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ROLE OF RET SIGNALING IN THE REGULATION OF THE NIGROSTRIATAL DOPAMINERGIC SYSTEM IN MICE

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ACADEMIC DISSERTATION

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) enhances survival of dopamine (DA) neurons *in vitro*, protects DAergic neurons in animal models of Parkinson's disease (PD), stimulates DA neuron function, regulates postnatal development and supports DA neurons during adulthood. GDNF utilizes a multireceptor complex consisting of a ligand-binding co-receptor GDNF family receptor alpha 1 (GFRα1) and a signal-transducing receptor. The major signaling receptor of GDNF is the receptor tyrosine kinase Ret, although alternative signaling receptors have been found. GDNF and its receptors are considered important targets for developing new therapies that would ameliorate the degeneration of DA neurons and loss of DA that underlie movement impairments in PD.

The present study investigated the role and significance of Ret signaling in the nigrostriatal DAergic system using mutant mice with a point mutation in Ret, which leads to constitutive activation of the Ret receptor tyrosine kinase and causes the cancer syndrome called multiple endocrine neoplasia type B (MEN2B). Immunohistochemical, neurochemical and molecular alterations in the DAergic system, as well as the behavior of the MEN2B mice, were studied. Particularly, we explored striatal DAergic neurotransmission in the MEN2B mice by using classical pharmacological tools as well as in vivo voltammetry. Also, in order to clarify the role of Ret in neuroprotection we studied effects of toxins on the DAergic system of MEN2B mice.

We found markedly increased tissue DA concentrations, increased number of DA cells in the substantia nigra, elevated TH and DAT levels in the striatum and increased sensitivity to the stimulatory effects of cocaine in the MEN2B mice with constitutively active Ret. Also, synthesis, storage, release and uptake of DA in the striatum were found to be enhanced in the MEN2B mice. Finally, constitutive Ret activity protected DA cell bodies against neurotoxicity but was ineffective in protecting their axonal terminals in the striatum.

In conclusion, this study showed that the constitutive Ret signaling supports and protects brain DA neurons, and promotes the DAergic phenotype, implicating Ret as an important signaling receptor for GDNF in the brain DAergic system.

ABBREVIATIONS

5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydorxytryptamine
6-OHDA	6-hydroxydopamine
ANOVA	Analysis of variance
ARTN	Artemin
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
DA	Dopamine
DAT	Dopamine transporter
DOPA	3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenyl acetic acid
GABA	γ-Aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligands
GFRα	GDNF family receptor alpha
GPe	Globus pallidus external segment
GPi	Globus pallidus internal segment
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
i.p.	Intraperitoneally
LC	Locus coeruleus
MAO	Monoamine oxidase
MEN2B	Multiple endocrine neoplasia type 2 B
MOPEG	3-Methoxy-4-hydroxy-phenylglycol
MPTP	1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine
M/+	Heterozygous knock-in Ret-MEN2B mouse
M/M	Homozygous knock-in Ret-MEN2B mouse
NA	Noradrenaline
NCAM	Neural cell adhesion molecule
NRTN	Neurturin
PD	Parkinson's disease
PCR	Polymerase chain reaction
Ret	Rearranged during transfection
SEM	Standard error of mean
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area
Wt	Wild type littermate

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications (I-III):

I Mijatovic J, Airavaara M, Planken A, Auvinen P, Raasmaja A, Piepponen TP, Costantini F, Ahtee L, Saarma M (2007) Constitutive Ret activity in knock-in multiple endocrine neoplasia type B mice induces profound elevation of brain dopamine concentration via enhanced synthesis and increases the number of TH-positive cells in the substantia nigra. Journal of Neuroscience 27:4799-4809.

II Mijatovic J, Patrikainen O, Yavich L, Airavaara M, Ahtee L, Saarma M, Piepponen TP (2008) Characterization of the striatal dopaminergic neurotransmission in MEN2B mice with elevated cerebral tissue dopamine. Journal of Neurochemistry 105:1716-1725.

III Mijatovic J, Piltonen M, Alberton P, Männistö PT, Saarma M, Piepponen TP (2009) Constitutive Ret signaling is protective for dopaminergic cell bodies but not for axonal terminals. Accepted for publication in Neurobiology of Aging

1. INTRODUCTION

Parkinson's disease (PD) is an age-related, progressive neurodegenerative disorder that manifests with impairments of motor function. The motor symptoms of PD result from progressive degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and a consequent reduction in striatal dopamine levels (Olanow, 2004). The therapy of PD is symptomatic, based on dopamine replacement, and currently no therapy that can stop or reverse progressive degeneration of DA neurons exists.

Neurotrophic factors are generally described as naturally occurring polypeptides that support the development and survival of neurons. The first neurotrophic factor, nerve growth factor (NGF) was discovered more than 50 years ago (Levi-Montalcini, 1987) and since then, as the field of neurotrophic factors has advanced, many new neurotrophic factors have been discovered (Hefti, 1997; Skaper and Walsh, 1998). One of them is the glial cell line-derived neurotrophic factor (GDNF), which has been shown to enhance the survival and differentiation of midbrain DA neurons *in vitro* (Lin et al., 1993) and to have prominent beneficial effects on the compromised DAergic system in the animal models of PD (Hoffer et al., 1994; Kirik et al., 2004). Thus, by the virtue of its neuroprotective properties for nigrostriatal DA neurons, GDNF attracted interest as a potential therapeutic agent for PD, eventually reaching clinical trials in PD patients (Gill et al., 2003; Nutt et al., 2003; Slevin et al., 2005; Lang et al., 2006). In addition, the identification of three GDNF-related proteins, neurturin, artemin and persephin, expanded the GDNF family of neurotrophic factors (Baloh et al., 2000).

In parallel, extensive research on the mechanisms of action and physiological role of GDNF was proceeding. It was found that GDNF exerts its physiological actions via a multicomponent receptor complex, which consist of the ligand-binding coreceptor GFRα1 and signal transducing receptor tyrosine kinase Ret (Airaksinen et al., 1999). However, more recently Ret-independent GDNF signaling has also been described (Paratcha et al., 2003). In addition to survival-promoting effects on DA neurons, GDNF appears to exert neurotrophic effects on various neuronal populations, and also to have various functions outside of the nervous system (Airaksinen and Saarma, 2002). However, as mutant mice lacking either GDNF or its receptors die at birth, and specific deletion of Ret from DA neurons revealed significant alteration only in the aging brain DAergic system, the physiological roles of GDNF and its signaling receptor Ret in the developing and adult brain DA neurons have remained largely unclear (Airaksinen and Saarma, 2002; Jain et al., 2006; Kramer et al., 2007). Thus, besides analyzing loss-of-function mutants, use of animal models with increased GDNF/Ret signaling might prove to be a useful and complementary approach.

2. REVIEW OF THE LITERATURE

2.1 Brain DA systems

In the mid 1950s, Carlsson and co-workers showed that DA functions as a transmitter in the CNS independently of its role as a precursor in the synthesis of noradrenaline and adrenaline (Carlsson et al., 1957; Carlsson, 1959). Since then, interest in DA has dramatically increased as a result of its involvement in a broad array of behaviors and disorders, such as control of motor behavior, attention, reward and in the pathologies of Parkinson's disease, addiction to drugs of abuse and schizophrenia.

DA neurons in the mammalian brain are localized in nine distinctive cell groups, from A8 to A16 (Dahlström and Fuxe, 1984; Hökfelt et al., 1984; Björklund and Dunnett, 2007). The primary dopaminergic nuclei in the brain are the substantia nigra pars compacta (A9), ventral tegmental area (VTA, A10), retrorubral area (A8), all of which reside in the ventral midbrain, and the arcuate nucleus (A12) of the hypothalamus (Figure 2.1*A*). In mice, there are about 20 000 - 30 000 DAergic cells in the A8, A9 and A10 cell groups, with about half of the cells located in the SNpc (Björklund and Dunnett, 2007). Cells of the SNpc project to the basal ganglia nuclei caudate and putamen, forming the nigrostriatal DAergic pathway. In addition, a minor part of the nigrostriatal DA pathway consists of axons from the retrorubral field (A8) projecting to the striatum. Axons of the nigrostriatal pathway run alongside fibers containing noradrenaline (NA) and serotonin (5-HT) via the medial forebrain bundle (MFB). The nigrostriatal system is involved in the control of motor behavior, learning of motor programs and habit formation. The A10 cells innervate limbic structures, such as the nucleus accumbens, amygdala, hippocampus, septum and olfactory tubercle, forming mesolimbic DA pathway. Also, the A10 cells project to cortical areas including medial prefrontal, cingulate and entorhinal cortices and constitute the mesocortical DA pathway. The mesolimbic pathway is involved in the control of emotions and reward as well as control of motor behavior. The mesocortical pathway regulates higher cognitive functions, learning and reward. It should be noted that this widely used early classification is rather an oversimplification, and with help of more advanced methods, it has been more recently shown that DA neurons projecting to the striatal, limbic and cortical areas are partially intermixed (Björklund and Dunnett, 2007).

The nigrostriatal, mesolimbic and mesocortical pathways are the so-called long, ascending DA pathways that link ventral midbrain with forebrain structures. In the hypothalamus, arcuate (A12) and periventricular (A14) DAergic nuclei project to the intermediate lobe of the pituitary and into the median eminence, forming the tuberoinfundibular pathway of intermediate length that regulates the pituitary gland function and prolactin release. Additionally, there are some DAergic interneurons in the olfactory cortex, medulla and retina (Rang et al., 1999; Cooper et al., 2003). This thesis is focused on the nigrostriatal DAergic pathway.

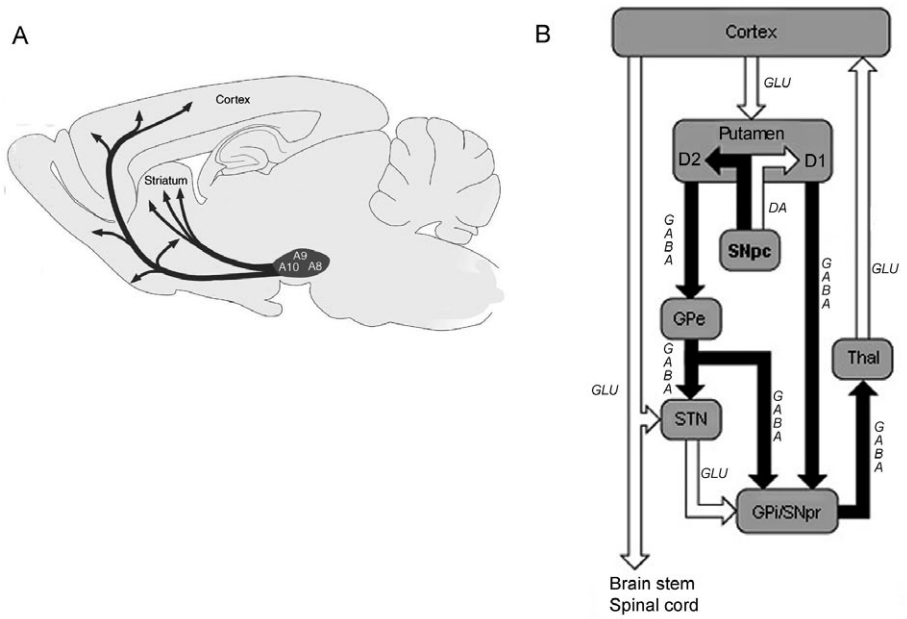

Figure 2.1. *(A) Schematic representation of the ascending DAergic pathways originating in the substantia nigra pars compacta (A9), ventral tegmental area (A10) and retrorubral field (A8). (B) Schematic diagram of basal ganglia-thalamocortical circuitry. Inhibitory connections are depicted as black arrows, and excitatory connections as white arrows. Putamen is a major input nucleus and GPi (globus pallidus internal segment) and SNpr (substantia nigra pars reticulata) are the major output nuclei of the basal ganglia; D1 and D2, dopamine receptors; GPe (globus pallidus external segment); STN (subthalamic nucleus); thalamus (Thal); SNpc (substantia nigra pars compacta). Neurons containing DA (dopamine), GABA (γ-aminobutyric acid) and GLU (glutamate) are shown as indicated. Modified from (Wichmann and DeLong, 1996; Björklund and Dunnett, 2007).*

2.1.1 DA synthesis, storage and metabolism

At the axonal terminal, DA is synthesized from the amino acid tyrosine, which is actively transported across the blood-brain barrier (Figure 2.2). The first and rate-limiting step in the biosynthesis of DA (and other catecholamines) is catalyzed by the enzyme tyrosine hydroxylase (TH) and results in the formation of 3,4-dihydroxyphenylalanine (DOPA) (Carlsson and Lindqvist, 1973, 1978). During the next step, DOPA is rapidly converted to DA by aromatic amino acid decarboxylase (AAADC; Figure 2.2).

Since tyrosine hydroxylase is the rate-limiting enzyme in biosynthesis of DA, regulation of its activity represents a central means of controlling the neuronal concentrations of DA. The activity of TH can be modulated by two mechanisms: medium- to long-term regulation of gene expression and short-term direct regulation of enzyme activity (feedback inhibition and phosphorylation) (Kumer and Vrana, 1996; Fujisawa and Okuno, 2005). End-product feedback inhibition of TH by intraneuronal DA, which

competes with cofactor tetrahydrobiopterin (BH4) for a binding site on TH, acts as a sensor of the local concentrations of DA and is predominant during low neuronal activity. Phosphorylation of TH by different protein kinases rapidly activates TH and increases DA synthesis during period of increased impulse flow. Phosphorylation causes a conformational change in the TH protein that leads to its increased affinity for cofactors and reduced affinity for dopamine (Kumer and Vrana, 1996; Dunkley et al., 2004). In contrast, activation of D2 autoreceptors by extraneuronal DA leads to inhibition of TH and decreased synthesis of DA (Missale et al., 1998).

Newly synthesized DA is sequestered from the cytoplasm into storage vesicles by vesicular monoamine transporter 2 (VMAT2; Figure 2.2). Within the nerve terminal, vesicles reside in distinct "pools" that include a releasable pool that maintains exocytosis during mild stimulation and storage pool that is depleted only after the stimulation surpasses normal limits (Rizzoli and Betz, 2005). The releasable pool of DA contains newly synthesized DA and is sensitive to blockade of TH, whereas the storage pool of dopamine serves as a depot that contains most of the intracellular dopamine and is sensitive to the blockade of VMAT2 (Yavich, 1996). The DA stores are not static, but exist in highly dynamic state where active and rapid packing of DA into vesicles counterbalances passive outward leakage of DA from vesicles. Cytosolic DA, resulting from leakage of DA from the vesicles, reuptake of released DA, and newly synthesized transmitter, if not packed in vesicles, are metabolized by monoamine oxidase (MAO) to yield 3,4-dihydroxyphenylacetic acid (DOPAC; Figure 2.2) (reviewed by Eisenhofer et al., 2004). Thus, short-term accumulation of DOPAC in mouse brain is taken as a reflection of the activity of DA neurons (Cooper et al., 2003). There are two forms of MAO, namely MAO-A and MAO-B. They are derived from distinct genes and differ in their substrate specificity and cellular localization. Both MAO-A and MAO-B have similar affinities for dopamine

DOPAC diffuses out of nerve terminals and is extraneuronally converted to homovanillic acid (HVA) by catechol-O-methyltransferase (COMT; Figure 2.2). Also, extracellular DA that escapes dopamine uptake is converted to 3-methoxytyramine (3-MT) by COMT in glial cells or postsynaptic neurons. In addition, part of HVA is derived from 3-MT in a reaction catalyzed by MAO (Figure 2.2). Tissue levels of 3-MT can be used as index of dopamine release, provided that postmortem activity of COMT is inhibited, whereas most HVA is derived from DOPAC, and is considered as a 'second metabolite' rather than a marker of extra-neuronal activity (Westerink, 1985). The role of COMT in clearance of extracellular DA is minor in areas with high DA uptake such as the striatum.

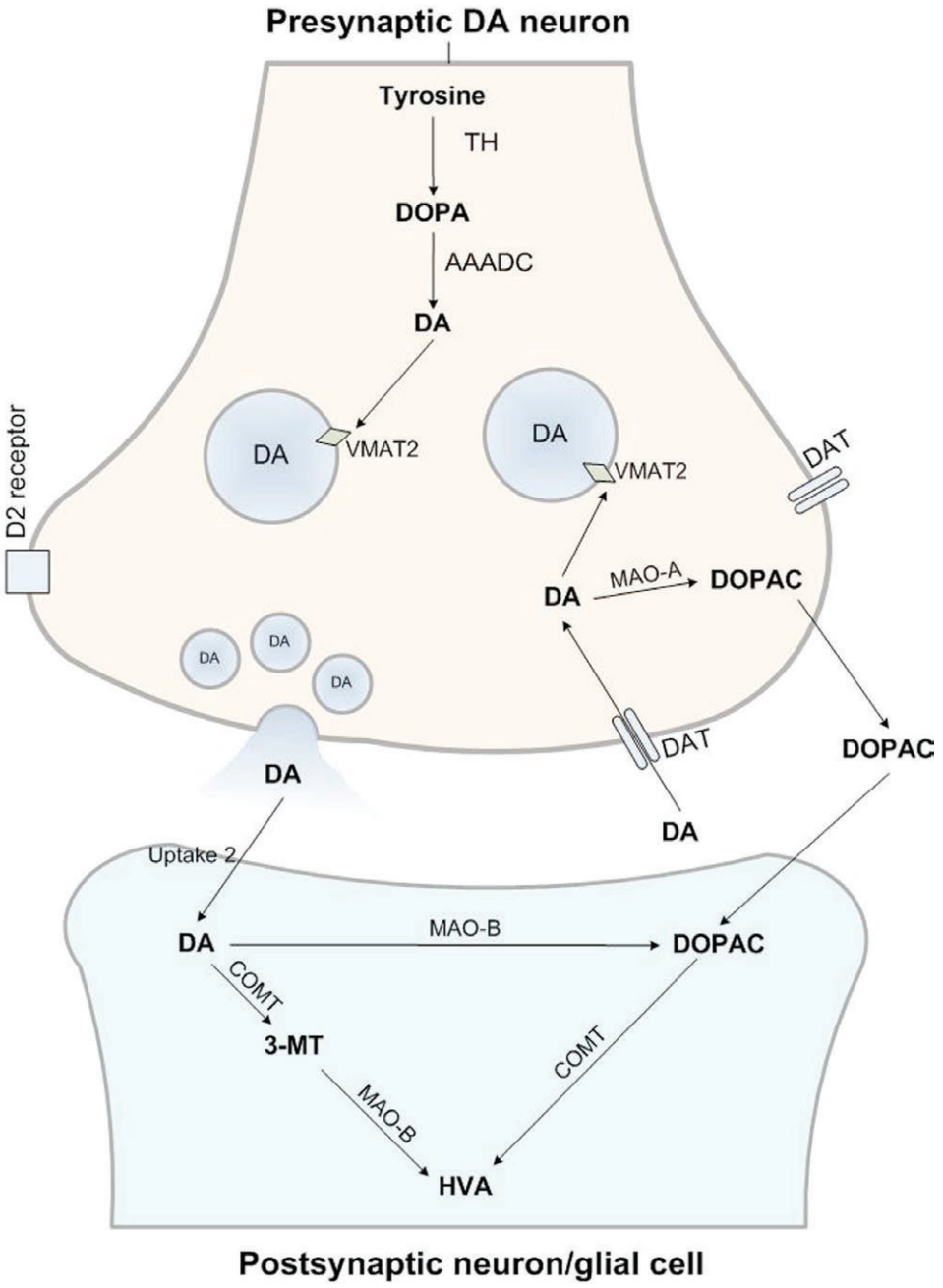

Figure 2.2. *Dopamine (DA) synthesis and metabolism pathways. TH (tyrosine hydroxylase), DOPA (3,4-dihydroxyphenylalanine), AAADC (aromatic amino acid decarboxylase), VMAT2 (vesicular monoamine transporter 2), DAT (dopamine transporter), MAO-A and MAO-B (monoamine oxidase types A and B), DOPAC (3,4-dihydroxyphenylacetic acid),COMT (catechol-O-methyltransferase), HVA (homovanillic acid), 3-MT (3-methoxytyramine).*

2.1.2 DA release and uptake

When an action potential arrives at the nerve terminal, DA is released from synaptic vesicles via Ca^{2+}-dependent exocytotic release. However, Ca^{2+}-independent release has also been reported. The main point of release of DA from axon terminal is represented by regular enlargements of the axon called axonal varicosities. Released DA can diffuse beyond synaptic cleft to reach targets far from the release site (volume transmission; reviewed by Zoli et al., 1998; Rice and Cragg, 2008).

Dopamine neurons display three main patterns of activity: an inactive, hyperpolarized state; a slow (2-10 Hz) single-spike firing and burst firing (15-30 Hz) (reviewed by Grace and Bunney, 1984a, b). Single-spike firing is driven by an intrinsic pacemaker potential (Grace and Bunney, 1983). Tonic DA transmission, resulting from single-spike firing, has been shown to underlie the basal, extrasynaptic DA concentration (e.g. 10-20 nM DA found within the striatum), which is mediated by an escape of DA from synapses (Floresco et al., 2003). Tonic DA transmission exhibits a slow time course of change and is under strong homeostatic control since it is maintained at basal levels even when up to 80% of the tissue DA levels are depleted (Abercrombie et al., 1990; Castaneda et al., 1990). In contrast, burst firing of DA neurons is thought to mediate phasic DA transmission and induces a high amplitude signal. In this case, intrasynaptic DA concentrations reach a low millimolar range (Grace et al., 2007). This high concentration of DA release is restricted both spatially and temporally by powerful, immediate reuptake into presynaptic terminals (Grace et al., 2007). Burst firing is activated by behaviorally meaningful stimuli and is believed to signal important events to postsynaptic neurons. Tonic extracellular DA is usually measured by microdialysis, a neurochemical technique that offers high sensitivity and a long timescale. On the other hand, phasic extracellular dopamine can be measured by *in vivo* voltammetry, which has subsecond temporal resolution and is most often used in combination with electrical stimulations to probe the effects of drugs on DA release and uptake (reviewed by Robinson et al., 2003).

As already mentioned, following release, DA is rapidly taken up by the dopamine transporter (DAT) into the presynaptic DA terminals (Sotnikova et al., 2006). Plasma membrane DAT is the most important and highly efficient mechanism controlling functional extracellular DA concentrations in the dorsal and ventral striatum (Benoit-Marand et al., 2000). DAT is localized in the soma, dendrites, axons and axon terminals of DA midbrain neurons (Nirenberg et al., 1996). Ultrastructural immunohistochemical studies have demonstrated that DAT is mostly present extrasynaptically (Nirenberg et al., 1996), supporting the idea of extrasynaptic DA transmission. DAT is a major target of psychostimulant drugs, such as cocaine, which induces dramatic increase in extracellular DA concentrations by interfering with DAT function. Also, the selectivity of neurotoxins such as 6-OHDA and MPTP for DAergic neurons depends on their high affinity for DAT (Gainetdinov et al., 1997; Takahashi et al., 1997)

2.1.3 DA receptors

Various actions of DA are mediated by five distinct subtypes of G protein-coupled receptors. On the basis of their pharmacology and coupling to adenylyl cyclase, the dopamine receptors are classified into two populations: D1-like receptors, which stimulate adenylyl cyclase and D2-like receptors which inhibit adenylyl cyclase (Jaber et al., 1996). The D1-like DA receptor population includes D1 and D5 receptors and the D2-like receptor population includes D2, D3 and D4 receptors. D1 and D2 receptors are involved in the reinforcing properties of different drugs of abuse, control of locomotion as well as in the mediation of the effects of DA on learning and memory. D1 receptors are the most widely expressed DA receptors in the brain, with the highest level of expression in the projection areas of midbrain DA neurons. In the striatum, D1 receptors are expressed on the spiny dendrites and perikarya of medium spiny neurons that are part of the direct pathway of basal ganglia. Also, D1 receptors are found on the axons of striatonigral pathway and both axons and terminals in the SNpr and entopeduncular nucleus (Yung et al., 1995). D2 receptors are found in the olfactory tubercle, nucleus accumbens and in the medium spiny GABA neurons of the striatum that are part of the indirect pathway of the basal ganglia. It should be emphasized that this segregation in the expression of D1 and D2 receptors in the striatum is not exclusive, and many neurons that predominantly express one type of the receptor may also express small amounts of the other receptor subtype (Aizman et al., 2000). Interestingly, two recent studies demonstrated the existence of a complete segregation between the D1- and D2-receptor-expressing striatal neurons in response to pharmacological stimuli (Bateup et al., 2008; Bertran-Gonzalez et al., 2008).

Part of the D2 receptors that are found in striatum, substantia nigra pars compacta and ventral tegmental area are expressed by the dopaminergic neurons themselves. These dopaminergic D2 receptors serve as autoreceptors and have an important role in the regulation of presynaptic function in a feedback-inhibitory manner (Jaber et al., 1996; Missale et al., 1998). D2 autoreceptors are localized on the soma, dendrites and nerve terminals. Activation of the somatodendritic D2 receptors slows the firing rate of DA neurons, while activation of autoreceptors on the nerve terminals inhibits dopamine synthesis and release. Thus, D2 receptors can be defined as synthesis-regulating, release-regulating and impulse-regulating autoreceptors. In addition to inhibition of adenylyl cyclase, D2 autoreceptors inhibit DA neurons by activation of inwardly-rectifying K^+ channels and inhibition of voltage-gated Ca^{2+} channels (Missale et al., 1998; Schmitz et al., 2003).

2.2 Basal ganglia

The basal ganglia are a group of subcortical nuclei involved in the control of movement. The basal ganglia include the caudate and putamen (striatum in rodents), the external segment of globus pallidus (GPe), the internal segment of globus pallidus (GPi; its equivalent in rodents is called entopeduncular nucleus), the subthalamic nucleus (STN) and substantia nigra pars reticulata (SNpr; Figure 2.1*B*) (reviewed by Smith et al., 1998b).

The striatum is the major division of basal ganglia that receives afferent inputs from cerebral cortex, and is a principal site where information processing occurs. More than 90% of all of the cells in the striatum are GABAergic medium spiny neurons and are classified into two subclasses by different neuropeptides and dopamine receptors subclasses that they express. The remaining 5-10% of neurons in the striatum are either GABAergic or cholinergic interneurons (Tepper and Bolam, 2004). The globus pallidus internal segment and substantia nigra pars reticulata are the principal output nuclei of the basal ganglia that provide inhibitory (GABAergic) connections to the targets of the basal ganglia located in the thalamus and brain stem (those include the ventral tier of thalamus, the lateral habenula, the superior colliculus, the mesopontine tegmentum and the reticular formation). Target nuclei of basal ganglia send glutamatergic, excitatory projections back to the striatum and to the motor cortex, closing the feedback loop (Figure 2.1*B*).

Information from the cortex is processed and transmitted to the output nuclei of the basal ganglia via two routes: either directly from the striatum to SNpr/GPi or indirectly, in a circuit which involves an inhibitory projection from striatum to GPe, an inhibitory projection from GPe to STN, and an excitatory projection from STN to the output nuclei (Smith et al., 1998a, b). The direct and indirect pathways arise from two different populations of medium spiny neurons. Medium spiny neurons that express D1 dopamine receptors and contain substance P are part of the direct pathway, while those that contain enkephalin and D2 dopamine receptors give rise to the indirect pathway. The activation of the direct pathway results in disinhibition of the targets of the basal ganglia, leading to the facilitation of the movement, whereas activation of the indirect pathway increases the levels of tonic inhibition of the targets of the basal ganglia and leads to the suppression of movements (Smith et al., 1998b). An imbalance in the activity of the direct and indirect pathways is thought to be the cause of movement disorders such as Parkinson's and Huntington's disease. Furthermore, pharmacological manipulation or surgical interventions that restore the balance between the two pathways alleviate the abnormal motor activity.

The striatum is the most important target of midbrain DA neurons and dopamine released there has an important regulatory function in the circuitry of the basal ganglia (Figure 2.1*B*). The nigral neurons provide excitatory inputs mediated by D1 type DAergic receptors on the spiny neurons of the direct pathway and inhibitory inputs mediated by D2 receptors on the spiny neurons that are part of the indirect pathway. Thus, different actions of DA in the striatum produce the same effect; that is, a decrease in the inhibitory outflow of the basal ganglia and a resultant facilitation of movements. In addition, SNpc DA neurons innervate the other basal ganglia nuclei, such as GPi and GPe and STN, and send projections to the thalamus affecting directly thalamo-cortical activity. Additionally, dendritic DA release in the SN is a mechanism by which nigral DA neurons regulate not only their own activity but also facilitate GABA release from the axons of striatonigral GABAergic neurons in the SNpr via activation of D1 receptors (Gauthier et al., 1999; Smith and Kieval, 2000; Smith and Villalba, 2008). Taken together, DA regulates the function of the basal ganglia by acting at multiple sites within the circuitry.

2.2.1 Parkinson's disease

Parkinson's disease (PD) is an age-related, progressive neurodegenerative disorder. Clinically, PD manifests mainly by impairments of motor function, including resting tremor, rigidity, bradykinesia and gait disturbances with postural instability. However, with time, PD patients develop nonmotoric impairment such as dementia, depression and sleep disturbances (Lang and Lozano, 1998a, b; Olanow, 2004). Pathologically, PD is characterized by the progressive death of DAergic neurons in the SNpc and a consequent reduction in striatal DA, which accounts for most of the classical motor symptoms of PD. There is a quite long presymptomatic period; at the onset of parkinsonian signs, about 70-80% of striatal DA nerve terminals and about 50-60% of DA cell bodies in SNpc have already been lost. However, other neurotransmitter systems, such as noradrenergic, serotonergic and cholinergic systems, also degenerate to a lesser extent. This may account for the nonmotor symptoms of PD. Another important pathological feature of the disease is the presence of intracellular inclusions called Lewy bodies that contain proteins such as α-synuclein (Spillantini et al., 1997). The factors thought to trigger neuronal degeneration and death in PD include oxidative stress, mitochondrial dysfunction, excitotoxins, accumulation of damaged proteins and deficiency of trophic factors (Lotharius and Brundin, 2002; Schulz, 2008). Recently discovered PD-associated genes suggest that aberrations in ubiquitin–proteasome pathway and cellular stress resulting from mitochondrial dysfunction are involved in pathogenesis of PD (Jain et al., 2005). It should be noted, however, that familial PD accounts for a small minority of cases (Farrer, 2006).

2.2.2 Animal models of PD

To study the pathogenesis of PD and test therapeutic agents for its treatment, animal models for PD have been developed. The most commonly used are "classical" PD models utilizing toxins that selectively destroy DA neurons, such as the 6-hydroxy-dopamine (6-OHDA model) and the 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) models reviewed by (Schober, 2004). Use of MPTP replicates many of the biochemical and neuropathological features of PD. MPTP easily crosses the blood-brain barrier and is converted to MPP+ by MAO-B in glial cells. Thereafter, MPP+ enters DA neurons, where it impairs mitochondrial respiration and causes cell death. MPTP is mainly used in non-human primates and mice, since rats are almost completely insensitive to MPTP-induced neurodegeneration (Blum et al., 2001). Also, the level of DA degeneration in this paradigm depends on the dose and schedule of MPTP administration (Jackson-Lewis and Przedborski, 2007). The 6-OHDA is administered via intracerebral injection and it is taken into DA neurons by DAT. The 6-OHDA produces oxidative stress and mitochondrial dysfunction, inducing death of DA neurons (Glinka and Youdim, 1995; Blum et al., 2001; Ding et al., 2004; Berretta et al., 2005; Hanrott et al., 2006; Tanaka et al., 2006). However, both of the neurotoxins fail to replicate progressive degeneration of DA neurons, the motor symptoms of PD and generation of Lewy bodies.

Recently, the identification of PD-linked genes, such as α-synuclein, DJ-1, LRRK2, Parkin, UCH-L1 and PINK1, led to the creation of transgenic and knockout models of the disease in rodents (Devine and Lewis, 2008). These transgenic animal models are useful for understanding the cause and mechanisms of PD as they replicate many key features of the disease, including selective neurodegeneration, neurochemical deficits, Lewy body neuropathology and motor deficits.

Treatment of PD is symptomatic, with substitution therapy with dopaminergic drugs (levodopa, DA agonists and MAO inhibitors) as the main option for alleviating motor symptoms (Olanow, 2004; Schapira, 2009). Thus, the goal of current research, especially in the field of neurotrophic factors, is to find agents that can stop/slow progression of PD or reverse neurodegeneration.

2.3 Neurotrophic factors

'Neurotrophic factor' is a term generally applied to the naturally occurring polypeptides that support the survival and development of neurons. Neurotrophic factors are secreted molecules that produce their effects by activation of specific receptors on the cell surface. The first neurotrophic factor, nerve growth factor (NGF), was identified more than 50 years ago (Levi-Montalcini, 1987). The early studies with NGF were conducted in the peripheral nervous system and revealed that NGF is synthesized and secreted by the target organs of the nerves, taken up by the nerve terminals and then retrogradely transported to the cell bodies. Only neurons that received NGF signal from their targets kept connections with the target and survived. These findings lead to creation of the neurotrophic hypothesis, according to which neurons are born in excessive numbers during development (Burek and Oppenheim, 1996) and in order to survive and to establish appropriate connections developing neurons must compete for a limiting amount of neurotrophic factor derived from the target tissue (Lewin and Barde, 1996). The target-derived neurotrophic factor pattern appears to predominate in the peripheral system. In the central nervous system, in addition to being target-derived, neurotrophic factors are released from glia or neurons and may act on neighboring neurons in the absence of synaptic connections (paracrine mode) as well as on the very neurons that release them (autocrine mode; (Bothwell, 1995). In addition to being transported retrogradely, neurotrophic factors are anterogradely transported to axon terminals, where they are secreted and act on the cell bodies or nerve terminals of other nerves (Lewin and Barde, 1996; Nestler et al., 2001).

Most neurotrophic factors seem to have broader roles than were originally postulated. Thus, in addition to regulation of developmental survival, the principal roles of neurotrophic factors in the developing CNS include biochemical and morphological differentiation, stimulation of axonal growth and synaptogenesis. In the adult nervous system, neurotrophic factors are involved in the maintenance of target innervations and cell survival, as well as in the regulation of synaptic plasticity (Hefti, 1997; Sariola and Saarma, 2003). Also, several neurotrophic factors that act through different signaling pathways can provide trophic support to the same neuronal population. Growth factors and

their signaling proteins thus represent potential drug targets for the treatment of neurological diseases in which neuronal function is diminished or disturbed, e.g. in Alzheimer's disease, PD, amyotrophic lateral sclerosis (ALS), Huntington's disease, addiction, and depression. The traditional neurotrophic factors belong to families of structurally and functionally related molecules, which include neurotrophins (NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), (Huang and Reichardt, 2001)), glial cell-line derived neurotrophic family ligands (GFLs) (Baloh et al., 2000; Airaksinen and Saarma, 2002), neurokines (Sariola et al., 1994) and the MANF/CDNF family of neurotrophic factors (mesencephalic astrocyte-derived neurotrophic factor/conserved dopaminergic neurotrophic factor) (Petrova et al., 2003; Lindholm et al., 2007).

2.4 The GDNF family of neurotrophic factors

GDNF (glial cell-line derived neurotrophic factor) was originally purified from a rat glioma cell-line, and was characterized as a glycosylated, disulfide-bonded homodimer that enhanced survival and morphological differentiation of midbrain DAergic neurons (Lin et al., 1993). GDNF and the subsequently discovered, structurally related factors neurturin (NRTN), persephin (PSPN) and artemin (ARTN), comprise the GDNF family of neurotrophic factors (Lin et al., 1993; Kotzbauer et al., 1996; Baloh et al., 1998; Milbrandt et al., 1998; Baloh et al., 2000; Airaksinen and Saarma, 2002). In spite of low sequence homology, GFLs represent a distinct subclass within the transforming growth factor-β superfamily because they, like the other TGF-β family members, have seven conserved cysteines with similar spacing, and contain three disulfide bonds arranged in a typical configuration known as 'cysteine knot' (reviewed by Airaksinen and Saarma, 2002). GFLs are synthesized as pre-proGFLs and then processed to mature proteins upon secretion or extracellularly after secretion.

Because of the powerful neurotrophic effects it has on DA neurons, GDNF has raised interest as a potential therapeutic agent for the treatment of PD. In addition to DA neurons, GDNF has been shown to promote survival of wide variety of neurons. In the central nervous system, GDNF promotes survival of spinal and central motor neurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995), basal forebrain cholinergic neurons (Williams et al., 1996), central noradrenergic neurons (Arenas et al., 1995) and cerebellar Purkinje cells (Mount et al., 1995). Furthermore, GDNF supports sympathetic, parasympathetic, enteric and peripheral sensory neurons (Baloh et al., 2000; Airaksinen and Saarma, 2002). Likewise, NRTN and ARTN promote the survival of several neuronal populations. NRTN was discovered as a survival factor for sympathetic neurons (Kotzbauer et al., 1996). It is also able to support survival of DA neurons *in vitro* and it can effectively regenerate DA neurons *in vivo* (Horger et al., 1998; Åkerud et al., 1999, reviewed by Chiocco et al., 2007). However, unlike GDNF it does not induce axonal growth or hypertrophy. PSPN protects the brain from ischemic insults (Tomac et al., 2002), and has been demonstrated to support the survival of midbrain DA neurons (Milbrandt et al., 1998) in vitro as well as preventing the loss of DA neurons in a mouse

model of PD (Akerud et al., 2002). However, PSPN does not support peripheral neurons (Milbrandt et al., 1998). ARTN, the most distant member of the family, can support the survival of sensory neurons and sympathetic neurons both *in vitro* and *in vivo*, and supports DA neurons *in vitro*, although its expression in human brain is very low (Baloh et al., 1998). Outside of the nervous system, GDNF has been found to regulate morphogenesis of ureteric branching (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) and spermatogenesis (Meng et al., 2000).

2.4.1 The GDNF family signaling

GFRα receptors All GDNF family members signal through a common signaling receptor, the Ret tyrosine kinase, but first they bind to one of the glycosyl phophatidylinositol (GPI)-linked GDNF family co-receptors α (GFRα1-4; Airaksinen et al., 1999). GDNF primarily binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4, although there is a certain amount of promiscuity in ligand binding. The original model of GFL signaling proposed stepwise formation of a GFL-receptor complex. Thus, GDNF, a homodimer, complexes with either a monomeric or dimeric GFRα1 co-receptor. Next, this ligand-co-receptor complex brings two Ret transmembrane molecules together, which leads to trans-autophosphorylation of specific tyrosine residues and the induction of intracellular signaling (Jing et al., 1996; Sariola and Saarma, 2003).

Ret (**re**arranged during **t**ransfection) was considered an orphan, oncogenic receptor tyrosine kinase (Takahashi and Cooper, 1987) until its physiological role as a signaling receptor for GFLs was uncovered (Durbec et al., 1996; Treanor et al., 1996; Trupp et al., 1996). The Ret receptor consists of an extracellular part with four cadherin-like domains and a cysteine-rich region important for GFL/GFRα binding, a transmembrane part, and an intracellular part containing a tyrosine kinase domain and a C-terminus that have a function in the activation of downstream signaling pathways (Figure 2.3; Runeberg-Roos and Saarma, 2007). There are two major splice variants of Ret protein that differ from each other in the length of the C-terminus tail: a short isoform with 9 amino acids (Ret9) and a long isoform containing 51 amino acids (Ret51). The Ret9 isoform contains 14 tyrosine residues whereas Ret51 possesses two additional tyrosines within this 51 amino acid stretch. There are indications that the two isoforms might fulfill different biological functions; Ret9 may be important for enteric innervation and renal development while Ret51 is required for growth and metabolism of mature sympathetic neurons (de Graaff et al., 2001; Tsui-Pierchala et al., 2002; Runeberg-Roos and Saarma, 2007).

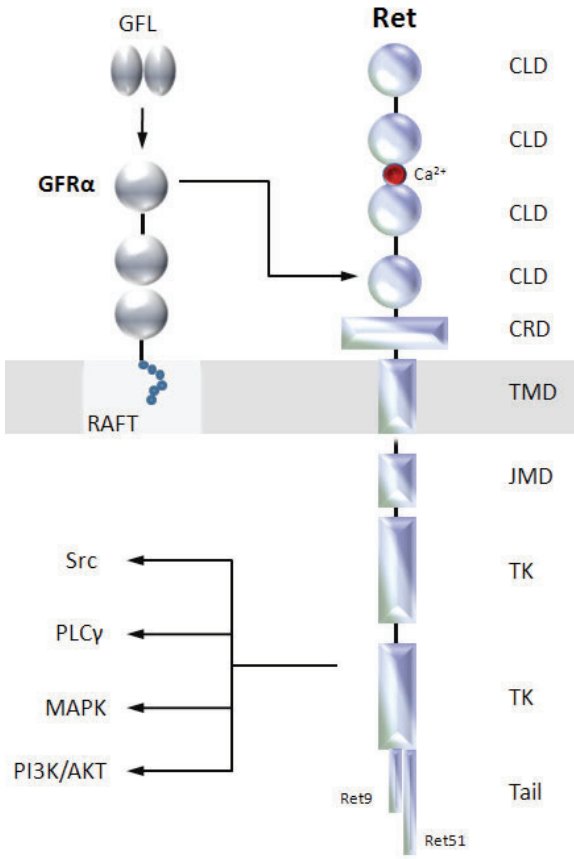

Figure 2.3. *Schematic drawing of the Ret protein containing four cadherin-like domains (CLD), a cysteine-rich domain (CRD), a transmembrane domain (TMD), juxtamembrane domain (JMD) and an intracellular tyrosine kinase (TK). Also, the two alternatively spliced forms, RET9 and RET51, are indicated. Ret is stimulated by complexes of GFL with GPI-anchored GFRα co-receptors that are distributed within lipid rafts. The signaling pathways triggered upon Ret activation are indicated. These include the phosphoinositide 3-kinase (PI3K)/AKT pathway, mitogen activated protein kinase pathway (MAPK), Src-family kinase and phospholipase Cγ (PLCγ). Modified from (Santoro et al., 2004).*

Ret receptor activation leads to the phosphorylation of the key tyrosine residues such as Tyr^{905}, Tyr^{981}, Tyr^{1015}, Tyr^{1062} and Tyr^{1096} (only in the long isoform), which function as the docking sites for several adaptor proteins (Airaksinen and Saarma, 2002). This then leads to activation of downstream signal transduction pathways typical of receptor tyrosine kinase signaling (Figure 2.3), including the phosphoinositide 3-kinase (PI3K)/AKT pathway, the Jun N-terminal kinase pathway (JNK), the mitogen activated protein kinase pathway (MAPK), the Src-family kinases and phospholipase Cγ (PLCγ). These signalling pathways regulate cell survival, proliferation, differentiation, migration, neuritogenesis, branching morphogenesis and synaptic plasticity (Figure 2.3; Sariola and Saarma, 2003;

Santoro et al., 2004; Arighi et al., 2005). For example the MAPK and Src-kinase pathways are indicated to be involved in neurite outgrowth and neuronal survival as well as ureteric branching (Kaplan and Miller, 2000; Fisher et al., 2001; Sariola and Saarma, 2003). The PI3K pathway is crucial for both neuronal survival (Soler et al., 1999) and neurite outgrowth (van Weering and Bos, 1998) and is thought to be required for the differentiation of dopaminergic neurons (Pong et al., 1998). Accordingly, it has been demonstrated that the constitutively active form of Akt has pronounced trophic effects on DA neurons in mice, including increases in neuronal size, phenotypic markers and sprouting, and that it provides protection to DA neurons against neurotoxic insults *in vivo* (Ries et al., 2006). Recently, *in vivo* data from knock-in animals bearing targeted mutations of the key tyrosine residues as well as the serine 697 residue in the Ret tyrosine kinase domain clarified the roles of different Ret signaling pathways (Asai et al., 2006; Encinas et al., 2008).

2.4.2 Ret-independent signaling pathways

Ample evidence suggests that GDNF signaling is more complex than it was initially proposed to be, and co-operation of the neurotrophin with other proteins has been reported (shown in figure 2.4).

GFRα1 Although both GFRα1 and Ret can mediate GDNF signaling, their expression patterns in the CNS do not completely overlap (Trupp et al., 1997; Yu et al., 1998). The expression of GFRα1 in cells lacking Ret suggested an alternative role for this protein beyond being a co-receptor for Ret. Furthermore, this finding suggested that GDNF may signal independently of Ret, presumably in collaboration with novel transmembrane proteins. Firstly, it has been shown that GFRα1 can modulate Ret signaling in non-cell-autonomous fashion (trans signaling; Paratcha et al., 2001). Additionally, in cells that do not express Ret, GDNF via GFRα1 can activate Src-family kinases, which further leads to phosphorylation of ERK/MAPK and PLC-γ, activation of the transcription factor CREB and the induction of Fos (Poteryaev et al., 1999; Trupp et al., 1999).

Met receptor tyrosine kinase In a cell line lacking endogenous Ret, GDNF/GFRα1 was found to activate Met receptor tyrosine kinase via Src-family kinases (Popsueva et al., 2003). However, the *in vivo* significance of GDNF/Met signaling is still unclear. The finding that the phenotypes of GDNF and GFRα1 knockout mice were similar to that of Ret knockout mice, and that all die at birth (Baloh et al., 2000), suggested that Ret-independent signaling via GFRα1 may play a role in neuronal functions postnatally rather than during embryogenesis.

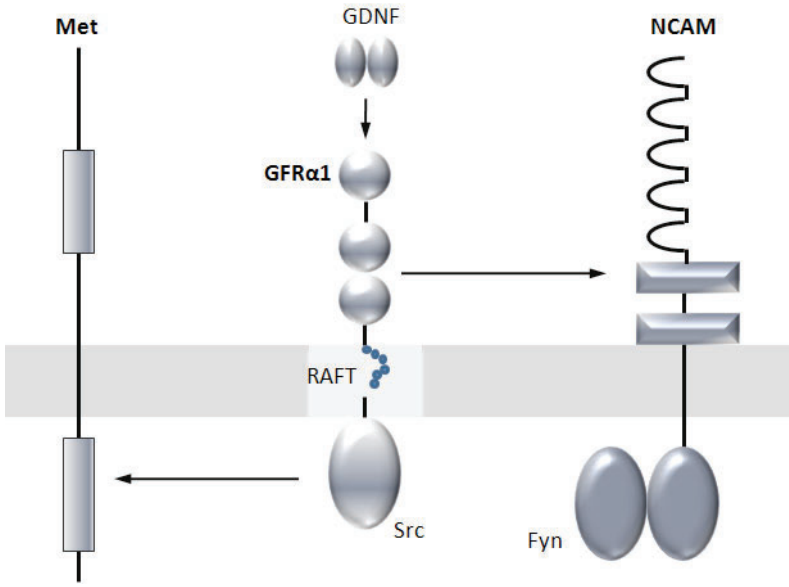

Figure 2.4. *Ret-independent signaling of GDNF. The GDNF-GFRα1 complex binds to neural cell adhesion molecule (NCAM) and induces signaling and activation of Fyn kinase, and via Src kinase activation further activates Met receptor tyrosine kinase. Modified from (Santoro et al., 2004).*

NCAM The neural cell adhesion molecule (NCAM), a prominent cell adhesion molecule, was found to be widely co-expressed with GFRα receptors, including the cells that do not express Ret (Crossin and Krushel, 2000). The fact that in the non-Ret expressing cell lines, the intracellular pathways of the GDNF signaling were strikingly similar to those triggered by NCAM prompted researchers to examine the role of NCAM in GDNF signaling. Paratcha et al (2003) discovered that, in the presence of GFRα1, GDNF binds with high affinity to NCAM and causes activation of the tyrosine kinase Fyn and focal adhesion kinase (FAK) in the cytoplasm in a Ret-independent fashion. GDNF, through NCAM, stimulates Schwann cell migration and differentiation as well as axonal growth in primary hippocampal and cortical neurons (Paratcha et al., 2003; Iwase et al., 2005). Furthermore, NCAM-deficient mice, otherwise viable and fertile, have impairments in spatial learning (Cremer et al., 1994) and heterozygous GDNF knockout mice exhibited a similar phenotype (Gerlai et al., 2001). These findings suggested that GDNF/GFRα1/NCAM signaling indeed has physiological relevance *in vivo*. Even before NCAM was suggested as a signaling receptor for GDNF, Chao and colleagues observed that NCAM function-blocking antibodies antagonized the effects of GDNF both on survival and outgrowth of DA neurons derived from normal adult rats (Chao et al., 2003). However, most of the data on GDNF/GFRα1/NCAM signaling were obtained *in vitro*. When Enomoto and colleagues (2004) studied the physiological relevance of Ret-independent GDNF/GFRα1 signaling in mutant mice that expressed GFRα1 only in Ret

bearing cells, and thus lack trans GFRα1, they found no structural abnormalities in the peripheral nervous system, the central nervous system or the kidney in these so-called *cis* only mice (Enomoto et al., 2004). Thus, this study suggested that Ret-independent GFRα1 signaling appears to play only a minor role in organogenesis and nerve regeneration *in vivo*. However, a recent *in vivo* study showed that NCAM is indeed involved in mediating the effects of GDNF on neurite outgrowth in lesioned DA neurons (Cao et al., 2008). Interestingly, GDNF was shown to regulate morphological differentiation and tangential migration of GABAergic cells in the cortex via GFRα1, but neither Ret nor NCAM appeared to be important in this process, suggesting the existence of an additional transmembrane signaling receptor for this factor (Pozas and Ibanez, 2005).

2.4.3 Cooperation of GDNF with other proteins

TGF-β (transforming growth factor-β) It has been shown that GDNF exerts its trophic activity on a variety of cultured neurons, including DA neurons, only in the presence of cooperating TGF-β factor (Krieglstein et al., 1998). TGF-β is believed to be involved in the clustering of GFRα1 at the cell membrane, which may help the ligand to recognize the co-receptors (Peterziel et al., 2002). Also, the importance of cooperation between GDNF and TGF-β has been confirmed in a mouse model of PD, where it was shown that TGF-β is essential for the neuroprotective effects of exogenous GDNF (Schober et al., 2007). A recent study reported a normal number of midbrain TH-positive neurons in TGF-β2/GDNF double mutant mice at embryonic day 18.5, indicating that cooperation of GDNF and TGF-β2 is not essential for development of DAergic neurons (Rahhal et al., 2009).

Heparan sulfate Cell-surface associated heparan sulfate glycosaminoglycans are required for GDNF to activate Ret and induce axonogenesis in neurons (Barnett et al., 2002). Interestingly, the effect of a rather non-specific chemical deprivation of heparan sulfate from kidneys is similar to that of genetically deleting GDNF and Ret (Bullock et al., 1998). It was proposed that heparan sulfate may modulate GDNF signaling by concentrating the ligand (Barnett et al., 2002).

2.4.4 Ret as an oncogenic protein

Beside its role as a growth factor receptor, Ret can function as an oncogenic protein when mutated. Mutations resulting in Ret receptor dysfunction cause Hirschprung's disease, which is characterized by megacolon agangliosis. In contrast, missense mutations leading to constitutive activation of Ret give rise to multiple endocrine neoplasia types 2A and 2B and familial medullary thyroid carcinoma (FMTC) (reviewed by Manie et al., 2001). MEN2A and MEN2B are autosomal dominant cancer syndromes characterized by medullary thyroid carcinoma and pheochromocytoma. MEN2A patients additionally suffer from hyperparathyroidism while MEN2B patients, on the other hand, develop more aggressive and complex phenotype including developmental abnormalities such as

marfanoid habitus, thickened corneal nerve and ganglioneuromatosis of the gastrointestinal tract and mucosa (reviewed by Takahashi, 2001). FMTC is characterized by medullary thyroid carcinoma only. Also, papillary thyroid carcinoma (PTC), associated with genomic rearrangements in the Ret gene, is caused by Ret activation. In MEN2A and FMTC patients, several germline missense mutations that induce disulfide-linked homodimerization and activation of the Ret receptor are found in the cysteine-rich extracellular domain.

More than 95% of MEN2B cases are caused by a single germline mutation that results in a substitution of threonine for the normal methionine at codon 918 (Met918Thr) in the Ret kinase domain (Eng et al., 1996). The Met918Thr mutation has been predicted to induce a conformational change of the tyrosine kinase catalytic core, leading to activation of Ret in a monomeric form in a ligand-independent manner (Eng et al., 1996). However, it was subsequently shown that ligand can still induce dimerization of the MEN2B Ret and additionally increase the activity of the mutated receptor (Bongarzone et al., 1998). Initial reports suggested that the MEN2B mutation alters phosphorylation and the substrate specificity of Ret so that it preferentially binds substrates of cytoplasmic kinases that would lead to activation of signaling pathways distinct from Wt Ret pathways (Santoro et al., 1995; Songyang et al., 1995; Liu et al., 1996; Bocciardi et al., 1997). However, most of these studies compared downstream signaling pathways of the MEN2B Ret with those of the Wt, inactive Ret (in the absence of ligand activation). Gujral and co-workers showed that under stimulation with GDNF, there was no difference in the nature of Wt and MEN2B Ret substrates and that Met918Thr mutation leads to an increase in the intrinsic kinase activity of Ret, as well as ligand-independent dimerization and autophosphorylation (Gujral et al., 2006). Also, although it was reported that MEN2B Ret precursor is active already in the endoplasmic reticulum before reaching the cell surface, no qualitative differences in the signaling between Wt and MEN2B Ret were observed (Runeberg-Roos et al., 2007). Nevertheless, some alterations in the signaling properties of the MEN2B Ret receptor cannot be completely ruled out.

2.4.5 GDNF, GFRα1 and Ret distribution in the CNS

Using *in situ* hybridization and reverse transcription-PCR, GDNF mRNA was localized in many different regions of the developing and adult brain, including the striatum, nucleus accumbens, globus pallidus, olfactory tubercle, hippocampus, granular layer of cerebellum, parietal and piriform cortices, thalamus and olfactory bulb (Schaar et al., 1993; Springer et al., 1994; Choi-Lundberg and Bohn, 1995; Nosrat et al., 1997). The expression of GDNF mRNA in the targets of DA neurons in the SNpc is accordant with its role of a target-derived neurotrophic factor for these neurons. In fact, when injected into the rat striatum, GDNF is retrogradely transported to the dopaminergic cell bodies in the SNpc (Tomac et al., 1995). Reports on the expression of GDNF mRNA in the SN are somewhat controversial. While some studies did not detect GDNF mRNA in the SNpc (Nosrat et al., 1997), others reported the presence of GDNF mRNA in the SN and suggested an autocrine and paracrine role for GDNF (Golden et al., 1998; Cho et al., 2004;

Oo et al., 2005). On the other hand, it has been shown that only the GDNF protein without mRNA expression, is found in the rostromedial part of the SN and the VTA in the adult rat (Barroso-Chinea et al., 2005). Furthermore, the same group has demonstrated that under physiological conditions, and not only after being injected or overexpressed, GDNF is retrogradely transported from the striatum to the ventral midbrain.

During development, GDNF mRNA expression in the rat striatum was first detected around E15 (Nosrat et al., 1997). During subsequent days of prenatal development, levels of GDNF mRNA expression increase, reaching their highest levels during early postnatal life and peaking at postnatal day 0 (P0) and postnatal days 10-14 (Schaar et al., 1993; Stromberg et al., 1993; Choi-Lundberg and Bohn, 1995; Nosrat et al., 1997). Likewise, protein levels of GDNF in the striatum are highest during the first postnatal week; thereafter, levels of GDNF decrease in the striatum of the adult rat (Lopez-Martin et al., 1999; Oo et al., 2005). The greatest GDNF expression in the striatum coincides with a developmental period of natural cell death of DAergic neurons and suggests a crucial role for GDNF in this event. Oo and colleagues (Oo et al., 2005) demonstrated that medium-sized neurons in the developing striatum express GDNF mRNA and confirmed previous findings that GDNF protein is localized in the striatal neuropil (Lopez-Martin et al., 1999). In addition, several groups found GDNF protein in fibers and axons in the principal striatal efferent targets, including SNpr, GP and the endopeduncular nucleus (Kawamoto et al., 2000; Oo et al., 2005). This is in line with studies reporting the anterograde transport of GDNF along neuronal projections, from the striatum to SNpr and the globus pallidus (Kordower et al., 2000; Georgievska et al., 2004). However, in the dorsal striatum of an adult rat and in the human caudate putamen (CPu), most of the GDNF-positive cells were identified as interneurons (Bizon et al., 1999; Kawamoto et al., 2000).

The widespread expression of GDNF is consistent with previous findings that GDNF supports multiple neuronal populations in addition to its known effects on nigrostriatal DAergic neurons. Thus, the cortex and hippocampus, which express GDNF mRNA, may serve as sources of GDNF for a number of neuronal populations projecting to these areas. For example, cell bodies of the noradrenergic neurons that reside in the locus coeruleus and project to the cortex and hippocampus express both GFRα1 and Ret (Trupp et al., 1997; Glazner et al., 1998). Intriguingly, some GDNF mRNA expression was detected in the locus coeruleus during prenatal development, but it was lost by birth (Nosrat et al., 1997).

Ret mRNA is often accompanied by GFRα1 receptor mRNA in brain tissues such as the SN, dorsal raphe nucleus, locus coeruleus, hypothalamic nuclei, Purkinje and molecular layers of cerebellum and brainstem nuclei that innervate skeletal muscles (Trupp et al., 1997; Golden et al., 1998; Yu et al., 1998). Thus, it is likely that in these areas, GDNF and GFRα1 activate Ret in the *cis* mode. Expression of both receptors has been shown to be predominantly neuronal (Golden et al., 1998). Ret protein is present on perikarya and proximal dendrites of DA neurons of the SN (pars compacta, predominantly) and VTA (Nosrat et al., 1997; He et al., 2008), and is also found in the striatum, on axonal projections of DAergic neurons (Jain personal communication, Hirata and Kiuchi, 2007). No Ret mRNA was found in DAergic neuronal target areas (Nosrat et al., 1997). However, GFRα1 protein and mRNA are found both in the ventral midbrain

and in the striatum (Bresjanac and Antauer, 2000; Cho et al., 2004). The role of GFRα1 in the striatum is not known, although in theory, it could act *in trans* to regulate development of the nigrostriatal projections. As mentioned earlier, expression of GFRα1 in rodent brain was found to be more widespread than that of Ret (Trupp et al., 1997; Glazner et al., 1998). Thus, GFRα1 is found in areas such as hippocampus, cortex and medial habenula that contain no or very little Ret, but are the targets of systems abundant in Ret, which further suggests activation of Ret *in trans* (Yu et al., 1998). Additionally, this discrepancy in expression patterns suggests the existence of alternative signaling receptors for GDNF or an alternative role for GFRα1 in addition to being a co-receptor for GDNF. A recent study implicated a role for GFRα1 in establishing precise synaptic contacts and inducing presynaptic differentiation (Ledda, 2007).

2.5 GDNF/Ret and the brain DAergic system

2.5.1 Physiological role of GDNF/Ret signaling in the nigrostriatal DA system

During embryogenesis, neurons are created in excess numbers and undergo a period of natural, apoptotic cell death that determines their final number (reviewed by Burke, 2004). In rats, cell death can be detected in the SN just before birth, peaks on postnatal days 2 and 14 (the so-called first and second phase of ontogenic cell death, respectively), and ceases on postnatal day 28. Much evidence indicates that these death events are most likely regulated by secretion of a limited amount of neurotrophic factors, for which developing neurons compete, from target tissues (Burke, 2004). The GDNF, GFRα1 and Ret knockout mice, which die at birth due to kidney agenesis, show no reduction in the number of DA neurons in the ventral midbrain (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enomoto et al., 1998). Also, we have found that brain tissue DA levels are similar among GDNF-/-, GDNF +/- and GDNF +/+ mice at embryonic day 18 (E18) (Mijatovic et. al, unpublished). This indicates that GDNF/GFRα1/Ret signaling is not important for fetal development of DA neurons. Alternatively, compensatory modifications may have occurred during development, masking the role of GDNF-Ret signaling.

Nevertheless, the expression of GDNF in a target tissue of DA neurons, its developmental regulation and retrograde transport to SN suggest that GDNF may serve as physiological, limiting target-derived factor for DA neurons throughout the natural cell death period. Indeed, Granholm and colleagues found that GDNF is essential for postnatal survival of midbrain DA neurons by grafting fetal neuronal tissue from GDNF knockout mice into the brain of wildtype mice (Granholm et al., 2000). Also, it was shown that during postnatal development, exogenously provided GDNF diminished natural cell death by inhibiting apoptosis while neutralizing antibodies against GDNF augmented it (Burke et al., 1998; Burke, 2006). The effects of GDNF appeared to be restricted to the early postnatal development of nigral DAergic neurons, more precisely to the first phase of cell

death (Beck et al., 1996; Oo et al., 2003). Thus, a transgenic mouse model in which overexpression of GDNF was sustained in the striatum throughout development showed an increased number of DA neurons surviving the first phase of natural cell death (Kholodilov et al., 2004). However, this increase in the number of DA neurons did not persist into adulthood, suggesting that neurotrophic factors other than GDNF also act to regulate the number of the DA neurons in the mature SN.

Heterozygous GDNF knockout mice are viable and by appearance similar to their wild-type littermates. Thus, they were used to study the effects of chronic reduction of GDNF levels (by 35-50%) in maturation and aging of the nigrostriatal DAergic system (Gerlai et al., 2001; Airavaara et al., 2004; Boger et al., 2006). In young adult mice, the number of TH-positive cells in the nigra, tissue concentration of DA in the striatum as well as TH and DAT levels in the striatum were normal, suggesting that GDNF may not be critical for the development and function of the nigrostriatal DAergic system (Gerlai et al., 2001; Airavaara et al., 2006); Mijatovic et al., unpublished). However, Airavaara and colleagues reported increased striatal extracellular DA levels as well as postsynaptic activity in GDNF $^{+/-}$ mice, suggesting that there may have been compensatory alterations in their DA system (Airavaara et al., 2004). A deficiency in GDNF was reported to be associated with an accelerated decline in motor activity and a decrease in TH immunostaining in SN during the aging process (Boger et al., 2006) as well as an increased susceptibility to methamphetamine-induced neurotoxicity (Boger et al., 2007). Similarly, reduction of GFRα1 co-receptor in heterozygous GFRα1 knockout mice resulted in exacerbation of aging-related decline in function of the DAergic system and an increased sensitivity to neurotoxins (Boger et al., 2008; Zaman et al., 2008). Thus, GDNF appears to be involved in the long-term maintenance of the nigrostriatal DAergic system.

To investigate the postnatal, physiological role of Ret for the maintenance and function of the nigrostriatal DA neurons, conditional Ret knockout mice have been created (Jain et al., 2006; Kramer et al., 2007). DAT-Cre-Ret mice contained no Ret in their midbrain DA neurons, starting from mid embryonic development. The data obtained from these conditional Ret knockouts suggested that Ret is not important for the establishment, development and maturation of the nigrostriatal DA system. Aged conditional Ret knockout mice, on the other hand, showed loss of midbrain DA neurons, degeneration of DA terminals and glial activation in the striatum indicating an important role of Ret in long-term maintenance of the nigrostriatal DA system. Also, when MPTP was given to conditional Ret knockouts, it was found that physiological Ret signaling is important for resprouting of DAergic fiber but its loss did not affect the vulnerability of DA cell bodies (Kowsky et al., 2007). However, in spite of the creation of a large number of animal models, role of endogenous GDNF-Ret pathway in the postnatal life, maturation and aging of brain DA system has not been ascertained.

Pascual and colleagues recently reported that the deletion of GDNF in 2-month-old mice resulted in a marked and progressive reduction of ventral midbrain DA neurons and an almost complete ablation of noradrenergic neurons of the locus coeruleus (Pascual et al., 2008). These data demonstrated that GDNF is critical for the survival of adult catecholaminergic neurons and stress the importance of a continuous neurotrophic support for the maintenance of adult neuron survival. The reason for this dramatic discrepancy

between previous mouse models with reduced GDNF/GFRα/Ret signaling and the conditional GDNF knockout mice is suspected to be related to the timing of the gene ablation, compensatory mechanisms independent of GDNF and the existence of alternative signaling receptors (Andressoo and Saarma, 2008; Ibanez, 2008).

2.5.2 Effects of exogenous GDNF on the injured DA system

Neuroprotection The most robust effect of GDNF in the CNS is the protection of DAergic neurons exposed to toxins and other insults. Early experiments with GDNF revealed that it promotes survival of DA neurons and protects them against toxin-induced death *in vitro* (Lin et al., 1993). Afterwards, numerous studies further investigating the neuroprotective effects of GDNF were performed in animal models of PD, rodents or primates in which DAergic neuron degeneration was induced by mechanical insult or by DA neuron-specific neurotoxins (with MPTP used in mice and primates, and 6-OHDA in rats). The main findings of these studies were that GDNF prevented the loss of nigral DA neurons, the decrease of DA nerve terminal density and the depletion of dopamine levels in the striatum, as well as lesion-induced motor deficits, if it was administered before or around the time of DA neuron injury (Beck et al., 1995; Sauer et al., 1995; Tomac et al., 1995). In animal models of PD, GDNF consistently protects DA cell bodies, provided that it is delivered at the site of toxic insult in regions such as the striatum, the SN or the lateral ventricle, (Beck et al., 1995; Kearns and Gash, 1995; Sauer et al., 1995). In contrast, GDNF will protect DAergic axonal terminals if it is administered in the striatum (Tomac et al., 1995; Kirik et al., 2004). Thus, in rats with striatal 6-OHDA lesion, GDNF application into the striatum leads to preservation of the entire nigrostriatal pathway whereas GDNF administration into SN does not protect DAergic axonal terminals in the striatum (Kirik et al., 2000a; Kirik et al., 2000b; Kirik et al., 2004). Furthermore, rescue of DA bodies in SN without protection of DAergic striatal innervation, as seen after nigral GDNF delivery, is insufficient for the preservation of motor function in rats with striatal 6-OHDA lesion (Sauer et al., 1995; Winkler et al., 1996; Rosenblad et al., 1999; Kirik et al., 2000a, b).

Neurorestoration However, even more important for the treatment of PD are the effects of GDNF on dysfunctional neurons that have already started to degenerate. These restorative effects have been studied by administering GDNF into an already lesioned, compromised nigrostriatal DA system. In these paradigms, GDNF is usually delivered as single or repeated bolus injection or continuous infusion into the lateral ventricle, SN or putamen of parkinsonian primates or extensively 6-OHDA-lesioned rats. There it restored the phenotype of quiescent DA neurons, resulting in increased number and size of TH-positive cells and TH-positive fiber density in the SN (Hoffer et al., 1994; Gash et al., 1996; Lapchak et al., 1997a; Gerhardt et al., 1999; Grondin et al., 2002). In addition, GDNF treatment elevated tissue levels of DA and its metabolites, enhanced DA turnover and stimulated DA release in the SN and GP of MPTP-lesioned monkeys (Gash et al., 1996; Gerhardt et al., 1999; Gash et al., 2005) or 6-OHDA MFB lesioned rats (Hoffer et

al., 1994; Hoffman et al., 1997; Lapchak et al., 1997a). The effects of nigral GDNF delivery on recovery of DA function in the striatum were unremarkable(Tomac et al., 1995; Gash et al., 1996; Gerhardt et al., 1999). On the other hand, GDNF administered into the striatum or lateral ventricle of advanced Parkinsonian monkeys was able to increase DA levels and DA turnover in the striatum and GP (Grondin 2002). In any case, even in the absence of improvement of the striatal DA function in severely-lesioned animals, GDNF promoted recovery of toxin-induced motor impairments (Hoffer et al., 1994; Hoffman et al., 1997; Lapchak et al., 1997b; Gerhardt et al., 1999). It has been suggested that GDNF attenuates toxin-induced behavioral impairments by increasing DAergic neurotransmission in the SN or by affecting some other system within the basal ganglia. Indeed, Xin and colleagues showed that the globus pallidus plays a critical role in GDNF-induced functional improvements in PD monkeys (Xin et al., 2008).

Rats with intrastriatal 6-OHDA lesions provide a model of progressive dopamine neuron degeneration and are considered a partial lesion model of PD (Björklund et al., 1997). In this partial lesion model of PD, ventricular GDNF infusion starting 2 weeks postlesion blocked the delayed death of DAergic cells in the SN and improved the motor impairments, whereas the striatal GDNF infusion was ineffective. However, both administration routes were ineffective in restoring degenerated fiber terminals in the striatum (Kirik et al., 2001). On the other hand, Rosenblad and coworkers (1998) showed that striatal GDNF delivery leads to axonal sprouting and reinnervation of a partially deafferated striatum, and subsequently results in functional recovery of the animals with striatal 6-OHDA lesion (Rosenblad et al., 1998). Controversially, the same group showed that GDNF induced axonal sprouting, although only around the needle site, that was not followed by striatal reinnervation and functional recovery (Rosenblad et al., 2000). Also, aberrant sprouting in 6-OHDA lesioned rats was observed near the site of GDNF injection (Rosenblad et al., 1999; Kirik et al., 2000a). Likewise, in primate models of PD as well as in clinical studies, GDNF was shown to induce sprouting of compromised DA fibers (Kordower et al., 2000; Love et al., 2005). In DA neurons of the developing midbrain, as well as in mature ventral mesencephalic grafts *in oculo* , GDNF caused TH-positive fiber outgrowth and increased the number of synaptic sites (Lin et al., 1993; Johansson et al., 1995; Åkerud et al., 1999; Bourque and Trudeau, 2000). All in all, restorative actions of GDNF in animal models of PD are not as dramatic as neuroprotective actions.

2.5.3 Effects of exogenous GDNF on intact DA system

In the intact nigrostriatal system of rats, a single injection of GDNF into the SN or striatum has a direct stimulatory effect on DA neuron function. This so -called “pharmacological effect” of GDNF on DAergic neurotransmission includes an increase in tissue DA and DA turnover in the ipsilateral SN and striatum, and an increase of striatal release of DA and its metabolites (Hudson et al., 1995; Beck et al., 1996; Hebert et al., 1996; Martin et al., 1996b; Hebert and Gerhardt, 1997; Xu and Dluzen, 2000; Salvatore et al., 2004). Also, acute GDNF has been shown to increase expression of TH (Hudson et al., 1995) and/or to augment phosphorylation and activity of TH in the striatum and SN

(Salvatore et al., 2004). These neurochemical changes have fully manifested by 1-3 weeks after GDNF administration and may persist for up to 6 weeks (Martin et al., 1996b). Additionally, a single GDNF injection increases locomotor activity and amphetamine-induced turning (Hudson et al., 1995; Martin et al., 1996a; Kobayashi et al., 1998). This effect of GDNF was blocked by D1 and D2 receptor antagonists (Kobayashi et al., 1998) indicating that the improvements in motor behavior induced by GDNF in intact as well as in lesioned animals are to a great extent associated with an upregulation of DA function.

The administration site plays an important role on where in the nigrostriatal DA system GDNF exerts its pharmacological actions. Thus, intranigrally administered GDNF affected DA function both in the striatum and SN, whereas the stimulatory effects of striatally administered GDNF were limited to the striatum. The effects of GDNF on nigrostriatal DAergic function may be explained by findings from in vitro studies, which found that GDNF increased DA release (Pothos et al., 1998), DA uptake (Lin et al., 1993) and enhanced DA neuron excitability by modulating ion channels (Yang et al., 2001; Wang et al., 2003). In addition, GDNF augmented TH mRNA and protein levels (Xiao et al., 2002) and increased TH phosphorylation and activity in cell cultures (Kobori et al., 2004).

However, while continuous long-term GDNF administration to rats with an intact DA system initially resulted in an enhancement of the function of the DA system, after 6 weeks there was a downregulation of TH protein levels in the striatum and of TH mRNA in the SN (Rosenblad et al., 2003; Georgievska et al., 2004). This was not accompanied by an alteration in other striatal dopaminergic markers, such as DA, VMAT2 and DAT levels, and the number of D1- or D2- DA receptors. The TH downregulation was time- and dose-dependent and appeared to be a compensatory response to the initial GDNF-induced overactivity of DA neurons and its direct effects on TH gene expression. On the other hand, in both young and aged intact primates, long-term GDNF administration upregulated the function of the DA system (Kordower et al., 2000; Grondin et al., 2003). Eslamboli and colleagues showed that in primates, continuous GDNF delivery using viral vectors that resulted in the expression of high levels of GDNF upregulated DA transmission in the striatum, whereas expression of low levels of GDNF produced only minimal effects (Eslamboli et al., 2005).

2.5.4 GDNF in clinical studies

The encouraging results in animal models raised hopes that GDNF could be successfully used for the treatment of PD. However, in a human clinical study in which GDNF protein was delivered at a high concentration into the cerebral ventricles of PD patients, no beneficial effects were seen, probably because of the limited penetration to the target brain areas, and significant side effects occurred (Nutt et al., 2003).

In contrast, in two open-label trials of continuous intraputamenal GDNF infusion in PD patients, Gill and colleagues reported excellent tolerance, few side effects and clinical benefits associated with a significant increase in dopamine storage and fiber sprouting in the putamen, as well as an improvement in the motor function of the patients (Gill et al.,

2003; Love et al., 2005; Slevin et al., 2005). In a randomized, controlled clinical trial conducted to confirm the open-label results, however, no significant improvement was seen (Lang et al., 2006). It has been suggested that different delivery protocols and dosing regimes used in these trials led to different outcomes, and until the methodological questions are resolved, it is premature to conclude that GDNF is ineffective as a treatment for PD (Sherer et al., 2006).

Also, in a phase I clinical trial, viral delivery of neurturin resulted in good tolerability and safety as well as in mild improvement of parkinsonian symptoms (Marks et al., 2008). In a phase II study, even more promising results have been obtained (unpublished, Kordower et al., personal communication).

3. AIMS OF THE STUDY

In spite of intensive research, the physiological roles of both GDNF and its signaling receptor Ret in the development and maintenance of brain DAergic system remain largely unknown. Complicating matters even more, Ret-independent GDNF signaling has recently been described. Thus, the purpose of the present study was to elucidate the physiological role and significance of GDNF-Ret signaling in the brain DAergic system by using mice with constitutively active Ret, MEN2B knock-in mice.

The specific aims of this study were:

1. To investigate whether and how continuous Ret activity affects brain DA systems with emphasis on the nigrostriatal system. Thus, we studied neurochemical, molecular and behavioral changes in the MEN2B mice.
2. To characterize in more detail compensatory alterations in the striatal DAergic transmission in the MEN2B mice.
3. To explore whether continuous Ret activity protects the nigrostriatal DAergic system against neurotoxins.

4. MATERIALS AND MAIN METHODS

A more detailed description of the materials and methods used in the present study is given in the respective original publications I-III.

4.1 Animals

In the present studies we used homozygous (M/M) and heterozygous (M/+) MEN2B knock-in mice and their wild-type (Wt) littermates. The Met918Thr point mutation in humans leads to the substitution of normal methionine by threonine at codon 918 in the Ret kinase domain resulting in activation of the Ret signaling (Takahashi, 2001). The MEN2B mouse model was generated by introducing corresponding mutation, Met919Thr, into the murine *Ret* gene (Smith-Hicks et al., 2000). Therefore, MEN2B mice, that carry constitutively active Ret, represent a mouse model for increased Ret signaling. However, although not confirmed, difference in the signaling pathways between Wt and MEN2B Ret might exist, which needs to be taken into consideration when interpreting the data obtained from the MEN2B mice. The MEN2B mice were originally generated on a mixed C57BL/6 X 129Sv background (Smith Hicks et al., 2000) and in the Laboratory Animal Center, University of Helsinki, the mice were backcrossed on a pure C57BL/6 background. Male mice were used at 8-16 weeks of age. The mice were housed in groups of 2-8 to a cage and had free access to mouse chow and water. They were maintained under 12:12 h light/dark cycle with lights on from 06:00 to 18:00 at an ambient temperature of 20-22°C. The chief veterinarian of the county administrative board or the Committee on Animal Experiments at the University of Helsinki approved all the experiments. The experiments were conducted according to the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purposes". The mice were genotyped by conducting a PCR on DNA samples taken from the mouse tail (described in more details in Study I). As described by Smith Hicks at al. (2002) homozygous M/M mice display an earlier onset and increased severity of C-cell and chromaffin cell hyperplasia (that progressed to pheochromocytoma), male reproductive defects, ganglioneuromas of adrenal medulla and enlargement of sympathetic ganglia, thus more closely matching the human MEN2B syndrome. Heterozygous M/+ mice are less severely affected, displaying hyperplasia of thyroid C-cells and adrenal chromaffin cells. These abnormalities are minimal at the age of 8-16 weeks when the mice were used in our experiments.

4.2 Monoamine analysis of brain tissue samples (Studies I, II and III)

The mice were killed by decapitation, their brains rapidly removed from the skull, placed on an ice-cooled brain matrix and dissected. The brain dissection method was described in details in the Study I. The dissected brain areas include whole striatum, dorsal and ventral striatum, hypothalamus, cortex, hippocampus and lower brain stem. The tissue pieces were immediately placed into frozen microcentrifuge tubes, weighed and stored at -80°C until assayed. Monoamines analyzed from brain tissue samples were DA, DOPAC, HVA, 5-HT, 5-HIAA, NA, MOPEG (in the Studies I, II and III) and DOPA (in the Study II). Monoamine concentrations were determined using HPLC combined with electrochemical detector and their concentrations reported in ng/g wet weight of tissue (described in details by Airavaara et al., 2005).

4.3 Immunohistochemistry (Study I and III)

4.3.1 Tissue preparation

The mice were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brains were removed, postfixed for 4 h and stored in sodium phosphate buffer containing 20% sucrose at 4ºC. Coronal striatal and nigral sections were cut and saved individually in serial order at -20ºC until used for either TH or DAT immunostaining.

4.3.2 Immunolabelling

TH immunohistochemistry. Brains from animals of all the genotypes were included in every run. In brief, after quenching with 3% hydrogen peroxide and 10% methanol for 5 min, sections were blocked in blocking buffer (2% normal goat serum (NGS, Chemicon, S-1000), 0.3% Triton X-100 in PBS) for 1 h and thereafter incubated in the primary rabbit anti-TH antibody (1:2000, Chemicon) overnight. The next day the sections were incubated for 2 h with the biotinylated goat anti-rabbit secondary antibody (1:200, Vector). The standard avidin-biotin reaction was performed using Vectastain® Elite ABC peroxidase Kit and the immunoreactivity was revealed using diaminobenzidine (DAB) and H_2O_2. The sections were mounted on gelatin/chrome alume-coated slides, air-dried overnight, dehydrated through graded ethanols, cleared in xylene and coverslipped.
DAT immunohistochemistry. Striatal free-floating sections used for densitometry measurements (40 µm) were processed as described above for TH, with the exception that the primary antibody was rat anti-DAT monoclonal antibody at 1:2000 (Chemicon) and secondary antibody was biotinylated rabbit anti-rat antibody at 1:200 and normal rabbit

serum (Vector Laboratories) was used instead of normal goat serum. Striatal sections (30 µm) used for stereological estimation of DAT-positive varicosities were incubated for 48 h (at +4ºC) and the concentration of primary antibody was increased to 1:1000 (Chemicon).

4.3.3 Quantification of immunoreactivity

Striatal densitometry measurements. Digitalized images of TH and DAT immunostained striatal sections were collected and optical density (OD) of the stainings was measured using Image-Pro-Plus TM software (Version 3.0.1., Media Cybernetics, Silver Spring, MD). For each animal, the OD was measured from three striatal coronal sections and the final reading was calculated as an average of those three values. The nonspecific background correction in each section was done by subtracting OD value of corpus callosum from the striatal OD value obtained from the same section.
Stereological analysis of TH-positive cells and DAT-positive varicosities. From each animal, three TH-immunostained midbrain sections were selected, and TH-positive somas lateral to the medial terminal nucleus (MTN) were counted. The counts were made at 60X under oil, and with help of the StereoInvestigator software with optical fractionator according to optical dissector rules (Gundersen et al., 1988). After SNpc estimations were performed, the same sections were used for counting of TH-positive cells in the VTA. TH staining was used to define the outline of the VTA with the MTN as the lateral border. The number of DAT-immunoreactive (DAT-IR) punctuate structures in the striatum was determined using a stereology approach similar to that described previously (Parish et al., 2001). From each animal, four DAT-immunostained striatal sections were analysed at 100X under oil. The DAT-positive terminals were identified as round swellings in association with axons and used as counting units. The total number of the DAT-positive punctuate structures on one side for each striatum was determined by the StereoInvestigator program. The coefficient of error was calculated as an estimate of precision and values under 0.1 were accepted. The person performing counting was blinded as to the identity of the samples.

4.4 Immunoblotting (Study I)

Western blotting experiments were performed from individual dorsal striatal tissues and SN/VTA tissues pooled from five mice of the same genotype. Individual striata were homogenized in lysis buffer (5mM HEPES, pH 7.4, 320mM sucrose, 1mM EDTA, 0.1mM PMSF and 0.1% SDS), the homogenates were centrifuged at 900 X g for 3 minutes and the supernatants were further centrifuged at 16 000 X g for 20 minutes. Thereafter, the supernatant was collected and protein concentration was determined by a commercial protein assay BCA kit (Pierce, Rockford, IL). The samples containing equal amounts of protein (15µg) were loaded on and electrophoresed in a 7.5% SDS-polyacrylamide gel and then transferred onto Protran® nitrocellulose transfer membranes

(Schleicher & Schuell Bioscience GmbH, Daseel, Germany). After blocking of nonspecific binding with 5% nonfat dry milk, the membranes were then probed with sheep anti-TH polyclonal antibody (at 1:1000; Chemicon) and rat anti-DAT monoclonal antibody (at 1:500; Chemicon). Next, membranes were treated with appropriate secondary antibody (1:2000) conjugated with horseradish peroxidise (HRP), and blots were visualized with chemiluminescent substrate (Pierce, Rockford) detected and estimated using GeneGnome chemiluminescent detector and its software (SynGene, Synoptics LTD, UK). The membranes were then stripped of antibodies and afterwards re-probed with mouse anti-actin antibody at 1: 10000 (Sigma) and appropriate anti-mouse HRP-conjugated secondary antibody (1:2000, Zymed). The stripping protocol included shaking the membranes in 40% methanol for 30 min at room temperature, followed by submersion in stripping buffer containing: 2% SDS, 62.5mM Tris HCl, 100mM β-mercaptoethanol, at 55°C for 30 min. Five-percent nonfat milk was used for blocking.

After the homogenization of the SN/VTA samples, normalized amounts of protein sample (10µg) were loaded on a 10% SDS-polyacrylamide gel, transferred onto Hybond ECL Nitrocellulose membrane (Amersham Biosciences) which was then probed with anti-TH antibody and subsequently treated with horseradish peroxidase conjugated secondary antibody. The blots were visualized by chemiluminescent substrate (Pierce). The images were taken using LAS 3000 CCD Camera from Fujifilm and quantified using the AIDA Image Analyzer software.

4.5 Drugs and treatments

NSD-1015 (Study II, BioChemika, Sigma, China), alpha-methyl-paratyrosine (Study II, α-MT, Labkemi AB, Sweden), tetrabenazine (Study II, Tocris, Avonmouth, UK), cocaine (Studies I and II, University Pharmacy, Helsinki, Finland), haloperidol (Study II, Janssen Pharmaceutica N.V., Beerse, Belgium), MPTP (Study III, Toronto Research Chemicals) and d-amphetamine (University Pharmacy, Helsinki, Finland) were dissolved in saline (0.9% NaCl). Control animals received saline. All the injections were given i.p. in a volume of 10 ml/kg. Drug doses were calculated as free base.

Five µg of 6-OHDA (Study III, Sigma, USA) was injected into the left striatum using stereotaxic frame and injector. The mice were pretreated with desipramine (25 mg/kg, i.p.) 30 min before 6-OHDA. 6-OHDA·HCl was dissolved in 0.02% ascorbic acid solution and using a 10 µl Hamilton syringe was infused into the left striatum (total volume injected was 2 µl, rate of injection was 0.5 µl/min). The needle was slowly retracted 2 min from the cessation of injection.

4.6 Stereotaxic Surgery (Study II and III)

The mice were under general isoflurane (3.5% induction, 2% maintenance) and local lidocaine anesthesia while using the stereotaxic device (Stoelting, Wood Dale, IL, USA) to implant microdialysis guide cannulae or to inject 6-OHDA into striatum. Mice were given buprenorphine (0.1 mg/kg s.c.) to relieve pain. Guide cannulae were aimed in the striatum above the point (A/P = + 0.6, L/M = +1.8 and D/V = -2.3) and 6-OHDA was infused into left striatum at coordinates AP + 0.7, ML +1.8, DV -2.7 relative to bregma and dura according to mouse brain atlas (Franklin and Paxinos, 1997). After surgery, mice were placed into individual test cages and allowed to recover in the cages for 5-7 days before the microdialysis experiment whereas 6-OHDA-treated mice were used 3 weeks later for neurochemical, histological and behavioral analysis.

4.7 *In vivo* microdialysis (Study II)

On the previous afternoon, a microdialysis probe (AgnTho's, Lidingö, Sweden, 1 mm membrane, outer diameter 0.24 mm) was inserted into the guide cannula, and the probe was infused with Ringer solution at a low flow rate of 0.5 µl/min. In the morning of the experimental day, the flow rate was increased to 2 µl/min, and after a 2 h of stabilization period, the collection of actual microdialysis samples (every 20 min, 40 µl/sample) was started. The concentrations of DA, DOPAC and HVA were determined by HPLC with an electrochemical detector (Coulochem II; ESA Inc., Chelmsford, MA, USA). Basal steady-state extracellular monoamine concentrations were determined as mean of concentrations of four stable pre-drug samples collected during the first 80 minutes of sampling (variation < 20%). Post-drug neurotransmitter levels were converted to a percentage of this baseline value, which was defined as 100%. Potassium stimulation was performed by manually switching the perfusion medium, Ringer, to the one containing 100 mM KCl and 27.5 mM NaCl (remaining composition not changed). After completion of the experiments, the positions of the probes were verified histologically.

4.8 *In vivo* voltammetry (Study II)

4.8.1 Preparation of animals

The mice were anaesthetized with chloral hydrate (450 mg/kg, i.p.) and fixed to a stereotaxic frame. The carbon fiber working electrode was inserted through an opening in the skull to the caudate nucleus (A/P= +1.18 mm, L/M = +1.5 mm, H =3.4 mm vs. bregma) and a bipolar stimulating electrode was implanted in the medial forebrain bundle (A/P +2.1 mm, L/M +1.1 mm, H: 5.0–5.2 mm vs. bregma) according to mouse brain atlas (Paxinos and Franklin 2000). The exact placement of the stimulating electrode in the dorsoventral coordinate was adjusted for maximal dopamine release. A small leak-free Ag/AgCl reference electrode (AH 69-0023, Harvard Apparatus, Holliston, MA, USA) in a saline bridge was placed on the skull. A stainless steel screw was fixed onto the skull as the auxiliary electrode.

4.8.2 Electrochemical technique

Stimulated release of dopamine was measured using constant potential amperometry (CPA). A single Nafion-coated carbon fiber, 30 µm in diameter (WPI, Sarasota, FL, USA) was protruded in pulled capillary glass and insulated with epoxy glue. The exposed tip of the fiber was 300 µm. A custom-built three-electrode potentiostat held the working electrode at 0.4 V against an Ag/AgCl reference electrode. The current at the working electrode was converted to voltage at a headstage converter located near the working electrode. The data were digitized at 10 KHz and recorded with a personal computer for further off-line analysis. The electrochemical signals in response to stimulations were stabilized in about 90 min after implantation of the working electrodes. After the experiments, the working electrodes were rinsed with deionized water and calibrated for dopamine in phosphate-buffered saline (pH = 7.4).

4.8.3 Electrical stimulation and experimental protocol

A battery-operated constant current unit (A365, WPI) run by a personal computer was used for the stimulation. Constant current pulses (1 ms in duration) of 180–200 µA were delivered to the stimulation electrode at 10–60 Hz in 2 s bursts. The intervals between the bursts were 1–6 min. Longer intervals corresponded with higher stimulation frequencies. Another stimulation protocol was used to obtain peaks of DA overflow of different amplitudes, from which peaks of similar amplitudes can be directly compared. We used 50 Hz stimulations applied in the bursts of 0.2, 0.4, 0.8, and 1.6 s lengths at 1–4 min intervals.

The right slope of the dopamine overflow curve reflects dynamics of dopamine elimination from the extracellular space. This curve can be approximated with a good fit

by a standard exponential function $f(t) = Ae^{-t/\tau} + C$. Curve fitting was made with Clampfit v.8 (Axon Instruments/Molecular Devices Corporation, Sunnyvale, CA, USA) with peaks of similar amplitudes. We analysed and depicted dopamine decay from the extracellular space using parameter tau (τ) which is time constant of dopamine decay curve. The effect of cocaine was analyzed 25–45 min after the administration at different stimulation frequencies (10–60 Hz, 2 s burst length) and lengths (0.2–1.6 s, at 50 Hz).

4.9 Behavioral testing methods (Study I and III)

Locomotor activity. A detailed description can be found in the study I. In brief, the mice were put into transparent plastic cages, infrared photobeam interruptions caused by mice movement were registered by activity monitors and distance traveled in a period of time was measured (MED Associates, Open field activity monitor, ST. Albans, GA). Except when spontaneous locmotor activity was assessed, the mice were habituated to the test cages for 5 - 10 minutes.

Turning behavior. Amphetamine-induced turning behavior was measured using Roto-Rat™ test system (Med Accociates Inc., Georgia, VT, USA). On the morning of experiment the mice were placed into individual bowls and attached via collars (put on the previous evening) to the leashes that extend from the sensitive optical encoders which report movements in either direction. The animals were left to habituate to the bowls for 15min before single d-amphetamine injection (University Pharmacy, Helsinki, Finland; 3 mg/kg i.p.) and the number of full 360° ipsilateral (body turns toward the side of lesion) and contralateral turns (body turns away from the side of lesion) was recorded for a period of 90 min. The data were expressed as net ipsilateral turns.

4.10 Statistical analyses

Data are presented as mean ± S.E.M and a level of $p < 0.05$ was taken as a statistically significant effect. Data obtained from monoamine tissue measurements were analyzed with one-way or two-way ANOVA, followed by Tukey/Kramer *post hoc* comparisons (Study I and II) or Student-Newman-Keuls *post hoc* test (Study III). One-way ANOVA and Tukey/Kramer *post hoc* test were used to analyze statistically significant differences in steady-state extracellular concentrations of DA, DOPAC and HVA between genotypes (Study II). One-way ANOVA for repeated measures was used to analyze the drug effects on output of extracellular levels of DA and DOPAC in MEN2B mice and their wild-type littermates (Study I). Locomotor activity data were analyzed with two-way ANOVA for repeated measures and Tukey/Kramer post hoc test (Study I). In voltammetry experiments (Study II) the effects of cocaine on peak dopamine overflow after stimulations at increasing frequencies were statistically analyzed with multivariate analysis of variance for repeated measures (MANOVA). Also, MANOVA was used to compare time constants of uptake in two genotypes and after cocaine, and a t-test was used for comparison of

individual pairs. The numbers of TH-positive cells and those of DAT-positive varicosities in the Wt and homozygous M/M mice were compared by unpaired Student's *t*-test (Study I). The effects of 6-OHDA or MPTP on TH-positive cell numbers in the SN were analyzed with two-way ANOVA followed by Student-Newman-Keuls *post hoc* test (Study III).

5. RESULTS

5.1 Monoamine concentrations in different brain areas of the MEN2B mice (Study I)

The analysis of monoamine levels in the different brain tissues (striatum, cortex, hypothalamus, hippocampus and lower brain stem) revealed that in the MEN2B mice, dopamine concentrations were increased in the target areas of the dopaminergic neuronal pathways (Table 5.1 and Figure 5.1).

Table 5.1. Post mortem, cerebral tissue concentrations of DA, NA and 5-HT (ng/g of wet tissue) in the M/+ and M/M mice as compared with the Wt mice.

	DA	NA	5-HT
Striatum			
M/+	↑↑**	nd	↔
M/M	↑↑↑** ##	nd	↔
Cortex			
M/+	↑↑**	↔	↔
M/M	↑↑**	↔	↔
Hypothalamus			
M/+	↔	↔	↔
M/M	↑*	↔	↔
Hippocampus			
M/+	nd	↔	↔
M/M	nd	↔	↔
Lower brain stem			
M/+	↔	↔	↔
M/M	↑	↑*	↔

Arrows indicate the increase in the concentration of a monoamine in the MEN2B mice as compared with the Wt: ↑ indicates 20-35% increase, ↑↑ 40-80% and ↑↑↑ more than 80% increase. ↔ indicates no change; nd indicates that a neurotransmitter was not detected. One-way ANOVA and Tukey/Kramer *post hoc* test were used for *s*tatistical analysis: $^{**}p < 0.01$ vs. Wt mice; $^{\#}p < 0.05$, vs. M/+ mice

In the whole striatum, DA concentrations were increased in the heterozygous M/+ mice by 47% and in the homozygous M/M mice by 100%, as compared with the Wt mice. Cortical DA was similarly increased in both M/+ and M/M mice (by about 70%), whereas in the hypothalamus, DA was significantly elevated (by about 30%) only in the M/M mice (Table 5.1).

As shown in Figure 5.1, in the dorsal striatum the tissue content of DA was increased by 54% in the M/+ and by 94% in the M/M mice, as compared with Wt mice. In the ventral striatum, however, DA levels were comparable between M/+ and M/M mice, and by about 60% higher in both than in the Wt mice (Figure 5.1). In conjunction with DA, concentrations of DOPAC and HVA in the MEN2B mice were augmented in the aforementioned brain tissues in comparison to Wt mice (Figure 5.1). In the dorsal striatum of M/+ mice, DOPAC was up by 102% and HVA by 72%, while in the M/M mice, DOPAC was increased by 185% and HVA by 137%. Thus, as is the case with DA, concentrations of DOPAC and HVA in the dorsal striatum were significantly higher in the M/M than in the M/+ mice. In the ventral striatum, DOPAC and HVA were similarly increased in the M/+ and M/M mice (DOPAC: by 120-130% and HVA: by 80%; Figure 5.1).

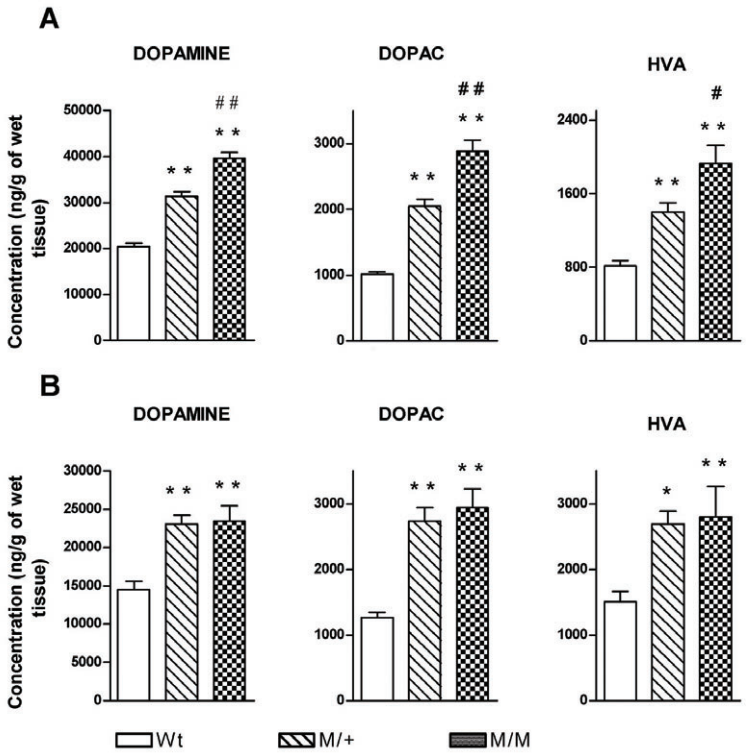

Figure 5.1. *Concentrations (nanograms per gram) of dopamine, DOPAC and HVA in the dorsal (A) and ventral (B) striatum of the M/+ and M/M mice were elevated as compared with their Wt littermates. Furthermore, in the dorsal striatum, dopamine and its metabolites were increased in the gene dosage-dependent manner whereas in the ventral striatum, the maximal effect could already be observed in the heterozygous mice. Statistical results were obtained with one-way ANOVA and Tukey-Kramer post-hoc analysis. *p < 0.05 vs. Wt mice, *p < 0.01 vs. Wt mice, $^{\#}p < 0.05$ vs. M/+ mice, $^{\#\#}p < 0.01$ vs. M/M mice.*

In contrast to DA, concentrations of serotonin were not changed in any of the analyzed tissues in both heterozygous M/+ and homozygous M/M mice. Likewise, the concentrations of noradrenaline did not differ among the mice of all three genotypes, except in the lower brain stem where NA was slightly increased (by 27%) in the homozygous M/M mice (Table 5.1).

5.2 TH and DAT expression in the nigrostriatal DAergic system of MEN2B mice (Study I)

Next, we examined whether other dopaminergic markers, such as TH and DAT, are altered in the MEN2B mice. Indeed, optical densities of the TH-immunostained striatal sections in the M/+ and M/M mice were found to be 55% and 75% larger than in the Wt mice, respectively (Table 5.2). Thus, we again found a dose-dependent effect of the gene mutation on DAergic system in the M/+ and M/M mice. Intensity of DAT-immunostaining in the striatum was increased by about 50% in both M/+ and M/M mice as compared with Wt mice (Table 5.2). Western blotting analysis confirmed that striatal TH and DAT levels are indeed increased in M/+ and M/M mice (Study I, Figure 3*A* and 3*B*). In the SN/VTA region, levels of TH protein were assessed by Western blotting analysis and found to be elevated by 60% and 180% in the M/+ and M/M mice, respectively, as compared with Wt mice (Study I, Figure 3*C*).

Table 5.2. Striatal optical density of TH- and DAT-immunostained striatal sections in the M/+ and M/M mice as compared with Wt mice

	TH OD	DAT OD
M/+	↑*	↑**
M/M	↑↑**	↑**

↑ indicates 50-60% increase and ↑↑ indicates more than 70% increase in the M/+ and M/M mice as compared with the Wt mice. One-way ANOVA and Tukey/Kramer *post hoc* test were used for statistical analysis: $*p < 0.05$ vs. Wt mice, $**p < 0.01$ vs. Wt mice.

When quantitative real-time PCR was used, about 2.5-fold increase in the levels of TH mRNA in the SN/VTA of the homozygous M/M mice was observed. Also, levels of DAT mRNA in the SN/VTA were raised in the M/M mice (by about 2.7 fold), but the difference did not reach statistical significance. The levels of other genes studied, such as GDNF, GFRα1 and NCAM in SN/VTA and GDNF in striatum, were not changed in the M/M mice.

5.3 The numbers of midbrain TH-positive cells and striatal DAT-positive varicosities in the MEN2B mice (Study I)

Stereological analysis of TH-stained sections revealed a 26% increase in the number of TH-positive neurons in the SNpc in the homozygous M/M mice compared with their Wt littermates (Table 5.3). No increase in the number of nigral TH-positive cells was found in the heterozygous M/+ mice. In the VTA, the number of TH-positive cells did not differ among Wt, M/+ and M/M mice (Table 5.3).

The number of DAT-positive punctate structures in the striatum of the MEN2B mice was counted in order to assess possible sprouting of dopaminergic fibers in the striatum under influence of constitutive Ret activity. A significant increase in the number of DAT-positive varicosities in the striatum was found only in the homozygous M/M (by about 23%) and not in heterozygous M/+ mice, as compared with their Wt littermates (Table 5.3).

Table 5.3. Numbers of TH-positive cells in the SNpc and VTA and of DAT-positive varicosities in the striatum of M/+ and M/M mice as compared with Wt mice.

	TH-positive cells		DAT-positive varicosities
	SNpc	VTA	Striatum
M/+	↔	↔	↔
M/M	↑*	↔	↑*

↑ indicates significant increase of about 20-25% and ↔ indicates no change in comparison with Wt mice. *$p < 0.05$ vs. Wt mice as revealed by Student's *t*-test

5.4 Brain dopamine concentration at different developmental stages in the MEN2B mice (unpublished)

The next objective was to assess the role of the Ret signaling during the development of the DAergic system. Therefore, we estimated brain DA levels in the MEN2B mice at different developmental stages (Table 5.4). At the embryonic day 18 (E18), there were no differences in the brain DA concentration between the genotypes. Also, at the postnatal day 7 (P7), no alterations in the brain DA levels in the MEN2B mice were observed, as compared to the Wt mice. However, at postnatal day 16 (P16), brain DA levels were significantly increased by about 55% in the homozygous M/M mice as compared with Wt mice. Also, at P16, brain DA in the heterozygous M/+ mice was elevated by about 22%, though the difference did not reach statistical significance.

Table 5.4. Brain DA concentration at embryonic day 18 (E18), postnatal day 7 (P7) and postnatal day 16 (P16) in the M/+ and M/M mice, as compared with the Wt mice

	E 18	P7	P16
M/+	↔	↔	↑
M/M	↔	↔	↑↑$^{**\ \#\#}$

↔ indicates no change, ↑ indicates 20-25% increase and ↑↑ indicates 50-55% increase of brain DA concentrations in the M/+ and M/M mice as compared with the Wt mice. One-way ANOVA and Tukey/Kramer *post hoc* test were used for *s*tatistical analysis: $^{**}p < 0.01$ vs. Wt mice, $^{\#\#}p < 0.01$ vs. M/+ mice. n = 6-14/ group.

5.5 Assessment of locomotor activity in the MEN2B mice (Study I)

Spontaneous locomotor activity in nonhabituated Wt, M/+ and M/M mice declined during the 60 min in a similar fashion. Nevertheless, both M/+ and M/M mice showed reduced locomotor activity as compared with the Wt mice, while no differences between M/+ and M/M mice were found (Study I, Figure 6). However, after the mice were habituated and locomotor activity was recorded over 24 h, no differences in the distances traveled or time points of activity peaks between the mice of the three genotypes were found (Study I, Figure 6).

Next, the mice were given cocaine (5, 10 or 20 mg/kg) or saline, and locomotor activities were recorded for 30 min. Cocaine significantly and dose-dependently increased locomotor activity in mice of all three genotypes, but its effects differed between the Wt and MEN2B mice. The effect of 5 mg/kg of cocaine was similar in all genotypes but doses of 10 and 20 mg/kg of cocaine increased locomotor activity significantly more in heterozygous M/+ and homozygous M/M mice than in Wt mice. Furthermore, the

homozygous mice treated with 20 mg/kg cocaine traveled a significantly longer distance than their correspondingly treated wild-type littermates. Locomotor activities of saline-treated MEN2B mice and their wild-type littermates did not differ significantly (Figure 5.6).

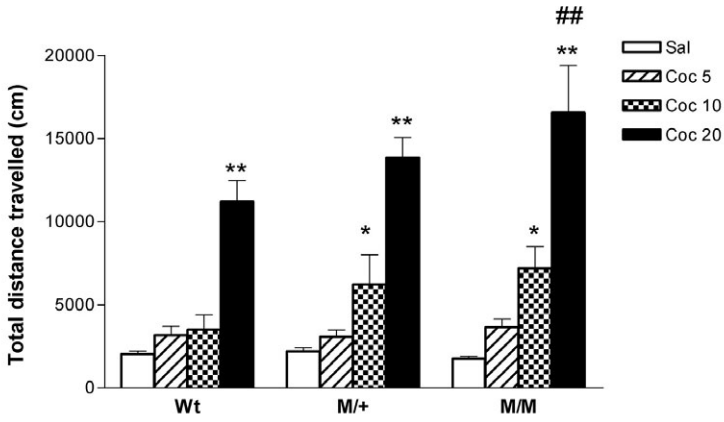

Figure 5.6. *The effects of acute cocaine administration on the locomotor activities of heterozygous M/+ mice, homozygous M/M mice and their wild-type littermates (Wt). Saline or cocaine (5 mg/kg, 10 mg/kg or 20 mg/kg) were given i.p. immediately before starting the recording. Columns give means ± SEM of total distances traveled during 30 min. *p < 0.05, **p < 0.01 as compared to corresponding saline treated mice; ##p < 0.01 as compared to cocaine 20 mg/kg treated Wt mice (Tukey/Kramer post hoc test)*

5.6 Synthesis and storage of DA in the MEN2B mice (Study II)

Based on the findings that the levels of both dopamine and TH were augmented in the striatum of M/+ and M/M mice, the next objective was to examine if synthesis and storage of dopamine were increased in the DA neuron terminals in the striatum in the MEN2B mice.

A good indicator of TH activity and DA synthesis is the degree of L-DOPA accumulation after blocking its conversion to DA. Following the inhibition of DOPA decarboxylation by NSD-1015 (150 mg/kg), striatal L-DOPA levels were increased by 41% in the M/+ and by 68% in the M/M mice as compared with their Wt littermates (Figure 5.7) showing that TH activity and DA synthesis are indeed enhanced in MEN2B mice.

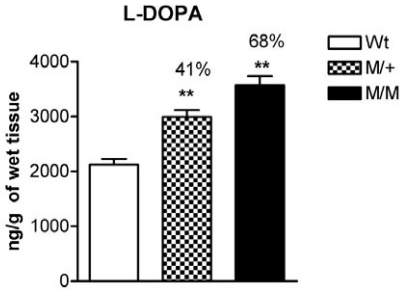

Figure 5.7*. Increased NSD-1015-induced L-DOPA accumulation in the M/+ and M/M mice as compared with Wt mice. The columns give the concentrations of L-DOPA (means ± SEM). Statistical results were obtained with one-way ANOVA and Tukey/Kramer post hoc test. **p < 0.01; n = 7-15/genotype.*

The α-methyl-para-tyrosine (αMT) is an inhibitor of TH and causes exhaustion of the newly synthesized, so-called releasable pool of intracellular dopamine. Tetrabenazine, a VMAT2 blocker, depletes the storage pool of intracellular dopamine, thereby causing subsequent accumulation of DOPAC. The elevated response of M/+ and M/M mice (striatal DA depletion was 33% and 40%, respectively) to αMT (250 mg/kg) compared to that of the Wt mice (DA depletion 27%) suggests that releasable pool of dopamine in M/+ and M/M mice is augmented in comparison to the Wt mice (Figure 5.8*A*). Also, it substantiates the finding that the activity of TH is increased in these mice. Likewise, after tetrabenazine (5 mg/kg), DA depletion was enhanced in M/+ and M/M mice (75% and 81%, respectively) as compared with Wt mice (69%, Figure 5.8*B*). Accordingly, tetrabenazine-induced accumulation of striatal DOPAC was larger in the M/+ and M/M mice (341% and 373%, respectively) than in the Wt mice (279%) (Study II, Figure 3.*B*).

Thus, the augmented tetrabenazine response indicates that storage pool of dopamine is larger in MEN2B mice that in the Wt mice.

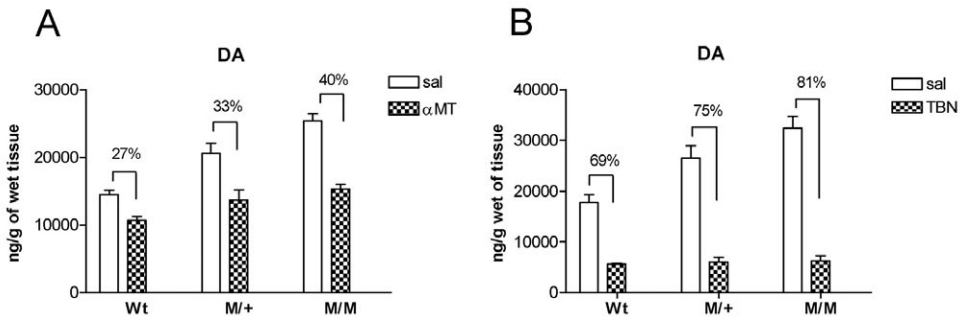

Figure 5.8. *The effects of alpha-methyl-paratyrosine (αMT, 250 mg/kg, i.p.) and tetrabenazine (TBN, 5 mg/kg, i.p.) on depletion of dopamine (DA) in the M/+ and M/M mice as compared with Wt mice. The columns give the concentrations of DA (means ± SEM).The αMT-induced DA depletion was larger in the M/+ and M/M mice (genotype X treatment interaction p = 0.0391, two-way ANOVA). Also, TBN depleted DA more in the M/+ and M/M mice (genotype X treatment interaction p = 0.0001, two-way ANOVA).*

5.7 Effects of cocaine, haloperidol and high K^+ stimulus on extracellular concentrations of dopamine, DOPAC and HVA in striatal dialysates in the MEN2B mice (Study II)

Basal extracellular DA concentrations in the dorsal striatum were similar among the three genotypes, as revealed by microdialysis (Table 5.5). However, concentrations of extracellular DOPAC were significantly higher in the dorsal striatum in the M/+ (by 58%) and M/M (by 71%) mice as compared with the Wt mice (Table 5.5). Basal levels of HVA in both M/+ and M/M mice were elevated by about 30%, but not significantly (Table 5.5).

Table 5.5. The basal, steady-state extracellular concentrations of DA, DOPAC and HVA in the striatal dialysates in the M/+ and M/M mice as compared with Wt mice

	DA	DOPAC	HVA
M/+	↔	↑↑**	↑
M/ M	↔	↑↑**	↑

↔ indicates no change, ↑ indicates 30-35% increase and ↑↑ indicate 50-80% increase in the concentration of DA or its metabolites in the M/+ and M/M mice as compared with the Wt mice. One-way ANOVA and Tukey/Kramer *post hoc* test were used for *s*tatistical analysis: $^{**}p < 0.01$ vs. Wt mice.

Effects of dopamine uptake blocker, cocaine (10 and 20 mg/kg, i.p.), D2-type receptor blocker, haloperidol (0.5 mg/kg, i.p.) and high K^+ (100 mM KCl) stimulus on striatal dopamine release were studied by microdialysis. Unexpectedly, there was no difference among the genotypes in the increase of extracellular dopamine concentrations in the response to either dose of cocaine (Study II, Figures 5.*A* and 5.*B*). High K^+ stimulation via microdialysis probe increased the output of DA similarly in all three genotypes (Study II, Figure 5.*B*). Also, no difference among genotypes was found in the effect of haloperidol on extracellular DA concentration in the dorsal striatum (Study II, Figure 6.*A*). Nevertheless, haloperidol increased extracellular DOPAC concentration significantly more in M/M mice than in the M/+ and Wt mice (Study II, Figure 6.*B*).

5.8 *In vivo* voltammetry assessment of uptake and release of dopamine in the dorsal striatum of the MEN2B mice (Study II)

As revealed by *in vivo* voltammetry, the M/M mice showed a much larger increase in stimulated peak dopamine overflow in comparison with the Wt mice (Study II, Figures 7.*A* and 7.*B*). Also, dopamine decay from extracellular space was three times faster in the M/M mice than in the Wt mice (Study II, Figure 8.*B*) and the effect of cocaine on dopamine elimination was significantly larger in the M/M mice (Study II, Figure 8.*C*). Taken together, the data obtained by *in vivo* voltammetry reveal that both release and uptake of dopamine are increased in the MEN2B mice.

5.9 Effects of systemic MPTP on the dopaminergic system of MEN2B mice (Study III)

Two weeks after MPTP treatment, there was a similar decline in dopamine concentrations (by about 45%) in the dorsal striatum in the Wt, M/+ and M/M mice as compared with their saline-treated controls (Table 5.6). Likewise, MPTP lowered striatal TH-immunostaining similarly in all three genotypes (by about 15-20 %). However, MPTP did not induce the loss of TH-positive cells in the substantia nigra pars compacta in any of the genotypes (Study III, Figure 3). Unlike in the Wt mice, we found no compensatory increase in striatal DOPAC/DA ratio in the MPTP-treated MEN2B mice as compared with their saline-treated controls (Table 5.6). No difference in the HVA/DA ratio among the genotypes was found.

Table 5.6. Summary of the effects of MPTP treatment on DA depletion, TH-immunostaining and DOPAC/DA ratio in the striatum of M/+ and M/M mice as compared with Wt mice

DA depletion	TH-immunostaining	DOPAC/DA ratio
↔	↔	$\downarrow^{*}$
↔	↔	$\downarrow^{*}$

↔ indicates that the response to MPTP was similar to the Wt mice, ↓ indicates 35-50% lower response of M/+ and M/M mice as compared with the Wt mice. $^{*}p < 0.05$ vs. Wt mice (one-way ANOVA and Student-Newman-Keuls *post hoc* test)

5.10 Effects of unilateral, striatal 6-OHDA on the dopaminergic system of MEN2B mice (Study III)

There was marked decrease in the striatal dopamine levels on the lesioned side of both Wt and M/M mice 3 weeks following unilateral, striatal 6-OHDA lesion. The 6-OHDA-induced DA depletion was substantial in mice of both genotypes. However, the degree of DA depletion was significantly smaller in the M/M mice (by about 73%) than in the Wt mice (by about 84%, Figure 5.9*A*). Morphometric analysis of TH-positive cells revealed that the M/M mice were much less affected by 6-OHDA than the Wt mice, as a significant loss of TH-positive cells in the substantia nigra pars compacta was found in the Wt mice (36%) while only a minor one was observed in the MM mice (9%, Figure 5.9*B*).

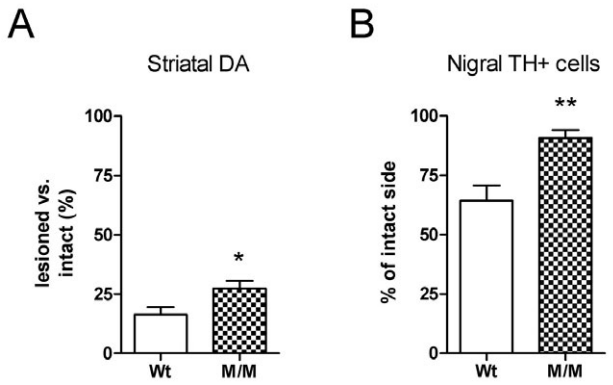

Figure 5.9*. The effects of intrastriatal, unilateral 6-OHDA lesion on DA levels in the striatum (A) and TH-positive cell number in the substantia nigra pars compacta (B) in M/M and Wt mice. The 6-OHDA-induced DA depletion in the striatum was less pronounced in the M/M than in the Wt mice. A significant decrease in TH-positive cells was found only in the Wt mice, whereas M/M mice were only slightly affected.*

In the M/M mice, the striatal DOPAC/DA ratio in the lesioned side was not augmented whereas in the control Wt mice the DOPAC/DA ratio in the lesioned side was increased 3-fold as compared with the intact side. Also, in the lesioned striatum of the Wt mice there was profound elevation of HVA/DA ratio (310%) whereas there was only a modest increase (137%) in the M/M mice (Study III, Figure 5). The number of amphetamine-induced turnings was increased in 6-OHDA-treated MEN2B mice by about 49% as compared with their 6-OHDA-lesioned Wt littermates (Study III, Figure 7).

6. DISCUSSION

6.1 The effects of constitutive Ret activity on brain DA concentrations, production and uptake of DA in the MEN2B mice

We found that tissue concentrations of DA and its metabolites in the target areas of brain dopaminergic systems were profoundly elevated in mice expressing a constitutively active Ret receptor (Study I). This is consistent with reports that exogenously administered GDNF increases DA levels and DA turnover in the striatum and SN of rodents (Hudson et al., 1995; Martin et al., 1996b; Lapchak et al., 1997b; Gash et al., 2005). Also, the levels of TH mRNA in SN/VTA and TH protein in the striatum and SN/VTA were increased in the MEN2B mice (Study I). This is in line with the outcome from a study showing that administration of GDNF promotes the transcription of the TH gene and the stability of TH mRNA (Xiao et al., 2002). In addition, GDNF treatment has been found to increase TH phosphorylation, enhancing its activity and increasing DA synthesis (Kobori et al., 2004; Salvatore et al., 2004). In contrast, after prolonged GDNF treatment of adult rats, no changes in DA concentrations were observed and mRNA and protein levels of TH were found to be decreased (Lu and Hagg, 1997; Rosenblad et al., 2003; Georgievska et al., 2004). This is thought to be a compensatory response of the already mature DA system to the initial stimulatory effects of exogenous GDNF on TH. In the MEN2B mice, on the other hand, Ret signaling is permanently activated from early development, when it is actively shaping the development of the DA system.

Increased TH levels suggested that elevated DA concentrations in the MEN2B mice are a consequence of increased DA production. We verified this assumption by blocking DOPA decarboxylase and inhibiting the conversion of DOPA to DA. The accumulation of DOPA after NSD-1015 treatment was found to be higher in the MEN2B mice than in the controls, and thus provided direct proof that TH activity and production of DA are indeed enhanced in the MEN2B mice (Study II).

We also found that the levels of DAT protein in the striatum were increased in the MEN2B mice (Study I). In accordance with the upregulation of DAT in the striatum, MEN2B mice were more sensitive to the behavioral effects of cocaine, a blocker of DAT. It has been shown that GDNF can increase dopamine uptake (Lin et al., 1993) and that Ret has an inducing effect on the expression of the DAT gene (Li et al., 2006; Li et al., 2009). These findings suggested that constitutive Ret activity in the MEN2B mice might have caused elevation of DAT levels in the nigrostriatal DAergic neurons in the first place. However, since the other brain DA systems that express much less or very little DAT, such as mesolimbic, mesocortical and tuberoinfundibular systems, have increased tissue DA levels and are apparently also affected by the Ret-MEN2B mutation, this is a less likely explanation. Thus, even though a direct effect of Ret-MEN2B mutation on DAT cannot be ruled out, it is more likely that constitutive Ret activity primarily affects TH and synthesis of dopamine and that increased DAT levels in the striatum in the MEN2B mice are probably a compensatory response to the increased DA levels.

6.2 The effects of constitutive Ret activity on development and maintenance of the nigrostriatal DA system in the MEN2B mice

We found that adult homozygous MEN2B mice had a higher number of TH-positive cells in the SNpc than the Wt mice, while no difference was found in the VTA. Previous studies on GDNF, GFRα1 and Ret knock-out mice suggested that GDNF-Ret signaling is not important for prenatal, embryonic development of nigral DA neurons (Marcos and Pachnis, 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enomoto et al., 1998). Also, our finding that MEN2B mice had normal brain DA levels at embryonic day 18 suggested that increased Ret signaling did not affect prenatal development of DA system. Thus, the increase in the number of SNpc TH-positive cells in the adult homozygous M/M mice is likely to arise during the postnatal development of the nigrostriatal DA system. GDNF has been shown to increase survival of postnatal DAergic neurons by suppressing apoptotic cell death (Oo et al., 2003), and overexpression of GDNF in the striatum of a transgenic mouse model resulted in an increased number of DA neurons in the SNpc surviving the first phase of natural cell death, although this increase in DA neurons is only maintained during adulthood in the VTA but not in the SN (Kholodilov et al., 2004). Thus, constitutive Ret activity in the MEN2B mice may have suppressed the physiological postnatal cell death and resulted in the increased number of DA neurons in adulthood. However, the normal levels of brain DA found in the MEN2B mice at postnatal day 7 suggest that increased Ret activity does not result in increased survival of DA neurons during the first phase of ontogenic cell death. Additionally, these data suggest a role for an alternative GDNF signaling receptor in the regulation of this early phase.

As majority of DA neurons appear during postnatal development, another possibility is that constitutively active Ret stimulates developing neurons to acquire a dopaminergic phenotype. Indeed, our data on brain DA levels at the different developmental stages revealed that constitutive Ret activity starts to have an effect on DA system of the MEN2B mice somewhere between postnatal days 7 and 16.

Deletion of GDNF in 2-month-old mice revealed a critical role for GDNF in maintaining adult DA neurons (Pascual et al., 2008). Thus, under the influence of constitutive Ret signaling, DA neurons display increased expression of DAergic markers such as TH and DAT and are stimulated to produce and accommodate more DA. Furthermore, because of increased neurotrophic support delivered by continuously active Ret, the DA neurons of the MEN2B mice are better preserved, which might have resulted in their increased number in adulthood. Taken together, our data suggest that GDNF supports DA neurons and maintains DAergic phenotype during postnatal life, perhaps via Ret signaling.

Interestingly, data obtained from conditional Ret knockout mice (Jain et al., 2006; Kramer et al., 2007) that expressed no Ret in the midbrain DA neurons starting from mid embryonic development suggested that Ret is not important for the establishment, development and maturation of the nigrostriatal DA system. Only after reaching one year of age did conditional Ret knockout mice exhibit progressive loss of midbrain DA neurons, degeneration of DA terminals and finally glial activation in the striatum (Kramer

et al., 2007). It is possible that deletion of Ret from DA neurons lead to the activation of compensatory mechanisms that resulted in the normal development and functioning of DA system. Thus, by using MEN2B mice we are able to avoid compensatory changes that mask the importance of Ret signaling for brain DAergic system.

6.3 Lack of the effects of constitutive Ret activity on DA fiber sprouting in the MEN2B mice

It has been shown that GDNF can induce DA fiber sprouting after injury (Åkerud et al., 1999; Kordower et al., 2000; Love et al., 2005) and Ret signaling is required for resprouting after MPTP treatment (Kowsky et al., 2007). Thus, we examined the possibility that increases in the levels of DA, TH and DAT in the striatum of MEN2B mice could be due to increased sprouting of DAergic fibers under the influence of constitutive Ret activity. We found about a 20% increase in the number of DAT-IR varicosities in the striatum of homozygous MEN2B mice (Study I). This increase in the number of DAT-IR punctate structures correlated with a 26% increase in the number of TH-positive cells in the SN. Therefore we concluded that no additional sprouting of DA neurons occurred because of constitutively active Ret in MEN2B mice. Also, in line with our finding, overexpression of GDNF in the target areas of mesencephalic DA neurons lead to the similar increases in the number of VTA DAergic neurons and their innervation of cortex (Kholodilov et al., 2004). Thus, increased Ret signaling is not sufficient to increase fiber sprouting under physiological conditions, but rather it supports repair of the DA fiber innervation in the striatum after injury (Kowsky et al., 2007).

6.4 The effects of constitutive Ret activity on noradrenergic system in the MEN2B mice

It has been found that the NA neuronal population expresses Ret receptor (Trupp et al., 1997; Glazner et al., 1998). Also, it has been shown that GDNF prevents 6-hydroxydopamine induced degeneration of NA neurons, promotes their phenotype *in vivo* (Arenas et al., 1995) and is important for the development of NA neurons in the LC-HIP pathway (Quintero 2004). In contrast to findings from conventional GDNF knockout mice, which suggest that GDNF does not promote the development and survival of LC NA neurons (Moore et al., 1996; Sanchez et al., 1996), a study using a conditional GDNF knockouts demonstrated that GDNF is essential for survival of adult LC NA neurons (Pascual et al., 2008). The concentration of NA was unaffected in target areas of LC noradrenergic neurons in the MEN2B mice (Study I), suggesting that constitutive Ret activity did not affect NA neurons. Also, adult conditional Ret knockout mice did not show loss of NA neurons in the LC (Kramer et al., 2007). Taken together, this may indicate that GDNF signaling via Ret is not involved in the development and maintenance of LC NA neurons, but that signaling receptors other than Ret may be important in the

regulation of the NAergic system. Nevertheless, it has been shown that A5 NA neurons in the lower brain stem are GDNF-responsive (Huang et al., 2005). This may explain moderately increased NA concentrations we found in the lower brain stem of homozygous MEN2B mice (Study I).

6.5 Striatal DAergic neurotransmission in the MEN2B mice

Numerous studies have proven that DAergic neurotransmission is under tight homeostatic control. Thus, the increases in the synthesis and tissue concentration of DA observed in the MEN2B mice imply that there are complex compensatory alterations occurring in DAergic transmission. Therefore, we investigated different aspects of DAergic transmission such as storage, release and re-uptake of DA in the nigrostriatal system of these mice.

Firstly, we found increased responses of the MEN2B mice to the effects of α-MT and tetrabenazine, suggesting that releasable (αMT–sensitive) and storage pools of DA (reserpine/tetrabenazine-sensitive) are larger in these mice. In other words, the data suggest that DAergic neurons in the MEN2B mice are very capable of accommodating large amounts of DA.

It has been shown that augmented DA synthesis and VMAT2 activity lead to increased DA release (Pothos, 2002). Therefore, we expected to find increased release of DA and higher extracellular DA concentrations in the MEN2B mice. However, in our microdialysis studies, no difference in basal concentration of extracellular DA was found, nor was there any difference in the K^+-evoked DA output between the genotypes (Study II). DAT provides one of the most efficient and important mechanisms to rapidly clear the released DA from extracellular space. Thus, increased striatal DAT levels in parallel with the stronger behavioral response to cocaine in the MEN2B mice compared to the Wt mice (Study I), suggested that increased DA uptake fully compensates for augmented DA release in the striatum. However, we were unable to confirm this since cocaine, a DAT blocker, similarly increased extracellular DA in striatal microdialysates in all genotypes. In addition to DAT, D_2-type dopamine autoreceptors control extracellular DA levels through the regulation of DA synthesis, DA release and neuronal activity of DAergic neurons. However, no differences in the effect of D2-receptor antagonist, haloperidol, on the DA concentrations in striatal microdialysates were found, indicating that the function of D_2 autoreceptors controlling the release/neuronal activity of DA is not altered in the MEN2B mice.

Because microdialysis is rather ineffective (or too slow) to identify the compensatory mechanism that regulates DAergic neurotransmission in MEN2B mice, we employed *in vivo* voltammetry, a method with high temporal resolution used for investigation of rapid events associated with neurotransmission. Indeed, data obtained with *in vivo* voltammetry (increased DA peak overflow and faster clearance of DA from extracellular space in the MEN2B mice) directly shows that both the release of DA and uptake of DA are increased in these animals (Study II). Taken together, our data indicate that enhanced synthesis of DA in the MEN2B mice is accompanied by larger storage of DA resulting in elevated

tissue DA concentrations in presynaptic DAergic terminals in striatum. These changes further resulted in greater release of DA, which is counteracted by higher DAT activity.

6.6 Locomotor activity of the MEN2B mice

Enhanced DAergic transmission is linked to motor hyperactivity. However, over a 24-h period, the MEN2B mice traveled distances similar to those of the Wt mice (Study I). We speculate that 24-h locomotion of MEN2B mice is not altered due to the substantial compensation in their DAergic transmission. Also, this finding suggested that there are no major deficits in the sensorimotor system of the MEN2B mice. The finding that MEN2B mice were more sensitive and responded more strongly to the stimulatory effects of cocaine than the Wt mice is in line with increased levels and activity of DAT. Thus, since DAergic ending in the striatum in the MEN2B mice are loaded with DA, blocking DAT should lead to greater DA output and subsequently increased hyperlocomotion. The cause of reduced exploratory activity of MEN2B mice remains unclear.

6.7 The effects of toxins on DAergic system of MEN2B mice - role of the constitutive Ret activity in the neuroprotection

Numerous studies *in vitro* and in animal models of PD have shown that GDNF is a very potent neurotrophic factor that protects DA neurons against neurotoxic injury. However, it is not known which of the neuroprotective properties of GDNF can be ascribed to signaling via Ret. Therefore, the next objective of the present work was to try to clarify the role of Ret in neuroprotection of the nigrostriatal DAergic system. The effects of the neurotoxins, 6-OHDA and MPTP, on the DAergic system of MEN2B mice were studied in order to examine whether continuous Ret signaling could protect DA neurons.

The 6-OHDA treatment only slightly affected the MEN2B mice, whereas it caused significant loss of TH-positive cell bodies in the substantia nigra pars compacta in the Wt mice (Study III). This indicates an increased resistance of DAergic neurons that are under influence of permanent Ret activity to neurotoxic insult. Sustained Ret activity probably slows down retrograde progression of the damage, leaving cell bodies in the substantia nigra mostly unaffected by 6-OHDA, and/or effectively rescues damaged TH-positive cells from dying. Our data are in agreement with previous studies showing that exogenously provided GDNF *per se* is neuroprotective against toxins (Tomac et al., 1995; Kordower et al., 2000; Schober et al., 2007). On the other hand, loss of physiological Ret signaling was shown not to increase the sensitivity against MPTP (Kowsky et al., 2007). Taken together, these data suggest that only elevated GDNF/Ret signaling is able to protect nigral DA neurons from neurotoxins, whereas physiological GDNF/Ret signaling is insufficient to do so.

While the number of TH-positive nigral cell bodies in the MEN2B mice was practically unaffected by 6-OHDA toxicity (showing only a 9% DA cell loss), the toxin

profoundly affected the DAergic terminals in the striatum, as revealed by marked DA depletion (73%). Also, we found that MPTP similarly reduced striatal DA concentration in Wt and MEN2B mice (Study III). Thus, effects of both 6-OHDA and MPTP in the striatum of MEN2B mice suggest that the survival-supporting effect of constitutively active Ret signaling does not occur at the level of DAergic terminals but most likely in the cell body. This view is supported by the findings that application of the GDNF protein into the substantia nigra leads to the protection of only the DAergic cell bodies whereas its delivery into the striatum leads to preservation of the entire nigrostriatal pathway (Tomac et al., 1995; Connor et al., 1999; Kirik et al., 2000a; Kirik et al., 2000b) . In addition, it has been proposed that neurotrophic factors may elicit different effects in relation to axonal growth and survival when applied locally at the axon or cell body (Kimpinski et al., 1997; Watson et al., 2001).

Previous studies have shown that GDNF does protect axonal terminals against 6-OHDA and MPTP (Tomac et al., 1995; Kirik et al., 2000a; Kirik et al., 2000b) Therefore, the lack of protection of the DA terminals by constitutively active Ret in the MEN2B mice suggests that Ret-independent GDNF signaling events might be involved in the neuroprotective effects of GDNF against toxin-induced degeneration of striatal DA terminals. Also, differences in the signaling properties between MEN2B and wildtype Ret could explain lack of protection of striatal DAergic endings in the MEN2B mice.

It should be noted that we did not examine the DAergic system of toxin-treated MEN2B mice at a later time point, and thus did not study the effects of constitutive Ret signaling on regeneration of the DAergic system. Therefore, our findings do not rule out a possible role for Ret signaling in the regeneration of injured DAergic fibers, as shown by Kowsky and co-workers (2007).

6.8 Increased DAergic transmission and vulnerability of the DA system in the MEN2B mice

There are reports suggesting that dopamine itself is toxic and may cause neuron degeneration (Ben-Shachar et al., 1995; Fumagalli et al., 1999; Rabinovic et al., 2000; Chen et al., 2008). Also, both MPTP and 6-OHDA enter DA neurons through DAT, which is critical for their neurotoxicity (Gainetdinov et al., 1997; Blum et al., 2001). Thus, high tissue concentrations of DA and increased DAT activity in the striatum of the MEN2B mice (Mijatovic et al., 2007; Mijatovic et al., 2008) could theoretically lead to increased vulnerability of DA neurons and even to increased sensitivity to neurotoxins. However, our striatal DA-depletion data show that increased tissue DA levels or uptake rate are not detrimental in MEN2B mice.It has been shown that VMAT2 plays an important role in neurotoxin sequestration into vesicles (Takahashi et al., 1997; Gainetdinov et al., 1998). Thus, increased dopamine transporter activity is probably balanced by increased VMAT2 activity we previously found in the MEN2B mice (Mijatovic et al., 2008). However, we cannot completely rule out the possibility that the extent of the striatal lesion may have initially been larger in the MEN2B mice, and subsequently counteracted by neurotrophic support of increased Ret activity.

The lack of a compensatory increase in metabolite/DA ratios that we found in the MEN2B mice after administration of neurotoxins could mean that DA terminals in MEN2B mice are capable of maintaining DAergic neurotransmission without additionally increasing their activity. Alternatively, it might suggest the inability of maximally activated DA neurons to further increase their activity.

Paradoxically, we found an increase in the number of amphetamine-induced rotations in the MEN2B mice compared to the Wt mice (Study III). The number of ipsilateral rotations after amphetamine challenge is not an accurate indicator of higher degree of the lesion. We speculate that amphetamine-induced DA release in the intact side of the MEN2B mice probably exceeds that in the Wt mice, thus exaggerating the DAergic imbalance between lesioned and intact sides in the MEN2B mice more than in the wild-type mice and finally resulting in increased rotational behavior in the MEN2B mice. Also, lack of a compensatory increase in the DAergic activity in the lesioned striatum of the MEN2B mice might contribute to larger imbalance between the sides.

7. CONCLUSIONS

Generally, this study showed that constitutive Ret signaling in mice supports and protects brain DA neurons and promotes DAergic phenotype, thus endorsing Ret as an important signaling receptor for GDNF in the nigrostriatal DAergic system.
The more specific conclusions drawn from the results of the present study are:

1. Continuous activity of Ret in the MEN2B mice leads to profound increase in brain DA levels and increase in the number of DA neurons in the SNpc. Constitutive Ret activity also stimulates synthesis of DA, as revealed by increased levels and enhanced activity of TH, the rate-limiting enzyme of DA synthesis. The effects of constitutive Ret signaling on the brain DA system start during the postnatal life and persist during adulthood.

2. Augmented DA production in the MEN2B mice is accompanied by its increased storage in the axonal ending in the striatum. This further leads to enhanced DA release, which is compensated for by increased uptake of DA.

3. Constitutive Ret activity in the MEN2B mice provides protection against neurotoxins only to the DA cell bodies in the substantia nigra pars compacta but not to the axon terminals in the striatum.

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