Death pathways activated in the neurotrophic factor-deprived neurons

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ABBREVIATIONS:

AIF	apoptosis inducing factor
Apaf-1	apoptotic protease activating factor-1
ATP	adenosine diphosphate
BAF	bocaspartyl-(OMe)-fluoromethyl-ketone
BDNF	brain-derived neurotrophic factor
BH domain	Bcl-2 homology domain
CARD	caspase recruitment domain
Caspase	cysteinyl aspartate-specific proteinases
CDNF	conserved dopamine neurotrophic factor
CNTF	ciliary neurotrophic factor
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DMEM	Dulbecco´s modified Eagle´s medium
DRG	dorsal root ganglion
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FADD	Fas-associated protein with death domain
$FAIM_L$	long isoform of Fas apoptosis inhibitory molecule
FGF	the fibroblast growth factor
GDNF	glial cell line-derived neurotrophic factor
HBSS	Hank's balanced salt solution
IAP	inhibitor of apoptosis protein
IGF	insulin-like growth factor
IL-6	interleukin 6
JNK	c-jun N-terminal kinase
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
MLK	mixed lineage kinase
NGF	nerve growth factor
NRIF	neurotrophin receptor interacting factor
NT-3	neurotrophin-3
6-OHDA	6-hydroxydopamine
PARP	poly-(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCD	programed cell death
Ret	rearranged during transfection
RT-PCR	reverse transcription-polymerase chain reaction
SCG	superior cervical ganglion
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Smac/DIABLO	second mitochondria-derived activator of caspase/Direct IAP binding protein

SN	substantia nigra
TGF-β	transforming growth factor beta
TH	tyrosine hydroxylase
TNF	tumor necrosis factor
TRADD	TNFR1 associated death domain protein
XIAP	X-linked inhibitor of apoptosis

LIST OF ORIGINAL PUBLICATIONS

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

I. **Li-Ying Yu**, Eija Jokitalo, Yun-Fu Sun, Patrick Mehlen, Dan Lindholm, Mart Saarma and Urmas Arumäe (2003). GDNF-deprived sympathetic neurons die via a novel nonmitochondrial pathway. J Cell Biol. 163(5):987-97.

II. Servane Tauszig-Delamasure, **Li-Ying Yu**, Jorge Ruben Cabrera, Jimena Bouzas-Rodriguez, Catherine Mermet-Bouvier, Catherine Guix, Marie-Claire Bordeaux, Urmas Arumäe, and Patrick Mehlen (2007**)**. The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm. Proc Natl Acad Sci U S A. 104(33):13361-6. (first two authors have equal contribution).

III. **Li-ying Yu** and Urmas Arumäe (2008). Survival assay of transiently transfected dopaminergic neurons. J Neurosci Methods. 169(1):8-15.

IV. **Li-ying Yu**, Mart Saarma, and Urmas Arumäe (2008). Death Receptors and Caspases But Not Mitochondria Are Activated in the GDNF- or BDNF-Deprived Dopaminergic Neurons. J Neurosci. 28(30):7467-75.

ABSTRACT

Programed cell death (PCD) is a fundamental biological process that is as essential for the development and tissue homeostasis as cell proliferation, differentiation and adaptation. The main mode of PCD - apoptosis - occurs via specific pathways, such as mitochondrial or death receptor pathway. In the developing nervous system, programed death broadly occurs, mainly triggered by the deficiency of different survival-promoting neurotrophic factors, but the respective death pathways are poorly studied. In one of the best-characterized models, sympathetic neurons deprived of nerve growth factor (NGF) die via the classical mitochondrial apoptotic pathway. The main aim of this study was to describe the death programs activated in these and other neuronal populations by using neuronal cultures deprived of other neurotrophic factors.

First, this study showed that the cultured sympathetic neurons deprived of glial cell line-derived neurotrophic factor (GDNF) die via a novel non-classical death pathway, in which mitochondria and death receptors are not involved. Indeed, cytochrome *c* was not released into the cytosol, Bax, caspase-9, and caspase-3 were not involved, and Bcl-xL overexpression did not prevent the death. This pathway involved activation of mixed lineage kinases and c-jun, and crucially requires caspase-2 and -7. Second, it was shown that deprivation of neurotrophin-3 (NT-3) from cultured sensory neurons of the dorsal root ganglia kills them via a dependence receptor pathway, including cleavage of the NT-3 receptor TrkC and liberation of a pro-apoptotic dependence domain. Indeed, death of NT-3-deprived neurons was blocked by a dominant-negative construct interfering with TrkC cleavage. Also, the uncleavable mutant of TrkC, replacing the siRNA-silenced endogeneous TrkC, was not able to trigger death upon NT-3 removal. Such a pathway was not activated in another subpopulation of sensory neurons deprived of NGF. Third, it was shown that cultured midbrain dopaminergic neurons deprived of GDNF or brain-derived neurotrophic factor (BDNF) kills them by still a different pathway, in which death receptors and caspases, but not mitochondria, are activated. Indeed, cytochrome *c* was not released into the cytosol, Bax was not activated, and Bcl-xL did not block the death, but caspases were necessary for the death of these neurons. Blocking the components of the death receptor pathway - caspase-8, FADD, or Fas - blocked the death, whereas activation of Fas accelerated it. The activity of Fas in the dopaminergic neurons could be controlled by the apoptosis inhibitory molecule $FAIM_L$. For these studies we developed a novel assay to study apoptosis in the transfected dopaminergic neurons. Thus, a novel death pathway, characteristic for the dopaminergic neurons was described. The study suggests death receptors as possible targets for the treatment of Parkinson's disease, which is caused by the degeneration of dopaminergic neurons.

REVIEW OF THE LITERATURE

1.1. Programed cell death

The term "programed cell death" (PCD) was introduced by Richard Lockshin to denote the death of intersegmental muscles during insect metamorphosis (Lockshin and Williams, 1964). The term was coined to emphasize that the death was developmentally programed not accidentally. PCD is also often called ontogenetic cell death. By current understanding, PCD plays a pivotal role in the development by sculpting the shape of organs and achieving the proper cell number by removal of unwanted and superfluous cells, which are initially overproduced during development (Vaux and Korsmeyer, 1999; Ameisen, 2002). It also plays a critical role in pathologic processes by eliminating damaged or infected cells (Thompson, 1995). Cell death has attracted extensive research interest, mainly because of the potential for understanding oncogenesis and the possibility of exploiting the cell death program for therapeutic purposes. For example, tumor cells bear cell death-blocking mutations in cellular mechanisms that would otherwise have eliminated them. On the other hand, intentional induction of cell death might provide the means for eliminating unwanted cells (*e.g.* tumor cells).

1.1.1. Programed cell death in the development of the nervous system

PCD controls cell number in multicellular organisms and is particularly important for the proper development of the nervous system. During development of the nervous system, neurons are initially produced in excess and the surplus is removed during the critical PCD periods that coincide with target innervation establishment (Oppenheim, 1991). Thus, cell death acts as a kind of biological sculpturing process giving rise to the tissue shape, sharpens of the borders of the brain compartments, retention of the proper neuronal population, and matches to the neuronal number to the size of peripheral targets (Oppenheim, 1991).

Massive PCD of postmitotic neurons was first detected during the development of interactions between neurons and their targets (Oppenheim, 1981). This line of studies led to the discovery of the first neurotrophic factor, NGF, and formulation of the neurotrophic hypothesis (Hamburger, 1992; Hamburger, 1993; Levi-Montalcini, 1998). In its original form, this hypothesis stated that neurons compete for a limited amount of survival-promoting factors provided by the targets as means of attaining optimal, quantitative innervation of their targets (Barde, 1989). More recently, this hypothesis has been extended to also include competition for support from afferent inputs and other cellular partners such as glia (Oppenheim, 1996). The best characterized neurotrophic factors are those of the neurotrophin family, especially in the peripheral nervous system. Gene knockout studies show that specific populations of sensory or sympathetic neurons are lost in the mice lacking a particular neurotrophin or neurotrophin receptor. The neurons in the central nervous system are less affected in these knockout mice (Huang and Reichardt, 2001).

Several hypotheses have been proposed to account for the biological significance of neuronal death. Because the operation of the nervous system is unique in its

establishment and refinement of the synaptic network and neural circuitry, error-correction functions (*e.g*. deletion of harmful cells, negative selection) is an attractive hypothesis that provides an adaptive rationale for neuronal PCD. In fact, some have argued that it may be the major role for the PCD of differentiating neurons as they form connections (Clarke et al., 1998; Finlay and Pallas, 1989; Lamb, 1988). Also, it has been hypothesized that controlling of the number of innervating neurons by the target, during the ontogenesis (at PCD), is the only chance to regulate the neuronal number, as during the rest of life the number of almost all postmitotic neurons can only diminish (Buss et al., 2006).

Thus, PCD of developing neurons can serve a variety of different roles depending on the stage of development, neuronal subtypes, and species.

1.2. Neurotrophic factors

The classic conceptuation of mechanisms underlying the regulation of the neuron survival, the neurotrophic factor concept (Levi-Montalcini, 1987b; Thoenen *et al*., 1987), was primarily the result of embryological studies carried out during the 20th century. The target-derived neurotrophic factor concept originally postulated a neurotrophic molecule that was secreted by the innervated tissue and taken up by the axon terminal (Barde, 1989; Oppenheim, 1991). Such neurotrophic molecules were hypothesized to be available in a limited amount only to those neurons that had successfully established synaptic contacts with their target cells. Neurons without the trophic support were supposed to die by apoptosis. This interdependence of neurogenesis and factor support presumably helped adjust neuronal numbers to the size of the innervation targets. The period the number of neurons was established, by target-derived neurotrophic factors, was called the "programed or ontogenetic cell death period" (Oppenheim, 1991). The experimental evidence on which the neurotrophic factor concept was built came mainly from the studies of the first discovered and purified neurotrophic factor, NGF, and its capacity to promote survival, neurite outgrowth, and neurotransmitter synthesis of paravertebral sympathetic neurons (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1987b). Several families of neurotrophic factors later have been found, such as the neurotrophin family, GDNF family, members of the transforming growth factor beta (TGF-β) superfamily, and cytokine family. The cytokine family consists of numerous family members including the ciliary neurotrophic factor (CNTF) (Arakawa *et al*., 1990), interleukin 6 (IL-6) (Gadient and Otten, 1997), leukemia inhibitory factor (LIF) (Kim *et al*., 2005), cardiotrophin, and several other growth factors. They have multiple functions on both the peripheral and central nervous system. In addition, the hepatocyte growth factor, the insulin-like growth factor (IGF), the fibroblast growth factor (FGF) family, and some other members of the cytokine family also have neutrophic activity (Mitsumoto and Tsuzaka, 1999b; Mitsumoto and Tsuzaka, 1999a).

Although originally developed for the peripheral nervous system, the neurotrophic factor concept has also been adapted to the central nervous system. Unlike the peripheral neurons, which mostly depend on a single neurotrophic factor, however, the central neurons seem to depend on several survival-promoting factors, as no massive death of the neurons during PCD occurs in the respective knockout mice (Oppenheim, 1996). Extensions and

modifications of the classic concept have been made over the past few decades. Recruitment of trophic support from diverse sources, rather than the innervated target only, is now increasingly accepted. Neurotrophic support may even come in an autocrine manner, as with sensory neurons (Acheson *et al.*, 1995). Moreover, evidence exists showing the requirement of multiple neurotrophic factors acting simultaneously and/or sequentially on the same neuronal population (Davies, 1998).

1.2.1. Neurotrophin family

The neurotrophin family consists of several members: NGF, BDNF, and neurotrophin-3, -4/5, -6, and -7 (NT-3, NT-4/5, NT-6 and NT-7) (Hallböök *et al*., 1991; Gotz *et al*., 1994; Nilsson *et al*., 1998; von Boyen *et al*., 2002). NT-6 and NT-7 are only expressed in fish species. Neurotrophins contain a cysteine knot that is formed by three disulfide bonds, and they act exclusively as homodimers. The core structure is formed by two pairs of intertwined two-strand β-sheets, assembled by three disulfide bonds. This core structure is conserved in all members of the neurotrophin family (Butte *et al*., 1998; Butte, 2001). Neurotrophins are first synthesized as precursors (pre-pro-form). The pre-region is cleaved in the endoplasmic reticulum (ER) during secretion. The pro-form of the immature protein is then cleaved either in the Golgi or in the secretory granules into mature proteins (neurotrophic factors) (Seidah *et al*., 1996). Recently, the pro-NGF and pro-BDNF were also shown to be secreted without being cleaved and to have a pro-apoptotic activity of their own (Nykjaer *et al*., 2005). Neurotrophin receptors consist of two types of receptors: the Trk tyrosine kinase receptor family and the neurotrophin receptor p75 ($p75^{NTR}$). The Trk receptor family includes three receptors: TrkA, TrkB, and TrkC. Each of them binds a set of neurotrophins: TrkA

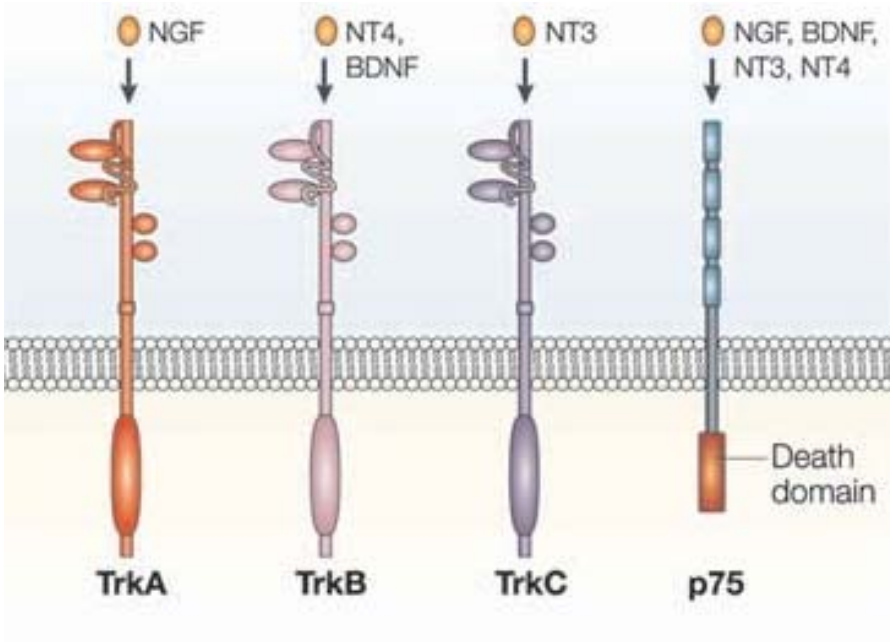

Figure 1. Neurotrophins and their receptors. Trk receptors bind specific neurotrophins, whereas p75 binds all neurotrophins. Modified from Chao (2003).

binds NGF, TrkB binds BDNF and NT-4/5, whereas TrkC binds preferentially NT-3 (Figure 1). $P75^{NTR}$, belonging to the tumor necrosis factor (TNF) receptor family, binds all neurotrophins either alone or in association with Trk receptors. $P75^{NTR}$ enhances the affinity and specificity of the neurotrophins binding to the Trk receptors (Kalb, 2005; Zampieri and Chao, 2006). Upon binding of the pro-neurotrophins, $p75^{NTR}$ can also activate apoptosis through its intracellular domain (Teng *et al*., 2005; Nykjaer *et al*., 2004). Thus, these two types of neurotrophin receptors can either consort or antagonize each other to mediate the effects of neurotrophins (Chao, 1992; Miller and Kaplan, 2001). $P75^{NTR}$ has also been implicated in other processes. For example, myelin-based growth inhibitors can bind to $p75^{NTR}$ in combination with Nogo-R and LINGO-1 as a complex to restrict axonal regeneration (Nykjaer *et al*., 2005)

1.2.1.1. Nerve growth factor

NGF is a small secreted protein that induces the differentiation and survival of particular target neurons. It was the first discovered neurotrophic factor by Levi-Montalcini and Hamburger in 1951. NGF is critical for the survival and maintenance of sympathetic and sensory neurons (Levi-Montalcini, 1987b). It, however, has no direct survival-promoting effects on motor neurons (Henderson *et al*., 1993). It is released from the target cells, binds to and activates its high-affinity receptor (TrkA) at the neurite terminals, and is internalized by the responsive neuron. NGF binds two receptors on the surface of cells that respond to this growth factor, TrkA and $p75^{NTR}$. Beside the developmental PCD periods, NGF and its receptors are produced throughout adult life by many different cell types. The dynamically regulated expression of NGF and its receptors throughout adult life suggests multiple functions for NGF signaling. The importance of these relationships is emerged through experiments disrupting the genes for NGF and the receptors. Mice deficient in NGF lose most small nociceptive dorsal root ganglion (DRG) neurons and sympathetic neurons in the peripheral nervous system (Crowley *et al*., 1994). In the central nervous system of animals heterozygous for disruption of the NGF gene, there is a clear reduction in the number of basal forebrain cholinergic neurons, atrophy of these cells, and reduction in the cholinergic innervation of the hippocampus (Chen *et al*., 1997). Both basal forebrain and striatal cholinergic neurons, however, are reduced in the size and number in TrkA null mutant animals (Fagan *et al*., 1997). Also, there is a marked reduction of both small DRG neurons and sympathetic neurons in TrkA knockout animals (Smeyne *et al*., 1994). Changes are also detected with $p75^{NTR}$ gene disruption in both the peripheral and central nervous system (Lee *et al*., 1992; Lee *et al*., 1994; Brennan *et al*., 1999; Bamji *et al*., 1998). NGF plays an important role as an intercellular signaling molecule throughout development. It influences a wide range of cell types and takes part in numerous functions.

1.2.1.2. Brain-derived neurotrophic factor

BDNF is a member of the neurotrophin family of growth factors (Barde *et al*., 1982; Leibrock *et al*., 1989). It acts on certain neurons of both the central and peripheral nervous system, helping to support the survival of existing neurons and enhance the growth and differentiation of new neurons and synapses (Acheson *et al*., 1995; Huang and Reichardt,

2001). In the brain, it is expressed in the hippocampus, cortex, and basal forebrain areas vital to learning, memory, and higher cognitive functions (Yamada and Nabeshima, 2003). Two receptors, TrkB and $p75^{NTR}$, are capable of binding on the surface and responding to this growth factor (Patapoutian and Reichardt, 2001). BDNF knockout mice develop a severe deletion in the peripheral sensory nervous system and lose vestibular function. TrkB null mutant animals also exhibit similar deficits. It suggests that BDNF is not only required for the survival of particular neurons, but also for collateral branching and innervation of some targets (Jones *et al*., 1994; Ernfors *et al*., 1994a; Liu and Jaenisch, 2000). Recent studies have revealed that proBDNF can induce neuronal apoptosis when it binds to $p75^{NTR}$ and sortilin receptor complex (Teng *et al*., 2005). Pro-neurotrophins are produced in the brain in the pathological conditions and contribute to the neuronal loss in the disease (Volosin *et al*., 2006).

1.2.1.3. Neurotrophin-3

NT-3 is the third member of the neurotrophin family (Maisonpierre *et al*., 1990). NT-3 and its receptor, called neurotrophic tyrosine kinase receptor type 3 (TrkC), are widely expressed, although primarily in the nervous system where they are presumed to have multiple functions during development (Lamballe *et al*., 1991; Merlio *et al*., 1992; Tessarollo *et al*., 1997). In primary cultures, NT-3 promotes the survival and/or differentiation of cells from different populations of the peripheral and central nervous system, including neural crest cells and oligodendrocyte precursors (Maisonpierre *et al*., 1990; Kalcheim *et al*., 1992; Barres *et al*., 1994; Birren *et al*., 1993; DiCicco-Bloom *et al*., 1993; Averbuch-Heller *et al*., 1994). *In vivo* studies indicate that NT-3 might have a role in the early neurogenesis before target innervation. In particular, TrkC is expressed by virtually all precursors of the sensory neurons before they differentiate into subpopulations that express other Trks, and NT-3 mutants have early embryonic loss of sensory precursors (Tessarollo *et al*., 1993; Farinas *et al*., 1994). Blocking the biological activity of NT-3 by injection of anti-NT-3 antibodies into the animals induces sensory neuron loss during gangliogenesis (Gaese *et al*., 1994). Several other studies argue that effects *in vivo* are indirect, because they illustrate that Trk proteins are only detected in neurons not in neural crest cells or neuronal precursors, and the defects in NT-3 mutants are due to the death of the neurons not the precursors (Farinas *et al*., 1998; Huang *et al*., 1999). Importantly, mice deficient in NT-3 have much greater deficiency in spinal sensory neurons (about 70%) than the TrkC knockouts (about 30%) (Tessarollo *et al*., 1994; Tessarollo *et al*., 1997). This discrepancy has been explained by the ability of NT-3 to also activate TrkA and TrkB (Farinas *et al*., 1998). Alternatively, or in addition, the sensory neurons could be lost in the NT-3 knockouts due to death actively triggered by de-liganded TrkC (paper II).

1.2.2. Glial cell line-derived neurotrophic factor family

The GDNF family consists of four members: GDNF (Lin *et al*., 1993), neurturin (NRTN) (Kotzbauer *et al*., 1996), persephin (PSPN) (Milbrandt *et al*., 1998) and artemin (ARTN) (Baloh *et al*., 1998), which belong to the TGF-β superfamily. These four factors have their own preferential receptors, the GDNF family receptor alphas (GFRαs). There are four GFRαs: GFRα1 (Jing *et al*., 1996;

Treanor *et al.*, 1996), GFRα2 (Baloh *et al*., 1997; Klein *et al*., 1997), GFRα3 (Jing *et al*., 1997; Worby *et al*., 1998; Masure *et al*., 1998), and GFRα4 (Thompson *et al*., 1998; Lindahl *et al*., 2001), plus a common receptor tyrosine kinase called Ret (rearranged during transfection) (Takahashi *et al*., 1985). Ret is the signaling receptor, whereas GFRs give the ligand-specificity for Ret.

GDNF was originally purified from a rat glial cell line as a neurotrophic factor to promote the survival of embryonic dopaminergic neurons (Lin *et al*., 1993). It is expressed in many tissues and many cell types including neurons (Schaar *et al*., 1993; Stromberg *et al*., 1993; Springer *et al*., 1994; Trupp *et al*., 1995; Suvanto *et al*., 1997). It is broadly expressed in the peripheral and central nervous system, and has multiple neuronal targets. GDNF signals through a receptor complex consisting of a ligand-specific glycosyl-phosphatidylinositol-linked binding molecule (GFRα1) and the membrane-spanning Ret (Durbec *et al*., 1996; Suvanto *et al*., 1997). The binding of GDNF-GFRα1 to the extracellular domain of Ret leads to activation of its intracellular tyrosine kinase domain (Airaksinen and Saarma, 2002; Bespalov and Saarma, 2007). Mice deficient in GDNF die soon after birth (Moore *et al*., 1996). These mice completely lack the enteric nervous system. GDNF also has a crucial role in kidney development and spermatogenesis. $GDNF^{-/-}$ mice display complete renal agenesis due to a lack of the induction of the ureteric bud formation, an early step of kidney development (Moore *et al*., 1996; Pichel *et al*., 1996; Sanchez *et al*., 1996; Meng *et al*., 2000). The phenotypes of GDNF and GFRα1 null mice are strikingly similar. For example, about 20-40% of spinal and cranial motoneurons are missing in $GFR\alpha1^{-/-}$, similar to that of GDNF mutant mice (Cacalano *et al*., 1998; Garces *et al*., 2000). In the peripheral nervous system, no effect on the number of sensory neurons of spinal and trigeminal ganglia is detected in $GFR\alpha1^{-/-}$ newborns, nor loss of SCG neurons. But there are subtle deficits of SCG in GDNF null mice (Airaksinen *et al*., 1999; Cacalano *et al*., 1998; Enomoto *et al*., 1998). GFRα1 inactivation *in vivo* induces enteric neuron death through a non-apopototic path (Uesaka *et al*., 2007) (Table 1). $Ret^{-/-}$ mice, like knockouts from GDNF or GFRα1 die soon after birth, the phenotypes are also similar. However, the migration of SCG precursor cells and initial axon growth defect are observed in $Ret^{-/-}$ mice (Enomoto *et al*., 2001).

GDNF has also received attention as a potential therapeutic agent for the treatment of certain neurological diseases such as Parkinson´s disease.

1.3. GDNF as a neuroprotective trophic factor for midbrain dopaminergic neurons

Parkinson's disease is a degenerative disorder of the central nervous system (Burke, 2007), characterized by muscle rigidity, tremor, slowing of physical movement, and in extreme cases loss of physical movement. The major symptoms of Parkinson's disease result from the loss of dopaminergic neurons in the pars compacta region of the substantia nigra (SN). Some common causes of Parkinson's disease include genetic alterations, toxins, head trauma, cerebral anoxia, and drug-induced Parkinson's disease, but in most of the cases the actual reason for the disease is not known. A number of specific genetic mutations causing Parkinson's disease have been discovered, but these constitute a small minority of all cases (Burke, 2008).

Table 1. Phenotypes of mice that lack GDNF or its receptors

Gene knockout	Ret	GDNF or GFRα1	conditional ablation of GFRα1
Gross phenotype	Lethal at birth	Lethal at birth	Viable, fertile
Viscerosensory	Breathing defect	PG: 40% loss of neurons; breathing defect	ND
Somatosensory	DRG: NS	endings in whisker follicle DRG: neuron number NS, reduced soma size	ND
Sympathetic	SCG: migration and initial axon growth defect; subtle deficits in other ganglia	SCG neurons: NS or subtle loss	ND
Parasympathetic	No SPG or OG; reduced number and soma size in SMG and other ganglia	No SPG or OG; reduced number and soma size in SMG and other ganglia	ND
Enteric	No neurons in bowel below stomach	No neurons in bowel below stomach	the colon; enteric neuron death induced by GFRα1 inactivation is non-apoptotic, caspase-3.-7 and Bax are not involved.
Motor	Loss in various nuclei	Moderate loss in various nuclei	ND
Brain	SN: NS	learning in adult GDNF+/- mice	ND
Other tissues	No kidneys, moderate C-cell loss	No kidneys, testis degeneration in adult GDNF+/- mice	ND

The corresponding references for this table are: Ret knockout (Taraviras *et al.,* 1999; Enomoto *et al.,* 2001); GDNF or GFRα1 knockout (Erickson *et al*., 2001; Oppenheim *et al.*, 2000; Cacalano *et al*., 1998; Garces *et al*., 2000); conditional ablation of GFRα1 (Uesaka *et al*., 2007). DRG: dorsal root ganglion; ND: not determined; NS: not significantly different from wild type; OG: otic ganglion; PG: petrosal ganglion; SCG: superior cervical ganglion; SMG: submandbular ganglion; SPG: sphenopalatine ganglion; SN: substantia nigra; TG: trigeminal ganglion. Modified from Airaksinen and Saarma (2002).

No cure for Parkinson's disease exists, and all the current treatments retard but do not block the progression of the disease (Dauer and Przedborski, 2003; Gandhi and Wood, 2005).

GDNF and another recently discovered factor, conserved dopamine neurotrophic factor (CDNF), are the most promising neurotrophic factors for the treatment of Parkinson's disease (Gill *et al.,* 2003; Lindholm *et al*., 2007). When applied *in vivo*, GDNF is a potent treatment factor in the animal models of the Parkinson's disease. Indeed, treatment with GDNF causes significant improvements in the critical symptoms of Parkinsonian

monkeys (Gash *et al*., 1996). GDNF or CDNF, when delivered to the striatum of the mice in the 6-hydroxydopamine (6-OHDA)-induced Parkinsonian model, significantly improved the survival of dopaminergic neurons. Importantly, these factors also rescued the dopaminergic neurons when applied after 6-OHDA treatment (Lindholm *et al*., 2007). GDNF has also been applied to the human Parkinsonian patients in the clinical studies, but again with contradictory results. In one study, a significant and long-lasting improvement of the symptoms followed GDNF treatment, making GDNF a very promising treatment factor (Gill *et al*., 2003). Another study, however, failed to show a significant improvement in the patients and also claimed that GDNF treatment has several side effects (Lang *et al*., 2006). Again, the reasons for such discrepancy remain to be clarified. For example, difference in the methods of GDNF delivery into the brain should be critically considered as the cause for discrepancy.

Some studies also suggest an essential role for GDNF in the ontogenetic death of the dopaminergic neurons. Injecting GDNF into the striatum postnatally suppresses naturally-occurring cell death of dopaminergic neurons, whereas neutralizing antibodies to GDNF augments it (Burke, 2004; Oo *et al*., 2003; Oo *et al*., 2005). Transgenic mice that overexpress GDNF exclusively in regions of mesencephalic neurons have an increased surviving number of substantia nigra (SN) dopamine neurons (Burke, 2006). On the other hand, mice with a conditional deletion of the GDNF receptor Ret in the dopaminergic system do not reveal any defect in the development of the dopaminergic system but, instead, a degeneration phenotype in the adult animals (Jain *et al*., 2006; Kramer *et al*., 2007). The reasons for such discrepancies are not known, but may involve the differences in the experimental paradigms (acute manipulations versus genetic germline changes). Also, GDNF could exert its trophic effect via receptors other than Ret, such as N-CAM (Paratcha *et al*., 2003).

Despite these different *in vivo* results, GDNF and related factors are promising candidates for preventing the degeneration of dopaminergic neurons and promoting fuctional reinnervation. Understanding the GDNF-related death pathways in dopaminergic neurons could give new ideas of how GDNF treatment may help the disease.

1.4. Classification of cell death: apoptosis and necrosis

Cell death has classically been divided into two broad categories: apoptosis, in which the cell plays an active role, and necrosis where the cell dies passively due to overwhelming stress. In some classifications, apoptosis is called type I death and necrosis as type III (or cytoplasmic) death, whereas an additional mode - autophagic death is distinguished as type II death (Clarke, 1990). More modes of death, however, exist.

Some forms of nonapoptotic cell death, previously labelled necrotic and thus assumed to be passive, have turned out to be programed, therefore, some have referred to these as “necrosis-like” (Vande Velde *et al*., 2000), whereas others prefer the term “programed necrosis” (Zong and Thompson, 2006). A recently generated Nomenclature Committee on Cell Death is working to create a more systematic classification of the cell death modes (Kroemer *et al*., 2008).

The term apoptosis was coined in 1972 by Kerr to describe the phenomenon of a form of cell death (Kerr *et al*., 1972). The terms apoptosis and programed cell death are often used as synonyms, meaning cell-intrinsic biochemical programs controlled by the doomed cell, *i.e.* the apoptotic pathways. It would be more appropriate, however, to use the term PCD in its original sense: cell death processes that occur at a precise location and time according to a developmental program, *i.e.* ontogenetic death (Lockshin and Williams, 1964). It is only during the last two decades that apoptosis has attracted extensive research interests, mainly because of the establishment of the molecular pathway of apoptosis and the identification of several key apoptosis regulators in nematode *Caenorhabditis elegans* and subsequently their counterparts in mammals. Apoptosis accounts for most physiological cell death and is characterized by several distinct morphological and biochemical features including membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation (Williams *et al*., 1974) and the formation of apoptotic bodies that are engulfed by macrophages or neighboring cells (Wyllie *et al*., 1980; Fiers *et al*., 1999). During the early phase of apoptosis, cellular organelles remain relatively intact, preventing leakage of cellular content into the surrounding tissues where it can evoke inflammatory responses (Kerr *et al*., 1995). Thus, the original criteria to define apoptosis were purely morphological. Presently, additional biochemical criteria are also used to define apoptosis. These include caspase activation, consumption of energy-adenosine diphosphate (ATP), and exposure of phosphatidylserine on the cell surface. These are not fully sufficient to define apoptosis, however, For example, caspases can be also activated in non-apoptotic vital processes, and some non-apoptotic programs also require energy. Thus, the exact definition of apoptosis is not yet available. It was recommended that apoptosis (and other cell death modalities) be defined by integrating morphological, enzymatic, immunological, etc. criteria (Galluzzi *et al*., 2007).

Necrosis is usually induced in a pathological situation by accidental and acute damage to the cell. It begins with cell swelling, chromatin digestion, and disruption of the plasma membrane and organelle membranes. Late necrosis is characterized by extensive DNA hydrolysis, vacuolation of the endoplasmic reticulum, organelle breakdown, and cell lysis. The release of intracellular contents after plasma membrane rupture is the cause of inflammation and immune response in necrosis (Figure 2) (Ravichandran and Lorenz, 2007; Chen *et al*., 2007).

Such binary distinction of apoptosis and necrosis, however, is clearly an oversimplification. Several non-apoptotic death programs exist (see below). Also, the necrotic mode of death may include several apoptotic features. Many examples show the features, to various extents, of both death types in the same cell. It is believed that at least some aspects of necrosis may be programed, although perhaps to a lesser extent than in apoptosis. Intracellular Ca^{2+} homeostasis is critical for the induction of both necrosis and apoptosis (Xu *et al*., 2001; Schwab *et al*., 2002; Leist *et al*., 1997; Crompton, 1999; Proskuryakov *et al*., 2003). Thus, apoptosis and necrosis may occur in response to the same death stimuli, and the induced mode of death is interchangable under certain circumstances.

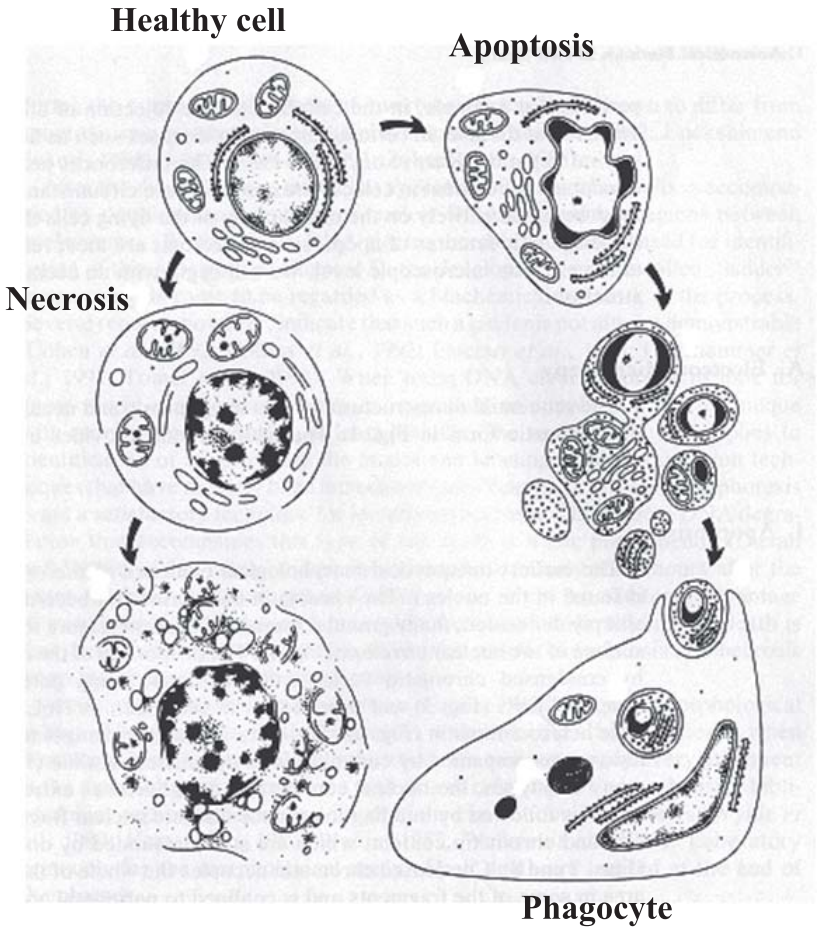

Figure 2. Morphological differences between apoptosis and necrosis. A healthy cell can die either apoptotically (right) or necrotically (left). Morphological changes of apoptosis include: I. Cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation. II. Fragmentation of the cell into the apoptotic bodies. III. Engulfment of the apoptotic bodies. Morphological changes of necrosis include: I. Swelling of the cell, partial chromatin digestion. II. Disruption of the plasma membrane and organelle membranes. Modified from Kerr (1995).

1.5. Apoptotic pathways

Based on the mode of cell death induction and execution, apoptosis can occur via two main pathways: the intrinsic, or mitochondrial pathway and the extrinsic, or the death receptor pathway. The Bcl-2 family proteins are the best characterized regulators involved in the regulation of apoptotic death pathways. Both pathways culminate in the activation of caspases, which cleave key cellular substrates at specific aspartate residues, leading to the features of apoptosis (Vander Heiden *et al*., 2001). Several novel apoptotic pathways have also been described, including those presented in this study.

1.5.1. Bcl-2 family

The Bcl-2 family is a group of proteins that make critical survival/death decisions in cells. Proteins of the Bcl-2 family share

one or more of the four characteristic domains of homology entitled the Bcl-2 homology (BH) domains (named BH1-4), which control the ability of these proteins to dimerize and function as regulators of apoptosis (Gross *et al*., 1999; Cory and Adams, 2002). Functionally, Bcl-2 family proteins can be divided into anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, BOO/DIVA, A1/Bfl-1, and NR-13, and pro-apoptotic proteins. These can be further divided into two subgroups: the multidomain proteins Bax, Bak, and Bok /Mtd and the BH-3-only subfamily members including Bid, Bim, Bad, DP5/ HrK, Bcl-xS, Blk, Bik, BNip3, Nix, Mcl-1s, Bcl-Gs, Noxa, Puma, and N-Bak. The multidomain proteins have three common domains (BH1, BH2, BH3), whereas BH3-only proteins possess only the BH3 domain. This is shown in Figure 3.

The precise mechanisms by which the Bcl-2 family proteins co-ordinately regulate programed cell death are extensively studied, but not fully clear. It seems that the activity of Bcl-2 family proteins is regulated through formation of homo- and heterodimers. Interactions between pro- and anti-apoptotic members neutralize the activity of each other (Korsmeyer, 1999; Adams and Cory, 2001; Bouillet and Strasser, 2002; Van Delft and Huang, 2006). Anti-apoptotic Bcl-2 family proteins associate and integrate with the mitochondrial outer membrane, endoplasmic reticulum (ER), or nuclear

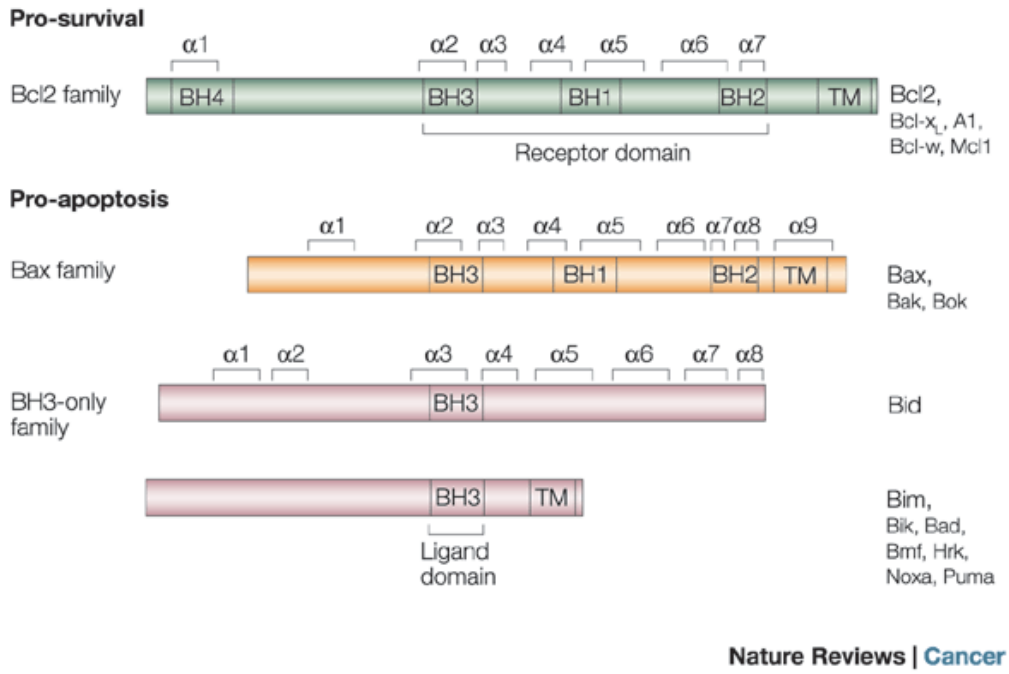

Figure 3. A diagrammatic representation of the BCL-2 family proteins. The main functional domains are shown: BH1, BH2, BH3, BH4, and the transmembrane (TM) domain. The proteins are grouped as pro-survival and pro-apoptosis according to their function. The pro-apoptotic members of the BCL-2 family can be subdivided into at least two groups: the proteins containing two or three distinct BH domains and the BH3-only proteins. Modified from Cory and Adams (2002).

membrane (Krajewski *et al*., 1993; Lithgow *et al*., 1994; Hsu *et al*., 1997; O'Reilly *et al*., 2001; Nutt *et al*., 2002; Scorrano *et al*., 2003). Pro-apoptotic proteins, such as Bax or Bak, are debatable for the dysfunction of mitochondria or ER and release of apoptogenic factors (Lindsten *et al*., 2000; Zong *et al*., 2001; Wei *et al*., 2001). BH3-only proteins are shown to be essential initiators of apoptosis (Huang and Strasser, 2000). Thus, in healthy cells, pro-apoptotic proteins are suppressed or sequestered to a distinct subcellular compartment and kept inactive by diverse posttranslational mechanisms (Puthalakath and Strasser, 2002). In apoptotic cells, however, the BH3-only proteins are activated by different individual modes. Subsequently, the active BH3-only proteins can bind to and inactivate the relevant anti-apoptotic proteins, and allow the activation of pro-apoptotic members, Bax and Bak (Willis *et al*., 2007).

Bax and Bak are critical pro-apoptotic proteins in most cell types (in neurons, Bak is replaced by its splice variant N-Bak (Sun *et al*., 2001)). In apoptotic cells, the conformation of Bax is changed and it is translocated from the cytosol to the mitochondrial outer membrane where it, together with Bak, oligomerizes and participates in the formation of pores in the mitochondrial outer membrane (Hsu *et al*., 1997; Wolter *et al*., 1997; Gross *et al*., 1998; Goping *et al*., 1998; Desagher and Martinou, 2000; Desagher *et al*., 1999; Korsmeyer *et al*., 2000; Smaili *et al*., 2001; Nechushtan *et al*., 2001). It is not clear, however, how the conformational change of Bax or Bak is induced in apoptotic cells and what is the mechanism of Bax translocation into mitochondria, although in several models, the involvement of tBid and Bim have been proposed (Desagher *et al*., 1999; Huang and Strasser, 2000; Eskes *et al*., 2000; Putcha *et al*., 1999; Whitfield *et al*., 2001; Brustovetsky *et al*., 2003). It has been shown that Bax translocation and the apoptotic activity is mediated by its C-terminal transmembrane region (Nechushtan *et al*., 1999; Schinzel *et al*., 2004).

The crystal structure of Bax and Bcl-xL revealed a similarity to the pore-forming domains of bacterial toxins, suggesting a pore-forming property of these proteins (Muchmore *et al*., 1996; Petros *et al*., 2004). The multi-BH-domain Bcl-2 family proteins can indeed generate channels into the mitochondrial outer membrane that allow the release of macromolecules to the cytosol (Martinou and Green, 2001; Waterhouse *et al*., 2002).

The mechanism by which Bax and/or Bak mediate the release of apoptogenic factors is also not fully understood (Martinou and Green, 2001; Robertson *et al*., 2003). It could be mediated by the channel formation and directly activated by Bax and/or Bak with (Marzo *et al*., 1998a; Marzo *et al*., 1998b; Marzo *et al*., 1998c; Narita *et al*., 1998; Schendel *et al*., 1998; Crompton, 1999; Belzacq *et al*., 2003; Roucou *et al*., 2002) or without the involvement of other mitochondrial proteins (Eskes *et al*., 1998; Basanez *et al*., 1999; Saito *et al*., 2000; Shimizu and Tsujimoto, 2000; Antonsson *et al*., 2000). Another model involves the swelling of the mitochondrial matrix and the subsequent disruption of the mitochondrial outer membrane (Vander Heiden *et al*., 2001), but this model has not been confirmed in most of the studies. It is also reported that the interaction of activated Bax with mitochondrial lipids, in particular cardiolipin, in the absence of other proteins is sufficient to generate openings in the membrane and release cytochome c (Epand *et al*., 2002; Ott *et al*., 2002; Kuwana *et al*., 2002). Cytochrome *c*

release is proposed to occur in two steps: the detachment from the outer surface of the mitochondrial inner membrane, followed by permeabilization of the mitochondrial outer membrane. Cardiolipin interaction with cytochrome *c* limits the amount of cytochrome *c* release, and reducing the cardiolipin level decreases the cytochrome *c* binding to the mitochondrial inner membrane (Ott *et al*., 2007).

Gene targeting studies have revealed that Bcl-xL and Bax are the key anti- or pro-apoptotic Bcl-2 family members during brain development (Kuan *et al*., 2000). Within the anti-apoptotic Bcl-2 family only the deletion of Bcl-x has been reported to result in a clear phenotype in neurodevelopment. These mice die around embryonic day 13 and had massive immature hematopoietic cells and neuronal death, whereas Bcl-2 is only crucial for the maintenance of some specific populations of neurons during the early postnatal period (Motoyama *et al*., 1995; Michaelidis *et al*., 1996). Bax is a crucial pro-apoptotic Bcl-2 family member during brain development, as disruption of the gene dramatically inhibits apoptosis in the nervous system and causes resistance to trophic factor deprivation (Knudson *et al*., 1995; Ockel *et al*., 1996b; Deckwerth *et al*., 1996). Bax/Bcl-xL double knockout mice show that the lack of Bax can neutralize the neuronal loss caused by Bcl-xL deficiency (Shindler *et al*., 1997). While Bak knockout mice develop normally, most Bax/Bak double knockout mice die during embryogenesis, indicating that Bax and Bak have overlapping roles in the regulation of apoptosis during development (Lindsten *et al*., 2000). Bim and DP5 also play an important role in neuronal cell death *in vivo* (Strasser *et al*., 2000; Imaizumi *et al*., 2004; Coultas *et al*., 2007).

1.5.2. Caspase family

Despite the diversity of death stimuli and immediate signal transduction pathways, the execution of apoptosis is always implemented by a family of cysteine proteases, called caspases (cysteinyl aspartate-specific proteinases) (Thornberry *et al*., 1997a; Honglin and Junying, 1999; Earnshaw *et al*., 1999; Denault and Salvesen, 2002). Caspases cleave cellular substrate after specific aspartate residues, while three amino acid residues upstream of the aspartate residue determine the substrate specificity of individual caspases (Cerretti *et al*., 1992; Lazebnik *et al*., 1994; Takahashi *et al*., 1996; Thornberry *et al*., 1997a; Sakahira *et al*., 1998). Recent studies demonstrate that current knowledge of preferred sites for individual caspases is not fully correct and the issue requires further studies (Van Damme *et al*., 2005; McStay *et al*., 2007; Timmer and Salvesen, 2006). Activation of caspases above a certain threshold presents a point of no return during apoptosis. The morphological and biochemical features of apoptosis are a collective consequence of cellular degradation by caspases. Caspases can be divided into inflammatory caspases (caspase-1, -4, -5, and -11) (Yuan *et al*., 1993; Wang *et al*., 1998) that are essential for the maturation of cytokines, and apoptotic caspases (caspase-2, -3, -6, -7, -8, -9, -10, and -12). Apoptotic caspases can again be divided into initiator caspases (caspase-2, -8, -9, -10, and -12) that have a long prodomain (Slee *et al*., 1999) and effector caspases (caspase-3, -6, and -7) that have a short prodomain. The long prodomains of caspase-8 and caspase-10 have two tandem repeats of death effect domains (DEDs), which mediate their homophilic interactions with the DED-containing adaptor proteins like FADD

(Vincenz and Dixit, 1997). The prodomain of caspase-9 and caspase-2 contain caspase recruitment domains (CARDs) that mediate the interaction of these caspases with other CARD-containing molecules such as Apaf-1 (Zou *et al*., 1997; Li *et al*., 1997; Hofmann *et al*., 1997) (Figure 4).

Caspases are generated as inactive zymogens or pro-caspases. Activation of a caspase involves two proteolytic steps: first cleavage at the site between the small and large subunit and then cleavage at the site between the prodomain and large subunit. Two large and two small subunits form a heterotetramer representing an active caspase (Figure 5.2). Therefore, the prodomain of caspase functions as an interaction domain and inhibitory domain. In response to apoptotic stimulus, initiator caspases become activated by a proximity-induced dimerization without cleavage. Cleavage is neither required nor sufficient for activation initiator caspases but only enhances their activity (Logue and Martin, 2008; Riedl and Salvesen, 2007; Boatright *et al*., 2003; Bao and Shi, 2007).

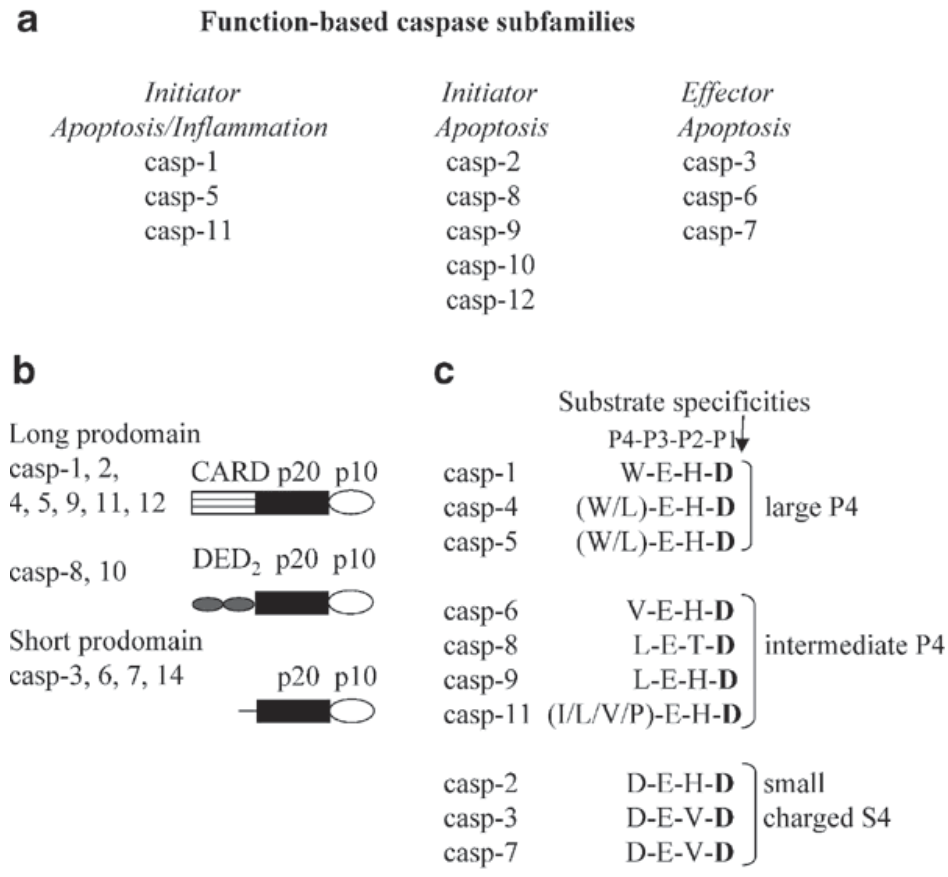

Figure 4. Classification of caspases. (**a**) Classification of the caspase family based on the functions. (**b**) General structure of caspases and classification based on the prodomain length. (**c**) Caspase substrate specificities. Data are based on (Thornberry *et al*., 1997b). Preferred amino acids in P4-P1 positions are shown. Based on the size of the S4 subsite and P4 residue, caspases can be divided into three subfamilies. Modified from Degterev (2003).

In the death receptor pathway, pro-caspase-8 or -10 is recruited to the death-inducing signaling complex (DISC) via the DED domain, and activated by induced proximity (Figure 5). In the mitochondrial pathway, release of cytochrome *c* from the mitochondria to the cytosol leads to assembly of the apoptosome that recruits pro-caspase-9 via the CARD domain (Figure 5). Similarly to pro-caspase-8, pro-caspase-9 can also be activated without processing because of its unusally long linker between its large and small subunits. Most of the active caspase-9 remains complexed with apoptotic protease activating factor-1 (Apaf-1) (Stennicke and Salvesen, 1999; Acehan *et al*., 2002). The effector caspases such as caspase-3, -6, and -7 are activated through proteolysis by upstream initiator caspases (Muzio *et al*., 1998; Salvesen and Dixit, 1999; Strasser *et al*., 2000). Collective action of the executioner caspases finally brings about the apoptotic death of the cell.

Caspase-2 is a CARD-containing caspase with effector-caspase substrate cleavage preference, suggesting that it may function as both an initiator and effector caspase. Accumulating evidence indicates that caspase-2 could be activated as an initiator caspase upstream or independent of mitochondria (Lassus *et al*., 2002; Robertson *et al*., 2002; Guo *et al*., 2002; Baliga and Kumar, 2003; Blaschke *et al*., 1996; Read *et al*., 2002). Caspase-2 is localized in the Golgi complex and its cleavage is dependent on Apaf-1 and caspase-9 (Mancini *et al*., 2000). It is also localized in the nuclei and triggers cytochrome *c* release from the nuclei in turn to induce apoptosis (Parone *et al*., 2002). How caspase-2 becomes activated is still poorly known. Recently several studies have shown that during stress-induced apoptosis, activation of caspase-2 occurs in a complex called PIDDosome, consisting of a death domain-containing protein PIDD, RAIDD, and caspase-2 (Tinel and Tschopp, 2004; Park *et al*., 2007) (Figure 5.1). Apoptosis induced by overexpression of RAIDD in sympathetic neurons is dependent on caspase-2 (Jabado *et al*., 2004). Delayed death of CA1 neurons after transient ischemia is mediated by a complex that includes PIDD, RAIDD, and caspase-2 (Niizuma *et al*., 2008). We recently described a novel death pathway in the GDNF-deprived sympathetic neurons that is most probably initiated by activation of caspase-2 without apoptosome or DISC (paper I). Further elucidation of caspase-2 activation would probably shed light on whether the PIDDosome formation is involved after GDNF deprivation.

Caspases are highly specific endoproteinases, which generate discrete fragments that are not further processed. Some proteins are activated by cleavage, whereas others are inactivated. For example, caspases themselves are the substrates of activated caspases. A caspase substrate concept has been proposed based on a relatively few proteins that match the consensus substrate specificity of caspase (Timmer and Salvesen, 2006). More than 280 caspase substrates have been identified and several of them act as transducers and amplifiers that determine the apoptosis (Fischer *et al*., 2003).

Targeted gene disruption of individual caspases has revealed that they perform essential functions in development, immune regulation, proliferation, and apoptosis (Puthalakath and Strasser, 2002; Wang and Lenardo, 2000). But for a given death signal, a specific caspase may be essential in one cell type and dispensable in another. Female mice deficient in caspase-2 had overaccumulation of germ cells, indicating that caspase-2 was

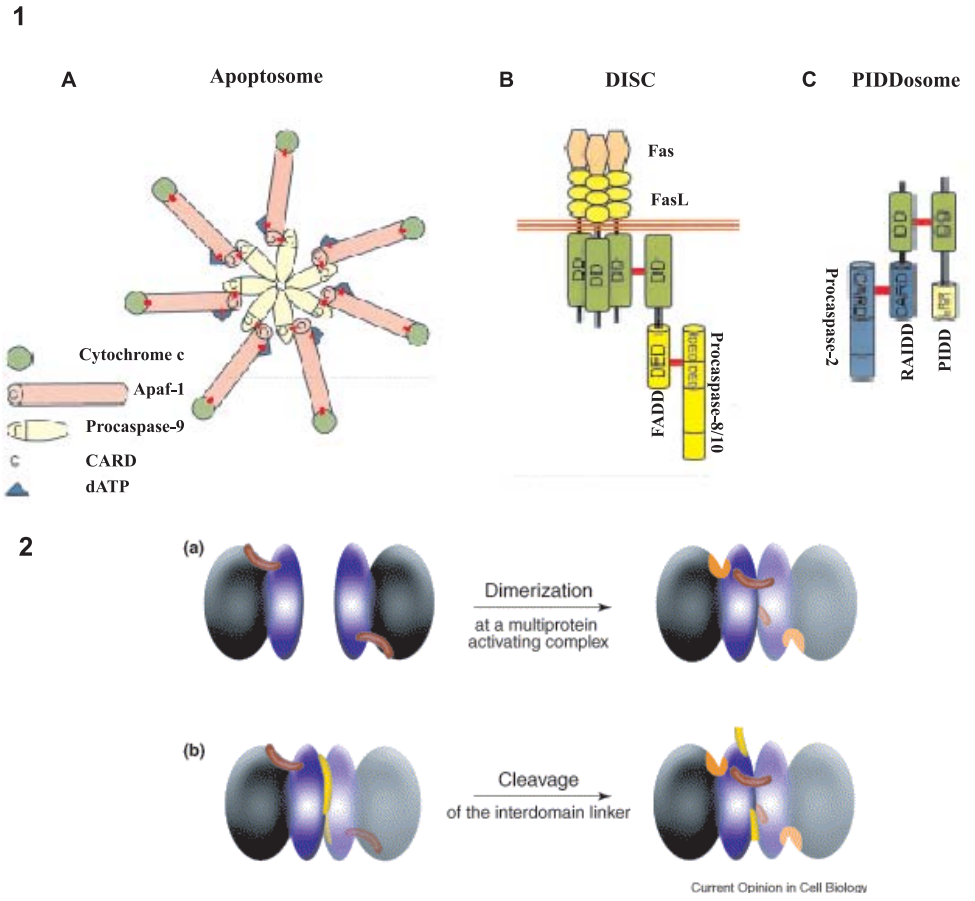

Figure 5. 1. **Protein complexes responsible for the activation of initiator caspases.** The activation of caspase-9, caspase-8, and caspase-2 in mammalian cells is mediated by the apoptosome (A), the DISC (B), and the PIDDosome **(**C). The apoptosome is composed of seven molecules of Apaf-1 bound to cytochrome *c* in the presence of ATP/dATP. DISC is assembled following the binding of death ligand to its receptor and contains FADD and caspase-8 (or –10). The PIDDosome contains at least three components, PIDD, RAIDD, and caspase-2. Modified from Ho and Hawkins (2005). 2. Cartoon representation of the two molecular mechanisms of procaspase activation. (a) Activation of initiator caspases. The zymogens of initiator caspases exist as latent monomers. These monomers are activated by dimerization, which allows translocation of the activation loop (depicted as a red 'sausage') into the accepting pocket of the neighboring dimer. The active site is represented by an orange patch. (b) Activation of executioner caspases. The zymogens of executioner caspases exist as preformed dimers. Their zymogen latency is maintained by steric hindrances imposed by the interdomain linker (depicted as a yellow 'banana'). Cleavage of this linker permits translocation of the activation loop, facilitating formation of the active site. Adapted from Boatright and Salvesen (2003).

critically required for apoptosis of oocytes. These mice also had a decreased number of facial motor neurons (Bergeron and Yuan, 1998). Sympathetic neurons from caspase-2 deficient mice showed increased sensitivity to NGF deprivation induced apoptosis, but remained resistant to apoptosis induced by β-amyloid treatment. The effects of caspase-2 deletion on sympathetic neurons were attributed to the compensatory overexpression of caspase-9 and Smac/DIABLO (Troy *et al*., 2001). Apoptosis in the neural progenitor cells of the forebrain requires caspase-9 and -3 or Apaf-1. Mice deficient in caspase-3 (Kuida *et al*., 1996), caspase-9 (Hakem *et al*., 1998) or Apaf-1 (Yoshida *et al*., 1998) show developmental defects in the central nervous system and exhibit prominent forebrain malformation (Zaidi *et al*., 2001; Roth and D'Sa, 2001; D'Sa-Eipper *et al*., 2001). Caspase-8 knockout mice died around embryonic day 12.5 due to the defect in heart development. The number of hematopoietic precursors is dramatically reduced due to a reduced proliferative capacity. Moreover, fibroblasts from caspase-8 deficient mice are completely resistant to death receptor mediated apoptosis. Thus, besides its critical role in apopotosis, caspase-8 is also essential for the growth and differentiation of heart muscle and hematopoietic progenitors cells (Varfolomeev *et al*., 1998).

1.5.3. Intrinsic or mitochondrial apoptotic pathway

Death signals, such as cellular stress or deprivation of survival promoting factors, trigger apoptosis by releasing of the mitochondrial death machinery (Desagher and Martinou, 2000; Ferri and Kroemer, 2001; Kroemer and Reed, 2000). Mitochondria play a central role in this pathway by releasing of mitochondrial proteins, such as cytochrome *c* (Liu *et al*., 1996); apoptosis inducing factor (AIF) (Susin *et al*., 1999), and Smac/DIABLO (Second mitochondria-derived activator of caspase/Direct IAP binding protein) (Du *et al*., 2000; Verhagen *et al*., 2000), normally residing in the intermembrane space into the cytosol. The Bcl-2 family of proteins critically regulate the mitochondrial apoptotic function. Following death stimulus, pro-apoptotic Bcl-2 family proteins become activated, inducing mitochondrial membrane permeabilization and releasing cytochrome *c* and other apoptotic factors into the cytosol. Once released, cytochrome *c* binds to Apaf-1 in the presence of ATP/dATP and induces the assembly of an apoptosome. Pro-caspase-9 binds to the apoptosome and is activated there by dimerization to the induced proximity mode (Pop *et al*., 2006). Activated caspase-9 proteolytically activates effector caspases such as caspase-3, -6, and -7, which cleave the cellular substrates leading to cell death (Figure 6) (Liu *et al*., 1996; Li *et al*., 1997; Zou *et al*., 1997; Slee *et al*., 1999). On the other hand, the inhibitor of the apoptosis protein (IAP) family also critically regulates the programed cell death by binding and inhibiting caspases (Takahashi *et al*., 1998; Sun *et al*., 1999; Sun *et al*., 2000; Deveraux and Reed, 1999; Salvesen and Duckett, 2002; Fesik, 2000). To ensure the full activation of the caspases both Smac/DIABLO and Omi/HtrA antagonize the activity of IAPs (Slee *et al*., 1999; Verhagen *et al*., 2000; Wu *et al*., 2000; Liu *et al*., 2000; Chai *et al*., 2000; Verhagen *et al*., 2002; Verhagen and Vaux, 2002).

1.5.4. Extrinsic or death receptor apoptotic pathway

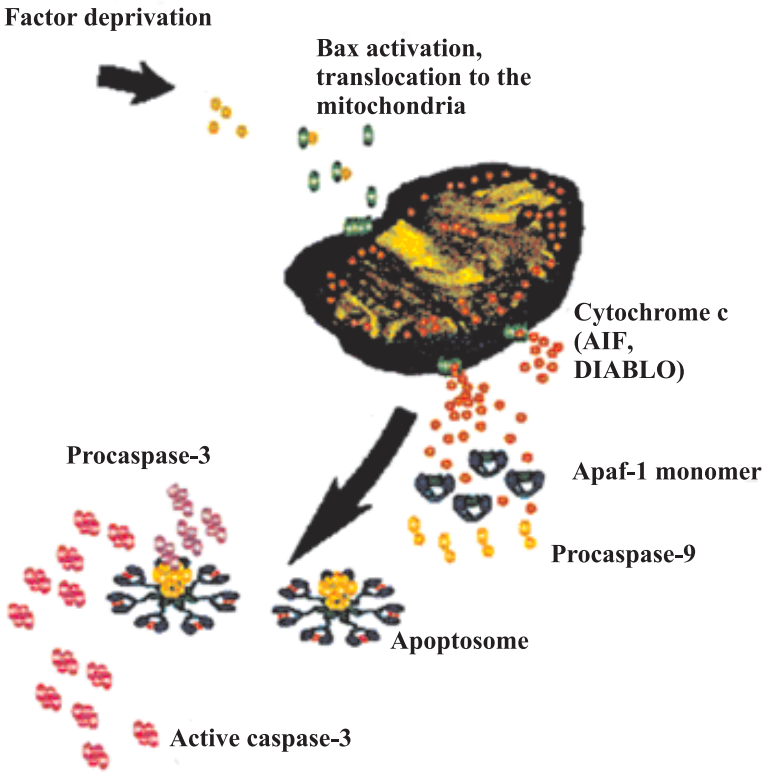

Figure 6. Mitochondrial death pathway: Following apoptotic stimuli, including cellular stress or deprivation of survival promoting factors, Bax is translocated to the mitochondria, causing cytochrome *c* and other mitochondrial proteins such as AIF and DIABLO to be released into the cytosol as a result of mitochondrial outer membrane permeabilization. This is followed by the formation of the apoptosome complex and caspase-9 activation. Modified from Green and Evan (2002).

The extrinsic pathway triggers apoptosis through the engagement of the cell surface death receptors by their ligand. This activates the caspase cascade, which carries out numerous proteolytic events that mediate the apoptotic cell death program.

1.5.4.1 Death receptors

Death receptors form a subgroup in the large TNF receptor superfamily and are cell surface receptors that transmit apoptotic signals initiated by specific death ligands (Smith *et al*., 1994; Ameyar-Zazoua *et al*., 2002). They contain the death domain, which enables death receptors to mediate the extrinsic cell death pathway (Tartaglia *et al*., 1993; Nagata, 1997). The best characterized death receptors are Fas (CD95/Apo1) (Itoh *et al*., 1991) and TNFR1 (CD120a/p55) (Tartaglia and Goeddel, 1992). Also TRAIL and its receptors TRAILR1 and TRAILR2 have

attracted considerable attention (Johnstone *et al*., 2008). Death receptor ligands are also transmembrane proteins of the tumor necrosis factor superfamily and can activate the receptors mainly through cell-cell contacts.

Fas is expressed broadly in various tissues, particularly thymocytes and activated T cells (Debatin *et al*., 1993; Katsikis *et al*., 1995). Ligation of Fas by FasL or cross-linking of Fas by agonistic antibodies induces apoptosis of Fas-bearing cells (Itoh *et al*., 1991; Trauth *et al*., 1989). Several studies show that cells expressing IAP or p35 are resistant to death induced by Fas. In addition, caspase inhibitors can block Fas-induced apoptosis. This indicates that caspases are mediators of Fas-induced apoptosis (Beidler *et al*., 1995; Enari *et al*., 1995; Enari *et al*., 1996). The mechanism of caspase activation by Fas/DISC has been extensively studied. Fas ligation leads to the formation of its clusters. An adaptor protein FADD, which also contains a death domain (DD), binds to the clustered receptor through its own death domain (Boldin *et al*., 1996; Chinnaiyan and Dixit, 1996). FADD also has a death effector domain (DED) at its N-terminus that can bind to pro-caspase-8. Formation of the DISC induces the oligomerization and subsequently self-cleavage of pro-caspase-8 (Martin *et al*., 1998; Muzio *et al*., 1998; Yang *et al*., 1998). Caspase-8 can then activate a downstream effector caspase. Ligated TNFR1 also assembles DISC with a different composition, including adapter TNFR-associated death-domain (TRADD), FADD, and pro-caspase-8 (Micheau and Tschopp, 2003). Negative regulators of death receptor signaling such as vFLIPs and cFLIP also exist (Thome *et al*., 1997; Shu *et al*., 1997; Hu *et al*., 1997; Srinivasula *et al*., 1997). Several Fas regulatory molecules are expressed in the nervous system such as, lifeguard and PEA15 (Boldin *et al*., 1995; Somia *et al*., 1999; Fernandez *et al*., 2007). Recently an apoptosis inhibitory molecule $FAIM_L$ (long isoform of Fas apoptosis inhibitory molecule) was described as interacting with and blocking the apoptotic activity of Fas, specifically in neurons (Segura *et al*., 2007). Studies with Fas-deficient mice reveal that Fas has physiological and pathological roles in the immune system (Cohen and Eisenberg, 1991).

Death receptors such as TNFR1, as well as Fas, can trigger both survival and apoptotic pathways. At the cell surface, the ligated death receptors assemble a complex (complex I) that includes TRADD, the kinase RIP1, and TRAF2, but not pro-caspase-8, which can activate survival-promoting NF-*k*B pathway. It is only in the cytosol, after the receptors are endocytosed, that DISC (complex II) is collected, leading to caspase-8 activation and cell death (Micheau and Tschopp, 2003; Schutze *et al*., 2008). Thus, the death receptors can trigger different, even opposite signals that are regulated by receptor endocytosis.

1.5.4.2. Death receptor apoptotic pathway

The "extrinsic" cell death signals, such as those mediated by death receptors of the TNF receptor superfamily, activate the caspase cascade more directly. This direct cell death occurs through ligation and activation of the plasma membrane death receptors on target cells and is very important for the immune system (Krammer, 2000). The best characterized death receptor Fas contains an extracellular cysteine-rich domain and an intracellular death domain essential for interacting

and recruiting adaptor molecules. Death ligands belong to the TNF superfamily and signal by inducing trimerization of their cognate death recptors. This trimerization usually leads to apoptosis (Schulze-Osthoff *et al*., 1998; Ashkenazi and Dixit, 1998; Nagata, 1997). Ligand binding to the death receptor, for example Fas or TRAIL/apo-2L, causes the aggregation of the death receptors on the plasma membrane and recruitment of a cytoplasmic adaptor FADD (Vincenz and Dixit, 1997), forming a death-inducing signaling complex (DISC) (Figure 7) (Kischkel *et al*., 1995; Chinnaiyan *et al*., 1995; Medema *et al*., 1997; Papoff *et al*., 1999; Chan *et al*., 2000; Walczak and Sprick, 2001). Pro-caspase-8 or -10 is recruited to DISC by binding to Fas-associated protein with a death domain (FADD) via their death effector domains (DEDs) and activated by an induced-proximity manner (Muzio *et al*., 1998; Fernandes-Alnemri *et al*., 1996; Vincenz and Dixit, 1997; Wang *et al*., 2001). Activated caspase-8 or -10 can in turn initiate the activation of the caspase cascade by direct cleavage of effector caspases such as caspase-3, -6, and -7 (type 1 cells). TNFR-1 also activates caspase-8 and -10 through FADD, but TNFR-1

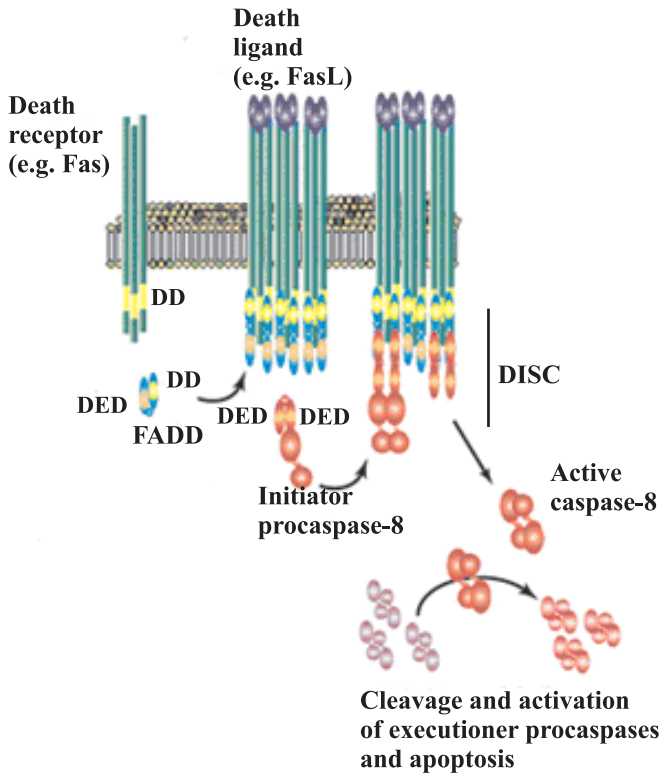

Figure 7. Death receptor pathway. The extrinsic pathway is triggered by the binding of death ligands to their receptors on the cell surface. This results in the recruitment of the adaptor proteins, such as FADD, and formation of the death inducing signaling complex (DISC) at their intracellular regions. The DISC promotes the activation of initiator caspases, most prominently caspase-8, that in turn cleave and activate the effector caspases. DD: death domain. Modified from Green and Evan (2002).

requires TRADD (TNFR1 associated death domain protein) to act as an adaptor between the receptor and FADD (Hsu *et al*., 1995). In some cases the activation of caspase-8 is very slow, thus the death signal needs to be amplified by the mitochondrial pathway (type 2 cells). In these cells, caspase-8 cleaves p22 Bid, a BH3-only Bcl-2 family member protein, into P15 tBid, which then binds to and activates Bax and/or Bak, the pro-apoptotic Bcl-2 family proteins, inducing mitochondrial permeabilization and subsequent cell death (Scaffidi *et al*., 1998; Li *et al*., 1998; Luo *et al*., 1998; Scaffidi *et al*., 1999). Thus, in type 2 cells, a crosstalk between the intrinsic and extrinsic pathways can occur.

1.6. Non-apoptotic programed cell death pathways

Apoptosis has been studied extensively. Although programed cell death has often been equated with apoptosis, it has become more and more clear that several non-apoptotic forms of programed cell death occur (Galluzzi *et al*., 2007; Bredesen, 2007).

1.6.1. Autophagic cell death

Autophagy was first described in the 1960s as a process whereby a cell digests its own components. Autophagy can be broadly separated into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy involves the formation of a *de novo*-formed membrane sealing on itself to engulf cytosolic components (proteins and/or whole organelles), which are degraded after fusion with the lysosome, whereas microautophagy is the direct invagination of materials into the lysosome. A variety of autophagic processes exist, all of which involve the degradation of intracellular components via the lysosome.

Autophagy is best characterized in yeast cells as a process facilitating cell survival in hard conditions. During nutrient starvation, the increased levels of autophagy lead to the breakdown of non-vital components and the release of nutrients, ensuring that vital processes can continue (Yorimitsu and Klionsky, 2005). Mutant yeast cells that have a reduced autophagic capability rapidly perish in nutrition-deficient conditions. Autophagy also plays a role in the destruction of some bacteria within the cell and helps to get rid of pathogens. Recently, autophagy has also been related to cell death, either as a part of apoptosis (Xue *et al*., 1999) or as a separate, non-apoptotic death mode that is independent of caspases. For example, the cells of embryonic interdigital tissue are dying by an autophagic ultrastructure (Lockshin and Zakeri, 2001). Autophagic features have also been reported in several pathological tissues, such as excitotoxically dying neurons (Portera-Cailliau *et al*., 1997). Importantly, autophagic death is often triggered in cells where caspases are blocked or can not be activated. Indeed, the PCD in most tissues of caspase-3, caspase-9, or Apaf-1 knockout mice occurs with autophagic ultrastructure (Oppenheim *et al*., 2001; Oppenheim *et al*., 2008). Also, cells in which caspases are blocked (*e.g.* Bax/Bak double-deficient fibroblasts) die with autophagic features when exposed to apoptotic stimuli (Shimizu *et al*., 2004). Death of such cells is dependent on autophagic genes Atg5 and beclin-1, and can be inhibited by the autophagy inhibitor 3-methyladenine (Shimizu *et al*., 2004).

Thus, autophagy can be both a survival-promoting and death-promoting process. It is not yet fully clear when and to what

extent autophagy really participates in the programed cell death in mammals. Most probably there could be a threshold at which autophagy switches from survival-promoting to a death-promoting process (Baehrecke, 2005; Maiuri *et al*., 2007).

1.6.2. Other non-apoptotic cell death pathways

Several other non-apoptotic programed death pathways have been described, but these are very poorly studied.

Paraptosis Hyperactivation of the tyrosine kinase receptor insulin-like growth factor I receptor induce a non-apoptotic form of cell death called paraptosis, whose activity is mediated by mitogen-activated protein kinases (MAPKs) and inhibited by AIP-1/Alix (Sperandio *et al*., 2000; Sperandio *et al*., 2004). The cells dying paraptotically exhibit extensive cell vacuolization and swelling of the mitochondria and ER, thereby resembling necrosis like death (Clarke, 1990). Caspases were not activated during paraptosis and Bcl-xL did not block the death in either the primary culture or cell lines, suggesting that this type of cell death is fundamentally different from apoptosis. At present, it is unclear whether paraptosis represents a route of cell death that is really distinct from all others.

PARP-dependent cell death Dawsons and colleagues demonstrated that after the activation of poly-(ADP-ribose) polymerase-1 (PARP-1) and the translocation of AIF from the mitochondria to the nucleus, the death appears to be caspase-independent (Yu *et al*., 2002). Agents that induced DNA damage can induce this form of programed cell death. PARP-dependent cell death displays a morphology and biochemistry that is distinct from the classic programed cell death.

Oncosis Oncosis has been defined as a form of cell death accompanied by cellular swelling, organelle swelling, and increased membrane permeability. It has been regarded as passive cell death, occurring only after severe tissue injury brought about by ischemia (Trump *et al*., 1997). It is thought to be mediated by the failure of the plasma membrane ionic pumps. One potential mediator of oncosis could be a calpain-family protease, which suggests that oncosis maybe related to calcium-activated necrosis-like cell death.

Necroptosis Recently a novel non-apoptotic death mode termed necroptosis was described in cultured cells (Vercammen *et al*., 1998; Holler, 2000; Kawahara *et al*., 1998; Degterev *et al*., 2005). This type of cell death does not involve the pro-apoptotic proteins such as cytochrome *c*, caspases, or Bcl-2 family members. Morphologically it has many features of classical necrosis, and is therefore described as a sort of programed necrosis. Necroptosis cannot be blocked by the inhibitors of apoptosis but, instead, by small molecule compounds called necrostatins (that, in turn, do not block apoptosis) (Degterev *et al*., 2005; Wang *et al*., 2007; Zheng *et al*., 2008; Degterev *et al*., 2008). RIP1, one member of the RIP kinase family bears a C-terminal death domain, and an intermediate domain. The death domain of RIP1 is important for binding to death receptors such as TNF-receptor 1, TRAIL-receptor 1, and TRAIL-receptor 2, and also to death domain-containing adaptor proteins such as TRADD and FADD. Necrostatins directly bind and inhibit RIP1 showing that it is a component of necroptototic

pathway (Vandenabeele et al., 2008).

In the pathological situations, the cells often die via mixed death morphologies that include both apoptotic and non-apoptotic pathways, in particular necrotic and/or autophagic features, depending on the nature and intensity of the disease or trauma (Golstein and Kroemer, 2007; Levine and Kroemer, 2008). Most probably these cannot be defined as separate cell death modalities (Kroemer *et al*., 2008). Cells can die many ways and new modes are likely to still be described. The pathways that mediate the non-apoptotic death, as well as their biological meaning are, however, still very poorly understood.

1.7. Model of neurotrophic factor-deprived neurons

The target-derived neurotrophic factor model supposes that during PCD, the neurons are intrinsically apoptotic, and the apoptosis is actively suppressed by target-derived neurotrophic factors. Neonatal sympathetic neurons from the superior cervical ganglion (SCG) are well-suited for studying this model. The PCD of these neurons occurs during the first postnatal week when about half of the neurons die. This survival depends almost completely on NGF, as virtually all SCG neurons are lost in the NGF or TrkA knockout mice (Crowley *et al*., 1994; Fagan *et al*., 1997). Deprivation of NGF from neonatal SCG neurons *in vitro* recapitulates their ontogenetic death *in vivo*. Deprivation of NGF from cultured sympathetic neurons leads to the following events. Transcription factor c-jun becomes phosphorylated and the protein level of c-jun also increases (Estus *et al*., 1994; Ham *et al*., 1995; Virdee *et al*., 1997; Eilers *et al*., 1998). Inhibition of the JNK (c-jun N-terminal kinase) pathway protects the neurons from death (Harris *et al*., 2002). The proapoptotic protein Bax is translocated from the cytosol to the mitochondria (Deckwerth *et al*., 1996; Putcha *et al*., 1999) and cytochrome *c* is released from the mitochondria into the cytosol (Deshmukh and Johnson, 1998; Neame *et al*., 1998; Martinou *et al*., 1999) to form a complex with Apaf-1 and procaspase-9. As a result, caspase-9 and -3 (Deshmukh *et al*., 2000; Deshmukh *et al*., 2002), but also caspase-2 (Troy *et al*., 2001) are activated. All of these events are critically required for the NGF deprivation-induced death *in vitro*. The neurons then exhibit several characteristic morphological changes, including cytoplasmic shrinkage, soma degeneration, neurite fragmentation, and chromatin condensation (Martin *et al*., 1988; Pittman *et al*., 1993; Edwards and Tolkovsky, 1994; Xue *et al*., 1999), and they finally die in the culture by secondary necrosis. Thus, the NGF-deprived SCG neurons basically die via the classical intrinsic pathway (Wright *et al*., 2006). Several neuron-specific features have also been described in the death of NGF-deprived SCG neurons. For example, differently from non-neuronal cells, the mere cytosolic localization of cytochrome *c* in the non-apoptotic NGF-maintained neurons is not able to trigger apoptosis. Microinjected cytochrome *c* kills the neurons only when they are also deprived of NGF. This phenomenon, called "competence", is most probably related to strong interaction of caspases with a natural inhibitor XIAP (X-linked inhibitor of apoptosis), and also with the low levels of Apaf-1 in SCG neurons (Deshmukh and Johnson, 1998; Potts *et al*., 2003; Potts *et al*., 2005). Also, these neurons (as all neurons) do not express Bak but, instead, a BH3-only splicing variant N-Bak (Sun *et*

al., 2001; Sun *et al*., 2003).

Although most of peripheral neurons that die during PCD are thought to do so due to of lack of neurotrophic support, deprivation of SCG neurons from neurotrophic factors other than NGF or death mechanisms of other neurotrophic factor-derived neurons are less studied. Deprivation of trophic factors from motoneurons and sensory neurons has been studied (Raoul *et al*,. 1999; Dolcet *et al*., 1999; Freeman *et al*., 2004). Serum deprivation is also a potent inducer of apoptosis *in vitro* for many different cell types including neurons (Atabay *et al*., 1996; Huang *et al*., 2000; Charles *et al*., 2005), but the underlying mechanism is also poorly understood.

1.8. Active modes of death

The death of NGF-deprived SCG neurons seems to in pricinple, be a passive process. The death program of these neurons is believed to lack some unknown constraints that normally keep the program repressed, and thus requires continuous suppression by NGF. Therefore, the signaling of NGF-bound TrkA by activated Akt kinase, B-Raf (Encinas *et al*., 2008), and other survival-promoting proteins somehow repress the

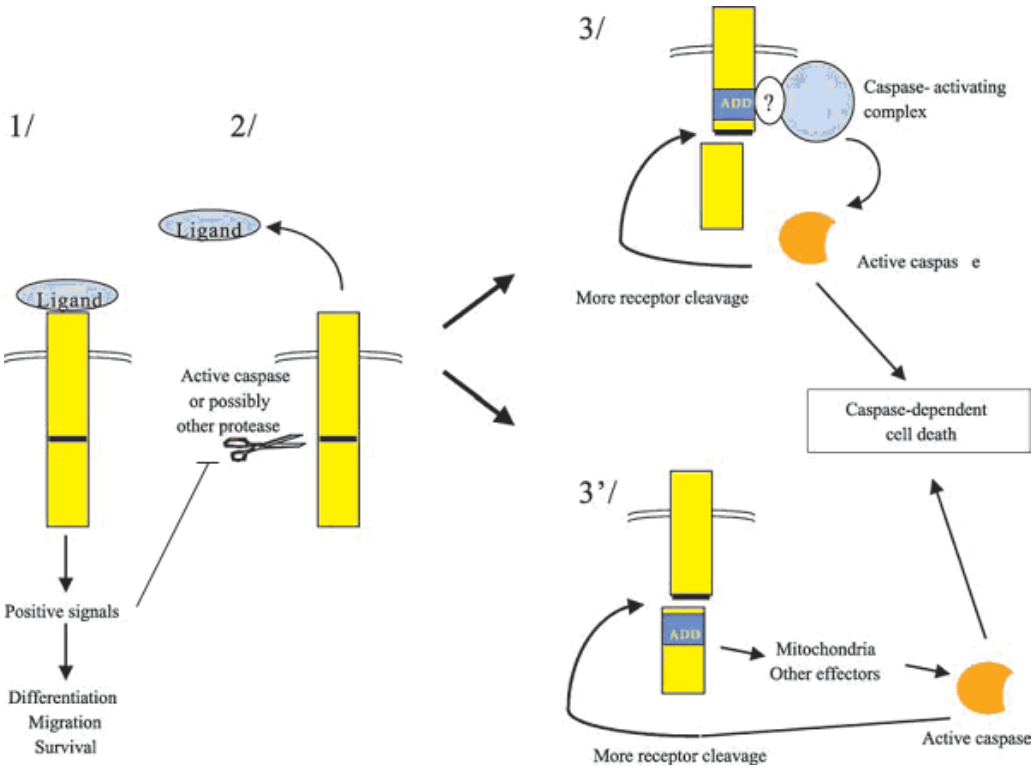

Figure 8. Model for apoptosis induced by the dependence receptor. When in the presence of ligand, dependence receptors mediate signal transduction that affects either differentiation or migration but may also inhibit the pro-apoptotic activity of these receptors. When unbound (or bound by a non-trophic ligand), however, a pro-apoptotic signal occurs via negative signal transduction. In at least some cases, this is mediated by a conformational change in the receptor, proteolytic cleavage, resulting in the release of pro-apoptotic dependence peptides. Adapted from Mehlen and Bredesen (2004).

death machinery. NGF withdrawal, causing cessation of the survival signaling, just passively releases the death program. Such release of apoptosis clearly opposes the theory that death receptors actively initiate death at the DISC. Recently, several other active modes of death triggering have been described, several also in neurons. The best studied of these are the dependence receptors and pro- neurotrophins.

1.8.1. Dependence receptors

Recently, a new family of functionally related receptors has been described as dependence receptors (Mehlen and Bredesen, 2004; Bredesen *et al*., 2004; Mehlen and Thibert, 2004). These proteins induce two completely opposite signals depending on the ligand availability. In the presence of the ligand, these receptors have positive effects on the cell survival, proliferation, differentiation etc., however when the ligand is absent, these receptors actively induce programed cell death. More than 10 receptors have been shown to display these two opposite activities: $p75^{NTR}$ (Rabizadeh *et al*., 1993), the netrin-1 receptors DCC (Forcet *et al*., 2001), UNC5H1, UNC5H2 and UNC5H3 (Llambi *et al*., 2001), the androgen receptor (Ellerby *et al*., 1999), the receptor for GDNF family ligands, Ret (Bordeaux *et al*., 2000; Canibano *et al*., 2007), integrins (Stupack *et al*., 2001; Ruoslahti and Reed, 1994), the receptor for Sonic hedgehog, Patched (Thibert *et al*., 2003) and the receptor for neogenin, RGM (Matsunaga *et al*., 2004). All of these receptors when deliganded, trigger cell death in a novel active manner that, in most cases, involves proteolytic cleavage of their intrcellular domain and release of the dependence domain that can actively trigger death. Although the details of activation of the deliganded receptor are still poorly understood, the current model proposes cleavage of the receptor from one or two sites by specific caspases. The released dependence domain can activate the apoptotic caspases that kill the cell. Deletion of this domain or mutation of the caspase cleavage sites is sufficient to block the killing activity (Figure 8). The cytoplasmic domain of netrin receptor DCC activates caspases in a cell-free system in the presence of cytosol that is depleted of cytochrome *c*, a novel mechanism (Furne *et al*., 2006). The caspase-activating mechanism of other dependence receptors is still poorly studied. Importantly, the deliganded dependence receptors are essential in killing the metastasing tumor cells that migrate out of the ligand-expressing area, and could be potential targets in tumor treatment (Mehlen and Thibert, 2004; Bernet and Mehlen, 2007).

1.8.2. Pro-neurotrophins and $P75^{NTR}$

A common-shared receptor of the neurotrophins is $p75^{NTR}$. It belongs to the superfamily of receptors that includes the tumor necrosis factor receptors and Fas (Chao, 1994). On one hand, $p75^{NTR}$ participates in the formation of high-affinity complexes with neurotrophins and the respective Trk receptors and thereby facilitates positive signaling of the neurotrophins. On the other hand, $p75^{NTR}$ can also actively induce cell death (Nykjaer *et al*., 2005). The mechanism of death-promoting activities of $p75^{NTR}$ can also be different (Bredesen *et al*., 2005). In some studies, $p75^{NTR}$ was shown to act as a dependence receptor, inducing cell death when not bound with the ligand, and death was blocked by binding of the ligand (Rabizadeh *et al*., 1993; Barrett and Bartlett, 1994; Rabizadeh *et al*., 1993). Importantly, as shown by overexpressed

p75^{NTR} without bound ligand, cleavage of the cytoplasmic domain of p75^{NTR} and release of a "chopper" domain in the juxtamembrane region mediates cell death (Coulson *et al*.**,** 2000). Increasing evidence also shows that p75^{NTR} can trigger death upon binding of a ligand, in particular pro-NGF or pro-BDNF (Lee *et al*., 2001; Teng *et al*., 2005), thus acting as a death receptor. Neurotrophins are synthesized as longer proforms. Earlier the pro-neurotrophins were considered inactive proteins that are activated by proteolytic removal of the pro-sequence. Recently it was shown, however, that the pro-neurotrophins can have an independent, pro-apoptotic function (Lee *et al*., 2001). In particular, the proforms activate p75^{NTR} to induce apoptosis, whereas mature forms mainly activate Trk receptors to promote survival. Sortilin is associated with p75^{NTR} and pro-neurotrophins to form a signaling complex essential for the induction of apoptosis (Nykjaer *et al*., 2004). Studies on sortilin knockout mice indicate that sortilin has distinct roles in pro-neurotrophin-induced apoptotic signaling in pathological conditions (Jansen *et al*., 2007). Interestingly, binding of pro-neurotrophins to p75^{NTR} can also cause the cleavage of its transmembrane domain by gamma-secretase. This cleavage facilitated nuclear translocation of the neurotrophin receptor interacting factor (NRIF). The cleavage of p75^{NTR} and nuclear translocation of NRIF were required for apoptosis of sympathetic neurons by pro-BDNF or BDNF binding in the absence of NGF (Kenchappa *et al*., 2006). Studies disagreeing with the pro-apoptotic function of p75^{NTR} also exist. For example, the cytoplasmic region of p75^{NTR} may not translocate to the nucleus, because it was not detected in the nucleus even after viral over-expression (Hébert *et al*., 2006). P75^{NTR} also has many other activities (Nykjaer *et al*., 2005, Bredesen *et al*., 2005). In summary, p75^{NTR} may induce death as a dependence receptor or as a death receptor, possibly depending on the cellular context.

1.9. One cell, multiple cell death pathways

It is clear that there are more programed death pathways than just the "two main" - extrinsic and intrinsic apoptotic pathways. Although currently poorly described, the "novel" pathways may also have important roles *in vivo*, and most likely more pathways will be discovered. The same death stimulus can trigger different death pathways in different cell types (*e.g.* deprivation of GDNF from sympathetic and dopaminergic neurons, paper I and IV). Also, the same cell can die differently in response to different death stimuli. For example, treatment of lymphocytes with genotoxic compounds kills them via the mitochondrial pathway, whereas activation of Fas activates the death receptor pathway in the same cells. Thus, cells possess more than one death pathway. *In vivo* relevance of this concept is well illustrated in mice deficient of the main components of the intrinsic apoptotic pathway: Apaf-1, caspase-3, caspase-9, or knock-in mice with non-apoptotic cytochrome *c*. In these mice, the developmental apoptosis in most cells of the nervous system (except in the forebrain precursors) still occurred but via different, non-apoptotic pathway that includes cytoplasmic vacuolization and increased autophagy (Cecconi *et al*., 1998; Roth *et al*., 2000; Zaidi *et al*., 2001; Hao *et al*., 2005; Oppenheim *et al*., 2008). Cultured sensory neurons switch to a death pathway that uses lysosomal proteases instead of caspases, when the caspase inhibitor is applied (Isahara *et al*.,

1999; Agerman *et al*., 2000). Studies of Bcl-xL/caspase-9 double knockout also give evidence of multiple death pathways in the same cells (Zaidi *et al*., 2001). Thus, upon failure of the default apoptotic pathway, most of the neurons switched to an alternative death mode, showing that they possess and can use several death machineries.

A notable example of non-apoptotic cell death, necroptotic cell death was also discovered in the cells that were under constant death stimulus of TNFα but had the main apoptotic pathway blocked by caspase inhibitors. In these conditions, the cells switched to a non-apoptotic pathway and died despite of caspase inhibition. Necroptosis can be blocked by specific compounds, necrostatins that do not block classical apoptosis. Conversely, necroptosis cannot be blocked by the inhibitors of apoptosis, such as Bcl-2 family members or caspase inhibitors. Importantly, necroptosis seems to be involved in many forms of pathological neuronal death (Vandenabeele *at al*., 2008; Festjens *at al*., 2007; Degterev *et al*., 2008),

The reason why just one death pathway in all cells in all situations is not sufficient is not understood. Most probably the different cell types have their "default" death pathway in response to the death stimuli that they normally encounter in their natural environment. The other pathways could be "spare pathways" that are used when the main one fails or is not available. The kinetic parameters are also one reason for the existence of different death programs. For example, the death receptor pathway, with fewer checkpoints, kills the cells more rapidly than the mitochondrial pathway. Such rapid action is required to fight against invading pathogens, whereas the slower intrinsic pathway gives the cell more opportunities to cope with the death-causing situation. The same could also be true for other active modes of cell death. Also, the differentiation status of cells could dictate the preference of the death program. Some differentiation programs may include or exclude the expression of the death-mediating proteins that could determine the pathway choice in the death inducing situation. Indeed, there are increasing bodies of evidence that many, if not all, death-mediating proteins also (or even primarily) have vital, non-apoptotic functions and could therefore be differentially expressed or regulated in different cell types (Galluzzi *et al*., 2008; Peter, 2007; Wallach *et al*., 2008).

2. AIMS OF THE STUDY

Neurotrophic factors promote the survival of both peripheral and central neurons *in vivo* and *in vitro*. Deficiency of these factors leads to ontogenetic death of these neurons. The well-characterized model for studying neuronal death is deprivation of NGF from the cultured sympathetic neurons. Other types of neurons as well as deprivation of other neurotrophic factors, are poorly studied in this respect. The aim of this study was to compare the death machineries triggered in different neuronal populations by removal of different neurotrophic factors.

1. To compare the death programs triggered by withdrawal of two different neurotrophic factors, GDNF or NGF, from the sympathetic neurons.

2. To test the hypothesis that TrkC acts as a dependence receptor in the death of NT-3-deprived sensory neurons.

3. To develop a new assay technique for studying the death pathways in apoptotic dopaminergic neurons.

4. To study the death pathways activated in the dopaminergic neurons by the deprivation of GDNF or BDNF.

3. MATERIALS AND METHODS

3.1. Cell cultures and survival assays (I, II, III and IV)

SCG from postnatal day 1-2 Han/Wi strain rats or NMRI strain mice were digested with collagenase (2.5mg/ml; Worthington), dispase (5mg/ml; Roche Molecular Biochemicals) and trypsin (10mg/ml; Worthington) for 45 minutes at 37°C and dissociated mechanically with a siliconized glass Pasteur pipette. Non-neuronal cells were removed by four hours preplating. The neurons were grown 5-6 days *in vitro* on poly-ornithine/ laminin-coated plastic dishes or glass coverslips in a 1:1 ratio of mixture F-12 and Dulbecco´s modified Eagle´s medium (DMEM) (Life Technologies,Inc.) containing 3% fetal calf serum (Hyclone), serum substitute and 30ng/ml NGF (Promega) or 100ng/ml GDNF (PeproTech). To reduce the number of non-neuronal cells, 1 μM cytosine arabinoside (Sigma-Aldrich) was added to the culture medium.

DRG neurons were prepared from embryonic day 16-17 NMRI strain mice, treated with 1% trypsin (Worthington) for 15 min and dissociated mechanically. The neurons were grown on polyornithine-laminin-coated dishes with either 10 ng/ml of human NT-3 (PeproTech) or 30 ng/ml of 2.5S mouse NGF (Promega).

The midbrain floors were dissected from the ventral mesencephali of 13-day NMRI strain mouse embryos. Tissues were incubated with 0.5% trypsin (ICN Biomedical, Inc.) in HBSS (Ca^{2+}/Mg^{2+} free) (Invitrogen/Gibco) for 15 min at 37°C, then mechanically dissociated using a large fire-polished Pasteur pipette. The cells were then plated onto culture dishes coated with poly-L-ornithine (Sigma-Aldrich) and were grown in DMEM/F12 medium (InVitrogen/Gibco) containing N2 supplement (Invitrogen/Gibco) for 5 days with GDNF (100 ng/ml) (Amgen Inc, CA) or BDNF (50 ng/ml) (R&D systems, Inc.).

Mouse embryonic fibroblast 3T3 cells were grown in DMEM medium with 10% fetal calf serum. The cells with 50-80% confluency were used for transfection or other experimental treatments.

To remove the trophic factors, the cultures were washed gently three times with trophic factor-free medium. In addition, function-blocking antibodies were added. The compounds of interest were added and the initial number of neurons counted immediately after neurotrophic factor deprivation. Living neurons were counted daily by a "blind" experimenter who was not aware of the identity of experimental groups.

3.2. Reverse transcription-polymerase chain reaction (RT-PCR) (IV)

Dopaminergic neurons were cultured for 5 days *in vitro* and deprived or not deprived of the factors for 24 hours with or without BAF. Total RNA from dopaminergic neurons, 3T3 (positive control, expressing both Fas and Fas ligand) or CHO (negative control) cells was isolated by Micro Scale RNA Isolation kit (Ambion) including DNase I digestion, according to the manual instructions. 200-500 ng of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and the cDNA was used directly for PCR. 2 μl of cDNA from each sample was amplified by PCR with the Expand High Fidelity PCR system (Roche). The following primers were used. Fas: forward: 5'-GTGTTCGCTGCGCCTC-3';

reverse 5'- GGTTCTGCGACATTCGGC-3' (Lesne *et al*., 2002); Fas ligand: forward 5'- TTTCATGGTTCTGGTGGCTCTGGT-3'; reverse 5'-AGCGGTTCCATATGTGTCTTCCCA-3'. PCR was performed after an initial denaturation of 5 min at 95°C, followed by 36 cycles of 30 s denaturation at 95°C, 30 s annealing at 60°C, and 30 s extensions at 72°C. As the negative controls, RT reaction was omitted from the 3T3 sample, or the whole procedure was performed in water without adding cDNA.

3.3. Microinjection and transfections (I, II, III and IV)

SCG or DRG neurons were pressure-microinjected with 50 ng/µl of expression plasmids together with 10 ng/µl of reporter plasmid for an enhanced green fluorescent protein (eGFP) (Clontech, France) as an indicator of successful injection. The corresponding empty vector (pcDNA3, PCR3.1 or pMV2B) as well as an un-injected control were always included. When the neurotrophic factor-deprived neurons were analyzed, the factor-maintained un-injected neurons were always included to show that the neurons do not die due to poor culture conditions. Neurons surviving after injection were counted 4-6 h later according to the map drawn with the help of squares scratched to the bottom of the culture dish and considered as initial neurons. The next morning, the few living injected neurons that did not show eGFP fluorescence were subtracted from the initial neurons. On average, 25-80 neurons were successfully injected per experimental point. All experiments were repeated at least three times on independent cultures.

Midbrain cultures grown 5 days *in vitro* with GDNF or BDNF were transfected with the calcium phosphate co-precipitation technique using the Ca-P kit (InVitrogen, CA) according to manufacturer's instructions. Plasmids of interest at 1 µg/ml were co-transfected with eGFP at 0.2 µg/ml. At that ratio, virtually all eGFP-expressing neurons co-express the co-transfected plasmid. The relevant empty vectors (pCR3.1, pMV2B or pCDNA3) without the insert were always included as mock-controls. The factors were deprived the day after transfection. The number of fluorescent (eGFP-expressing) neurons was counted "blindly" from each dish immediately after factor deprivation (initial) and at the third day (final). The results were expressed as percent of initial fluorescent neurons. To exclude eGFP-expressing non-dopaminergic neurons, the cultures were stained with tyrosine hydroxylase (TH) antibodies at the end of experiment and the number of eGFP-positive, TH-negative neurons were subtracted from the initial and final number. All experiments were repeated at least three times on independent cultures.

The 3T3 cells at 50-80% confluence were transfected with the indicated plasmids or with the mock vector using Lipofectamine 2000 (Life Technologies, Inc.).

3.4. Immunochemistry (I, IIIand IV)

Rat or mouse neurons were grown on glass coverslips, fixed with 4% PFA in phosphate-buffered saline (PBS) for 15 min, permeabilized with 0.3% Triton X-100 (Sigma-Fluka, Switzerland) and stained with the indicated antibodies. The specimens were mounted in Vectashield (Vector Laboratories, UK). The images were captured at RT with epifluorescence or confocal microscopy, and processed with Adobe Photoshop software.

3.5. Immunoblot and co-immunoprecipitation (II, III and IV)

Midbrain cultures grown with GDNF or BDNF for 5 days *in vitro* or NT-3 dependent DRG neurons were lyzed in the 2 % SDS lysis buffer. Lysates from the 3T3 cells or cortical cultures of embryonic mice were prepared in the same way. The proteins were separated on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose filters by standard procedures. The filters were probed with the indicated antibody.

For the co-immunoprecipitation experiments, midbrain neurons were cultured for 5 days *in vitro* with GDNF or BDNF and transfected with an expression plasmid for mouse Fas together with eGFP according to Yu and Arumäe (2008). After two days the cells were lyzed as described previously by Segura *et al*. (2007). Fas was precipitated by biotin-coupled anti-Fas antibody (BD Biosciences) or control biotin-coupled anti-GFRα2 antibody (BD Biosciences) and the immunocomplexes were collected with Neutravidin (Thermo, MA). Immunoblot analysis was performed right after immunoprecipitation.

3.6. Electron microscopy analysis (I)

SCG cultures were fixed two days after factor deprivation with 2% of glutaraldehyde. The cultures were processed for transmission electron microscopy. To estimate the size of the mitochondria from GDNF- or NGF-deprived neurons, mitochondria, at a final magnification of 33,000x, were manually traced onto transparencies that were scanned. A cross-sectional area of the mitochondria was measured using Image-Pro Plus version 3.0.

3.7. Statistical analysis (I, II, III and IV)

Statistical significance of the differences was estimated by one-way ANOVA and post hoc Tukey's test or by two-tailed Student´s t test with two-sample unequal variance. The null hypothesis was rejected at $p<0.05$.

4. RESULTS

Although neuronal death caused by neurotrophic factor deficiency is most probably a broadly occurring developmental phenomenon (Oppenheim, 1991), its molecular and cellular pathways have been systematically described mainly for NGF-deprived sympathetic neurons. Other neuronal populations, as well as deprivation of other neurotrophic factors are poorly studied. The main goal of this study was to describe the death pathways activated by the withdrawal of neurotrophic factors other than NGF in different neuronal populations.

4.1. GDNF-dependent sympathetic neurons die via a non-mitochondrial pathway which requires caspase activation (I)

Although almost all sympathetic neurons from the superior cervical ganglion require NGF for the survival, a subpopulation is additionally dependent on GDNF. These neurons are, however, very poorly studied. We studied the death of GDNF-deprived sympathetic neurons cultured *in vitro* and compared them to NGF-deprived sister cultures. Removal of GDNF or NGF from the rat sympathetic neurons for 72 hours leads to the death of about 80% of the respective factor-dependent neurons. To study how these neurons die, we checked the components of the core mitochondrial apoptotic machinery, such as cytochrome *c*, Bax, caspase-9, and caspase-3. Staining the neurons with cytochrome *c* antibody showed that removal of NGF dramatically reduced the number of the neurons with punctate cytochrome *c* localization, as shown by others (Deshmukh and Johnson, 1998; Neame *et al*., 1998; Martinou *et al*., 1999). In contrast, only a small fraction of GDNF-deprived neurons had diffuse cytosolic cytochrome *c* staining, whereas the vast majority had punctate, mitochondrial cytochrome *c* staining. Overexpression of Ku70, a protein which binds the NH2 terminus of Bax, and thereby blocks its translocation to the mitochondria (Sawada *et al*., 2003), did not protect GDNF-deprived neurons, although, it significantly blocked the death of NGF-deprived neurons. To check the involvement of caspases, we overexpressed the respective dominant-negative mutants in factor-deprived neurons. Blocking of caspase-9 and caspase-3, the central caspases of the mitochondrial pathway, failed to inhibit the death of GDNF-deprived neurons, although the death of NGF-deprived neurons was significantly blocked, as expected (Deshmukh *et al*., 2002; Deshmukh *et al*., 2000; Deshmukh *et al*., 2002; Harris *et al*., 2002; Troy *et al*., 2001). These data showed that the core elements of classical mitochondrial pathway are not activated in GDNF-deprived sympathetic neurons. To confirm this conclusion, we also overexpressed anti-apoptotic Bcl-2 family member Bcl-xL, which is shown to block mitochondrial death pathway (Tsujimoto, 1998). Bcl-xL did not protect GDNF-deprived neurons, but rescued most of the NGF-deprived neurons. The absence of cytochrome *c* release, together with a failure to observe the role of Bax, caspase-9, -3, and Bcl-xL, suggests that the mitochondrial pathway is not activated in GDNF-deprived sympathetic neurons.

The broad-range caspase inhibitor, BAF, almost completely blocked the death of both NGF- (Deshmukh *et al*., 1996, 2000; Martinou *et al*., 1999) and GDNF-deprived neurons. To clarify the relevant

caspases in the GDNF-deprived neurons, we overexpressed dominant-negative mutants of caspase-2, -6, -7, and -8 in the GDNF-deprived and NGF-deprived neurons. Blocking caspase-2,-3, and -9, and to lesser extent, caspase-6 and -7 significantly inhibited the death of NGF-deprived neurons. But only dominant negative mutants of caspase-2 and, to lesser extent, caspase-7 inhibited the death of GDNF-deprived neurons.

To study the activation of the transcription factor c-jun, we stained GDNF- or NGF-deprived neurons with antibodies to c-jun phosphorylated at serines 63 or 73. Removal of GDNF significantly increased the number of nuclei immunopositive for phosphorylated serine 73. The number of nuclei positive for phosphorylated serine 63 remains unchanged. In sister cultures, deprivation of NGF dramatically induced phosphorylation of serine 63 of c-jun as previously shown (Ham *et al*., 1995; Virdee *et al*., 1997; Eilers *et al*., 1998; Harris *et al*., 2002). Phosphorylation of c-jun is also catalyzed by the c-jun NH-2 terminal kinases whose activity is in turn regulated by several upstream kinases, including the mixed lineage kinases (MLK). We treated GDNF- or NGF-deprived neurons with CEP-1347, which is a selective inhibitor of MLK (Maroney *et al*., 2001). Almost all NGF-deprived neurons were rescued by CEP-1347 at 72 hours after deprivation as previously described (Maroney *et al*., 1999; Harris *et al*., 2002). CEP-1347 also rescued GDNF-deprived neurons with the same efficiency. Finally we tested whether c-jun was necessary for the death of factor-deprived neurons. We overexpressed a dominant-negative c-jun mutant FLAGΔ169 in GDNF- or NGF-deprived neurons. After 72 hours about 60% of NGF-deprived neurons were rescued as previously described (Ham *et al*., 1995; Eilers *et al*., 1998). A similar percentage of GDNF-deprived neurons was rescued by overexpressed FLAGΔ169. In summary, activation of c-jun is necessary for the death of GDNF-deprivation.

Overexpression of a dominant-negative mutant of FADD, an adapter that links pro-caspase-8 to most death receptors (Strasser and Newton, 1999; Vincenz, 2001; Peter and Krammer, 2003; Peter *et al*., 2007) protected neither GDNF- nor NGF-deprived neurons. In addition, overexpression of dominant-negative caspase-8 did not affect the death of these two neuronal populations. Thus, we did not find any evidence for the activation of death receptor pathway in GDNF- or NGF-deprived sympathetic neurons.

We also investigated the ultrastructural changes caused by GDNF- or NGF-deprivation. We found that the number of autophagic profiles was greatly increased in GDNF-deprived neurons, but not in NGF-deprived neurons. The mitochondrial structure became apoptotic in NGF-deprived neurons, but in GDNF-deprived neurons, the mitochondria remained orthodox.

In summary, we found that GDNF-deprived sympathetic neurons die via a novel non-mitochondrial, MLK, c-jun, and caspase-dependent pathway that had not yet been described. Removal of NGF from sympathetic neurons in the same conditions, however, activates the classical mitochondrial death pathway.

4.2. The dependence receptor activity of TrkC is necessary for the death of NT-3-deprived sensory neurons (II)

As another pair of neurotrophic factor and neurons, we turned to NT-3-deprived sensory neurons from the embryonic dorsal root ganglia. This study was carried

out in collaboration with Patrick Mehlen and colleagues (Centre Léon Bérard, Lyon), and was inspired from the model of dependence receptors developed by that group (Mehlen and Bredesen, 2004; Mehlen and Thibert, 2004). Our main goal here was to test the hypothesis that removal of NT-3 (deligation of its receptor TrkC) leads to the caspase-mediated cleavage of the cytoplasmic domain of TrkC and the cleaved fragment activates apoptotic caspases. If so, then TrkC is a novel dependence receptor. The group in Lyon tested this model on non-neuronal cells (not shown here), whereas we addressed it in the primary sensory neurons.

DRG neurons from the embryonic mice were cultured with NT-3 or NGF for 5 days. Deprivation of these factors leads to death of about 60-70% of the neurons after 72 hours. By immunoblot we found that deprivation of NT-3 can induce cleavage of TrkC, and this cleavage can be blocked by the caspase inhibitor BAF. The fragment was recognized by the antibodies to the extracellular domain of TrkC and is thus different from that predicted by the model. Most probably there are several cleavages of the receptor upon NT-3 removal. We then turned to a research tool that dominantly inhibits cleavage of de-ligated TrkC: a cytoplasmic domain of TrkC with mutated caspase cleavage site (TrkC IC D641N). Such constructs with similar design have been used to inhibit other dependence receptors (Ellerby *et al*., 1999; Forcet *et al*., 2001; Thibert *et al*., 2003). Overexpression of TrkC IC D641N dramatically increased survival of NT-3 deprived neurons, although it did not affect the death of NGF-deprived neurons. To exclude the possible role of tyrosine kinase domain, that could be auto-activated when separated from the rest of the protein, and provide the survival signal, we also microinjected the TrkC IC D641N bearing an additional kinase-inactivating mutation D679N. This mutation did not abolish the death-suppressing activity of TrkC IC D641N in NT-3-deprived neurons. To further demonstrate the role of TrkC cleavage in the death of NT-3-deprived neurons, we suppressed the endogeneous receptor by a specific RNAi and replaced it by ectopic expression of uncleavable or wild-type TrkC. Replacement of endogenous TrkC by uncleavable TrkC significantly inhibited the death of NT-3-deprived neurons, whereas replacement with wild-type TrkC had no effect.

These data together with the data provided by the Lyon group on the non-neuronal cells (not shown in this thesis) prove that TrkC is a new dependence receptor. Thus, NT-3 deprivation-induced cell death is not only due to the loss of survival signals, but also to active cell death stimulus triggered by unbound TrkC. These data also show that NT-3-deprived sensory neurons die differently than the NGF-deprived ones.

4.3. A survival assay of transiently transfected dopaminergic neurons to analyze the apoptotic proteins in these neurons (III)

Our results with GDNF-deprived sympathetic neurons prompted us to turn to another population of GDNF-dependent neurons, the dopaminergic neurons from the embryonic midbrain. Our main goal was to test whether the removal of GDNF triggers the same death pathway in different neuronal types, *i.e* does the de-ligation of GDNF receptor Ret always trigger the same pathway. Moreover, the dopaminergic neurons degenerate in Parkinson's disease and in several studies, GDNF considerably improved

the symptoms (Oo *et al*., 2003; Oo *et al*., 2005; Gill *et al*., 2003; Slevin *et al*., 2005; Lang *et al*., 2006; Patel and Gill, 2007). Understanding the mechanism of death due to GDNF deprivation could potentially suggest some treatment strategies.

To perform the study we required a relevant *in vitro* assay. In the midbrain cultures, the dopaminergic neurons constitute only a subpopulation of neurons. Moreover, our experiments often require transfection of the neurons and consequently recognition of transfected dopaminergic neurons among non-transfected and non-dopaminergic neurons to monitor their survival/death after neurotrophic factor deprivation. Such an assay was not available, so we had to first develop it.

We cultured the neurons with GDNF on a standard small area of the culture dish that enables us to count all of the neurons, and co-transfected the cultures with the plasmid of interest, together with the reporter plasmid for eGFP using the calcium phosphate co-precipitation technique. Such an approach enabled the visualization of the same transfected neurons before and after GDNF deprivation, allowing us to calculate the percentage of surviving transfected neurons for each dish (experimental point) individually. Because both dopaminergic neurons and non-dopaminergic neurons get transfected in the mixed culture, we stained the culture with antibodies to TH, the specific marker for dopaminergic neurons at the end of the experiment, and counted the number of neurons positive for both eGFP and TH. This number was normalized to the number of initially transfected neurons counted from the same dish. We also counted the number of transfected non-dopaminergic (TH-negative) neurons that did not die due to GDNF-deprivation, and subtracted this number from the initial and final experimental counts. Thereby we got the relevant estimation of the fraction of transfected dopaminergic neurons that survived after the deprivation of GDNF.

4.4. GDNF- or BDNF-deprived dopaminergic neurons die via a non-mitochondrial pathway which requires activation of death receptors and caspases (IV)

Having a reliable assay, we then studied the death pathways activated in the dopaminergic neurons by deprivation of GDNF. For comparison we chose another neurotrophic factor, BDNF that also promotes survival of the dopaminergic neurons, but by a different receptor, TrkB (a homologue of NGF receptor TrkA, chosen for the comparison in the study of sympathetic neurons).

We cultured dopaminergic neurons with GDNF or BDNF for 5 days *in vitro*. Deprivation of the factors induced death in about half of the dopaminergic neurons within three days. To study the involvement of the mitochondrial death pathway, we stained GDNF- or BDNF-deprived cultures with antibodies to cytochrome *c* and TH. In the vast majority of GDNF- or BDNF-deprived TH-positive neurons, cytochrome *c* localization was punctate, the same mitochondrial localization as in factor-maintained neurons, showing that it is not released to the cytosol. We overexpressed Ku70 in GDNF- or BDNF-deprived neurons to check the role of Bax in the death pathway. Ku70 had no effect on the death of these factor-deprived neurons, showing that Bax is not required for this process. Immunostaining of GDNF- or BDNF-deprived neurons with Bax antibody also revealed its diffuse, cytosolic localization. Overexpressed anti-

apoptotic Bcl-2 family member Bcl-xL did not block the death of GDNF- and BDNF-deprived neurons. Thus, we conclude that the mitochondrial pathway is not activated in GDNF- or BDNF-deprived dopaminergic neurons.

Since caspase inhibitor BAF can completely block the death of GDNF- and BDNF- deprived dopaminergic neurons, it was clear that caspases are absolutely required. We then checked which caspases are involved. We overexpressed dominant-negative mutants of caspase-2, -3, -7, and -9 in the GDNF- or BDNF-deprived neurons. Blocking of caspase-3, -7, and -9 significantly protected the neurons from death induced by GDNF- or BDNF-deprivation, whereas blocking caspase-2 had no statistically significant effect. Thus, different caspases are activated in the GDNF-deprived sympathetic and dopaminergic neurons.

To check the involvement of another classical apoptotic pathway, the death receptor pathway, we first checked the Fas receptor and FasL expression in our cultures. By RT-PCR and immunostaining, we clearly verified the expression of both Fas receptor and FasL in the dopaminergic neurons. We then transfected the neurons with dominantly blocking mutants of caspase-8 and FADD, both constructs blocked the death of GDNF- and BDNF-deprived dopaminergic neurons, showing that the death receptor pathway was activated. To get further evidence for this claim we applied agonistic and antagonistic research tools used in other studies of Fas. Antagonistic Fas-Fc, a chimaeric decoy containing the extracellular domain of Fas, shown to prevent interaction of Fas and FasL in motoneurons (Raoul *et al*., 2002), significantly blocked both GDNF- and BDNF- deprivation induced neuronal death. Furthermore, ligation of Fas by agonistic Jo2 anti-Fas antibody killed GDNF- and BDNF-maintained neurons. Thus, in contrast to GDNF (and NGF) -deprived sympathetic neurons (study I), the Fas-like death receptors are activated in GDNF- and BDNF-removed dopaminergic neurons.

Our immunostaining study shows that both Fas and FasL are constitutively expressed in the midbrain cultures. This differs from the immune system where FasL is induced on the killer cells only upon stimulation, to avoid unwanted killing of the cells (Ashkenazi and Dixit, 1998). We hypothesized the existence of a constitutive inhibitor of Fas in the dopaminergic neurons that would keep Fas apoptotically inactive in the normal situation, but could be removed upon neurotrophic factor deprivation. One such candidate is the long form of Fas apoptosis inhibitory molecule $FAIM_L$, which has been shown to associate with Fas and prevent its apoptotic activity specifically in the neurons (Segura *et al*., 2007). By immunoblot, we demonstrated its expression in our mixed neuronal culture. Endogenous $FAIM_L$ was coprecipitated with transfected Fas from the GDNF- and BDNF-maintained cultures, suggesting physical association of these proteins. Overexpressed Fas significantly killed GDNF- and BDNF- maintained neurons. In contrast, overexpressed $FAIM_L$ significantly blocked the death of GDNF- and BDNF-deprived neurons, and this protection was abolished upon cotransfection with Fas. Thus, Fas and $FAIM_L$ can functionally and physically interact with each other in the dopaminergic neurons. We were not able to check, however, whether $FAIM_L$ could be degraded upon GDNF deprivation. It is also expressed in other, non-dopaminergic neurons that constitute the majority in our cultures and overwhelm the possible

changes in the dopaminergic neurons in our tests.

We conclude that GDNF- and BDNF-deprived dopaminergic neurons die via a non-mitochondrial pathway, which activates death receptors and caspases. Withdrawal of the same factor GDNF triggers different death pathways in the sympathetic and dopaminergic neurons, whereas withdrawal of different factors (GDNF or BDNF) trigger the same death machinery in the dopaminergic neurons.

5. DISCUSSION

Neurons from both the peripheral and central nervous systems need trophic support from different sources for survival at specific development stages. The same type of neurons can be responsive to several factors at different times during development. For example, neurotrophins can promote the survival of largely separate sub-populations of somatic embryonic sensory neurons both *in vivo* and *in vitro* (Levi-Montalcini, 1966; Levi-Montalcini, 1987a; Smeyne *et al*., 1994; Ockel *et al*., 1996b; Liebl *et al*., 1997; Ockel *et al*., 1996a; Maisonpierre *et al*., 1990). Lack of this support leads to ontogenetic death of these neurons (Oppenheim, 1991, Huang and Reichardt, 2001). Apoptotic death of neurons, even the same type of neurons, can be evoked by different initiating events. Multiple pathways of apoptotic death may exist in cells. It is still poorly understood how and why deprivation of different factors from the same neuron could sometimes trigger different pathways.

The NGF-dependent sympathetic neurons have been extensively used to study the mechanism of neuronal PCD. Removal of NGF from these neurons induces cell death via the well-known mitochondrial pathway: releasing of cytochrome *c* (Deshmukh and Johnson, 1998; Martinou, 1999; Neame *et al*., 1998; Martinou *et al*., 1999), Bax translocation (Deckwerth *et al*., 1996; Putcha *et al*., 1999), activation of caspase-9 and -3 (Deshmukh *et al*., 2000; Deshmukh *et al*., 2002), and inhibition of the death by Bcl-xL (Gonzalez-Garcia *et al*., 1995). The death pathways activated by withdrawal of other neurotrophic factors have been much less explored.

We compared the death machineries triggered in the same neuron (sympathetic neurons in paper I; DRG neurons in paper II; dopaminergic neurons in paper IV) by removal of two different factors (NGF or GDNF in paper I; NGF or NT-3 in paper II; GDNF or BDNF in paper IV). To our surprise, we found that the death pathways differed considerably in different cell types and after deprivation of different trophic factors.

Compared to NGF-deprived sympathetic neurons, removal of GDNF from these neurons activated a novel death pathway: cytochrome *c* was not released; Bax and Bcl-xL were not involved; caspase-3 and -9 were not activated and the ultrastructure of mitochondria was unchanged. The way deprivation of SCG neurons of two different neurotrophic factors triggers the two different death programs is currently unknown. Loss of survival signals, like trophic support, passively releases the death program, but active death triggering may also occur. Recently, the concept of dependence receptors has been developed. Receptors of this family transmit positive signals of survival, proliferation, differentiation, or migration in the presence of ligand, but actively trigger apoptosis in the absence of ligand via a novel mechanism that involves receptor cleavage (Bredesen *et al*., 2004). Ret was suggested to be a dependence receptor, since unligated Ret can be cleaved by caspase-3 and generate an apoptotic fragment (dependence domain) that can trigger apoptosis in some cell lines (Bordeaux *et al*., 2000; Canibano *et al*., 2007). Thus, one possibility is that in the SCG neurons, the dependence domain of deliganded Ret itself actively triggers death via a non-mitochondrial pathway, whereas deliganded TrkA that is not a

dependence receptor (II), only passively releases the mitochondrial apoptotic machinery. Such triggering of a non-classical death pathway has been described for the dependence domain of DCC (Forcet *et al*., 2001). It has been shown, however, that Ret does not act as a dependence receptor in enteric neurons *in vivo* (Uesaka *et al*., 2008). Overexpression of Ret, or the apoptotic fragment of Ret, in both NGF- and GDNF-dependent sympathetic neurons did not induce death in our hands (unpublished data), suggesting that the active death stimulus from unbound Ret is not generated in the sympathetic neurons. The mechanisms by which deliganded Ret and TrkA activate different death programs in cultured sympathetic neurons require further studies. We speculate that maintaining the neurons with GDNF for 5-6 days differentiates those that could, among other things, include expression of a separate death machinery (*e.g.* the machinery for caspase-2 activation) and/or render the mitochondrial pathway nonfunctional. Whether and when such a pathway is activated *in vivo* is not known. As the GDNF-responsive SCG neurons are most probably also under the influence of NGF, it is tempting to speculate that local removal of GDNF could give an opportunity to remove them via an alternative pathway, even in the presence of NGF.

We found, however, that another tyrosine kinase receptor-TrkC functions as a dependence receptor in DRG neurons. Because the death of the cells, induced by NT-3 removal, critically depends on the release of a pro-apoptotic dependence domain from TrkC. This pro-apoptotic function is not caused by abnormal autoactivation of TrkC, because a kinase-dead mutant still displays a similar pro-apoptotic activity. In contrast, the caspase cleavage sites were not found at the corresponding positions in TrkA or TrkB and the intracellular domains of TrkA and TrkB can not be cleaved by caspase-3. It suggested that even closely related receptors like TrkA, TrkB, and TrkC can act differently regarding cell survival/cell death. We propose that TrkA and TrkB can induce only positive signaling when liganded, but remain passive when deliganded leading to cessation of the survival signaling. Liganded TrkC also induces positive signaling that ceases upon ligand removal. Deliganded TrkC, however, also triggers an active negative (apoptotic) signaling. Thus, TrkC actively controls both survival and death depending on its ligation status and is therefore a dependence receptor. Analyses of *NT-3*$^{-/-}$ and TrkC mutant mice suggests that NT-3 is involved in multiple aspects of early sensory neuron development (Klein *et al*., 1994; Tessarollo *et al*., 1994; Tessarollo *et al*., 1997), and overexpression of NT-3 in developing neurons increases the number of DRG neurons *in vivo* (Ringstedt *et al*., 1997). Data from knockouts revealed that the same amount of noxiceptive sensory neurons are lost at birth in both TrkA and NGF null mice (Crowley *et al*., 1994). Similarly, inactivation of either the TrkB or BDNF gene in mice results in an equivalent loss of the respective mechanoceptive neurons (Minichiello *et al*., 1995; Ernfors *et al*., 1994a). Neuronal counts in sensory ganglia of the TrkC mutant mice present a 30% loss, whereas more severe losses of about 70% were found in NT-3 null mutant mice (Ernfors *et al*., 1994b; Tessarollo *et al*., 1994; Tessarollo *et al*., 1997; Klein *et al*., 1994). One explanation for this discrepancy could be that NT-3 most effectively activates the TrkC receptor tyrosine kinase, but it can also signal using the TrkA and TrkB receptor (White *et al*.,

1996). A critical role for NT-3 signaling via TrkA is well-established in the sympathetic neuron development (Francis *et al*., 1999; Kuruvilla *et al.*, 2004). TrkC as a dependence receptor, however, could be an alternative explanation. Indeed, the neuronal death of TrkC mutant mice could be the result of only the loss of the positive signaling kinase activity of TrkC, whereas neuronal death observed in NT-3 knockouts could be the result of both the loss of the positive signaling and the pro-apoptotic activity of deliganded TrkC. Further studies are needed to investigate this hypothesis. In particular, it would support the dependence receptor hypothesis if the neuronal losses in mice double-deficient for NT-3 and TrkC would be less severe than in mice deficient for only NT-3.

We have shown that in sympathetic neurons, GDNF-deprived neurons die via a caspase-dependent, but non-mitochondrial, death pathway. To check whether this pathway is a specific feature of GDNF deprivation (deligation of Ret) we analyzed another population of neurons, the dopaminergic neurons, which also depend on GDNF signaling via GFRα1/Ret for survival *in vitro*. The survival of dopaminergic neurons is also promoted by BDNF, via the receptor TrkB. We found that GDNF deprivation activates a different death program in the dopaminergic neurons than in the sympathetic neurons. The death receptors and caspases, but not the mitochondria, were activated. Furthermore, a completely different set of caspases was activated in the GDNF-deprived dopaminergic neurons compared to the sympathetic neurons.

One unexpected finding was that although cytochrome *c* was not released into the cytosol, caspase-9 was still activated in GDNF- and BDNF-deprived dopaminergic neurons. In the classical mitochondrial death pathway, caspase-9 is activated at the apoptosome that forms in the cytosol when cytochrome *c* and dATP bind the monomeric scaffold protein Apaf-1. Recruitment of pro-caspase-9 to the apoptosome activates it by dimerization (Pop *et al*., 2006; Logue and Martin, 2008; Boatright and Salvesen, 2003) and it is also cleaved to enhance its catalytic activity (Boatright and Salvesen, 2003; Bao and Shi, 2007; Logue and Martin, 2008). How caspase-9 is activated in our system without apoptosome is not clear. We propose that it is activated by caspase-8, previously activated at the DISC formed by the death receptor Fas. Consistent with this idea, McDonnell and colleagues have shown that caspase-9 can be cleaved and activated by active caspase-8 in response to TNF receptor activation in murine cells (McDonnell *et al*., 2003). Similarly, in murine embryonic fibroblasts, caspase-8 cleaves and activates caspase-9 independent of apoptosome in response to TNF (Gyrd-Hansen *et al*., 2006). Thus, in dopaminergic neurons, caspase-9 may also be cleaved and activated by caspase-8. We could not visualize the cleavage fragment of caspases due to the small mount of material, plus immunostaining of the neurons with antibodies to activate caspase also failed in our hands. Thus, how caspase-9 is activated requires further studies.

To our surprise, death receptors, in particular Fas were activated in GDNF- and BDNF- deprived dopaminergic neurons. Inhibition of caspase-8 or FADD, but also overexpression of Fas inhibitor $FAIM_L$ blocked death. Usage of Fas agonists and antagonists also confirmed this phenomenon. The biology of Fas and FasL have mainly been studied in the immune system, but there is increasing evidence of

their involvement in the nervous system (Raoul *et al*., 2006; Raoul *et al*., 1999), Although, Fas-mediated signaling is not involved in trophic factor deprivation-induced apoptosis of sympathetic neurons (Putcha *et al.,* 2002). Fas and FasL, however, are broadly expressed in the nervous system. Moreover, there is an increasing body of evidence that Fas and other members of the TNFR family can trigger cellular responses other than apoptosis, such as inflammatory responses, cell growth, differentiation, and proliferation (Magnusson and Vaux, 1999; Beyaert *et al*., 2002; Wallach *et al*., 1999; Peter *et al*., 2007). Our study favors the model that Fas performs other, non-apoptotic functions in the healthy nervous system, whereas its death-inducing potential is only released in the apoptotic situation, such as deprivation of neurotrophic factors. Indeed, we found FasL constitutively expressed in the midbrain cultures, suggesting a constitutive activation of Fas. Avoiding unwanted apoptosis requires an inhibitor that could normally block Fas, but it is removed in the apoptotic situation. Recently a neuron-specific inhibitory protein $FAIM_L$ was described as interacting with Fas, and blocking the apoptotic activity of Fas in neurons (Segura *et al*., 2007). We confirmed the interaction of $FAIM_L$ and Fas in our dopaminergic neuron model. In healthy dopaminergic neurons, Fas could have other functions than apoptosis, because its apoptotic function is blocked by $FAIM_L$. Whereas when an apoptotic stimulus, such as neurotrophic factor deprivation is received, $FAIM_L$ can be removed from Fas and induce cell death. We were, however, unable to check whether $FAIM_L$ is degraded by GDNF deprivation due to technical limitations of the model.

We found that unbouded Ret triggers different death pathways in sympathetic and dopaminergic neurons, whereas the same pathways were activated in the dopaminergic neurons by deligation of Ret and TrkB. We expected different death pathways triggered by GDNF and BDNF deprivation, because that was the case in sympathetic neurons deprived of NGF or GDNF (paper I). But in both cases, the dopaminergic neurons died by the same program, at least as judged by the proteins whose involvement we tested. Thus, which death pathway gets activated is determined by the type of the neurons, not by the type of deprived factor/deliganded receptor. We conclude that dopaminergic neurons responded differently to GDNF deprivation than sympathetic neurons, but in the same way as BDNF deprivation. It seems that upon removal of the trophic factor, neurons have their own neuronal type-specific way to die. Still, even in the same neuron, different death stimuli can trigger different death pathways. For example, dopaminergic neurons treated with MPP+ or 6-OHDA can activate different death pathways that are still different from factor deprivation-induced death program (Choi *et al*., 1999). It is becoming increasingly clear that a cell has more than one death pathway, which are stimulus-dependent, but different cell types have their own "main" pathway. The classic mitochondrial pathway is clearly important, but not the only one.

It remains to be studied whether the dopaminergic neurons die ontogenetically *in vivo* via that pathway. Moreover, the role of endogenous GDNF in the ontogenetic death is controversial. The studies by Burke and coworkers demonstrated that GDNF can regulate the death of dopaminergic neurons *in vivo* (Oo *et al*., 2003). They also showed that dopaminergic neurons in the SN undergo ontogenetic cell death at the peak of postnatal days 2 and 14 (Oo

and Burke, 1997). Injection of GDNF into the striatum during the biphasic period of ontogenetic cell death inhibited apoptosis of dopaminergic neurons, although only during the first period of cell death (Kholodilov *et al*., 2004; Burke, 2004). Thus, the experiments with acute *in vivo* manipulations suggest that GDNF is a bona fide target-derived neurotrophic factor for the dopaminergic neurons. On the other hand, the data from transgenic mice suggests different conclusions. The knockouts of GDNF or its receptors are not informative in that respect, since all these mice die at birth, *i.e*. before the ontogenetic death period of the dopaminergic neurons (Baloh *et al*., 2000; Pichel *et al*., 1996; Cacalano *et al*., 1998; Enomoto *et al*., 1998; Taraviras *et al*., 1999; Krieglstein, 2004). Two different lines of Ret conditional knockout mice, surviving to adulthood, however, did not reveal changes in the number of dopaminergic neurons at the time of ontogenetic death. Rather, these neurons were affected in the adult animals (Jain *et al*., 2006; Kramer *et al*., 2007). Thus, the role of GDNF and Ret in the developmental death of dopaminergic neurons requires further studies. GDNF, however, is still the most probable survival factor for these neurons *in vivo* (Pascual *et al*, 2008; Andressoo and Saarma, 2008).

Our data showing activation of Fas by GDNF deprivation suggests that suppression of death receptors could be one mechanism by which GDNF prevents the degeneration of dopaminergic neurons in Parkinson's patients. Indeed, although the activation of death receptors in the dopaminergic neurons during ontogenetic death *in vivo* is not yet described, they are shown to be activated in Parkinson's disease (Hartmann *et al*., 2002). Human postmortem studies have revealed an increased number of dopaminergic neurons that displayed caspase-8 activation (Hartmann *et al*., 2001), and a decrease in FADD-immunoreactive dopaminergic neurons in patients with Parkinson's disease (Hartmann *et al*., 2002). It is not certain that the shortage of GDNF has any role in the etiology of Parkinson's disease, but the potent neuroprotective ability of GDNF together with our results strongly suggest that GDNF acts, at least partially, via inhibition of death receptors in the affected dopaminergic neurons. It is tempting to speculate that the development of small molecule therapeutic Fas antagonistic mimetics could prevent death receptor activation, targeting Fas, FasL, $FAIM_L$, other components of DISC, or other death signaling components and prevent their activation, just as GDNF does. Such small molecules could be applied more conveniently than the recombinant protein factors. Currently only few examples of such mimetics exist (Hasegawa *et al.*, 2004) and the consequences of their application in the brain are currently not known. For example, the death receptors like Fas are reported to be engaged to neurite outgrowth in DRG and hippocampal cells (Desbarats *et al*., 2003; Kajiwara *et al*., 2004). Thus, such small molecules which can prevent the Fas activity, could also lead to neurite degeneration. Our results, however, encourage us to consider the death receptor pathway as a target for the treatment of neurodegenerative diseases.

6. CONCLUSIONS

1. The neuronal death induced by removal of GDNF or NGF is considerably different. GDNF-deprived sympathetic neurons die via a novel non-mitochondrial, c-jun, and caspase-dependent pathway, whereas NGF-deprived sympathetic neurons die via classical mitochondrial death pathway.

2. TrkC is a dependence receptor and the dependence receptor activity is necessary for the death of NT-3-deprived sensory neurons. NT-3 deprivation-induced cell death is not only due to the loss of survival signals, but also due to the active cell death stimulus triggered by unbound TrkC.

3. We established a survival assay of transiently transfected dopaminergic neurons to analyze the apoptotic proteins in these neurons.

4. GDNF-deprived dopaminergic neurons die via a non-mitochondrial pathway which involves the activation of death receptors and caspases. Withdrawal of different factors (GDNF or BDNF) triggers the same death machinery in the dopaminergic neurons.

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