), crystallized as a homodimer, in which the two monomers had adopted an open conformation thus facilitating lipid access to the hydrophobic cavity (Fig. 5C) (Ahn et al. 2003). In the presence of detergent, SapC monomer crystallized in an open conformation with a solvent-exposed hydrophobic pocket, which is likely to contact with membrane lipids (Hawkins et al. 2005). Also at pH 4, SapC crystallized in an open conformation, but as a domain-swapped dimer (Fig. 5D). This conformation of SapC may facilitate vesicle fusion (Rossmann et al. 2008). SapC and SapA form oligomers in the presence of a detergent at low pH 4.8 (Ahn et al. 2006). Thus, the lysosomal lipid-rich acidic environment may induce conformational changes in SapC and SapA that are needed for their activities (Ahn et al. 2006). In line, acidic pH also increases the ability of SapC and SapD to bind lipid vesicles and destabilize phospholipid-containing membranes (Vaccaro et al. 1995). Differently from saposins, which are acidic molecules with pI values 4.6 - 4.8, CDNF and MANF saposin-like proteins are slightly basic with pI values 7.7 and 8.6, respectively. Whether pH affects the membrane interactions of CDNF and MANF will be studied in the future.

The role of lipids and membrane interactions in the biology of CDNF and MANF are currently unknown. Does lipid binding induce dimerization of CDNF and MANF and subsequent activation? The mechanism of CDNF and MANF membrane/lipid interaction is not known. However, it does not seem likely that CDNF and MANF disrupt the target membranes like granulysin and NK-lysin, since we have not observed cell death induced by CDNF or MANF. Whether CDNF and MANF have lipid transfer properties similar with those of saposins is not known. Hypothetical modes of CDNF and MANF dimerization and membrane interaction are presented in Fig. 10.

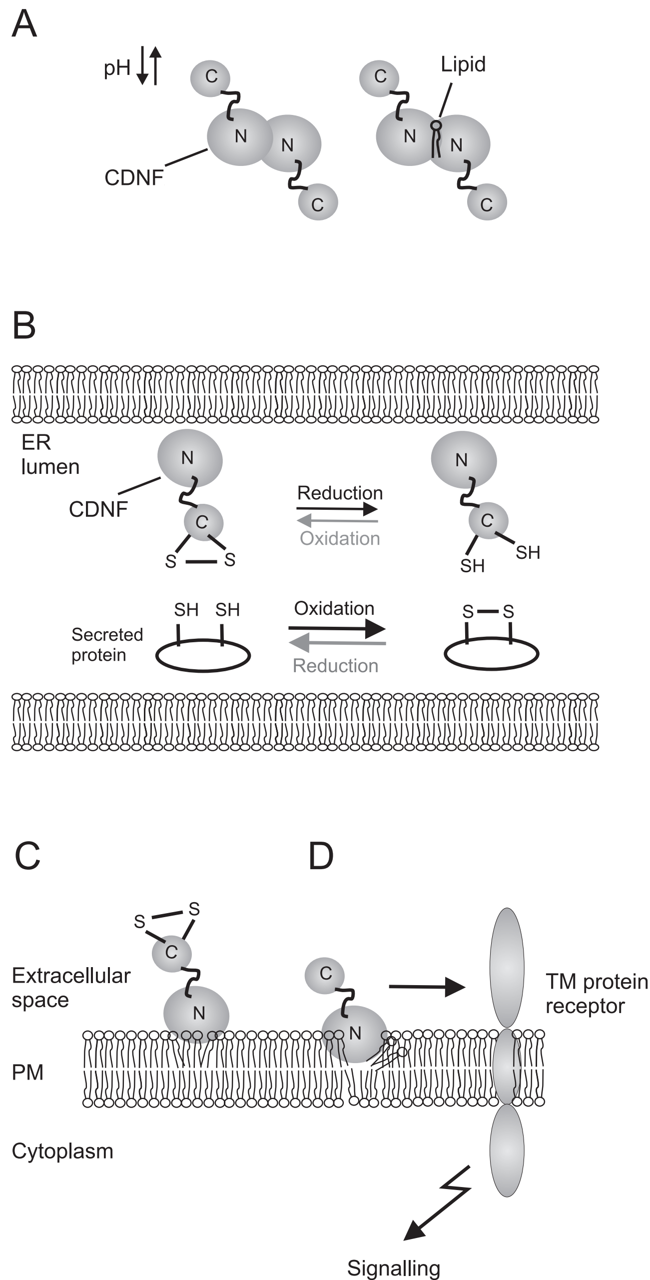

Figure 10. Hypothetical modes for CDNF (and MANF) action. **A**) Change in pH or lipid binding may trigger CDNF dimerization. **B**) CDNF may facilitate the correct formation of disulphide bonds on secretory proteins in the ER. –SH; thiol group; -S-S-, cysteine bond. **C**) The cysteine bridge in a CXXC motif may be redox active also on the cell surface. **D**) CDNF binding to membrane may lead to the activation of transmembrane protein receptor and subsequent intracellular signalling. Saposin-domain (N) and C-terminal domain (C) of CDNF is connected by a flexible linker region.

5.1.4. The C-terminal domain: Cytoprotection against ER stress?

In the MANF C-terminal–domain the two cysteines in ^{127}CKGC^{130} motif (^{132}CRAC^{135} in CDNF) form a C-terminal diulphide bridge (III). The C-terminal domain is presumably natively unfolded; the conformation seen in the crystal is stabilized by crystal contacts and apparently is one out of several possible conformations.

Interestingly, a CXXC active site motif is found in thiol/disulfide oxidoreductases which catalyze the formation of intramolecular disulphide bonds. These proteins include thioredoxins, like protein disulphide isomerases (PDIs), which function in the ER (Ellgaard and Ruddock 2005). The CXXC motif functions in reduction, oxidation and isomerisation reactions that are necessary for proper disulphide bond formation of target proteins in the ER (Horibe et al. 2004). The CXXC motif may also bind metal ions in metal-binding proteins, including PDI (Narindrasorasak et al. 2003). It has been shown that nitrosylation of the PDI active site motif inhibits activity of PDI, induces intracellular accumulation of polyubiquitinated proteins and cell death *in vitro*. Furthermore, nitrosylated PDI is found in brain samples derived from PD patients, indicating a link between PDI dysfunction and protein misfolding in neurodegenerative disorders (Uehara et al. 2006).

Oxidoreducatases are also secreted and they may be cell surface-associated (Jordan and Gibbins 2006). Interestingly, evidence suggests that the reduction and rearrangement of disulphide bonds is a mechanism of controlling protein function on the cell surface (Hogg 2003). Cell adhesion, uptake of bacterial toxins and viral fusion with a host cell membrane may depend on thiol-disuphide exchange reactions (Hogg 2003). Extracellular redox catalysts have been shown to regulate the functional forms of cell surface proteins as well as receptor-ligand interactions. For example, thioredoxin 1 (TRX1) mediates disulphide reduction in CD30 (tumor necrosis factor receptor superfamily member 8; TNFRS8), which induces a subsequent conformational change and affects functional properties of the CD30 ectodomain, thus regulating inflammatory response (Schwertassek et al. 2007). Extracellular TRX also activates TRPC5 (transient receptor potential, canonical 5) ion-channel located on the cell surface by breaking a disulphide bridge (Xu et al. 2008). Extracellular PDI is known to mediate entry of human immunodeficiency virus 1 (HIV-1) into lymphoid cells by catalysing redox changes of the HIV-1 envelope glycoprotein gp120 (Hogg 2003).

Recent studies have revealed that MANF (and presumably also CDNF) is cytoprotective against ER stress induced cell death (Apostolou et al. 2008; Tadimalla et al. 2008). Accumulation of unfolded proteins in the ER induces UPR, a signal-transduction pathway to counteract ER stress (Fig. 4) (Szegezdi et al. 2006b). If the ER stress is severe, apoptotic signalling is activated, leading to cell death. Inhibition of MANF expression by RNA interference (RNAi) increased tunicamycin-induced cell death and expression of UPR factors in HeLa cells (Apostolou et al. 2008). The crystal structure of MANF C-terminal domain supports the idea of MANF (and CDNF) as an “anti-ER stress” protein. MANF and CDNF may facilitate the formation of cysteine bridges and protein folding in the ER, thus reducing the ER stress caused

by unfolded or incorrectly folded proteins (III) (Fig. 10B).

The studies of Apostolou et al. (2008) and Tadimalla et al. (2008) were based on earlier reports showing *Armet* (*Manf*) upregulation in ER stress conditions (Holtz et al. 2005; Lee et al. 2003; Belmont et al. 2008; Mizobuchi et al. 2007). We found transient upregulation of *Manf* mRNA in adult rat brain after two pathological conditions, SE and global forebrain ischemia, suggesting that MANF may regulate neuronal survival and synaptic plasticity (II). In line with our data, ARMET protein was upregulated in cortical neurons after cortical ischemia induced by MCAO in rats (Apostolou et al. 2008). Whether the expression of *Cdnf* mRNA is regulated in SE or global forebrain ischemia is currently unknown. In contrast to *Manf*, *Cdnf* (*Armetl1*) expression was not upregulated by ER-stressors *in vitro*, suggesting that CDNF acts constitutively in the ER (Apostolou et al. 2008). However, the role of CDNF in the ER stress response needs further studies.

Interesting question is whether MANF and CDNF have oxidoreductase or metal binding activities, and if they do, what are the target molecules. Could cell-surface bound extracellular MANF or CDNF regulate disulphide bond breakage or formation on cell-surface proteins, including cell surface receptors (Fig. 10C)? Although the initial experiments did not detect oxidoreductase activity or bound metal ions in MANF (Mizobuchi et al. 2007), further studies are needed.

Since in the crystal structure of MANF the C-terminal amino acids 138-158 were not visible (III), further structural analysis of the C-terminal domain may give additional information about the molecular mechanism of MANF and CDNF action.

5.1.5. CDNF as potent as GDNF in the 6-OHDA model of PD

Since MANF was described as a survival factor for mDA neurons *in vitro* (Petrova et al. 2003), it was logical to ask whether CDNF has similar functions. We tested CDNF in a rat 6-OHDA model of PD (I), which is commonly used for preclinical studies of novel potential therapeutic agents. Several growth factors have neuroprotective effects in 6-OHDA or MPTP toxin models of PD, whereas only few molecules promote neurorestoration in these models. *In vivo* neurorestorative effects of GDNF and NRTN on midbrain dopaminergic neurons are well documented. However, clinical trials of GDNF protein and NRTN gene therapy in PD patients have not met expectations (see 5.4.). Therefore, novel candidates for therapeutic target molecules are needed for the treatment of neurodegenerative diseases. CDNF may be one of them.

The neuroprotective and neurorestorative activities of CDNF in the 6-OHDA rat model of PD were comparable to those of GDNF (I). We used a partial 6-OHDA lesion model, in which striatal 6-OHDA injection leads to protracted retrograde degeneration of the nigrostriatal pathway. In the neurorestorative experiments, 6-OHDA was injected into the striatum four weeks before a single injection of CDNF. The CDNF dose (10 µg) was chosen based on our dose-dependence studies, which showed that this was the most effective dose (I), and the same dose (10 µg) has been effective also for GDNF in the same model system (Aoi et al. 2000). After one week from the striatal 6-OHDA injection starts a rapid loss of nigral dopaminergic neurons (Sauer and Oertel 1994). This is completed within five weeks, and followed

by more protracted loss nigral neurons for several weeks (Sauer and Oertel 1994). At the time of CDNF injection the rapid loss of nigral dopaminergic neurons was presumably still ongoing. The CDNF injection induced recovery of motor function, which was significant at 8 weeks after injection (I). This suggests that CDNF prevented further degeneration of dopaminergic neurons and fibers, and maintained or increased the functionality of remaining neurons. Whether CDNF is able to induce sprouting of striatal dopaminergic fibers or upregulation of TH expression as has been reported for GDNF (Hoffer et al. 1994; Hudson et al. 1995) is not known, and needs further studies.

Although CDNF and GDNF both induced protection and functional restoration of nigral dopaminergic neurons, they are structurally unrelated proteins. GDNF receptor complexes and signalling mechanisms are well characterized, whereas the target molecules i.e. putative receptor(s) and signalling pathways activated by CDNF are unknown so far. Oxidative stress, mitochondrial dysfunction, apoptotic cell death and inflammation are related to the 6-OHDA toxicity (Schober 2004). Apparently CDNF is able to activate signalling pathways which counteract some of these phenomena. Does CDNF affect directly on mDA neurons, or is the protective effect mediated by other cell types? Does CDNF affect for example microglia and astrocytes, and reduce the inflammatory response related to 6-OHDA toxicity? Several growth factors which are protective in the rat 6-OHDA model of PD are also glial mitogens (Table 1), and their effect on dopaminergic neurons is presumably indirect. Whether this is true for CDNF or MANF is unclear. We did not detect statistically significant survival promoting effect of CDNF or MANF on embryonic dopaminergic neurons in culture differently from GDNF, which significantly promoted the survival and TH expression of embryonic dopaminergic neurons *in vitro* (data not shown). This suggests that the potential survival promoting effect of CDNF on embryonic dopaminergic neurons is indirect, and the cells mediating the survival were not present in sufficient amounts in the midbrain cultures. Alternatively, embryonic dopaminergic neurons do not express the target molecules for CDNF. It is currently unclear why we have not been able to reproduce the results by Petrova et al. (2003) showing the survival promoting effects of MANF on embryonic dopaminergic neurons *in vitro*.

ER stress and UPR are related to the 6-OHDA toxicity *in vitro*, although their role in the 6-OHDA toxicity *in vivo* is still unclear (Silva et al. 2005). Could extracellularly delivered CDNF inhibit ER stress induced by 6-OHDA? *Manf* mRNA expression was upregulated by 6-OHDA treatment in mouse dopaminergic MN9D cell line (Holtz et al. 2005). Whether 6-OHDA treatment affects to endogenous levels of CDNF or MANF expression in rat brain *in vivo* has not been studied. ER stress is also induced by simulated ischemia *in vitro* (Szegezdi et al. 2006a). Recombinant MANF protein was able to prevent ischemia-induced cell death on cardiac myocytes *in vitro* (Tadimalla et al. 2008). Thus, the neurotrophic activity of extracellular CDNF (I) or MANF (Voutilainen et al. submitted) on adult rat dopaminergic neurons may inhibit the ER stress-induced cell death related to 6-OHDA toxicity.

Since some of the SAPLIPs have neurotrophic activities (Bruhn 2005), it is possible that the saposin-like domain

of CDNF and MANF mediates their neurotrophic activities. The saposin-like domain may bind to cell surface and induce survival promoting signalling via a transmembrane receptor protein (Fig. 10D), whereas the C-terminal domain with the CXXC motif may mediate intracellular cytoprotective activity against ER stress (III). It is also possible that the neurotrophic and cytoprotective ("anti-ER stress") activities are mediated by the same structural motif. Could the C-terminal domain mediate the neurotrophic activity of CDNF and MANF? Could the C-terminal domain of the putative cell-surface bound CDNF or MANF catalyse reduction or oxidation of a disulphide bond on another cell surface protein e.g. receptor ectodomain, and induce survival promoting signalling in the cell? Alternatively, is extracellular CDNF or MANF taken up by the cell, where it induces cytoprotective effects? We have recently shown that CDNF is transported from the striatum to the SN *in vivo*, suggesting that CDNF is endocytosed (Merja H. Voutilainen and Mart Saarma, unpublished results). Future studies will give us a better understanding on the neurotrophic and cytoprotective activities of CDNF and MANF, and whether or how they are related.

5.2. Novel neurotrophic factors for midbrain dopaminergic neurons

Neurotrophic factors are, according the traditional view, secreted proteins provided by the target tissue which promote the survival of neurons during the developmental period of PCD. Although extensively studied, the role of neurotrophic factors in the target-innervation of dopaminergic neurons has remained unclear (Andressoo and Saarma 2008). Potential target-derived factors for mDA neurons include GDNF, NRTN, BDNF, NT-4 and FGF-2 (Krieglstein 2004). Are CDNF and MANF target-derived neurotrophic factors for midbrain dopaminergic neurons?

We have shown that CDNF is a secreted protein *in vitro* that prevents the death of adult rat dopaminergic neurons *in vivo* (I). In adult, *Cdnf* mRNA was expressed in the striatum and in the midbrain, and CDNF protein was detected in the SN, although not in the TH-positive dopaminergic neurons (I). At P1 and P10, *Cdnf* mRNA and protein was detected in the striatum and SN, although at low level (I). Thus, CDNF is present in the nigrostriatal system around the two postnatal PCD peaks of midbrain dopaminergic neurons at P2 and P14 (Burke 2004). *Gdnf* mRNA levels are relatively high in the striatum at the time of the second peak of PCD as compared to the levels in adult (Choi-Lundberg and Bohn 1995; Schaar et al. 1993; Strömberg et al. 1993). Differently from GDNF, our studies do not suggest transient postnatal upregulation of striatal CDNF expression in the mouse. Consistent with the neurotrophic hypothesis, GDNF is retrogradely transported via dopaminergic neurons from the striatum to SN (Tomac et al. 1995b). As already mentioned, CDNF is transported from the striatum to the SN; however, it is currently unclear whether the transport occurs via dopaminergic neurons (Merja H. Voutilainen and Mart Saarma, unpublished results). These data, although not conclusive, indicate that CDNF may function as a target-derived neurotrophic factor for mDA neurons. However, based on the expression analysis (I), it seems likely that endogenous CDNF secreted by other sources than the striatal target may

affect mDA neurons and support their survival.

Manf mRNA and protein were also detected in the developing nigrostriatal system. MANF was present in the striatum and midbrain postnatally at P1 and P10, and also in the striatum of adult mouse. In embryo, *Manf* mRNA was expressed in the midbrain at embryonic days E13 and E18, and MANF protein was detected in the midbrain at E16 by IHC (II). Thus, also MANF may have a role in the development of midbrain dopaminergic system. In adult, MANF protein was localized in TH-positive dopaminergic neurons in the SN (II). Similarly with CDNF, MANF promotes the survival of adult rat dopaminergic neurons in a 6-OHDA model of PD (Voutilainen et al. submitted). Whether MANF functions as a target-derived neurotrophic factor for midbrain dopaminergic neurons is currently unknown.

Neurotrophic factors with survival promoting activities on mDA neurons are known to affect other neuronal populations as well. GDNF supports the survival of different neuronal populations in the CNS and PNS, including motoneurons and enteric, sensory, parasympathetic and sympathetic neurons (Airaksinen and Saarma 2002). CDNF (I) or MANF (data not shown) did not promote the survival of embryonic motoneurons or DRG neurons, or postnatal SCG neurons *in vitro*. However, this does not exclude the possibility that CDNF and MANF affect these neurons *in vivo*, or other neuronal types in the CNS or PNS. Thus, additional studies are needed, for example using *in vitro* organotypic cultures, or *in vivo* models of neurodegeneration, including models of motoneuron degeneration (e.g. ALS models), models of Huntington's disease with striatal neurodegeneration, or models of AD.

5.3. Roles of CDNF and MANF in non-neuronal tissues?

CDNF and MANF are widely expressed in the brain and in non-neuronal tissues of mouse and human (I, II), suggesting that both proteins have important roles not only in the neuronal survival but also in the survival, proliferation and differentiation of non-neuronal cells and tissues. Non-neuronal roles are common for neurotrophic factors, e.g. GDNF regulates kidney development and spermatogenesis (Airaksinen and Saarma 2002), and BDNF has an important role in the heart development (Zacchigna et al. 2008).

In the tissue samples analyzed, high levels of *Manf* mRNA and protein were detected in the liver and testis, whereas *Cdnf* mRNA and protein levels were relatively high in the skeletal muscle, heart and testis (I, II). The skeletal muscle, heart and testis are tissues with high energy production by mitochondria and subsequent oxidative stress. Whether CDNF or MANF have a cytoprotective role against oxidative stress in these tissues is unknown. CDNF and MANF are also expressed in secretory organs and tissues, including the salivary gland (I, II), choroid plexus of brain ventricles (II), pancreas (II) and testis (I, II) (Mizobuchi et al. 2007), in accordance with the putative role of MANF (and CDNF) as intracellular chaperones that are needed in tissues of high protein synthesis and secretion. Whether endogenous CDNF and MANF proteins are secreted into body fluids, including the cerebrospinal fluid, serum or saliva has not been studied.

Interestingly, it was recently shown that MANF may regulate cell proliferation *in vitro* (Apostolou et al. 2008). Inhibiton of MANF expression by RNAi increased proliferation of HeLa cells, whereas overexpression of MANF in U2OS osteosarcoma cells inhibited proliferation (Apostolou et al. 2008). Whether CDNF affects cell proliferation *in vitro*, or whether MANF or CDNF have roles in the cell proliferation *in vivo*, is currently unknown.

5.4. CDNF and MANF as therapeutic proteins

Evidence for the potential trophic effects of CDNF and MANF is gradually accumulating. After the first publication of MANF as a survival promoting factor for midbrain dopaminergic neurons *in vitro* (Petrova et al. 2003), we showed that extracellular CDNF has neurotrophic effects on 6-OHDA-lesioned mDA system in a rat model of PD (I). Similarly, our recent studies showed neuroprotective and neurorestorative effects of MANF in a 6-OHDA model of PD (Voutilainen et al. submitted). An intrastriatal MANF (10 μg) injection 6 hours before a striatal 6-OHDA injection was able to prevent the degeneration of nigral dopaminergic neurons. Resembling the effects of CDNF (I), also MANF (10 μg) induced functional recovery of the mDA system when given four weeks after the 6-OHDA lesion (Voutilainen et al. submitted). Effects of recombinant MANF protein have also been studied in an experimental model of stroke in rats (Airavaara et al. 2009). MANF (6 μg) admistration into the cerebral cortex before MCAO significantly reduced the volume of infarction as measured after two days, and reduced markers of apoptosis in the ischemic cortex. MANF pre-treatment also improved motor recovery after stroke, although with some delay (Airavaara et al. 2009). Furthermore, MANF protein added to the culture medium of cardiac myocytes prevented cell death induced by simulated ischemia *in vitro* (Tadimalla et al. 2008).

CDNF (I) is clearly a potential novel protein for the treatment of PD. Several growth factors have been reported as a survival factors for mDA neurons, but only GDNF and NRTN have well-established neurorestorative effects in preclinical models of PD. However, recent clinical studies with GDNF (Lang et al. 2006) and NRTN (Ceregene Inc. press release at 26th of November 2008) have not been successful; thus, novel potential molecules are needed. Protein misfolding and aggregation is seen in many neurodegenerative diseases, including PD, AD and ALS. Since MANF and presumably also CDNF has cytoprotective role against ER stress *in vitro*, they may have protective effects against several neurodegenerative diseases.

Since neurotrophic factor proteins cannot cross the BBB, the method of delivery has to be carefully considered when designing preclinical trials for NTF therapy. In the recent clinical trials, GDNF protein was delivered by an intraputamenal infusion (Gill et al. 2003; Lang et al. 2006; Patel et al. 2005; Slevin et al. 2005), whereas neurturin was delivered by a gene therapy approach using AAV2 vector (Marks et al. 2008). Cell therapy with encapsulated or naked NTF-secreting cells implanted into the brain is also a potential method for NTF therapy (Aebischer and Ridet 2001; Lindvall and Wahlberg 2008). Recently it was shown that intravenous delivery of GDNF plasmid using Trojan horse liposomes which bind transferrin receptor induced functional recovery in a rat 6-OHDA model of PD (Zhang

and Pardridge 2009). The expression of GDNF transgene was regulated by a TH promoter (Zhang and Pardridge 2009). Alternative therapeutic strategies could include receptor agonists or inhibitors of NTF degradation. Whether some of these therapeutic strategies are applicape for CDNF and MANF will be revealed in the future.

An important question in NTF therapy is the potential side effects. GDNF was originally introduced as a specific neurotrophic factor for mDA neurons (Lin et al. 1993), but the claim is no longer valid. Although CDNF and MANF have potent effects on adult midbrain dopaminergic system (I) (Voutilainen et al. submitted), we do not know which other CNS or PNS neuronal types or non-neuronal cells CDNF may affect *in vivo*. Further preclinical studies are needed to assess the potency of CDNF and MANF as therapeutic agents for PD and other neurodegenerative diseases, and their possible side effects.

5.5. Receptors for CDNF and MANF are unknown

Molecular mechanism of the neurotrophic effect of CDNF (I) and MANF (Petrova et al. 2003; Voutilainen et al. submitted) on the nigral dopaminergic neurons, and the protective effect of MANF against stroke (Airavaara et al. 2009) is currently unknown. What could be the interacting and/or signalling partner(s) of CDNF and MANF on the plasma membrane?

Endocytosis of extracellular prosaposin is mediated by low-density lipoprotein receptor-related protein 1 (LRP1), a multifunctional transmembrane cell surface receptor, which is present in most cells and is involved in various functions including lipoprotein metabolism and activation of lysosomal enzymes (Hiesberger et al. 1998). LRP1 has multiple ligands, including platelet-derived growth factor (PDGF) and TGFβ, and mediates intracellular signalling events (Lillis et al. 2008). It has been suggested that PSAP and SapC bind to a G-protein coupled cell surface receptor (Hiraiwa et al. 1997). PSAP also interacts with gangliosides which apparently participate in the receptor binding and signalling (Misasi et al. 1998; Hiraiwa et al. 1997).

Whether CDNF and MANF bind a transmembrane protein receptor which subsequently activates intracellular signalling cascades, is not known. The possible binding of CDNF and MANF to LRP1, or the involvement of a G-protein coupled receptor in their signalling is not known. Would the lipid binding of CDNF and MANF be essential for subsequent activation of a transmembrane receptor protein? Do CDNF and MANF interact with glycolipids (gangliosides) which mediate their interaction with a transmembrane receptor protein and subsequent signaling? It is also unknown whether secreted CDNF and MANF proteins interact with extracellular matrix components. Whether CDNF and MANF use the same signalling receptors, or not, is also unknown. Differing surface residues between human CDNF, and human MANF and DmMANF proteins were identified (III), but their functional relevance remains to be studied in the future.

5.6. Evolutionarily conserved function?

In CDNF and MANF proteins, the N-terminal saposin-like domain and the C-terminal putative “anti ER-stress” domain are combined in a unique way (III). In the early evolution a MANF/CDNF gene has presumably emerged as

a combination of an ancestral saposin-like gene and an "anti-ER stress" gene, with putative membrane binding and cytoprotective activities, respectively. Since this functional combination has been an evident success, the invertebrate MANF/CDNF gene has duplicated during evolution, creating the two paralogs CDNF and MANF found in the vertebrates.

Interestingly, studies with *Drosophila* MANF (DmMANF) deficient flies indicate that DmMANF functions as a neurotrophic factor in the fly (Palgi et al. 2009). The genomic-null flies die in early larval stage, and mutant embryos show axonal degeneration, cuticular defects, and non-apoptotic cell death. Importantly, DmMANF is needed for the maintenance of dopaminergic neurites and dopamine levels (Palgi et al. 2009). Based on rescue experiments, human MANF is an orthologue of DmMANF (Palgi et al. 2009).

Gene ablation studies will presumably reveal the roles of CDNF and MANF *in vivo*. Our group has recently created CDNF deficient mice, which to our surprise (and excitement) die at early embryonic stage (Maria Lindahl and Mart Saarma, personal communication). This clearly shows that CDNF, as DmMANF, is crucially important for the embryonic development.

6. CONCLUSIONS

1) CDNF and MANF proteins form a novel evolutionarily conserved protein family. Paralogous CDNF and MANF are found in the vertebrates, whereas a single CDNF/ MANF ortholog is found in the invertebrates.

2) Mammalian CDNF and MANF are widely expressed in the brain, including midbrain and striatum, as well as in several non-neuronal tissues.

3) CDNF and MANF are secreted proteins. Protein production and purification schemes for recombinant CDNF and MANF proteins have been established.

4) Neuroprotective and neurorestorative activity of CDNF protein on adult rat midbrain dopaminergic neurons was studied in a partial 6-OHDA lesion model of PD. Intrastriatal CDNF protein was neuroprotective, when given before intrastriatal 6-OHDA. Importantly, CDNF induced functional recovery of nigrostriatal dopaminergic system, when given after 6-OHDA lesion. CDNF was as efficient as GDNF in both model systems. CDNF is a potent trophic factor for the treatment of PD.

5) The crystal structure of human MANF and CDNF was resolved. The structure consists of two domains and is consistent with the putative bifunctionality. The N-terminal saposin-like domain presumably interacts with membranes and mediates neurotrophic activities. The C-terminal domain is presumably unfolded, and contains a CXXC motif, which may have disulphide oxidoreductase or isomerase activities. Thus, the C-terminal domain may have cytoprotective activities against ER stress.

This study gave the first insights into the biology and therapeutic potential of the novel CDNF/MANF proteins. What are the molecular interactions and signalling pathways related to the potential neurotrophic and cytoprotective activities of CDNF and MANF will be revealed by future studies.

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REFERENCES

Abeliovich, A., and R. Hammond. 2007. Midbrain dopamine neuron differentiation: factors and fates. *Dev Biol* 304 (2):447-454.

Aebischer, P., and J. Ridet. 2001. Recombinant proteins for neurodegenerative diseases: the delivery issue. *Trends Neurosci* 24 (9):533-540.

Ahlskog, J. E. 2007. Beating a dead horse: dopamine and Parkinson disease. *Neurology* 69 (17):1701-1711.

Ahn, V. E., K. F. Faull, J. P. Whitelegge, A. L. Fluharty, and G. G. Prive. 2003. Crystal structure of saposin B reveals a dimeric shell for lipid binding. *Proc Natl Acad Sci U S A* 100 (1):38-43.

Ahn, V. E., P. Leyko, J. R. Alattia, L. Chen, and G. G. Prive. 2006. Crystal structures of saposins A and C. *Protein Sci* 15 (8):1849-1857.

Airaksinen, M. S., L. Holm, and T. Hätinen. 2006. Evolution of the GDNF family ligands and receptors. *Brain Behav Evol* 68 (3):181-190.

Airaksinen, M. S., and M. Saarma. 2002. The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci* 3 (5):383-394.

Airavaara, M., H. Shen, C. C. Kuo, J. Peränen, M. Saarma, B. Hoffer, and Y. Wang. 2009. Mesencephalic astrocyte-derived neurotrophic factor reduces ischemic brain injury and promotes behavioral recovery in rats. *J Comp Neurol* 515 (1):116-124.

Åkerud, P., J. Alberch, S. Eketjäll , J. Wagner, and E. Arenas. 1999. Differential effects of glial cell line-derived neurotrophic factor and neurturin on developing and adult substantia nigra dopaminergic neurons. *J Neurochem* 73 (1):70-78.

Åkerud, P., P. C. Holm, G. Castelo-Branco, K. Sousa, F. J. Rodriguez, and E. Arenas. 2002. Persephin-overexpressing neural stem cells regulate the function of nigral dopaminergic neurons and prevent their degeneration in a model of Parkinson's disease. *Mol Cell Neurosci* 21 (2):205-222.

Alexi, T., and F. Hefti. 1993. Trophic actions of transforming growth factor alpha on mesencephalic dopaminergic neurons developing in culture. *Neuroscience* 55 (4):903-918.

Anderson, D. H., M. R. Sawaya, D. Cascio, W. Ernst, R. Modlin, A. Krensky, and D. Eisenberg. 2003. Granulysin crystal structure and a structure-derived lytic mechanism. *J Mol Biol* 325 (2):355-365.

Andersson, M., H. Gunne, B. Agerberth, A. Boman, T. Bergman, R. Sillard, H. Jörnvall, V. Mutt, B. Olsson, H. Wigzell, and et al. 1995. NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. *EMBO J* 14 (8):1615-1625.

Andressoo, J. O., and M. Saarma. 2008. Signalling mechanisms underlying development and maintenance of dopamine neurons. *Curr Opin Neurobiol*.

Aoi, M., I. Date, S. Tomita, and T. Ohmoto. 2000. The effect of intrastriatal single injection of GDNF on the nigrostriatal dopaminergic system in hemiparkinsonian rats: behavioral and histological studies using two different dosages. *Neurosci Res* 36 (4):319-325.

Apostolou, A., Y. Shen, Y. Liang, J. Luo, and S. Fang. 2008. Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. *Exp Cell Res* 314 (13):2454-2467.

Arancio, O., and M. V. Chao. 2007. Neurotrophins, synaptic plasticity and dementia. *Curr Opin Neurobiol* 17 (3):325-330.

Arenas, E., M. Trupp, P. Åkerud, and C. F. Ibáñez. 1995. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron* 15 (6):1465-1473.

Baker, S. A., L. E. Stanford, R. E. Brown, and T. Hagg. 2005. Maturation but not survival of dopaminergic nigrostriatal neurons is affected in developing and aging BDNF-deficient mice. *Brain Res* 1039 (1-2):177-188.

Baloh, R. H., M. G. Tansey, P. A. Lampe, T. J. Fahrner, H. Enomoto, K. S. Simburger, M. L. Leitner, T. Araki, E. M. Johnson, Jr., and J. Milbrandt. 1998. Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. *Neuron* 21 (6):1291-1302.

Baquet, Z. C., P. C. Bickford, and K. R. Jones. 2005. Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. *J Neurosci* 25 (26):6251-6259.

Barde, Y. A., D. Edgar, and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. *EMBO J* 1 (5):549-553.

Bauer, S., B. J. Kerr, and P. H. Patterson. 2007. The neuropoietic cytokine family in development, plasticity, disease and injury. *Nat Rev Neurosci* 8 (3):221-232.

Beck, K. D., J. Valverde, T. Alexi, K. Poulsen, B. Moffat, R. A. Vandlen, A. Rosenthal, and F. Hefti. 1995. Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* 373 (6512):339-341.

Belmont, P. J., A. Tadimalla, W. J. Chen, J. J. Martindale, D. J. Thuerauf, M. Marcinko, N. Gude, M. A. Sussman, and C. C. Glembotski. 2008. Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. *J Biol Chem* 283 (20):14012-14021.

Bender, A., K. J. Krishnan, C. M. Morris, G. A. Taylor, A. K. Reeve, R. H. Perry, E. Jaros, J. S. Hersheson, J. Betts, T. Klopstock, R. W. Taylor, and D. M. Turnbull. 2006. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat Genet* 38 (5):515-517.

Berkemeier, L. R., J. W. Winslow, D. R. Kaplan, K. Nikolics, D. V. Goeddel, and A. Rosenthal. 1991. Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* 7 (5):857-866.

Bespalov, M. M., and M. Saarma. 2007. GDNF family receptor complexes are emerging drug targets. *Trends Pharmacol Sci* 28 (2):68-74.

Björklund, A., and S. B. Dunnett. 2007. Dopamine neuron systems in the brain: an update. *Trends Neurosci* 30 (5):194-202.

Blum, D., S. Torch, N. Lambeng, M. Nissou, A. L. Benabid, R. Sadoul, and J. M. Verna. 2001. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog Neurobiol* 65 (2):135-172.

Boger, H. A., L. D. Middaugh, P. Huang, V. Zaman, A. C. Smith, B. J. Hoffer, A. C. Tomac, and A. C. Granholm. 2006. A partial GDNF depletion leads to earlier age-related deterioration of motor function and tyrosine hydroxylase expression in the substantia nigra. *Exp Neurol* 202 (2):336-347.

Booth, G. E., E. F. Kinrade, and A. Hidalgo. 2000. Glia maintain follower neuron survival during Drosophila CNS development. *Development* 127 (2):237-244.

Bornhauser, B. C., and D. Lindholm. 2005. MSAP enhances migration of C6 glioma cells through phosphorylation of the myosin regulatory light chain. *Cell Mol Life Sci* 62 (11):1260-1266.

Bornhauser, B. C., P. A. Olsson, and D. Lindholm. 2003. MSAP is a novel MIR-interacting protein that enhances neurite outgrowth and increases myosin regulatory light chain. *J Biol Chem* 278 (37):35412-35420.

Bothwell, M. 2006. Evolution of the neurotrophin signaling system in invertebrates. *Brain Behav Evol* 68 (3):124-132.

Bove, J., D. Prou, C. Perier, and S. Przedborski. 2005. Toxin-induced models of Parkinson's disease. *NeuroRx* 2 (3):484-494.

Bowenkamp, K. E., A. F. Hoffman, G. A. Gerhardt, M. A. Henry, P. T. Biddle, B. J. Hoffer, and A. C. Granholm. 1995. Glial cell line-derived neurotrophic factor supports survival of injured midbrain dopaminergic neurons. *J Comp Neurol* 355 (4):479-489.

Braak, H., K. Del Tredici, U. Rub, R. A. de Vos, E. N. Jansen Steur, and E. Braak. 2003. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 24 (2):197-211.

Braak, H., E. Ghebremedhin, U. Rub, H. Bratzke, and K. Del Tredici. 2004. Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res* 318 (1):121-134.

Bruhn, H. 2005. A short guided tour through functional and structural features of saposin-like proteins. *Biochem J* 389 (Pt 2):249-257.

Buj-Bello, A., V. L. Buchman, A. Horton, A. Rosenthal, and A. M. Davies. 1995. GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* 15 (4):821-828.

Burbach, J. P., and M. P. Smidt. 2006. Molecular programming of stem cells into mesodiencephalic dopaminergic neurons. *Trends Neurosci* 29 (11):601-603.

Burke, R. E. 2004. Ontogenic cell death in the nigrostriatal system. *Cell Tissue Res* 318 (1):63-72.

Burke, R. E., W. T. Dauer, and J. P. Vonsattel. 2008. A critical evaluation of the Braak staging scheme for Parkinson's disease. *Ann Neurol* 64 (5):485-491.

Cacalano, G., I. Fariñas, L. C. Wang, K. Hagler, A. Forgie, M. Moore, M. Armanini, H. Phillips, A. M. Ryan, L. F. Reichardt, M. Hynes, A. Davies, and A. Rosenthal. 1998. GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* 21 (1):53-62.

Campana, W. M., M. Hiraiwa, K. C. Addison, and J. S. O'Brien. 1996. Induction of MAPK phosphorylation by prosaposin and prosaptide in PC12 cells. *Biochem Biophys Res Commun* 229 (3):706-712.

Casper, D., C. Mytilineou, and M. Blum. 1991. EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture. *J Neurosci Res* 30 (2):372-381.

Chao, M. V., and M. Bothwell. 2002. Neurotrophins: to cleave or not to cleave. *Neuron* 33 (1):9-12.

Chao, M. V., R. Rajagopal, and F. S. Lee. 2006. Neurotrophin signalling in health and disease. *Clin Sci (Lond)* 110 (2):167-173.

Chauhan, N. B., G. J. Siegel, and J. M. Lee. 2001. Depletion of glial cell line-derived neurotrophic factor in substantia nigra neurons of Parkinson's disease brain. *J Chem Neuroanat* 21 (4):277-288.

Chen, L., B. Cagniard, T. Mathews, S. Jones, H. C. Koh, Y. Ding, P. M. Carvey, Z. Ling, U. J. Kang, and X. Zhuang. 2005. Age-dependent motor deficits and dopaminergic dysfunction in DJ-1 null mice. *J Biol Chem* 280 (22):21418-21426.

Choi-Lundberg, D. L., and M. C. Bohn. 1995. Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. *Brain Res Dev Brain Res* 85 (1):80-88.

Chung, W. H., S. I. Hung, J. Y. Yang, S. C. Su, S. P. Huang, C. Y. Wei, S. W. Chin, C. C. Chiou, S. C. Chu, H. C. Ho, C. H. Yang, C. F. Lu, J. Y. Wu, Y. D. Liao, and Y. T. Chen. 2008. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. *Nat Med* 14 (12):1343-1350.

Cochrane, C. G., and S. D. Revak. 1991. Pulmonary surfactant protein B (SP-B): structure-function relationships. *Science* 254 (5031):566-568.

Conover, J. C., J. T. Erickson, D. M. Katz, L. M. Bianchi, W. T. Poueymirou, J. McClain, L. Pan, M. Helgren, N. Y. Ip, P. Boland, and et al. 1995. Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* 375 (6528):235-238.

Costa, S., M. M. Iravani, R. K. Pearce, and P. Jenner. 2001. Glial cell line-derived neurotrophic factor concentration dependently improves disability and motor activity in MPTP-treated common marmosets. *Eur J Pharmacol* 412 (1):45-50.

Coulpier, M., and C. F. Ibáñez. 2004. Retrograde propagation of GDNF-mediated signals in sympathetic neurons. *Mol Cell Neurosci* 27 (2):132-139.

Dauer, W., and S. Przedborski. 2003. Parkinson's disease: mechanisms and models. *Neuron* 39 (6):889-909.

Davies, A. M. 2003. Regulation of neuronal survival and death by extracellular signals during development. *EMBO J* 22 (11):2537-2545.

Davis, E. G., Y. Sang, B. Rush, G. Zhang, and F. Blecha. 2005. Molecular cloning and characterization of equine NK-lysin. *Vet Immunol Immunopathol* 105 (1-2):163-169.

de Alba, E., S. Weiler, and N. Tjandra. 2003. Solution structure of human saposin C: pH-dependent interaction with phospholipid vesicles. *Biochemistry* 42 (50):14729-14740.

Deumens, R., A. Blokland, and J. Prickaerts. 2002. Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Exp Neurol* 175 (2):303-317.

Ebert, A. D., A. J. Beres, A. E. Barber, and C. N. Svendsen. 2008. Human neural progenitor cells over-expressing IGF-1 protect dopamine neurons and restore function in a rat model of Parkinson's disease. *Exp Neurol* 209 (1):213-223.

Eigenbrot, C., and N. Gerber. 1997. X-ray structure of glial cell-derived neurotrophic factor at 1.9 A resolution and implications for receptor binding. *Nat Struct Biol* 4 (6):435-438.

Ekstrand, M. I., M. Terzioglu, D. Galter, S. Zhu, C. Hofstetter, E. Lindqvist, S. Thams, A. Bergstrand, F. S. Hansson, A. Trifunovic, B. Hoffer, S. Cullheim, A. H. Mohammed, L. Olson, and N. G. Larsson. 2007. Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. *Proc Natl Acad Sci U S A* 104 (4):1325-1330.

Ellgaard, L., and L. W. Ruddock. 2005. The human protein disulphide isomerase family: substrate interactions and functional properties. *EMBO Rep* 6 (1):28-32.

Endsley, J. J., J. L. Furrer, M. A. Endsley, M. A. McIntosh, A. C. Maue, W. R. Waters, D. R. Lee, and D. M. Estes. 2004. Characterization of bovine homologues of granulysin and NK-lysin. *J Immunol* 173 (4):2607-2614.

Engele, J. 1998. Changing responsiveness of developing midbrain dopaminergic neurons for extracellular growth factors. *J Neurosci Res* 51 (4):508-516.

Engele, J., and M. C. Bohn. 1991. The neurotrophic effects of fibroblast growth factors on dopaminergic neurons in vitro are mediated by mesencephalic glia. *J Neurosci* 11 (10):3070-3078.

Enomoto, H., T. Araki, A. Jackman, R. O. Heuckeroth, W. D. Snider, E. M. Johnson, Jr., and J. Milbrandt. 1998. GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* 21 (2):317-324.

Ernfors, P., C. F. Ibáñez, T. Ebendal, L. Olson, and H. Persson. 1990. Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proc Natl Acad Sci U S A* 87 (14):5454-5458.

Ernfors, P., K. F. Lee, and R. Jaenisch. 1994. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* 368 (6467):147-150.

Evron, E., P. Cairns, N. Halachmi, S. A. Ahrendt, A. L. Reed, and D. Sidransky. 1997. Normal polymorphism in the incomplete trinucleotide repeat of the arginine-rich protein gene. *Cancer Res* 57 (14):2888-2889.

Fallon, J., S. Reid, R. Kinyamu, I. Opole, R. Opole, J. Baratta, M. Korc, T. L. Endo, A. Duong, G. Nguyen, M. Karkehabadhi, D. Twardzik, S. Patel, and S. Loughlin. 2000. In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. *Proc Natl Acad Sci U S A* 97 (26):14686-14691.

Farkas, L. M., and K. Krieglstein. 2002. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) regulates survival of midbrain dopaminergic neurons. *J Neural Transm* 109 (3):267-277.

Fontan, A., A. Rojo, R. Sanchez Pernaute, I. Hernandez, I. Lopez, C. Castilla, J. Sanchez Albisua, A. Perez Higueras, I. Al-Rashid, A. Rabano, I. Gonzalo, M. Angeles Mena, A. Cools, S. Eshuis, P. Maguire, J. Pruim, K. Leenders, and J. Garcia de Yebenes. 2002. Effects of fibroblast growth factor and glial-derived neurotrophic factor on akinesia, F-DOPA uptake and dopamine cells in parkinsonian primates. *Parkinsonism Relat Disord* 8 (5):311-323.

Fujita, N., K. Suzuki, M. T. Vanier, B. Popko, N. Maeda, A. Klein, M. Henseler, K. Sandhoff, and H. Nakayasu. 1996. Targeted disruption of the mouse sphingolipid activator protein gene: a complex phenotype, including severe leukodystrophy and wide-spread storage of multiple sphingolipids. *Hum Mol Genet* 5 (6):711-725.

Gao, H. M., J. Jiang, B. Wilson, W. Zhang, J. S. Hong, and B. Liu. 2002. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. *J Neurochem* 81 (6):1285-1297.

Gash, D. M., Z. Zhang, Y. Ai, R. Grondin, R. Coffey, and G. A. Gerhardt. 2005. Trophic factor distribution predicts functional recovery in parkinsonian monkeys. *Ann Neurol* 58 (2):224-233.

Gash, D. M., Z. Zhang, A. Ovadia, W. A. Cass, A. Yi, L. Simmerman, D. Russell, D. Martin, P. A. Lapchak, F. Collins, B. J. Hoffer, and G. A. Gerhardt. 1996. Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* 380 (6571):252-255.

Gautier, C. A., T. Kitada, and J. Shen. 2008. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proc Natl Acad Sci U S A* 105 (32):11364-11369.

Genc, S., F. Kuralay, K. Genc, M. Akhisaroglu, S. Fadiloglu, K. Yorukoglu, M. Fadiloglu, and A. Gure. 2001. Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. *Neurosci Lett* 298 (2):139-141.

Georgievska, B., D. Kirik, and A. Björklund. 2002. Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. *Exp Neurol* 177 (2):461-474.

Giasson, B. I., J. E. Duda, S. M. Quinn, B. Zhang, J. Q. Trojanowski, and V. M. Lee. 2002. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* 34 (4):521-533.

Gill, S. S., N. K. Patel, G. R. Hotton, K. O'Sullivan, R. McCarter, M. Bunnage, D. J. Brooks, C. N. Svendsen, and P. Heywood. 2003. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med* 9 (5):589-595.

Glebova, N. O., and D. D. Ginty. 2005. Growth and survival signals controlling sympathetic nervous system development. *Annu Rev Neurosci* 28:191-222.

Glinka, Y. Y., and M. B. Youdim. 1995. Inhibition of mitochondrial complexes I and

IV by 6-hydroxydopamine. *Eur J Pharmacol* 292 (3-4):329-332.

Goldberg, M. S., S. M. Fleming, J. J. Palacino, C. Cepeda, H. A. Lam, A. Bhatnagar, E. G. Meloni, N. Wu, L. C. Ackerson, G. J. Klapstein, M. Gajendiran, B. L. Roth, M. F. Chesselet, N. T. Maidment, M. S. Levine, and J. Shen. 2003. Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *J Biol Chem* 278 (44):43628-43635.

Goldberg, M. S., A. Pisani, M. Haburcak, T. A. Vortherms, T. Kitada, C. Costa, Y. Tong, G. Martella, A. Tscherter, A. Martins, G. Bernardi, B. L. Roth, E. N. Pothos, P. Calabresi, and J. Shen. 2005. Nigrostriatal dopaminergic deficits and hypokinesia caused by inactivation of the familial Parkinsonism-linked gene DJ-1. *Neuron* 45 (4):489-496.

Golden, J. P., R. H. Baloh, P. T. Kotzbauer, P. A. Lampe, P. A. Osborne, J. Milbrandt, and E. M. Johnson, Jr. 1998. Expression of neurturin, GDNF, and their receptors in the adult mouse CNS. *J Comp Neurol* 398 (1):139-150.

Granholm, A. C., M. Reyland, D. Albeck, L. Sanders, G. Gerhardt, G. Hoernig, L. Shen, H. Westphal, and B. Hoffer. 2000. Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J Neurosci* 20 (9):3182-3190.

Grondin, R., Z. Zhang, A. Yi, W. A. Cass, N. Maswood, A. H. Andersen, D. D. Elsberry, M. C. Klein, G. A. Gerhardt, and D. M. Gash. 2002. Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys. *Brain* 125 (Pt 10):2191-2201.

Grothe, C., and M. Timmer. 2007. The physiological and pharmacological role of basic fibroblast growth factor in the dopaminergic nigrostriatal system. *Brain Res Rev* 54 (1):80-91.

Hagg, T., and S. Varon. 1993. Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons in vivo. *Proc Natl Acad Sci U S A* 90 (13):6315-6319.

Hallböök, F., C. F. Ibáñez, and H. Persson. 1991. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron* 6 (5):845-858.

Hanke, M., L. M. Farkas, M. Jakob, R. Ries, J. Pohl, and A. M. Sullivan. 2004. Heparin-binding epidermal growth factor-like growth factor: a component in chromaffin granules which promotes the survival of nigrostriatal dopaminergic neurones in vitro and in vivo. *Neuroscience* 124 (4):757-766.

Hanson, D. A., A. A. Kaspar, F. R. Poulain, and A. M. Krensky. 1999. Biosynthesis of granulysin, a novel cytolytic molecule. *Mol Immunol* 36 (7):413-422.

Harpster, M. H., S. Bandyopadhyay, D. P. Thomas, P. S. Ivanov, J. A. Keele, N. Pineguina, B. Gao, V. Amarendran, M. Gomelsky, R. J. McCormick, and M. M. Stayton. 2006. Earliest changes in the left ventricular transcriptome postmyocardial infarction. *Mamm Genome* 17 (7):701-715.

Harvey, B. K., A. Mark, J. Chou, G. J. Chen, B. J. Hoffer, and Y. Wang. 2004. Neurotrophic effects of bone morphogenetic protein-7 in a rat model of Parkinson's disease. *Brain Res* 1022 (1-2):88-95.

Hashino, E., M. Shero, D. Junghans, H. Rohrer, J. Milbrandt, and E. M. Johnson, Jr. 2001. GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development. *Development* 128 (19):3773-3782.

Hawkins, C. A., E. de Alba, and N. Tjandra. 2005. Solution structure of human saposin C in a detergent environment. *J Mol Biol* 346 (5):1381-1392.

Hebert, M. A., C. G. Van Horne, B. J. Hoffer, and G. A. Gerhardt. 1996. Functional effects of GDNF in normal rat striatum: presynaptic studies using in vivo electrochemistry and microdialysis. *J Pharmacol Exp Ther* 279 (3):1181-1190.

Hecht, O., N. A. Van Nuland, K. Schleinkofer, A. J. Dingley, H. Bruhn, M. Leippe, and J. Grotzinger. 2004. Solution structure of the pore-forming protein of Entamoeba histolytica. *J Biol Chem* 279 (17):17834-17841.

Hefti, F. F., A. Rosenthal, P. A. Walicke, S. Wyatt, G. Vergara, D. L. Shelton, and A. M. Davies. 2006. Novel class of pain drugs based on antagonism of NGF. *Trends Pharmacol Sci* 27 (2):85-91.

Heinrich, P. C., I. Behrmann, S. Haan, H. M. Hermanns, G. Muller-Newen, and F. Schaper. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374 (Pt 1):1-20.

Henderson, C. E., H. S. Phillips, R. A. Pollock, A. M. Davies, C. Lemeulle, M. Armanini, L. Simmons, B. Moffet, R. A. Vandlen, L. C. Simpson, and et al. 1994. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* 266 (5187):1062-1064.

Henning, J., U. Strauss, A. Wree, J. Gimsa, A. Rolfs, R. Benecke, and U. Gimsa. 2008. Differential astroglial activation in 6-hydroxydopamine models of Parkinson's disease. *Neurosci Res* 62 (4):246-253.

Herrera, A. J., A. Castano, J. L. Venero, J. Cano, and A. Machado. 2000. The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. *Neurobiol Dis* 7 (4):429-447.

Herzog, C. D., B. Dass, J. E. Holden, J. Stansell, 3rd, M. Gasmi, M. H. Tuszynski, R. T. Bartus, and J. H. Kordower. 2007. Striatal delivery of CERE-120, an AAV2 vector encoding human neurturin, enhances activity of the dopaminergic nigrostriatal system in aged monkeys. *Mov Disord* 22 (8):1124-1132.

Hiesberger, T., S. Huttler, A. Rohlmann, W. Schneider, K. Sandhoff, and J. Herz. 1998. Cellular uptake of saposin (SAP) precursor and lysosomal delivery by the low density lipoprotein receptor-related protein (LRP). *EMBO J* 17 (16):4617-4625.

Hiraiwa, M., W. M. Campana, B. M. Martin, and J. S. O'Brien. 1997. Prosaposin receptor: evidence for a G-protein-associated receptor. *Biochem Biophys Res Commun* 240 (2):415-418.

Hirate, Y., and H. Okamoto. 2006. Canopy1, a novel regulator of FGF signaling around the midbrain-hindbrain boundary in zebrafish. *Curr Biol* 16 (4):421-427.

Hirsch, E. C., S. Hunot, and A. Hartmann. 2005. Neuroinflammatory processes in Parkinson's disease. *Parkinsonism Relat Disord* 11 Suppl 1:S9-S15.

Hoane, M. R., A. G. Gulwadi, S. Morrison, G. Hovanesian, M. D. Lindner, and W. Tao. 1999. Differential in vivo effects of neurturin and glial cell-line-derived neurotrophic factor. *Exp Neurol* 160 (1):235-243.

Hofer, M., S. R. Pagliusi, A. Hohn, J. Leibrock, and Y. A. Barde. 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J* 9 (8):2459-2464.

Hoffer, B. J., A. Hoffman, K. Bowenkamp, P. Huettl, J. Hudson, D. Martin, L. F. Lin, and G. A. Gerhardt. 1994. Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. *Neurosci Lett* 182 (1):107-111.

Hogg, P. J. 2003. Disulfide bonds as switches for protein function. *Trends Biochem Sci* 28 (4):210-214.

Hohn, A., J. Leibrock, K. Bailey, and Y. A. Barde. 1990. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* 344 (6264):339-341.

Holtz, W. A., and K. L. O'Malley. 2003. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J Biol Chem* 278 (21):19367-19377.

Holtz, W. A., J. M. Turetzky, and K. L. O'Malley. 2005. Microarray expression profiling identifies early signaling transcripts

associated with 6-OHDA-induced dopaminergic cell death. *Antioxid Redox Signal* 7 (5-6):639-648.

Horger, B. A., M. C. Nishimura, M. P. Armanini, L. C. Wang, K. T. Poulsen, C. Rosenblad, D. Kirik, B. Moffat, L. Simmons, E. Johnson, Jr., J. Milbrandt, A. Rosenthal, A. Björklund, R. A. Vandlen, M. A. Hynes, and H. S. Phillips. 1998. Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. *J Neurosci* 18 (13):4929-4937.

Horibe, T., M. Gomi, D. Iguchi, H. Ito, Y. Kitamura, T. Masuoka, I. Tsujimoto, T. Kimura, and M. Kikuchi. 2004. Different contributions of the three CXXC motifs of human protein-disulfide isomerase-related protein to isomerase activity and oxidative refolding. *J Biol Chem* 279 (6):4604-4611.

Howells, D. W., M. J. Porritt, J. Y. Wong, P. E. Batchelor, R. Kalnins, A. J. Hughes, and G. A. Donnan. 2000. Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra. *Exp Neurol* 166 (1):127-135.

Huang, E. J., and L. F. Reichardt. 2001. Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 24:677-736.

Hudson, J., A. C. Granholm, G. A. Gerhardt, M. A. Henry, A. Hoffman, P. Biddle, N. S. Leela, L. Mackerlova, J. D. Lile, F. Collins, and et al. 1995. Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits in vivo. *Brain Res Bull* 36 (5):425-432.

Hurley, F. M., D. J. Costello, and A. M. Sullivan. 2004. Neuroprotective effects of delayed administration of growth/differentiation factor-5 in the partial lesion model of Parkinson's disease. *Exp Neurol* 185 (2):281-289.

Hwang, D. Y., S. M. Fleming, P. Ardayfio, T. Moran-Gates, H. Kim, F. I. Tarazi, M. F. Chesselet, and K. S. Kim. 2005. 3,4-dihydroxyphenylalanine reverses the motor deficits in Pitx3-deficient aphakia mice: behavioral characterization of a novel genetic model of Parkinson's disease. *J Neurosci* 25 (8):2132-2137.

Hyman, C., M. Hofer, Y. A. Barde, M. Juhasz, G. D. Yancopoulos, S. P. Squinto, and R. M. Lindsay. 1991. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* 350 (6315):230-232.

Hyman, C., M. Juhasz, C. Jackson, P. Wright, N. Y. Ip, and R. M. Lindsay. 1994. 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* 14 (1):335-347.

Hynes, M. A., K. Poulsen, M. Armanini, L. Berkemeier, H. Phillips, and A. Rosenthal. 1994. Neurotrophin-4/5 is a survival factor for embryonic midbrain dopaminergic neurons in enriched cultures. *J Neurosci Res* 37 (1):144-154.

Ibáñez, C. F. 1998. Emerging themes in structural biology of neurotrophic factors. *Trends Neurosci* 21 (10):438-444.

Imai, Y., M. Soda, and R. Takahashi. 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J Biol Chem* 275 (46):35661-35664.

Iwakura, Y., Y. S. Piao, M. Mizuno, N. Takei, A. Kakita, H. Takahashi, and H. Nawa. 2005. Influences of dopaminergic lesion on epidermal growth factor-ErbB signals in Parkinson's disease and its model: neurotrophic implication in nigrostriatal neurons. *J Neurochem* 93 (4):974-983.

Jaaro, H., G. Beck, S. G. Conticello, and M. Fainzilber. 2001. Evolving better brains: a need for neurotrophins? *Trends Neurosci* 24 (2):79-85.

Jackson-Lewis, V., M. Vila, R. Djaldetti, C. Guegan, G. Liberatore, J. Liu, K. L. O'Malley, R. E. Burke, and S. Przedborski. 2000. Developmental cell death in dopaminergic neurons of the substantia nigra of mice. *J Comp Neurol* 424 (3):476-488.

Jenner, P. 2008. Functional models of Parkinson's disease: a valuable tool in the development of novel therapies. *Ann Neurol* 64 Suppl 2:S16-29.

Jin, G., H. Kubo, M. Kashiba, R. Horinouchi, M. Hasegawa, M. Suzuki, T. Sagawa, M. Oizumi, A. Fujisawa, H. Tsukamoto, S. Yoshimura, and Y. Yamamoto. 2008. Saposin B is a human coenzyme q10-binding/transfer protein. *J Clin Biochem Nutr* 42 (2):167-174.

Johnston, L. C., X. Su, K. Maguire-Zeiss, K. Horovitz, I. Ankoudinova, D. Guschin, P. Hadaczek, H. J. Federoff, K. Bankiewicz, and J. Forsayeth. 2008. Human interleukin-10 gene transfer is protective in a rat model of Parkinson's disease. *Mol Ther* 16 (8):1392-1399.

Jones, K. R., I. Fariñas, C. Backus, and L. F. Reichardt. 1994. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* 76 (6):989-999.

Jongstra, J., T. J. Schall, B. J. Dyer, C. Clayberger, J. Jorgensen, M. M. Davis, and A. M. Krensky. 1987. The isolation and sequence of a novel gene from a human functional T cell line. *J Exp Med* 165 (3):601-614.

Jordan, J., M. Böttner, H. J. Schluesener, K. Unsicker, and K. Krieglstein. 1997. Bone morphogenetic proteins: neurotrophic roles for midbrain dopaminergic neurons and implications of astroglial cells. *Eur J Neurosci* 9 (8):1699-1709.

Jordan, P. A., and J. M. Gibbins. 2006. Extracellular disulfide exchange and the regulation of cellular function. *Antioxid Redox Signal* 8 (3-4):312-324.

Kadota, T., T. Shingo, T. Yasuhara, N. Tajiri, A. Kondo, T. Morimoto, W. J. Yuan, F. Wang, T. Baba, K. Tokunaga, Y. Miyoshi, and I. Date. 2009. Continuous intraventricular infusion of erythropoietin exerts neuroprotective/rescue effects upon Parkinson's disease model of rats with enhanced neurogenesis. *Brain Res* 1254:120-127.

Kassabov, S. R., Karl, K.A., Monje, F.J., Choi, Y.B., Balachandran, Y., Kandel, E. 2008. A novel Aplysia neurotrophin ApNT1 and its cognate ApTrk receptor constitute an invertebrate neurotrophic signaling system. Abstract at NGF 2008 / Katzir meeting on life and death in the nervous system, at Israel.

Kearns, C. M., W. A. Cass, K. Smoot, R. Kryscio, and D. M. Gash. 1997. GDNF protection against 6-OHDA: time dependence and requirement for protein synthesis. *J Neurosci* 17 (18):7111-7118.

Kearns, C. M., and D. M. Gash. 1995. GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo. *Brain Res* 672 (1-2):104-111.

Keinänen, K., A. Jouppila, and A. Kuusinen. 1998. Characterization of the kainate-binding domain of the glutamate receptor GluR-6 subunit. *Biochem J* 330 (Pt 3):1461-1467.

Keller, F., W. Hanke, D. Trissl, and T. Bakker-Grunwald. 1989. Pore-forming protein from Entamoeba histolytica forms voltage- and pH-controlled multi-state channels with properties similar to those of the barrel-stave aggregates. *Biochim Biophys Acta* 982 (1):89-93.

Kermani, P., and B. Hempstead. 2007. Brain-derived neurotrophic factor: a newly described mediator of angiogenesis. *Trends Cardiovasc Med* 17 (4):140-143.

Kervinen, J., G. J. Tobin, J. Costa, D. S. Waugh, A. Wlodawer, and A. Zdanov. 1999. Crystal structure of plant aspartic proteinase prophytepsin: inactivation and vacuolar targeting. *EMBO J* 18 (14):3947-3955.

Kim, I., W. Xu, and J. C. Reed. 2008. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* 7 (12):1013-1030.

Kirik, D., B. Georgievska, C. Rosenblad, and A. Björklund. 2001. Delayed infusion of GDNF promotes recovery of motor function in the partial lesion model of Parkinson's disease. *Eur J Neurosci* 13 (8):1589-1599.

Kishimoto, Y., M. Hiraiwa, and J. S. O'Brien. 1992. Saposins: structure, function, distribution, and molecular genetics. *J Lipid Res* 33 (9):1255-1267.

Kitada, T., A. Pisani, D. R. Porter, H. Yamaguchi, A. Tscherter, G. Martella, P. Bonsi, C. Zhang, E. N. Pothos, and J. Shen. 2007. Impaired dopamine release and synaptic plasticity in the striatum of PINK1-deficient mice. *Proc Natl Acad Sci U S A* 104 (27):11441-11446.

Knusel, B., P. P. Michel, J. S. Schwaber, and F. Hefti. 1990. 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* 10 (2):558-570.

Knusel, B., J. W. Winslow, A. Rosenthal, L. E. Burton, D. P. Seid, K. Nikolics, and F. Hefti. 1991. Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proc Natl Acad Sci U S A* 88 (3):961-965.

Kolter, T., and K. Sandhoff. 2005. Principles of lysosomal membrane digestion: stimulation of sphingolipid degradation by sphingolipid activator proteins and anionic lysosomal lipids. *Annu Rev Cell Dev Biol* 21:81-103.

Kolzer, M., K. Ferlinz, O. Bartelsen, S. L. Hoops, F. Lang, and K. Sandhoff. 2004. Functional characterization of the postulated intramolecular sphingolipid activator protein domain of human acid sphingomyelinase. *Biol Chem* 385 (12):1193-1195.

Kordower, J. H., C. D. Herzog, B. Dass, R. A. Bakay, J. Stansell, 3rd, M. Gasmi, and R. T. Bartus. 2006. Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. *Ann Neurol* 60 (6):706-715.

Kordower, J. H., S. Palfi, E. Y. Chen, S. Y. Ma, T. Sendera, E. J. Cochran, E. J. Mufson, R. Penn, C. G. Goetz, and C. D. Comella. 1999. Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. *Ann Neurol* 46 (3):419-424.

Kotani, Y., S. Matsuda, M. Sakanaka, K. Kondoh, S. Ueno, and A. Sano. 1996a. Prosaposin facilitates sciatic nerve regeneration in vivo. *J Neurochem* 66 (5):2019-2025.

Kotani, Y., S. Matsuda, T. C. Wen, M. Sakanaka, J. Tanaka, N. Maeda, K. Kondoh, S. Ueno, and A. Sano. 1996b. A hydrophilic peptide comprising 18 amino acid residues of the prosaposin sequence has neurotrophic activity in vitro and in vivo. *J Neurochem* 66 (5):2197-2200.

Kotzbauer, P. T., P. A. Lampe, R. O. Heuckeroth, J. P. Golden, D. J. Creedon, E. M. Johnson, Jr., and J. Milbrandt. 1996. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* 384 (6608):467-470.

Kramer, E. R., L. Aron, G. M. Ramakers, S. Seitz, X. Zhuang, K. Beyer, M. P. Smidt, and R. Klein. 2007. Absence of Ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. *PLoS Biol* 5 (3):e39.

Krensky, A. M., and C. Clayberger. 2005. Granulysin: a novel host defense molecule. *Am J Transplant* 5 (8):1789-1792.

Krieglstein, K. 2004. Factors promoting survival of mesencephalic dopaminergic neurons. *Cell Tissue Res* 318 (1):73-80.

Krieglstein, K., P. Henheik, L. Farkas, J. Jaszai, D. Galter, K. Krohn, and K. Unsicker. 1998. Glial cell line-derived neurotrophic factor requires transforming growth factor-beta for exerting its full neurotrophic potential on peripheral and CNS neurons. *J Neurosci* 18 (23):9822-9834.

Krieglstein, K., C. Suter-Crazzolara, G. Hotten, J. Pohl, and K. Unsicker. 1995. Trophic and protective effects of growth/differentiation factor 5, a member of the transforming growth factor-beta superfamily, on midbrain dopaminergic neurons. *J Neurosci Res* 42 (5):724-732.

Krieglstein, K., and K. Unsicker. 1994. Transforming growth factor-beta promotes survival of midbrain dopaminergic neurons and protects them against N-methyl-4-phenylpyridinium ion toxicity. *Neuroscience* 63 (4):1189-1196.

Lang, A. E., S. Gill, N. K. Patel, A. Lozano, J. G. Nutt, R. Penn, D. J. Brooks, G. Hotton, E. Moro, P. Heywood, M. A. Brodsky, K. Burchiel, P. Kelly, A. Dalvi, B. Scott, M. Stacy, D. Turner, V. G. Wooten, W. J. Elias, E. R. Laws, V. Dhawan, A. J. Stoessl, J. Matcham, R. J. Coffey, and M. Traub. 2006. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann Neurol* 59 (3):459-466.

Langston, J. W., P. Ballard, J. W. Tetrud, and I. Irwin. 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* 219 (4587):979-980.

Langston, J. W., L. S. Forno, C. S. Rebert, and I. Irwin. 1984. Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey. *Brain Res* 292 (2):390-394.

Lapchak, P. A., S. Jiao, F. Collins, and P. J. Miller. 1997. Glial cell line-derived neurotrophic factor: distribution and pharmacology in the rat following a bolus intraventricular injection. *Brain Res* 747 (1):92-102.

Lee, A. H., N. N. Iwakoshi, and L. H. Glimcher. 2003. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol* 23 (21):7448-7459.

Lee, F. S., and M. V. Chao. 2001. Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc Natl Acad Sci U S A* 98 (6):3555-3560.

Lee, M. K., W. Stirling, Y. Xu, X. Xu, D. Qui, A. S. Mandir, T. M. Dawson, N. G. Copeland, N. A. Jenkins, and D. L. Price. 2002. Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 --> Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proc Natl Acad Sci U S A* 99 (13):8968-8973.

Lee, T. J., O. Sartor, R. B. Luftig, and S. Koochekpour. 2004. Saposin C promotes survival and prevents apoptosis via PI3K/Akt-dependent pathway in prostate cancer cells. *Mol Cancer* 3:31.

Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y. A. Barde. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* 341 (6238):149-152.

Levi-Montalcini, R. 1987. The nerve growth factor 35 years later. *Science* 237 (4819):1154-1162.

Lewitt, P. A. 2008. Levodopa for the treatment of Parkinson's disease. *N Engl J Med* 359 (23):2468-2476.

Liang, G., T. E. Audas, Y. Li, G. P. Cockram, J. D. Dean, A. C. Martyn, K. Kokame, and R. Lu. 2006. Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element. *Mol Cell Biol* 26 (21):7999-8010.

Lieb, K., C. Andersen, N. Lazarov, R. Zienecker, I. Urban, I. Reisert, and C. Pilgrim. 1996. Pre- and postnatal development of dopaminergic neuron numbers in the male and female mouse midbrain. *Brain Res Dev Brain Res* 94 (1):37-43.

Liepinsh, E., M. Andersson, J. M. Ruysschaert, and G. Otting. 1997. Saposin fold revealed by the NMR structure of NK-lysin. *Nat Struct Biol* 4 (10):793-795.

Lillis, A. P., L. B. Van Duyn, J. E. Murphy-Ullrich, and D. K. Strickland. 2008. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiol Rev* 88 (3):887-918.

Lin, L. F., D. H. Doherty, J. D. Lile, S. Bektesh, and F. Collins. 1993. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260 (5111):1130-1132.

Lin, L. F., T. J. Zhang, F. Collins, and L. G. Armes. 1994. Purification and initial

characterization of rat B49 glial cell line-derived neurotrophic factor. *J Neurochem* 63 (2):758-768.

Lindahl, M., T. Timmusk, J. Rossi, M. Saarma, and M. S. Airaksinen. 2000. Expression and alternative splicing of mouse Gfra4 suggest roles in endocrine cell development. *Mol Cell Neurosci* 15 (6):522-533.

Lindfors, P. H., M. Lindahl, J. Rossi, M. Saarma, and M. S. Airaksinen. 2006. Ablation of persephin receptor glial cell line-derived neurotrophic factor family receptor alpha4 impairs thyroid calcitonin production in young mice. *Endocrinology* 147 (5):2237-2244.

Lindvall, O., and L. U. Wahlberg. 2008. Encapsulated cell biodelivery of GDNF: a novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease? *Exp Neurol* 209 (1):82-88.

Liu, J., C. Y. Wang, and J. S. O'Brien. 2001. Prosaptide D5, a retro-inverso 11-mer peptidomimetic, rescued dopaminergic neurons in a model of Parkinson's disease. *FASEB J* 15 (6):1080-1082.

Liu, Z., X. Wang, Y. Yu, X. Li, T. Wang, H. Jiang, Q. Ren, Y. Jiao, A. Sawa, T. Moran, C. A. Ross, C. Montell, and W. W. Smith. 2008. A Drosophila model for LRRK2-linked parkinsonism. *Proc Natl Acad Sci U S A* 105 (7):2693-2698.

Lotharius, J., and P. Brundin. 2002. Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci* 3 (12):932-942.

Love, S., P. Plaha, N. K. Patel, G. R. Hotton, D. J. Brooks, and S. S. Gill. 2005. Glial cell line-derived neurotrophic factor induces neuronal sprouting in human brain. *Nat Med* 11 (7):703-704.

Lu, B., P. T. Pang, and N. H. Woo. 2005. The yin and yang of neurotrophin action. *Nat Rev Neurosci* 6 (8):603-614.

Magal, E., P. Burnham, S. Varon, and J. C. Louis. 1993. Convergent regulation by ciliary neurotrophic factor and dopamine of tyrosine hydroxylase expression in cultures of rat substantia nigra. *Neuroscience* 52 (4):867-881.

Maisonpierre, P. C., L. Belluscio, S. Squinto, N. Y. Ip, M. E. Furth, R. M. Lindsay, and G. D. Yancopoulos. 1990. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science* 247 (4949 Pt 1):1446-1451.

Marks, W. J., Jr., J. L. Ostrem, L. Verhagen, P. A. Starr, P. S. Larson, R. A. Bakay, R. Taylor, D. A. Cahn-Weiner, A. J. Stoessl, C. W. Olanow, and R. T. Bartus. 2008. Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. *Lancet Neurol* 7 (5):400-408.

Mattson, M. P., M. Gleichmann, and A. Cheng. 2008. Mitochondria in neuroplasticity and neurological disorders. *Neuron* 60 (5):748-766.

Mattson, M. P., and T. Magnus. 2006. Ageing and neuronal vulnerability. *Nat Rev Neurosci* 7 (4):278-294.

McNaught, K. S., R. Belizaire, O. Isacson, P. Jenner, and C. W. Olanow. 2003. Altered proteasomal function in sporadic Parkinson's disease. *Exp Neurol* 179 (1):38-46.

Meng, X., D. G. de Rooij, K. Westerdahl, M. Saarma, and H. Sariola. 2001. Promotion of seminomatous tumors by targeted overexpression of glial cell line-derived neurotrophic factor in mouse testis. *Cancer Res* 61 (8):3267-3271.

Milbrandt, J., F. J. de Sauvage, T. J. Fahrner, R. H. Baloh, M. L. Leitner, M. G. Tansey, P. A. Lampe, R. O. Heuckeroth, P. T. Kotzbauer, K. S. Simburger, J. P. Golden, J. A. Davies, R. Vejsada, A. C. Kato, M. Hynes, D. Sherman, M. Nishimura, L. C. Wang, R. Vandlen, B. Moffat, R. D. Klein, K. Poulsen, C. Gray, A. Garces, E. M. Johnson, Jr., and et al. 1998. Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron* 20 (2):245-253.

Misasi, R., M. Sorice, T. Garofalo, T. Griggi, W. M. Campana, M. Giammatteo, A. Pavan, M. Hiraiwa, G. M. Pontieri, and J. S. O'Brien. 1998. Colocalization and complex formation between prosaposin and monosialoganglioside GM3 in neural cells. *J Neurochem* 71 (6):2313-2321.

Mizobuchi, N., J. Hoseki, H. Kubota, S. Toyokuni, J. Nozaki, M. Naitoh, A. Koizumi, and K. Nagata. 2007. ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element. *Cell Struct Funct* 32 (1):41-50.

Mogi, M., A. Togari, T. Kondo, Y. Mizuno, O. Komure, S. Kuno, H. Ichinose, and T. Nagatsu. 1999. Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. *Neurosci Lett* 270 (1):45-48.

Mohapel, P., H. Frielingsdorf, J. Häggblad, O. Zachrisson, and P. Brundin. 2005. Platelet-derived growth factor (PDGF-BB) and brain-derived neurotrophic factor (BDNF) induce striatal neurogenesis in adult rats with 6-hydroxydopamine lesions. *Neuroscience* 132 (3):767-776.

Moore, D. J., A. B. West, V. L. Dawson, and T. M. Dawson. 2005. Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci* 28:57-87.

Moore, M. W., R. D. Klein, I. Fariñas, H. Sauer, M. Armanini, H. Phillips, L. F. Reichardt, A. M. Ryan, K. Carver-Moore, and A. Rosenthal. 1996. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* 382 (6586):76-79.

Mufson, E. J., J. S. Kroin, T. Sobreviela, M. A. Burke, J. H. Kordower, R. D. Penn, and J. A. Miller. 1994. Intrastriatal infusions of brain-derived neurotrophic factor: retrograde transport and colocalization with dopamine containing substantia nigra neurons in rat. *Exp Neurol* 129 (1):15-26.

Murzin, A. G., S. E. Brenner, T. Hubbard, and C. Chothia. 1995. SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* 247 (4):536-540.

Narindrasorasak, S., P. Yao, and B. Sarkar. 2003. Protein disulfide isomerase, a multifunctional protein chaperone, shows copper-binding activity. *Biochem Biophys Res Commun* 311 (2):405-414.

Nikkhah, G., P. Odin, A. Smits, A. Tingstrom, A. Othberg, P. Brundin, K. Funa, and O. Lindvall. 1993. Platelet-derived growth factor promotes survival of rat and human mesencephalic dopaminergic neurons in culture. *Exp Brain Res* 92 (3):516-523.

Numan, S., and K. B. Seroogy. 1999. Expression of trkB and trkC mRNAs by adult midbrain dopamine neurons: a double-label in situ hybridization study. *J Comp Neurol* 403 (3):295-308.

Nutt, J. G., K. J. Burchiel, C. L. Comella, J. Jankovic, A. E. Lang, E. R. Laws, Jr., A. M. Lozano, R. D. Penn, R. K. Simpson, Jr., M. Stacy, and G. F. Wooten. 2003. Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology* 60 (1):69-73.

O'Brien, J. S., G. S. Carson, H. C. Seo, M. Hiraiwa, and Y. Kishimoto. 1994. Identification of prosaposin as a neurotrophic factor. *Proc Natl Acad Sci U S A* 91 (20):9593-9596.

O'Brien, J. S., G. S. Carson, H. C. Seo, M. Hiraiwa, S. Weiler, J. M. Tomich, J. A. Barranger, M. Kahn, N. Azuma, and Y. Kishimoto. 1995. Identification of the neurotrophic factor sequence of prosaposin. *FASEB J* 9 (8):681-685.

Oo, T. F., and R. E. Burke. 1997. The time course of developmental cell death in phenotypically defined dopaminergic neurons of the substantia nigra. *Brain Res Dev Brain Res* 98 (2):191-196.

Oppenheim, R. W. 1991. Cell death during development of the nervous system. *Annu Rev Neurosci* 14:453-501.

Oppenheim, R. W., L. J. Houenou, J. E. Johnson, L. F. Lin, L. Li, A. C. Lo, A. L.

Newsome, D. M. Prevette, and S. Wang. 1995. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* 373 (6512):344-346.

Palgi, M., R. Lindström, J. Peränen, T. P. Piepponen, M. Saarma, and T. I. Heino. 2009. Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. *Proc Natl Acad Sci U S A*.

Panchision, D. M., P. A. Martin-DeLeon, T. Takeshima, J. M. Johnston, K. Shimoda, P. Tsoulfas, R. D. McKay, and J. W. Commissiong. 1998. An immortalized, type-1 astrocyte of mesencephalic origin source of a dopaminergic neurotrophic factor. *J Mol Neurosci* 11 (3):209-221.

Parain, K., M. G. Murer, Q. Yan, B. Faucheux, Y. Agid, E. Hirsch, and R. Raisman-Vozari. 1999. Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. *Neuroreport* 10 (3):557-561.

Paratcha, G., F. Ledda, and C. F. Ibáñez. 2003. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* 113 (7):867-879.

Parkash, V., V. M. Leppänen, H. Virtanen, J. M. Jurvansuu, M. M. Bespalov, Y. A. Sidorova, P. Runeberg-Roos, M. Saarma, and A. Goldman. 2008. The Structure of the Glial Cell Line-derived Neurotrophic Factor-Coreceptor Complex: INSIGHTS INTO RET SIGNALING AND HEPARIN BINDING. *J Biol Chem* 283 (50):35164-35172.

Pascual, A., M. Hidalgo-Figueroa, J. I. Piruat, C. O. Pintado, R. Gomez-Diaz, and J. Lopez-Barneo. 2008. Absolute requirement of GDNF for adult catecholaminergic neuron survival. *Nat Neurosci* 11 (7):755-761.

Patel, N. K., M. Bunnage, P. Plaha, C. N. Svendsen, P. Heywood, and S. S. Gill. 2005. Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. *Ann Neurol* 57 (2):298-302.

Peña, S. V., D. A. Hanson, B. A. Carr, T. J. Goralski, and A. M. Krensky. 1997. Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. *J Immunol* 158 (6):2680-2688.

Peña, S. V., and A. M. Krensky. 1997. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. *Semin Immunol* 9 (2):117-125.

Peränen, J., M. Rikkonen, M. Hyvönen, and L. Kääriäinen. 1996. T7 vectors with modified T7lac promoter for expression of proteins in Escherichia coli. *Anal Biochem* 236 (2):371-373.

Petrova, P., A. Raibekas, J. Pevsner, N. Vigo, M. Anafi, M. K. Moore, A. E. Peaire, V. Shridhar, D. I. Smith, J. Kelly, Y. Durocher, and J. W. Commissiong. 2003. MANF: a new mesencephalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. *J Mol Neurosci* 20 (2):173-188.

Pichel, J. G., L. Shen, H. Z. Sheng, A. C. Granholm, J. Drago, A. Grinberg, E. J. Lee, S. P. Huang, M. Saarma, B. J. Hoffer, H. Sariola, and H. Westphal. 1996. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 382 (6586):73-76.

Piepoli, A., A. Gentile, M. R. Valvano, D. Barana, C. Oliani, R. Cotugno, M. Quitadamo, A. Andriulli, and F. Perri. 2006. Lack of association between UGT1A7, UGT1A9, ARP, SPINK1 and CFTR gene polymorphisms and pancreatic cancer in Italian patients. *World J Gastroenterol* 12 (39):6343-6348.

Ponting, C. P. 1994. Acid sphingomyelinase possesses a domain homologous to its activator proteins: saposins B and D. *Protein Sci* 3 (2):359-361.

Poulsen, K. T., M. P. Armanini, R. D. Klein, M. A. Hynes, H. S. Phillips, and A. Rosenthal. 1994. TGF beta 2 and TGF beta 3 are potent survival factors for midbrain dopaminergic neurons. *Neuron* 13 (5):1245-1252.

Prakash, N., and W. Wurst. 2006. Development of dopaminergic neurons in the mammalian brain. *Cell Mol Life Sci* 63 (2):187-206.

Puskovic, V., D. Wolfe, J. Wechuck, D. Krisky, J. Collins, J. C. Glorioso, D. J. Fink, and M. Mata. 2006. HSV-mediated delivery of erythropoietin restores dopaminergic function in MPTP-treated mice. *Mol Ther* 14 (5):710-715.

Qi, X., W. Qin, Y. Sun, K. Kondoh, and G. A. Grabowski. 1996. Functional organization of saposin C. Definition of the neurotrophic and acid beta-glucosidase activation regions. *J Biol Chem* 271 (12):6874-6880.

Quesada, A., and P. E. Micevych. 2004. Estrogen interacts with the IGF-1 system to protect nigrostriatal dopamine and maintain motoric behavior after 6-hydroxdopamine lesions. *J Neurosci Res* 75 (1):107-116.

Rahhal, B., S. Heermann, A. Ferdinand, J. Rosenbusch, M. Rickmann, and K. Krieglstein. 2009. In vivo requirement of TGF-beta/GDNF cooperativity in mouse development: focus on the neurotrophic hypothesis. *Int J Dev Neurosci* 27 (1):97-102.

Raykhel, I., H. Alanen, K. Salo, J. Jurvansuu, V. D. Nguyen, M. Latva-Ranta, and L. Ruddock. 2007. A molecular specificity code for the three mammalian KDEL receptors. *J Cell Biol* 179 (6):1193-1204.

Romero-Ramirez, L., H. Cao, D. Nelson, E. Hammond, A. H. Lee, H. Yoshida, K. Mori, L. H. Glimcher, N. C. Denko, A. J. Giaccia, Q. T. Le, and A. C. Koong. 2004. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. *Cancer Res* 64 (17):5943-5947.

Rosenblad, C., D. Kirik, and A. Björklund. 2000. Sequential administration of GDNF into the substantia nigra and striatum promotes dopamine neuron survival and axonal sprouting but not striatal reinnervation or functional recovery in the partial 6-OHDA lesion model. *Exp Neurol* 161 (2):503-516.

Rosenblad, C., D. Kirik, B. Devaux, B. Moffat, H. S. Phillips, and A. Björklund. 1999. Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. *Eur J Neurosci* 11 (5):1554-1566.

Rosenblad, C., A. Martinez-Serrano, and A. Björklund. 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 (1):129-137.

Rosenthal, A., D. V. Goeddel, T. Nguyen, M. Lewis, A. Shih, G. R. Laramee, K. Nikolics, and J. W. Winslow. 1990. Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* 4 (5):767-773.

Rossmann, M., R. Schultz-Heienbrok, J. Behlke, N. Remmel, C. Alings, K. Sandhoff, W. Saenger, and T. Maier. 2008. Crystal structures of human saposins C andD: implications for lipid recognition and membrane interactions. *Structure* 16 (5):809-817.

Roux, P. P., and P. A. Barker. 2002. Neurotrophin signaling through the p75 neurotrophin receptor. *Prog Neurobiol* 67 (3):203-233.

Ryu, E. J., H. P. Harding, J. M. Angelastro, O. V. Vitolo, D. Ron, and L. A. Greene. 2002. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J Neurosci* 22 (24):10690-10698.

Saarma, M. 2000. GDNF - a stranger in the TGF-beta superfamily? *Eur J Biochem* 267 (24):6968-6971.

Saarma, M., and H. Sariola. 1999. Other neurotrophic factors: glial cell line-derived neurotrophic factor (GDNF). *Microsc Res Tech* 45 (4-5):292-302.

Sanchez, M. P., I. Silos-Santiago, J. Frisen, B. He, S. A. Lira, and M. Barbacid. 1996. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* 382 (6586):70-73.

Sano, A., S. Matsuda, T. C. Wen, Y. Kotani, K. Kondoh, S. Ueno, Y. Kakimoto, H. Yoshimura, and M. Sakanaka. 1994. Protection by prosaposin against ischemia-induced learning disability and neuronal loss. *Biochem Biophys Res Commun* 204 (2):994-1000.

Santoro, M., R. M. Melillo, F. Carlomagno, G. Vecchio, and A. Fusco. 2004. Minireview: RET: normal and abnormal functions. *Endocrinology* 145 (12):5448-5451.

Sauer, H., and W. H. Oertel. 1994. Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience* 59 (2):401-415.

Sauer, H., C. Rosenblad, and A. Björklund. 1995. Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. *Proc Natl Acad Sci U S A* 92 (19):8935-8939.

Saura, J., M. Pares, J. Bove, S. Pezzi, J. Alberch, C. Marin, E. Tolosa, and M. J. Marti. 2003. Intranigral infusion of interleukin-1beta activates astrocytes and protects from subsequent 6-hydroxydopamine neurotoxicity. *J Neurochem* 85 (3):651-661.

Schaar, D. G., B. A. Sieber, C. F. Dreyfus, and I. B. Black. 1993. Regional and cell-specific expression of GDNF in rat brain. *Exp Neurol* 124 (2):368-371.

Schapira, A. H. 2008. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol* 7 (1):97-109.

Schapira, A. H., J. M. Cooper, D. Dexter, P. Jenner, J. B. Clark, and C. D. Marsden. 1989. Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* 1 (8649):1269.

Schober, A. 2004. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell Tissue Res* 318 (1):215-224.

Schober, A., H. Peterziel, C. S. von Bartheld, H. Simon, K. Krieglstein, and K. Unsicker. 2007. GDNF applied to the MPTP-lesioned nigrostriatal system requires TGF-beta for its neuroprotective action. *Neurobiol Dis* 25 (2):378-391.

Schwertassek, U., Y. Balmer, M. Gutscher, L. Weingarten, M. Preuss, J. Engelhard, M. Winkler, and T. P. Dick. 2007. Selective redox regulation of cytokine receptor signaling by extracellular thioredoxin-1. *EMBO J* 26 (13):3086-3097.

Sendtner, M., G. Pei, M. Beck, U. Schweizer, and S. Wiese. 2000. Developmental motoneuron cell death and neurotrophic factors. *Cell Tissue Res* 301 (1):71-84.

Shridhar, R., V. Shridhar, S. Rivard, J. M. Siegfried, H. Pietraszkiewicz, J. Ensley, R. Pauley, D. Grignon, W. Sakr, O. J. Miller, and D. I. Smith. 1996a. Mutations in the arginine-rich protein gene, in lung, breast, and prostate cancers, and in squamous cell carcinoma of the head and neck. *Cancer Res* 56 (24):5576-5578.

Shridhar, V., S. Rivard, R. Shridhar, C. Mullins, L. Bostick, W. Sakr, D. Grignon, O. J. Miller, and D. I. Smith. 1996b. A gene from human chromosomal band 3p21.1 encodes a highly conserved arginine-rich protein and is mutated in renal cell carcinomas. *Oncogene* 12 (9):1931-1939.

Shridhar, V., S. Rivard, X. Wang, R. Shridhar, C. Paisley, C. Mullins, L. Beirnat, M. Dugan, F. Sarkar, O. J. Miller, V. K. Vaitkevicius, and D. I. Smith. 1997. Mutations in the arginine-rich protein gene (ARP) in pancreatic cancer. *Oncogene* 14 (18):2213-2216.

Shults, C. W., J. Ray, K. Tsuboi, and F. H. Gage. 2000. Fibroblast growth factor-2-producing fibroblasts protect the nigrostriatal dopaminergic system from 6-hydroxydopamine. *Brain Res* 883 (2):192-204.

Signore, A. P., Z. Weng, T. Hastings, A. D. Van Laar, Q. Liang, Y. J. Lee, and J. Chen. 2006. Erythropoietin protects against 6-hydroxydopamine-induced dopaminergic cell death. *J Neurochem* 96 (2):428-443.

Silva, R. M., V. Ries, T. F. Oo, O. Yarygina, V. Jackson-Lewis, E. J. Ryu, P. D. Lu, S. J. Marciniak, D. Ron, S. Przedborski, N. Kholodilov, L. A. Greene, and R. E. Burke. 2005. CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an in vivo neurotoxin model of parkinsonism. *J Neurochem* 95 (4):974-986.

Silverman, W. F., J. M. Krum, N. Mani, and J. M. Rosenstein. 1999. Vascular, glial and neuronal effects of vascular endothelial growth factor in mesencephalic explant cultures. *Neuroscience* 90 (4):1529-1541.

Slevin, J. T., G. A. Gerhardt, C. D. Smith, D. M. Gash, R. Kryscio, and B. Young. 2005. Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. *J Neurosurg* 102 (2):216-222.

Smidt, M. P., and J. P. Burbach. 2007. How to make a mesodiencephalic dopaminergic neuron. *Nat Rev Neurosci* 8 (1):21-32.

Smits, S. M., J. P. Burbach, and M. P. Smidt. 2006. Developmental origin and fate of meso-diencephalic dopamine neurons. *Prog Neurobiol* 78 (1):1-16.

Sorice, M., S. Molinari, L. Di Marzio, V. Mattei, V. Tasciotti, L. Ciarlo, M. Hiraiwa, T. Garofalo, and R. Misasi. 2008. Neurotrophic signalling pathway triggered by prosaposin in PC12 cells occurs through lipid rafts. *FEBS J* 275 (19):4903-4912.

Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, S. A. Porcelli, B. R. Bloom, A. M. Krensky, and R. L. Modlin. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 282 (5386):121-125.

Strelau, J., A. Sullivan, M. Böttner, P. Lingor, E. Falkenstein, C. Suter-Crazzolara, D. Galter, J. Jaszai, K. Krieglstein, and K. Unsicker. 2000. Growth/differentiation factor-15/macrophage inhibitory cytokine-1 is a novel trophic factor for midbrain dopaminergic neurons in vivo. *J Neurosci* 20 (23):8597-8603.

Strömberg, I., L. Björklund, M. Johansson, A. Tomac, F. Collins, L. Olson, B. Hoffer, and C. Humpel. 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 (2):401-412.

Studer, L., M. Csete, S. H. Lee, N. Kabbani, J. Walikonis, B. Wold, and R. McKay. 2000. Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J Neurosci* 20 (19):7377-7383.

Sugaya, R., S. Ishimaru, T. Hosoya, K. Saigo, and Y. Emori. 1994. A Drosophila homolog of human proto-oncogene ret transiently expressed in embryonic neuronal precursor cells including neuroblasts and CNS cells. *Mech Dev* 45 (2):139-145.

Sullivan, A. M., J. Opacka-Juffry, and S. B. Blunt. 1998. Long-term protection of the rat nigrostriatal dopaminergic system by glial cell line-derived neurotrophic factor against 6-hydroxydopamine in vivo. *Eur J Neurosci* 10 (1):57-63.

Sullivan, A. M., J. Opacka-Juffry, G. Hotten, J. Pohl, and S. B. Blunt. 1997. Growth/differentiation factor 5 protects nigrostriatal dopaminergic neurones in a rat model of Parkinson's disease. *Neurosci Lett* 233 (2-3):73-76.

Sun, P. D., and D. R. Davies. 1995. The cystine-knot growth-factor superfamily. *Annu Rev Biophys Biomol Struct* 24:269-291.

Sun, Y., D. P. Witte, H. Ran, M. Zamzow, S. Barnes, H. Cheng, X. Han, M. T. Williams, M. R. Skelton, C. V. Vorhees, and G. A. Grabowski. 2008. Neurological deficits and glycosphingolipid accumulation in saposin B deficient mice. *Hum Mol Genet* 17 (15):2345-2356.

Szegezdi, E., A. Duffy, M. E. O'Mahoney, S. E. Logue, L. A. Mylotte, T. O'Brien, and

A. Samali. 2006a. ER stress contributes to ischemia-induced cardiomyocyte apoptosis. *Biochem Biophys Res Commun* 349 (4):1406-1411.

Szegezdi, E., S. E. Logue, A. M. Gorman, and A. Samali. 2006b. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 7 (9):880-885.

Tadimalla, A., P. J. Belmont, D. J. Thuerauf, M. S. Glassy, J. J. Martindale, N. Gude, M. A. Sussman, and C. C. Glembotski. 2008. Mesencephalic astrocyte-derived neurotrophic factor is an ischemia-inducible secreted endoplasmic reticulum stress response protein in the heart. *Circ Res* 103 (11):1249-1258.

Tanaka, H., Y. Shimada, H. Harada, M. Shinoda, S. Hatooka, M. Imamura, and K. Ishizaki. 2000. Polymorphic variation of the ARP gene on 3p21 in Japanese esophageal cancer patients. *Oncol Rep* 7 (3):591-593.

Teismann, P., K. Tieu, O. Cohen, D. K. Choi, D. C. Wu, D. Marks, M. Vila, V. Jackson-Lewis, and S. Przedborski. 2003. Pathogenic role of glial cells in Parkinson's disease. *Mov Disord* 18 (2):121-129.

Thomas, B., and M. F. Beal. 2007. Parkinson's disease. *Hum Mol Genet* 16 Spec No. 2:R183-194.

Tomac, A., E. Lindqvist, L. F. Lin, S. O. Ogren, D. Young, B. J. Hoffer, and L. Olson. 1995a. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* 373 (6512):335-339.

Tomac, A., J. Widenfalk, L. F. Lin, T. Kohno, T. Ebendal, B. J. Hoffer, and L. Olson. 1995b. 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 U S A* 92 (18):8274-8278.

Treanor, J. J., L. Goodman, F. de Sauvage, D. M. Stone, K. T. Poulsen, C. D. Beck, C. Gray, M. P. Armanini, R. A. Pollock, F. Hefti, H. S. Phillips, A. Goddard, M. W. Moore, A. Buj-Bello, A. M. Davies, N. Asai, M. Takahashi, R. Vandlen, C. E. Henderson, and A. Rosenthal. 1996. Characterization of a multicomponent receptor for GDNF. *Nature* 382 (6586):80-83.

Trupp, M., N. Belluardo, H. Funakoshi, and C. F. Ibáñez. 1997. Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *J Neurosci* 17 (10):3554-3567.

Trupp, M., M. Ryden, H. Jornvall, H. Funakoshi, T. Timmusk, E. Arenas, and C. F. Ibáñez. 1995. Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *J Cell Biol* 130 (1):137-148.

Uehara, T., T. Nakamura, D. Yao, Z. Q. Shi, Z. Gu, Y. Ma, E. Masliah, Y. Nomura, and S. A. Lipton. 2006. S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature* 441 (7092):513-517.

Ungerstedt, U., and G. W. Arbuthnott. 1970. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res* 24 (3):485-493.

Vaccaro, A. M., R. Salvioli, A. Barca, M. Tatti, F. Ciaffoni, B. Maras, R. Siciliano, F. Zappacosta, A. Amoresano, and P. Pucci. 1995. Structural analysis of saposin C and B. Complete localization of disulfide bridges. *J Biol Chem* 270 (17):9953-9960.

Vaccaro, A. M., R. Salvioli, M. Tatti, and F. Ciaffoni. 1999. Saposins and their interaction with lipids. *Neurochem Res* 24 (2):307-314.

Vaccaro, A. M., M. Tatti, F. Ciaffoni, R. Salvioli, A. Serafino, and A. Barca. 1994. Saposin C induces pH-dependent destabilization and fusion of phosphatidylserine-containing vesicles. *FEBS Lett* 349 (2):181-186.

Van den Heuvel, D. M., and R. J. Pasterkamp. 2008. Getting connected in the dopamine system. *Prog Neurobiol* 85 (1):75-93.

Van Laar, V. S., and S. B. Berman. 2009. Mitochondrial dynamics in Parkinson's disease. *Exp Neurol*.

Venero, J. L., M. L. Vizuete, M. Revuelta, C. Vargas, J. Cano, and A. Machado. 2000. Upregulation of BDNF mRNA and trkB mRNA in the nigrostriatal system and in the lesion site following unilateral transection of the medial forebrain bundle. *Exp Neurol* 161 (1):38-48.

Vila, M., and S. Przedborski. 2003. Targeting programmed cell death in neurodegenerative diseases. *Nat Rev Neurosci* 4 (5):365-375.

Vitt, U. A., S. Y. Hsu, and A. J. Hsueh. 2001. Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. *Mol Endocrinol* 15 (5):681-694.

Vogel, A., G. Schwarzmann, and K. Sandhoff. 1991. Glycosphingolipid specificity of the human sulfatide activator protein. *Eur J Biochem* 200 (2):591-597.

von Bohlen und Halbach, O. 2004. Synucleins and their relationship to Parkinson's disease. *Cell Tissue Res* 318 (1):163-174.

von Coelln, R., K. Unsicker, and K. Krieglstein. 1995. Screening of interleukins for survival-promoting effects on cultured mesencephalic dopaminergic neurons from embryonic rat brain. *Brain Res Dev Brain Res* 89 (1):150-154.

Voutilainen, M. H., S. Bäck, E. Pörsti, L. Toppinen, L. Lindgren, P. Lindholm, J. Peränen, M. Saarma, and R. K. Tuominen. Neurotrophic factor MANF is neurorestorative in rat model of Parkinson's disease. Manuscript submitted.

Wang, J., K. S. Bankiewicz, R. J. Plunkett, and E. H. Oldfield. 1994. Intrastriatal implantation of interleukin-1. Reduction of parkinsonism in rats by enhancing neuronal sprouting from residual dopaminergic neurons in the ventral tegmental area of the midbrain. *J Neurosurg* 80 (3):484-490.

Wang, X., R. H. Baloh, J. Milbrandt, and K. C. Garcia. 2006. Structure of artemin complexed with its receptor GFRalpha3: convergent recognition of glial cell line-derived neurotrophic factors. *Structure* 14 (6):1083-1092.

White, K., M. E. Grether, J. M. Abrams, L. Young, K. Farrell, and H. Steller. 1994. Genetic control of programmed cell death in Drosophila. *Science* 264 (5159):677-683.

Widenfalk, J., C. Nosrat, A. Tomac, H. Westphal, B. Hoffer, and L. Olson. 1997. Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *J Neurosci* 17 (21):8506-8519.

Winau, F., V. Schwierzeck, R. Hurwitz, N. Remmel, P. A. Sieling, R. L. Modlin, S. A. Porcelli, V. Brinkmann, M. Sugita, K. Sandhoff, S. H. Kaufmann, and U. E. Schaible. 2004. Saposin C is required for lipid presentation by human CD1b. *Nat Immunol* 5 (2):169-174.

Winkler, C., H. Sauer, C. S. Lee, and A. Björklund. 1996. Short-term GDNF treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. *J Neurosci* 16 (22):7206-7215.

Xu, S. Z., P. Sukumar, F. Zeng, J. Li, A. Jairaman, A. English, J. Naylor, C. Ciurtin, Y. Majeed, C. J. Milligan, Y. M. Bahnasi, E. Al-Shawaf, K. E. Porter, L. H. Jiang, P. Emery, A. Sivaprasadarao, and D. J. Beech. 2008. TRPC channel activation by extracellular thioredoxin. *Nature* 451 (7174):69-72.

Xue, Y. Q., L. R. Zhao, W. P. Guo, and W. M. Duan. 2007. Intrastriatal administration of erythropoietin protects dopaminergic neurons and improves neurobehavioral outcome in a rat model of Parkinson's disease. *Neuroscience* 146 (3):1245-1258.

Yamamoto, K., H. Yoshida, K. Kokame, R. J. Kaufman, and K. Mori. 2004. Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II. *J Biochem* 136 (3):343-350.

Yan, Q., C. Matheson, and O. T. Lopez. 1995. In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* 373 (6512):341-344.

Yasuhara, T., T. Shingo, K. Kobayashi, A. Takeuchi, A. Yano, K. Muraoka, T. Matsui, Y. Miyoshi, H. Hamada, and I. Date. 2004. Neuroprotective effects of vascular endothelial growth factor (VEGF) upon dopaminergic neurons in a rat model of Parkinson's disease. *Eur J Neurosci* 19 (6):1494-1504.

Yasuhara, T., T. Shingo, K. Muraoka, M. Kameda, T. Agari, Y. Wen Ji, H. Hayase, H. Hamada, C. V. Borlongan, and I. Date. 2005. Neurorescue effects of VEGF on a rat model of Parkinson's disease. *Brain Res* 1053 (1-2):10-18.

Yuan, W., X. Qi, P. Tsang, S. J. Kang, P. A. Illarionov, G. S. Besra, J. Gumperz, and P. Cresswell. 2007. Saposin B is the dominant saposin that facilitates lipid binding to human CD1d molecules. *Proc Natl Acad Sci U S A* 104 (13):5551-5556.

Yurek, D. M., and A. Fletcher-Turner. 2001. Differential expression of GDNF, BDNF, and NT-3 in the aging nigrostriatal system following a neurotoxic lesion. *Brain Res* 891 (1-2):228-235.

Zacchigna, S., D. Lambrechts, and P. Carmeliet. 2008. Neurovascular signalling defects in neurodegeneration. *Nat Rev Neurosci* 9 (3):169-181.

Zachrisson, O., A. Andersson, K. Dannaeus, R. Isacson, A. Mercer, E. Nielsen, C. Patrone, H. Rönnholm, L. B. Wikström, D. A. Di Monte, A. L. McCormack , M. Zhao, K. K. Delfani, A. M. Janson Lang, and A. Haegerstrand. 2008. Neurorestorative effects of PDGF-BB in primate models of Parkinson's disease through stimulation of cell proliferation. Abstract at Society for Neuroscience, 2008, at Washington DC.

Zhai, Y., and M. H. Saier, Jr. 2000. The amoebapore superfamily. *Biochim Biophys Acta* 1469 (2):87-99.

Zhang, Y., and W. M. Pardridge. 2009. Near complete rescue of experimental Parkinson's disease with intravenous, non-viral GDNF gene therapy. *Pharm Res* 26 (5):1059-1063.

Zhang, Z., Y. Miyoshi, P. A. Lapchak, F. Collins, D. Hilt, C. Lebel, R. Kryscio, and D. M. Gash. 1997. Dose response to intraventricular glial cell line-derived neurotrophic factor administration in parkinsonian monkeys. *J Pharmacol Exp Ther* 282 (3):1396-1401.

Zhou, C., C. Xiao, J. W. Commissiong, K. Krnjevic, and J. H. Ye. 2006. Mesencephalic astrocyte-derived neurotrophic factor enhances nigral gamma-aminobutyric acid release. *Neuroreport* 17 (3):293-297.

Zhou, D., C. Cantu, 3rd, Y. Sagiv, N. Schrantz, A. B. Kulkarni, X. Qi, D. J. Mahuran, C. R. Morales, G. A. Grabowski, K. Benlagha, P. Savage, A. Bendelac, and L. Teyton. 2004. Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. *Science* 303 (5657):523-527.

Zhu, B., J. A. Pennack, P. McQuilton, M. G. Forero, K. Mizuguchi, B. Sutcliffe, C. J. Gu, J. C. Fenton, and A. Hidalgo. 2008. Drosophila neurotrophins reveal a common mechanism for nervous system formation. *PLoS Biol* 6 (11):e284.