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GDNF/RET signalling in regulation of brain dopaminergic systems: significance for drug addiction

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ACADEMIC DISSERTATION

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ABSTRACT

Brain dopaminergic pathways are involved in the regulation of motor functions as well as processing of reward information. Glial cell line-derived neurotrophic factor (GDNF) has been shown to be a potent neurotrophic factor for the brain dopaminergic systems. Biological actions of GDNF are mediated by a receptor complex composed of the GDNF family receptor alpha (GFRα) 1 and signalling tyrosine kinase receptor RET. GDNF is an important candidate for drug targeting in Parkinson's disease, as it has been shown to protect and promote recovery of brain dopaminergic neurons. Almost all drugs of abuse increase dopamine release in mesolimbic and nigrostriatal dopaminergic pathways, and the dopaminergic systems are involved in the formation of associations between contextual stimuli and rewarding or aversive events. As brain dopaminergic pathways are important for perceiving drug reward, and as they undergo plastic changes during chronic drug use, interactions between GDNF and responses to abused drugs are of interest.

This study investigated the role of endogenous GDNF in the regulation of nigrostriatal and mesolimbic dopaminergic neurotransmission. The effects of acutely and repeatedly administered cocaine and morphine on striatal dopamine release, on behavioural sensitization and on drug reward were studied in heterozygous GDNF knockout mice. In addition, this study explored the effects of constitutive RET tyrosine kinase receptor activity on brain dopaminergic systems and on the locomotor enhancing effects of cocaine in mice.

Unexpectedly, it was found that extracellular dopamine concentrations were increased in striatal brain areas in heterozygous GDNF knockout mice. This was further supported by a clear increase in the number of FosB/ΔFosB positive nuclei in the caudate/putamen and nucleus accumbens in these mice. Thus, the present results indicate that dopaminergic transmission is increased in mice with reduced GDNF levels. In addition, heterozygous GDNF+/- mice were more sensitive to morphine's dopamine releasing effect, and reduced GDNF levels were associated with a shift in the bell-shaped dose-response curve of morphine to the left. Interestingly, it was found that after repeated morphine and cocaine treatment, the extracellular dopamine concentrations in the GDNF+/- mice were decreased to a level similar to their wild-type littermates. In addition, it was found that reduced GDNF levels are involved in a more rapid development of tolerance to locomotor enhancing effects of daily 30 mg/kg morphine injections, and in increased sensitivity to locomotor sensitization by a 5 mg/kg morphine challenge dose. Furthermore, the present results show that constitutive RET activity, caused by a single point mutation Met919Thr, robustly increased dopamine concentrations in the brain, whereas noradrenaline or serotonin concentrations were not affected. Increased dopamine concentrations were associated with increased tyrosine hydroxylase protein levels, indicating that dopamine synthesis is increased in these mice. An important finding was that increased RET activity increased the number of nigrostriatal dopamine neurons in the adult mice. In addition, acute cocaine administration increased the locomotor activity more in mice with increased RET activity than in their wild-type littermates.

All in all, the present findings emphasize the important role of GDNF/RET-signalling in the regulation of brain dopaminergic systems. These results suggest that reduced brain GDNF levels alter the response of dopaminergic systems to morphine and the results further substantiate the importance of RET tyrosine kinase as a signalling receptor of GDNF in the dopaminergic system.

ABBREVIATIONS

ANOVA	analysis of variance
ARTN	artemin
CNS	central nervous system
COMT	catechol-O-methyltransferase
CPP	conditioned place preference
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
FMTC	familial medullary thyroid carcinoma
GABA	γ-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GDNF+/-	heterozygous GDNF knockout mice
GFRα	GDNF family receptor alpha
5-HIAA	5-hydroxyindoleacetic acid
HVA	homovanillic acid
i.p.	intraperitoneal
L-DOPA	L-hydroxyphenylalanine
MAO	monoamine oxidase
MEN 2	multiple endocrine neoplasia type 2
MEN2B/+	heterozygous MEN2B mice with Met919Thr mutation
MEN2B/MEN2B	homozygous MEN2B mice with Met919Thr mutation
3-MT	3-methoxytyramine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NCAM	neural cell adhesion molecule
NRTN	neurturin
6-OHDA	6-hydroxydopamine
PSPN	persephin
RET	rearranged during transfection
s.c.	subcutaneous
SEM	standard error of the mean
SN	substantia nigra
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
TH	tyrosine hydroxylase
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
Wt	wild-type littermates

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, herein referred by their Roman numerals (I-IV):

I. Airavaara M, Planken A, Gäddnäs H, Piepponen TP, Saarma M, Ahtee L (2004) Increased extracellular dopamine concentrations and FosB/deltaFosB expression in striatal brain areas of heterozygous GDNF knockout mice. European Journal of Neuroscience 20: 2336-2344

II. Airavaara M, Mijatovic J, Vihavainen T, Piepponen TP, Saarma M, Ahtee L (2006) In heterozygous GDNF knockout mice the response of striatal dopaminergic system to acute morphine is altered. Synapse 59: 321-329

III. Airavaara M, Tuomainen H, Piepponen TP, Saarma M, Ahtee (2006) Effects of repeated morphine on locomotion, place preference and dopamine in heterozygous GDNF knockout mice. Genes, Brain and Behavior. Epub ahead of print, July 2006

IV. Mijatovic J *, Airavaara M*, Planken A, Auvinen P, Raasmaja A, Piepponen TP, Costantini F, Ahtee L, Saarma M (2006) Constitutive Ret activity in knock-in MEN2B mice induces profound elevation of brain dopamine concentration *via* enhanced synthesis and increases number of TH-positive cells in substantia nigra. Submitted
* equal contribution

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1. INTRODUCTION

Addiction to drugs of abuse is a major social and health care problem world-wide. In the United States and Western Europe, epidemiology surveys report that there are about 30 million individuals with drug or alcohol addiction (Pouletty, 2002). The economic costs of substance abuse are high. The cost of heroin addiction in the United States in 1996 was estimated to be US$21.9 billion, of which 53% was productivity loss, 24% criminal activities, 23 % medical care and only 0.1% social welfare costs (Mark et al., 2001). The economic costs of all abused drugs in the United States in 2002 was estimated to be US$180.9 billion of which 71.2% was loss of productivity (Office of National Drug Control Policy, 2004). To develop new drugs for addiction disorders, researchers face the important challenge of understanding the cellular and molecular mechanisms in the development of addiction. Novel approaches must be explored to find new treatment strategies. Neurotrophic factors are involved in plastic changes in the central nervous system (CNS). They may be involved in long-term responses to drug exposure, and are the focus of the studies reported upon herein.

Neurotrophic factors are endogenous proteins that have been studied for their role in neuronal growth, survival and differentiation during development. Nerve growth factor (NGF) was the first neurotrophic factor, identified by Rita Levi-Montalcini, Viktor Hamburger and Stanley Cohen in the 1950s (Levi-Montalcini, 1987). In the beginning of the 1990s, glial cell line-derived neurotrophic factor (GDNF) was purified from a rat glioma cell-line supernatant and was found to promote survival of mesencephalic dopamine neurons *in vitro* (Lin et al., 1993). Since then, the effects of GDNF on brain dopaminergic systems have been extensively studied, and it is an important candidate for drug targeting in Parkinson's disease (for review, see Kirik et al., 2004). Brain dopaminergic pathways are critically involved in drug reward and undergo changes during chronic drug use. GDNF has, therefore, been of interest also for treatment of drug addiction and there are indications that GDNF, administered to the brain, may be able to reduce the biochemical and behavioural changes induced by drugs of abuse (Green-Sadan et al., 2003; Green-Sadan et al., 2005; He et al., 2005; Messer et al., 2000). Thus, GDNF and its signalling pathway might be novel targets for developing drugs for addictive disorders.

Drug addiction is a complex disorder of the brain and has a variety of causes. Vulnerability to addiction is increased by certain genetic factors, environmental influences and by drug-induced changes in the brain (Kreek et al., 2005). Addiction is a behavioural response to the biochemical actions of drugs of abuse in the brain and its development requires repeated drug exposure. Addictive drugs alter behaviour, and casual drug use may progress to addiction. Transition from casual to compulsive patterns of drug use is thought to have its basis in the neural processes mediating drug-seeking behaviour (Robinson and Berridge, 2003). The transit from use to addiction involves many drug-induced changes in the brain and corresponding behavioural adaptations. The activation of dopaminergic pathways in the brain is considered to play a central role in the rewarding effects of drugs of abuse, and most importantly, the brain dopaminergic systems are involved in learning processes, such as in the formation of connections between contextual stimuli and rewarding or aversive events (Spanagel and Weiss, 1999).

The objectives of the present experiments were to study the involvement of GDNF/RET-signalling in the function and regulation of cerebral dopaminergic systems and its role in the development of drug addiction. The research aimed to clarify the physiological role of endogenous GDNF in the regulation of brain dopaminergic systems. The studies also explored how reduced endogenous GDNF levels are involved in the effects of acute and repeated administration of cocaine and morphine. Also, the studies explored the common GDNF-family ligand RET tyrosine kinase receptor and how its constitutive activity affects cerebral dopaminergic systems. This research was based on the hypothesis that reduced GDNF-RET-signalling will lead to similar changes in the brain, that are seen following repeated exposure to drugs of abuse, which will lead to a sensitization to the effects of cocaine or morphine. Alternatively, we hypothesized that the constitutive RET signalling and activity protects and maintains brain dopaminergic systems.

2. REVIEW OF THE LITERATURE

2.1. Brain dopaminergic systems

In the late 1950s, Carlsson and co-workers found that dopamine is an independent neurotransmitter in the brain and not just a precursor in the synthesis of adrenaline and noredrenaline (Carlsson et al., 1957; Carlsson et al., 1958; Carlsson, 1959). Dopamine is involved in many CNS functions including locomotor behaviour, emotions, congnition and endocrine regulation.

As shown in Figure 2.1, the cell bodies of the neurons forming major ascending dopaminergic pathways are located in the ventral midbrain, in the subtantia nigra pars compacta (SNpc, A9) and in the ventral tegmental area (VTA, A10). The A9 neurons project mainly to the caudate/putamen, also known as dorsal striatum, forming the nigrostriatal dopaminergic pathway (Fuxe et al. 1985). The A10 neurons project mainly to the ventral striatum (e.g. nucleus accumbens, and olfactory tubercle) and other limbic regions, such as amygdale, and hippocampus forming the mesolimbic dopaminergic pathway (Fuxe et al. 1985). The nucleus accumbens can be divided into two subdivisions: the shell and the core. The latter is surrounded on its medial and ventral sides by the shell (de Olmos and Heimer, 1999). The core is similar to the rest of the striatal complex, whereas the shell is similar to and a continuum of the extended amygdala. The extended amygdala is comprised of the shell of the nucleus accumbens, the bed nucleus of stria terminalis and the central nucleus of amygdala (Heimer et al., 1991). Also the mesocortical dopaminergic pathway originates from the A10 area. This pathway projects to limbic cortical areas, e.g. the medial prefrontal cortex, cingulate cortex and entorhinal areas.

In addition to the long ascending pathways described above, there are local ultrashort and intermediate length central dopaminergic pathways. Intermediate length central dopaminergic pathways include the tuberoinfundibular system, which originates from arcuate and periventricular nuclei (A12) and projects to median eminence and neurointermediate lobe of the pituitary. This tuberoinfundibular system is involved in the regulation of pituitary hormone release, particularly prolactin (for review, see Tuomisto and Männistö, 1985). Ultrashort systems include interplexiform amacrine-like neurons, which originate from the A17 area in the retina, and periglomerular dopamine cells, which originate from the A16 area in the olfactory bulb (Fuxe et al. 1985). This thesis is focused only on long ascending dopaminergic pathways described above.

The nigrostriatal pathway is involved in the control of motor behaviour, control of posture, maintaining automatic motor functions and learning of habits. Loss of striatal dopamine by destruction of nigrostriatal dopaminergic neurons causes extrapyramidal symptoms and signs of Parkinson's disease (Carlsson, 1959). The mesolimbic and mesocortical dopaminergic systems are involved in the control of motivation, emotions and reward, as well as in the control of motor behaviour. These two systems mediate the reinforcing actions of both natural rewards and abused drugs, such as cocaine, amphetamine, opioids and alcohol, which increase extracellular dopamine concentrations in the nucleus accumbens (Di Chiara and Imperato, 1988; Spanagel and Weiss, 1999). In addition, there is evidence that schizophrenia is associated

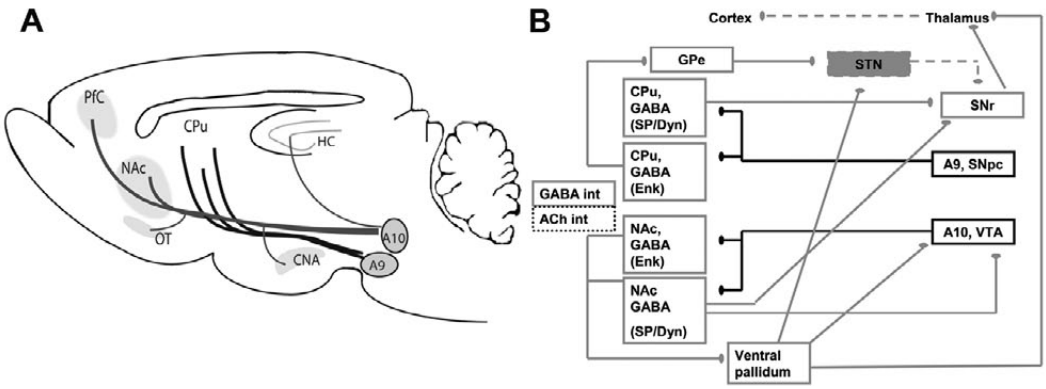

Figure 2.1. (A) Schematic representation of the ascending dopaminergic pathways originating from the substantia nigra pars compacta (SNpc, A9) and the ventral tegmental area (VTA, A10). (B) Simplified presentation of postsynaptic neurons in the striatum and projections of the medium-sized spiny GABAergic projection neurons. Continuous black lines represent dopaminergic pathways, grey lines represent GABAergic pathways, grey broken lines represent glutamatergic pathways. PfC= prefrontal cortex, NAc= nucleus accumbens, OT= olfactory tubercle, CPu= caudate putamen, CNA= central nucleus of amygdala. GPe= external segment of globus pallidus, STN= subthalamic nuclues, SNr= substantia nigra pars reticulata, SP= substance P, Dyn=dynorphin, enk=enkephalin, GABA int = aspiny GABAergic interneuron, Ach int= cholinergic interneuron. A is modified from Fuxe et al. 1985.; B from Alexander and Crutcher, 1990; Heimer et al., 1991; Kalivas et al., 1999; Zhou et al., 2003.

with abnormalities in the mesolimbic system (Carlsson et al., 2001). The hypothesis of excessive dopaminergic activity in limbic brain regions is based on the therapeutic effects of classical antipsychotic drugs, which block dopamine D2-like receptor, and that dopamine releasing drugs, such as amphetamine, can cause psychosis.

The vast majority of striatal neurons are medium-sized spiny projection neurons (Harlan and Garcia, 1998). They contain γ-aminobutyric acid (GABA) and can be subclassified according to the neuropeptides they produce. Approximately 50-60% express preproenkephalin which encodes a proenkephalin, which is cleaved into the active opioid neuropeptides methionine enkephalin and leucine enkephalin. A smaller proportion of medium-sized spiny projection neurons express preprotachykinin, which encodes the precursor of the neuropeptides substance P and neurokinin A. Many of these neurons also express preprodynorphin, which is the precursor of dynorphins A and B. Enkephalins and dynorphin are endogenous opioid peptides. They signal through μ-, δ- and κ-type opioid receptors. In the striatum about 90% of neurons have been classified as medium-sized spiny projection neurons and about 10% have been identified as aspiny interneurons, of which 1% can be classified as cholinergic and majority as GABAergic interneurons (Kawaguchi et al., 1995). GABAergic interneurons are local neurons that express, among others, calcium-binding proteins or neuronal nitric oxide synthase.

As shown in Figure 2.1, efferent GABAergic neurons project from the

dorsal striatum to the substantia nigra pars reticulata (SNpr) directly or indirectly *via* the external segment of the globus pallidus and subthalamic nucleus (Alexander and Crutcher, 1990). From the SNpr, there are projections to the thalamus, from which there are projections to the dorsal striatum and cortical areas. These projections constitute a cortico-striatal-pallido-thalamic loop. This circuitry is involved in the regulation of extrapyramidal motor processes. Efferent GABAergic projection neurons from the core and the shell of the nucleus accumbens differ. Both project to the ventral pallidum, but with a distinct topography. The core projection is located in the dorsolateral part of the ventral pallidum, whereas the shell projection is located in the medial part of the subcommissural ventral pallidum (Heimer et al., 1991). The projections from nucleus accumbens subdivisions to the ventral midbrain are also different, as the ones from the core project mainly to the substantia nigra (SN), while those from the shell project to the VTA (Heimer et al., 1991).

Besides dopaminergic inputs, the striatum is regulated by glutamatergic inputs. The dorsal striatum receives glutamatergic afferents from the entire neocortical area, whereas the nucleus accumbens receives glutamatergic afferents from several brain regions including prefrontal cortex, basolateral amygdala, hippocampus and thalamus (West et al., 2003). The main targets of glutamatergic inputs in the striatal area are the GABAergic medium-sized spiny projection neurons. In addition, glutamatergic and dopaminergic transmission interacts *via* pre- and postsynaptic mechanisms as a functionally related network to control striatal output (Descarries et al., 1996).

2.1.1. Synthesis and metabolism of dopamine

Dopamine and other catecholamines are synthesised from the amino acid tyrosine (Figure 2.2.). Tyrosine is first converted to L-dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme tyrosine hydroxylase (TH, Carlsson and Lindqvist, 1978; Levitt et al., 1965). Then L-DOPA is rapidly converted to dopamine by aromatic L-amino acid decarboxylase. Synthesized dopamine is taken by vesicular monoamine transporter (VMAT) and stored in nerve terminal vesicles or metabolized by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetic acid (DOPAC), which diffuses out of the neurons. From synaptic vesicles, dopamine is released in a calcium-dependent manner when an action potential reaches the nerve terminal. Most of the released dopamine is taken up into the dopaminergic neurons by the membrane dopamine transporter (DAT). Synaptic dopamine can interact with its receptors, and in addition, it has been suggested that there is another uptake system ($uptake_2$) located in the cell membranes of glial cells and possibly in the cell membranes of postsynaptic cells (Trendelenburg, 1989; Wilson et al., 1988). In the postsynaptic neurons or in the glial cells dopamine is converted into methoxytyramine (3-MT) by catechol-O-methyltransferase (COMT). COMT is found in soluble and membrane bound forms (Lundström et al., 1991; Salminen et al., 1990) and in the striatum COMT is found in the postsynaptic neurons, astro glial cells and also in the microglial cells (Kaakkola et al., 1987; Karhunen et al., 1995; Reenilä et al., 1997). In addition, dopamine is converted by MAO into DOPAC and, furthermore, 3-MT and DOPAC are converted into homovanillic acid (HVA, Figure 2.2). Both isoforms of MAO, MAO_A

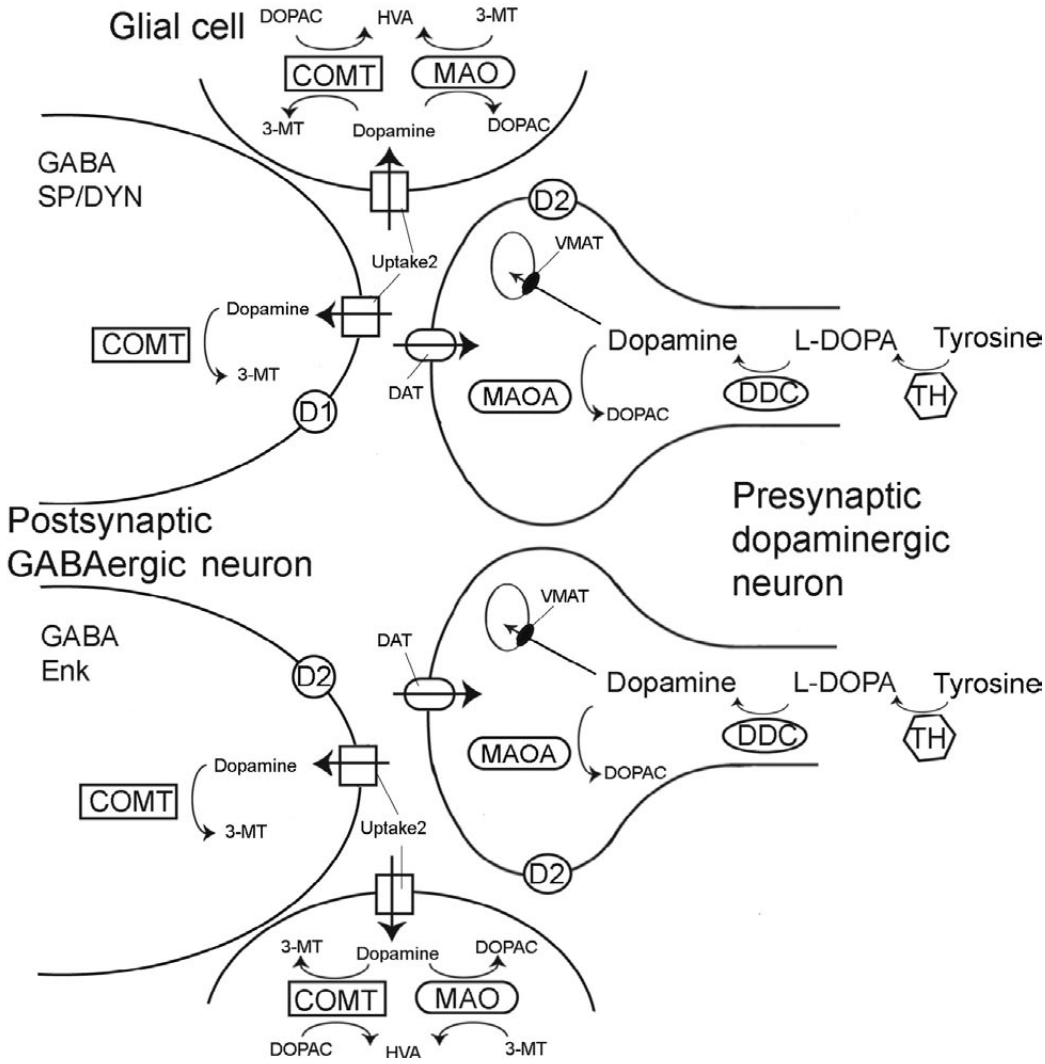

Figure 2.2. Schematic model of dopaminergic synapse and dopamine metabolism. Dopamine is synthesized from amino acid tyrosine. Tyrosine is first converted to L-DOPA by the rate limiting enzyme tyrosine hydroxylase (TH), and thereafter, L-DOPA is converted into dopamine by L-amino acid decarboxylase (DDC). In the nerve terminals, dopamine is stored in vesicles by vesicular monoamine transporters (VMAT) or metabolized by monoamine oxidase (MAO_A) to 3,4-dihydroxyphenylacetic acid (DOPAC). Synaptic dopamine may interact with pre- or postsynaptically located dopamine receptors. Most of the released dopamine is taken up by dopamine transporters (DAT) into presynaptic dopaminergic cells, but dopamine is also taken up by uptake systems associated with glial or postsynaptic cells (uptake2). Homovanillic acid (HVA) is metabolized sequentially from DOPAC or 3-methoxytyramine (3-MT) by catechol o-methyltransferase (COMT) and MAO, respectively. Dopamine D1 receptors are preferentially expressed in GABAergic neurons co-expressing substance P (SP) and dynorphin (DYN) whereas dopamine D2 receptors are present on dopaminergic terminals (autoreceptors) and preferentially on postsynaptic GABAergic neurons expressing enkephalins (enk). Figure modified from Törnwall 1994; Youdim et al. 2006.

and MAO_B, are involved in metabolism of dopamine and they are located intracellularly in the outer membrane of mitochondria (for review, see Youdim et al., 2006). In the striatam, MAO_A is found mainly in dopaminergic neurons and in glial cells and MAO_B is found found mainly in glial cells. In mice, dopamine is metabolized by MAO_A under basal conditions and by both MAO_A and MAO_B at high dopamine concentrations (Fornai et al., 1999). In humans and in primates, dopamine is also metabolized by both MAO forms (Di Monte et al., 1996; Glover et al., 1977).

2.1.2. Dopamine receptors

Dopamine receptors belong to the family of seven transmembrane domain G-protein coupled receptors and they are classified as D1- and D2-like receptors (for review, see Missale et al., 1998). The D1-like family contains D1 and D5 receptors and the D2-like family contains D2, D3 and D4 receptors. The activation of D1-like receptors increase adenylate cyclase activity whereas the activation of D2-like family receptors decrease adenylate cyclase activity, suppress Ca^{2+} currents and increase K^+ currents.

D1 receptors are the most widely expressed dopamine receptors in the brain. In the dopaminoceptive regions, they are highly expressed in caudate/putamen, nucleus accumbens, olfactory tubercle and amygdale. In the striatum, D1 receptors are located mainly on GABAergic neurons co-expressing substance P (Gerfen et al., 1990). Compared to D1 receptors, D5 receptors are much less widely expressed in the rat brain. D5 receptor mRNA is mainly expressed in forebrain regions. D2 receptors are widely expressed in the brain, overlapping with the distribution of D1 receptors. D2 receptors are highly expressed postsynaptically in the caudate/putamen, nucleus accumbens and in the olfactory tubercle, where they are mainly expressed in GABAergic medium-sized spiny projection neurons coexpressing enkephalins (Le Moine et al., 1990). In the nucleus accumbens, D2 receptors are mainly expressed in the core of the nucleus accumbens and only in restricted parts of the shell (Diaz et al., 1994). D3 receptors are absent from the dorsal striatum and are mainly expressed with high density in the shell of the nucleus accumbens on GABAergic medium-sized spiny projection neurons co-expressing neurotensin mRNA (Diaz et al., 1994).

Besides their postsynaptic localization, D2 receptors are also located presynaptically on dopamine neurons and referred to as autoreceptors. Dopamine autoreceptors in nerve terminals modulate synthesis and release of dopamine whereas autoreceptors located in somatodendritic regions modulate neuronal firing. Somatodendritically released dopamine (Cheramy et al., 1981) from the SNpc can either act on somatodendritic D2 autoreceptors or postsynaptically on D1 receptors in the SNpr. In SNpr, D1 receptors are on GABAergic cells and they control GABA release, which has an inhibitory effect on dopaminergic neurons. As D1 receptors are located mainly extrasynaptically (Caille et al., 1996), it has been proposed that more massive striatal dopamine output is needed for their activation than for that of D2 receptors.

2.1.3. Electrophysiological properties of dopaminergic neurons

Dopaminergic neurons originating from SNpc or from VTA exhibit two main patterns

of activity, single spike firing and burst firing. Dopamine release can be divided into high-amplitude transient phasic dopamine release mediated by burst firing of dopaminergic neurons and constant low-level tonic dopamine release regulated by baseline firing (Grace and Bunney, 1984a; Grace and Bunney, 1984b). The bursting mode leads to a much larger dopamine release than in the single spike mode (Gonon, 1988).

2.2. Neurotrophic factors and development of dopamine neurons

Neurotrophic factors are secreted proteins that bind to and activate their specific receptors on the cell surface. The classical neurotrophic factor concept states that limited amounts of available neurotrophic factors regulate the number of developing neurons (Levi-Montalcini, 1987). Neurotrophic factors are secreted by target tissues, are taken up by nerve terminals, and are transported in a retrograde manner to the cell bodies. Those neurons that receive survival-promoting neurotrophic proteins maintain synaptic connections with their neighbouring cells, while those that do not receive neurotrophic factors are not able to make synaptic connections and die largely by apoptosis. Originally, the neurotrophic factor hypothesis was proposed for the peripheral nervous system and it has been more recently adapted for the CNS. However, it is likely that in the CNS, the signalling cascades regulating cell survival are more complex. In the CNS, several neurotrophic factors may be involved in the regulation of certain neuronal phenotypes, and trophic support is delivered from diverse sources rather than only from the target. Besides regulating the number of neurons, neurotrophic factors have also been proposed to be involved in several aspects of neuronal functions, such as neurite branching, synaptogenesis, synaptic plasticity and electrophysiological properties (for review, see Sariola and Saarma, 2003). During the past several years, neurotrophic factors have been postulated as novel treatment strategies for diseases in which neuronal function is disturbed, such as spinal cord injury, neurodegenerative disorders (Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis), neuropathic pain, stroke and drug addiction.

In order to promote survival of dopaminergic neurons during maturation, the expression of neurotrophic factors should be detectable during critical periods of development. It has been suggested that dopaminergic neurons in the SN of rat undergo biphasic cell death during development, at postnatal days 2 and 14 (Oo and Burke, 1997). Thus, it is likely that neurotrophic factors regulate the number of dopaminergic neurons. Five established neurotrophic factors may be considered as a target-derived neurotrophic factor for dopaminergic neurons: Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), brain-derived neurotrophic factor (BDNF), neurotrophin-4 and fibroblast-growth factor-2 (FGF-2) (Krieglstein, 2004).

2.3. The GDNF family of neurotrophic factors

GDNF was identified from a rat glial cell line (B49) by its ability to increase dopamine uptake and to promote survival of dopaminergic neurons in embryonic midbrain cultures (Lin et al., 1993). GDNF is a glycosylated and disulfide-bonded protein having seven cysteine residues with the same

relative locations as the other members of the transforming growth factor (TGF) -β superfamily (Lin et al., 1993). Although it belongs to the TGF-β superfamily, its amino-acid sequence homology as compared with other members of the family is low. GDNF is synthesized in a prepro-form of 211 amino acids, and the mature protein consists of 134 amino acids. The GDNF-family consists of GDNF, neurturin (NRTN) (Kotzbauer et al., 1996), persephin (PSPN) (Milbrandt et al., 1998) and artemin (ARTN) (Baloh et al., 1998). All the GDNF-family ligands are basic and dimeric molecules and they are synthesized, secreted and activated by a variety of tissues in a prepro-form (Airaksinen et al., 1999).

As GDNF was first shown to promote survival of dopamine neurons, it has been the most extensively studied neurotrophic factor regulating the brain dopaminergic system. GDNF is not specific for dopaminergic neurons, and has effects on many types of cells inside and outside of the CNS. GDNF has been shown to promote the survival of central noradrenergic neurons (Arenas et al., 1995), central serotonergic neurons (Cass, 1996), spinal motor neurons (Henderson et al., 1994), peripheral sensory and autonomic neurons (Trupp et al., 1995) and forebrain cholinergic and GABAergic neurons (Williams et al., 1996). Gene knockout studies have shown that GDNF is essential for the development of the kidney, the enteric nervous system and sympathetic cervical ganglia (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Moreover, GDNF is crucially involved in spermatogenesis (Meng et al., 2000).

NRTN was originally identified because of its ability to promote the survival of sympathetic neurons (Kotzbauer et al., 1996), and, afterwards, it has been shown to promote the survival of spinal cord motoneurons, forebrain cholinergic neurons (Golden et al., 2003), retinal ganglion cells (Koeberle and Ball, 2002), enteric neurons (Heuckeroth et al., 1998) and substantia nigra dopaminergic neurons (Åkerud et al., 1999; Horger et al., 1998; Tseng et al., 1998). PSPN promotes the survival of ventral midbrain dopaminergic neurons and motor neurons but, in contrast to GDNF and NRTN, it appears to have no effects in the peripheral nervous system (Milbrandt et al., 1998). However, PSPN receptor, GDNF family receptor-α4, signalling is involved in regulation of calcitonin production in thyroid C cells. The most recently identified member of the GDNF-family, ARTN, promotes the survival of sensory and sympathetic neurons *in vitro* and *in vivo* (Baloh et al., 1998; Airaksinen and Saarma, 2002).

2.3.1. The GDNF family receptors and RET-dependent signalling

The common signalling receptor for all GDNF family ligands is the RET (**RE**arrangement during **T**ransformation) tyrosine kinase receptor (Durbec et al., 1996; Treanor et al., 1996; Trupp et al., 1996). All GDNF family ligands first bind to their corresponding GDNF family receptor-α (GFRα) protein forming a high affinity complex. GDNF, NRTN, ARTN and PSPN specifically bind to GFRα1, GFRα2, GFRα3 and GFRα4, respectively (Airaksinen and Saarma, 2002). The GFRα1-receptor has been shown to exist as bound to the plasma membrane by a glycosylphosphatidylinositol anchor, but it has also been shown to be biologically active in a soluble form (Paratcha et al., 2001). Homodimeric GDNF binds to either monomeric or dimeric GFRα1; the complex

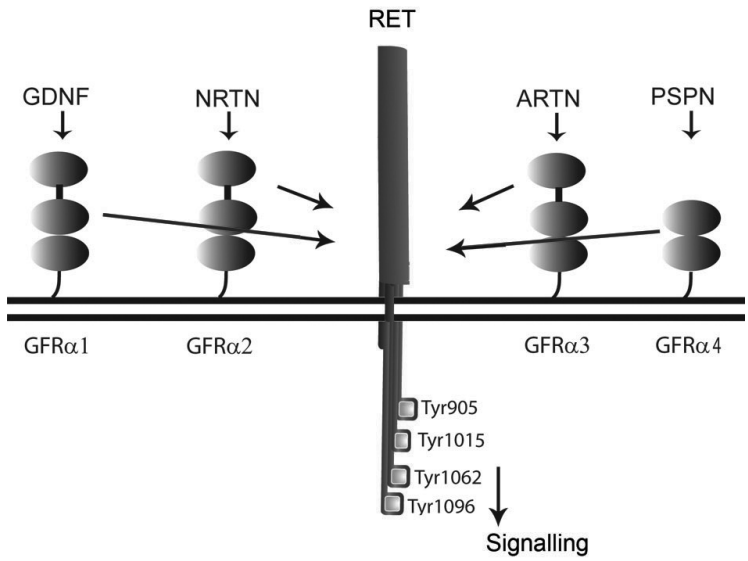

Figure 2.3. GDNF-family ligands and their receptors. Homodimeric GDNF-family ligand/GFRα-receptor complexes induce dimerization of two RET molecules, leading to transphosphorylation of their tyrosine kinase domains. All ligands activate RET *via* specific GFRα co-receptors. Four tyrosine residues (Tyr905, Tyr1015, Tyr1062, Tyr1096) serve as docking sites for different adaptors (Tyr1096 is only in the long isoform of the RET).

brings two RET molecules together, and tyrosine residues in the intracellular domains of the RET proteins are autophosphorylated (Figure 2.3).

Phenotypes of mice lacking GDNF, GFRα1 or RET are similar. Homozygous knockout mice die shortly after birth, lack kidneys and have abnormalities in their enteric nervous systems (Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994;). RET has been shown to have two spliced variants, the RET51 and RET9 isoforms (Tsui-Pierchala et al., 2002). It has been suggested that only RET9 is critical for life; it is important for normal development of kidneys and the enteric nervous system (de Graaff et al., 2001). However, the role of RET51 and RET9 in the regulation of kidney development is not clear, as recently it has been reported both homozygous RET9 and RET51 mice have normal kidney development (Jain et al., 2006). The intracellular domain of RET consists of 14 tyrosine residues, and phosphorylated tyrosine residues Tyr^{905}, Tyr^{1015}, Tyr^{1062} and Tyr^{1096} are docking sites for adaptor proteins (Airaksinen and Saarma, 2002). RET activates several intracellular signalling cascades, including the mitogen activated protein kinase pathway, phosphoinositide 3-kinase pathway, Src-family kinases and phospholipase Cγ that regulate cell survival, differentiation, proliferation, neurite outgrowth and synaptic plasticity (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003).

RET was originally described as an oncogene (Takahashi et al., 1985). In

humans, heterozygous activating mutations in the *RET* gene are found in a dominantly inherited cancer syndrome called multiple endocrine neoplasia type 2 (MEN2), and heterozygous inactivating mutations of *RET* cause Hirschsprung's disease (Edery et al., 1997; Santoro et al., 2004; Takahashi, 2001). There are three clinical subtypes of the MEN2-syndrome called MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC). MEN2A patients are affected by medullary thyroid carcinoma, and pheochromocytoma and parathyroid hyperplasia are present in about 50% and 15-30%, respectively, of the patients (Santoro et al., 2004). MEN2B patients suffer from medullary thyroid carcinoma, pheochromocytoma (about 50% of the patients) and, rarely, ganglioneuromas in the intestine. In MEN2A patients, mutations of *RET* affect the extracellular domain of the RET receptor and lead to its activation by covalent RET dimerization. The MEN2B-syndrome is associated primarily with a single activating, missense mutation of codon 918 (Met918Thr) affecting the catalytic intracellular tyrosine kinase domain (Takahashi, 2001). FMTC patients suffer from medullary thyroid carcinoma. Hirschsprung's disease is characterized by magacolon and absence of enteric ganglia.

2.3.2. Other mechanisms of GDNF signalling

GFRα receptors have been also found in brain regions where RET is not seen, and it has been proposed that GDNF/GFRα1 also signals independently of RET. In the adult midbrain of rodents, the expression patterns of GFRα1 and RET are similar, but, for example, in the cortex and GFRα1 is present but RET is absent (Golden et al., 1998; Trupp et al., 1997). In the nervous system, neural cell adhesion molecule (NCAM) is abundantly expressed in cell types that express GFRα1 but not RET, including cortical and hippocampal neurons (Crossin and Krushel, 2000). Indeed, it has been demonstrated that NCAM functions as an alternative signalling receptor for GDNF (Paratcha et al., 2003). In addition, ventral midbrain dopaminergic neurons also express NCAM, along with GFRα1 and RET, and neutralizing NCAM antibodies block the effects of GDNF on enhanced dopamine turnover and locomotor activity (Chao et al., 2003).

2.3.3. Co-operation of GDNF with other proteins

TGF-β has been shown to be important for the functional effects of GDNF. GDNF requires TGF-β for exerting its full neurotrophic potential on cultured peripheral autonomic, sensory and midbrain dopaminergic neurons (Krieglstein et al., 1998). The effects of GDNF are abolished when neutralizing antibodies to endogenous TGF-β are added. In TGF-β/GDNF synergistic signalling, TGF-β recruits GFRα1 to the plasma membrane and is involved in its glycosylphosphatidylinositol anchoring (Peterziel et al., 2002). In addition, brain-derived neurotrophic factor (BDNF) has been shown to be needed for the survival-promoting effect of GDNF on primary sensory neurons (Erickson et al., 2001). Also, GDNF signalling requires cell surface heparin sulphate glycosaminoglycans. Without heparin sulphate, GDNF does not fully activate the RET receptor, nor does it fully induce axonal growth or scattering of epithelial cells (Barnett et al., 2002). The idea of heparin sulphate/GDNF co-operation is also supported by the finding

that, similar to homozygous GDNF, GFRα and RET knockout mice, mice lacking heparin sulphate also lack kidneys (Bullock et al., 1998).

2.3.4. GDNF, GFRα1 and RET expression

Table 2.1 summarizes information on GDNF, GFRα1 and RET mRNA and protein expression in dopaminergic brain regions and in areas related to dopaminergic circuitry. During development, GDNF mRNA is widely expressed in the brain and outside the nervous system. GDNF mRNA is highly expressed in developing skin, whisker pad, kidney, stomach and testicle (Choi-Lundberg and Bohn, 1995; Trupp et al., 1995). In the striatum, the highest levels are on postnatal day (P) 0 and P10 (Choi-Lundberg and Bohn, 1995); in addition, GDNF mRNA is found in the hippocampus and in the globus pallidus during the development. The pattern of expression is similar in the adult brain, although the levels are lower. GFRα1 and RET are co-localized in developing and in the adult ventral midbrain. However, RET is not expressed in the striatum, ventral pallidum or globus pallidus. During development, the RET receptor is widely expressed in embryonic retina, kidney, autonomic and dorsal root ganglia, motor neurons of the spinal cord, enteric neurons, thyroid and testicle (Golden et al., 1998; Pachnis et al., 1993; Tsuzuki et al., 1995). In striatal brain areas, GFRα1 mRNA is not expressed, but in the dorsal striatum there are low levels of GFRα1 protein found. In the hippocampus and ventral pallidum, GFRα1 mRNA and protein are found and the protein has also been detected in the globus pallidus. GFRα1 mRNA and protein expression in the midbrain differ. GFRα1 mRNA is highly expressed in the SNpc and in the VTA, but protein is highly expressed in SNpc and SNpr and only at low levels in the VTA.

All GDNF-family ligands are produced in a prepro precursor form. The pre-sequence is cleaved during secretion, and the pro-form removal occurs by proteolytic cleavage (Airaksinen and Saarma, 2002). Regulation of GDNF synthesis is only partially known. In the nervous system, GDNF mRNA has been localized to both glial cells and neurons (Schaar et al., 1993), and, thus, GDNF might act on brain dopaminergic neurons as a paracrine factor and as a target-derived factor. Localization studies of GDNF in the striatum have shown that GDNF mRNA is localized mainly in striatal cholinergic interneurons, to a lesser extent in GABAergic interneurons, and also possibly in medium-sized GABAergic projection neurons (Bizon et al., 1999). This has also been supported by the finding that GDNF mRNA is expressed throughout the striatum in medium-sized neurons at low levels, whereas other striatal neurons express clearly higher levels (Trupp et al., 1997). However, in one study it was reported that in the developing postnatal brain of a rat, GDNF mRNA is expressed mainly in striatal GABAergic projection neurons (Oo et al., 2005). Studies exploring the transport of GDNF protein have shown that in adult brain, GDNF is retrogradely transported from striatum to SNpc (Georgievska et al., 2004; Tomac et al., 1995b), but only about half of the nigral dopaminergic neurons expressed GDNF (Tomac et al., 1995b). Thus, as a transport mechanism is present in adult brain, it is likely that GDNF is involved in maintenance of dopaminergic neurons.

There seems to be an interaction between dopamine D2 receptors and GDNF synthesis, since in D2 receptor knockout

mice, GDNF concentrations in the striatum are decreased (Bozzi and Borrelli, 1999). In addition, in mouse astrocytes, high concentrations of the dopamine D2 receptor agonist, bromocriptine, have been shown to decrease GDNF levels while the dopamine D1 receptor agonist SKF38393 has been shown to increase GDNF levels (Ohta et al., 2003). GDNF synthesis is differently regulated in glial and neuronal cells. Proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α have been shown to increase GDNF synthesis in glioblastoma cells and to decrease its synthesis in neuroblastoma cells (Verity et al., 1999). In animal models of seizures (Humpel et al., 1994; Kokaia et al., 1999) and ischemia (Kokaia et al., 1999), GDNF expression is increased. Seizures are associated with glutamate release and glutamate has been shown to regulate GDNF expression in striatal astrocytes (Ho et al., 1995). In addition, fibroblast growth factors-1, -2 and -9 have been shown to increase GDNF expression in glioblastoma

Table 2.1. mRNA and protein expression of GDNF, GFRα1 and RET in selected areas of developing and adult rodent brain. – indicates not detected, ± indicates barely detected, + low levels, ++ moderate levels, +++ high levels, empty cells indicate not measured; SNpc= substantia nigra pars compacta, SNpr= substantia nigra pars reticulata, VTA= ventral tegmental area, CPu= caudate putamen, NAc= nucleus accumbens, Hipp= hippocampus, VP= ventral pallidum, GP= globus pallidus

Developing brain	GDNF mRNA g, h, i	GDNF Protein c	GFRα1 mRNA d, f, i	GFRα1 protein	RET mRNA d, f, i	RET protein
SNpc			+++		+++	
SNpr			±		±	
VTA			+++		+++	
CPu	++	++	-		-	
NAc	++		-		-	
Hipp	++	++	++		+	
VP			++			
GP	++	++				
Adult	GDNF mRNA a, e	GDNF protein	GFRα1 mRNA a, e, f	GFRα1 Protein b,	RET mRNA a, e, f, j	RET Protein f
SNpc	-		+++	+++	+++	+
SNpr	-		+	+++	+	
VTA	-		+++	+	+++	+
CPu	+		-	+	-	
NAc	+		-	-	-	
Amygdale	±		+		±	
Hipp	++		++	+	+	
VP	+		++	+++	-	
GP	±		-	++	-	

Table combined from the following references: a: (Golden et al., 1998), b: (Matsuo et al., 2000), c: (Ikeda et al., 1999) d: (Burazin and Gundlach, 1999), e: (Trupp et al., 1997), f: (Nosrat et al., 1997), g; (Nosrat et al., 1996), h: (Strömberg et al., 1993), i: (Golden et al., 1999), j: (Kokaia et al., 1999)

cells (Suter-Crazzolara and Unsicker, 1996; Verity et al., 1999).

2.3.5. GDNF and the brain dopaminergic system

The physiological role of GDNF as a limiting, target–derived neurotrophic factor for dopaminergic neurons is not clear. Endogenous GDNF alone does not have a role in the embryonic development of dopaminergic systems as neither GDNF nor GFRα-1 homozygous knockout mice show any reduction in the number of dopaminergic neurons at birth (Enomoto et al., 1998; Granholm et al., 1997; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). However, as discussed above, during development, cell death of dopaminergic neurons is mainly postnatal (Oo and Burke, 1997). The role of GDNF as a rate-limiting factor for dopaminergic neurons is supported by the fact that GDNF mRNA is expressed in the striatum at moderate levels during early postnatal development (Choi-Lundberg and Bohn, 1995; Schaar et al., 1993; Strömberg et al., 1993). In line with the hypothesis, GDNF suppressed the first phase of natural cell death of dopaminergic neurons in a postnatal primary culture model (Burke et al., 1998), whereas injection of GDNF-activity blocking antibodies augments it (Oo et al., 2003). Transplanting ventral midbrain grafts from GDNF-/- foetuses into the brain of adult wild-type mice markedly reduced dopamine neuron numbers and fiber outgrowth (Granholm et al., 2000). Transgenic mice overexpressing GDNF in the striatum have increased numbers of dopaminergic neurons in the SNpc after the first phase of natural cell death (Kholodilov et al., 2004). However, the number of dopaminergic neurons in the SNpc was not maintained up to adulthood in these mice, although their number was increased in the VTA (Kholodilov et al., 2004). Thus, it has been shown that GDNF has a role in the regulation of the postnatal development and survival of dopaminergic neurons.

The effects of exogenous GDNF on intact dopaminergic neurons are widespread. It was first shown to promote the survival of mesencephalic dopamine neurons *in vitro* and to increase dopamine uptake (Lin et al., 1993). In addition, GDNF increases neurite outgrowth and cell size of TH-positive neurons *in vitro* (Lin et al., 1993) and increases sprouting and the number of synaptic terminals in isolated ventral tegmental dopaminergic neurons of rat (Bourque and Trudeau, 2000; Åkerud et al., 1999). In several studies it was found that GDNF enhances the release of striatal dopamine *in vitro* (Feng et al., 1999; Lin et al., 1993; Pothos et al., 1998). In several *in vivo* microdialysis studies with adult and aged rats and with aged primates, it was found that GDNF enhances stimulus-evoked release of striatal and nigral dopamine (Grondin et al., 2003; Hebert and Gerhardt, 1997; Hebert et al., 1996; Hoffman et al., 1997; Salvatore et al., 2004; Xu and Dluzen, 2000). In addition, a single GDNF injection into the SN or chronic perfusion into the lateral ventricle has been shown to increase motor activity (Grondin et al., 2003; Hebert and Gerhardt, 1997; Hebert et al., 1996; Hudson et al., 1995; Martin et al., 1996). As summarized in Table 2.2, the reported effects of GDNF on basal extracellular dopamine concentrations and on tissue dopamine concentrations are not all consistent. In several studies it has been shown that intranigral administration of GDNF decreases dorsostriatal tissue dopamine concentrations 2-3 weeks after the injection. However, when GDNF is

administered directly into the striatum the tissue dopamine content is increased after a latency period of 2-3 weeks. Interestingly, when GDNF is given unilaterally into the SN, the dopamine content has been found to be increased bilaterally in the nucleus accumbens of aged rats (Hebert and Gerhardt, 1997). In the developing postnatal brain, striatal GDNF injections clearly increase tissue dopamine concentrations in the striatum and in the SN (Beck et al., 1996).

In a neuroblastoma cell line, added GDNF increased TH mRNA expression, accompanied by increased protein levels, after 20-h incubation (Xiao et al., 2002). This is consistent with the findings that one week after intranigral GDNF administration TH-immunostaining in the striatum is increased and dopamine turnover is enhanced in the SN and in the striatum (Hudson et al., 1995; Martin et al., 1996). The GDNF-induced enhanced dopamine turnover and stimulus-evoked dopamine

Table 2.2. Effects of acute administration of GDNF into SN or striatum on extracellular and tissue dopamine, DOPAC and HVA concentrations in normal and aged rats; given is also the effect of chronic infusion into lateral ventricle in aged monkeys. Arrows indicate the increase or decrease of concentrations as compared with vehicle treatment;↔indicates that the concentration was not changed. Table combined from a: (Hebert and Gerhardt, 1997), b (Grondin et al., 2003), c: (Hudson et al., 1995), d: (Hebert et al., 1996), e: (Martin et al., 1996), f: (Xu and Dluzen, 2000). CPu= caudate putamen, NAc= nucleus accumbens, SN= substantia nigra.

Normal age rats, single administration							
Injection site, dose, latency period	Extra-cellular dopamine	Extra-cellular DOPAC	Extra-cellular HVA	Tissue dopamine	Tissue DOPAC	Tissue HVA	Reference
Intranigral, 10µg, 3 weeks				↑ CPu ipsilateral ↔ NAc ↓ SN ipsilateral			c
Intranigral, 10µg, 3 weeks	↔ CPu	↑ CPu	↑ CPu	↓ CPu ipsilateral ↔ SN	↔ CPu ↔ SN	↔ CPu ↔ SN	d
Intranigral, 100µg, 2 weeks				↓ CPu ipsilateral ↑ SN ipsilateral	↔ CPu ↑ SN ipsilateral	↔ CPu ↑ SN ipsilateral	e
Intrastriatal, 10/100µg, 2 weeks	↔ CPu	↑ CPu		↑ CPu ipsilateral	↑ CPu ipsilateral	↑ CPu ipsilateral	e, f
Aged rats, single administration							
Intranigral, 10µg, 3 weeks	↑ CPu ↑ NAc	↑ CPu ↑ NAc	↑ CPu ↑ NAc	↓ CPu ipsilateral ↑ NAc bilateral ↑ SN ipsilateral	↓ CPu ipsilateral ↔ NAc ↓ SN bilateral	↓ CPu ipsilateral ↑ NAc bilateral	a
Aged monkeys, chronic infusion							
Lateral ventricle, 7.5µg for 3 months	↔ CPu ↑ SN	↔ CPu ↔ SN	↑ CPu ↔ SN				b

release might be due to findings that GDNF increases TH phosphorylation (Kobori et al., 2004; Salvatore et al., 2004), potentiates excitability of cultured midbrain neurons by inhibiting transient A-type K^+-channels (Yang et al., 2001) and, in motor nerve terminals, increases Ca^{2+} influx in evoked transmission (Wang et al., 2001). The effects of GDNF on dopaminergic systems evoke compensatory changes, since long-term overexpression of GDNF has been shown to downregulate TH expression (Georgievska et al., 2004; Rosenblad et al., 2003) and also single intracranial injection of GDNF (100 µg) decreased striatal TH levels (Salvatore et al., 2004). However, long-term striatal GDNF overexpression does not affect the amount of DAT, VMAT, D1 receptors, D2 receptors or preproenkephalin expression in the striatum (Rosenblad et al., 2003).

In animal models of Parkinson's disease, GDNF has been shown to protect as well as to restore the dopaminergic system from drug-induced neurodegeneration. In rats, a single GDNF (10 µg) administration or prolonged GDNF administration over 4 weeks (totally 140 µg) to the SN protects dopaminergic neurons from nigral or striatal 6-hydroxydopamine (6-OHDA) lesions (Kearns and Gash, 1995; Sauer et al., 1995). In addition, an intrastriatal delivery of adenoviral vector encoding human GDNF protects rats from striatal 6-OHDA lesion (Choi-Lundberg et al., 1997; Kirik et al., 2000b). Moreover, a single GDNF (25 µg) administration into the striatum is more effective in protecting dopaminergic neurons from intrastriatal 6-OHDA lesions than injection of GDNF into the SN (25 µg) or into the lateral ventricle (50 µg), which was the least effective (Kirik et al., 2000a). In addition, both single administration and long-term expression of striatal GDNF induces robust sprouting of dopaminergic neurons in the striatum (Kirik et al., 2000a; Palfi et al., 2002). The neurorestorative effects of GDNF on the dopaminergic system are also prominent as GDNF (100 µg) injection into the SN of rats restores the dopamine content and behavioural consequences of medial forebrain bundle 6-OHDA injection (Hoffer et al., 1994). Also, GDNF given into the SN or striatum protects and restores the dopaminergic system in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (Tomac et al., 1995a). In MPTP-treated rhesus monkeys, intraventricular GDNF (300 µg) restores dopamine function (Gerhardt et al., 1999) and lentiviral GDNF delivery prevents MPTP-induced neurodegeneration and induces increased fluorodopa uptake in the striatum (Kordower et al., 2000). The outcome from studies done with animal models of Parkinson's disease and also from two clinical studies (Gill et al., 2003; Slevin et al., 2005), where GDNF was administered into putamen, strongly indicates that GDNF is a potent agent for the treatment of Parkinson's disease. However, there are two clinical studies with Parkinsonian patients, where GDNF was ineffective. In one study, where intraventricular GDNF administration was used, GDNF was shown to be ineffective (Nutt et al., 2003), but also in a randomized controlled clinical study, where intraputamenal GDNF administration was used, GDNF was shown to be ineffective (Lang et al., 2006).

2.4. Drug addiction

Drug addiction is a chronic relapsing disorder that is characterized by a compulsion to seek and take the drug, followed by a loss of control over drug intake. Drug craving and relapses can occur even after decades of abstinence. There are several causes for

drug addiction, but factors increasing vulnerability to addiction can be categorized as genetic factors, environmental influences and drug-induced changes in the brain (Kreek et al., 2005). There is a substantial number of people who become addicted to illicit drugs or alcohol, but repeated drug use does not inevitably lead to addiction. Only 15-16% of cocaine users become addicted within the first 10 years of cocaine use, corresponding values for marijuana and alcohol are 8% and 12-13%, respectively (Wagner and Anthony, 2002). Studies of genetic epidemiology provide evidence that individual differences in vulnerability to addiction may have a genetic component; for example genetic polymorphisms in the μ-opioid receptor, κ-opioid receptor, serotonin transporter, D4 dopamine receptor and COMT gene may alter vulnerability to opiate addiction and polymorphisms in preprodynorphin, D3 dopamine receptor, D4 dopamine receptor, cannabinoid receptor 1 and dopamine β-hydroxylase gene to cocaine addiction (for review, see Kreek et al., 2005).

The development of drug addiction involves a transition phase during which a casual use is shifted to compulsive patterns of drug use. There are four main theoretical explanations for the transition to addiction: 1. initial drug gives pleasure and subsequent withdrawal symptoms lead to development of addiction; 2. addiction is caused by aberrant learning in which strong stimulus-response habits are developed; 3. sensitization of neural systems causes wanting and motivation to take drugs; 4. normal decision making and inhibitory control over behaviour is disturbed due to dysfunction of frontal cortical systems (Robinson and Berridge, 2003). During repeated drug administration, tolerance develops to some effects of abused drugs, while other effects are sensitized. As discussed below, sensitization of dopaminergic systems appears to be involved in the development of drug addiction.

The traditional view of behavioural processes underlying addiction involves the term "reinforcement", which can be either positive or negative. The positive reinforcement theory assumes that drugs are used because of their rewarding effects, and positive stimulus increases the probability of behaviour leading to the following drug use. The negative reinforcement theory of addiction assumes that there is a desire to use drugs repeatedly in order to suppress physical and/or psychological withdrawal symptoms and subsequent emergence of a negative emotional state when access to the drug is prevented (Koob and Le Moal, 2001).

Another view of the mechanism of drug addiction is that a repeated use of drugs can take control of the behaviour. Repeated behavioral patterns related to repeated use of drug of abuse might lead to compulsive drug usage (Gerdeman et al., 2003). In this view, a repeated use of abused drugs causes strong repetitive and nearly automatic behaviours, habits that are centered on the addictive substance.

One theory underlying the development of addiction is the incentive-sensitization theory (Robinson and Berridge, 2003), which proposes that drug-induced sensitization of neuronal systems in the brain mediate incentive-motivational functions that cause drugs to become compulsively wanted. There are two different kinds of sensitization: 1. psychomotor sensitization 2. incentive motivational sensitization, which are both thought to be expressed by nucleus accumbens-related circuitry.

2.4.1. Drug addiction and dopamine

Cerebral dopaminergic systems are of prime importance among the neuronal systems involved in processing reward information. Natural rewards, e.g. food and sex, and most drugs of abuse including amphetamine, cocaine, opioids, alcohol and nicotine increase striatal dopamine release, especially in the nucleus accumbens (Di Chiara and Imperato, 1988; Wise and Rompre, 1989). In addition, when a drug of abuse is associated with neutral stimuli, the learning process leads to conditioning of accumbal and dorsostriatal dopamine release when just the neutral stimulus is presented (Ito et al., 2002). However, it has been shown that associative learning itself, without reward, is linked to enhanced dopamine output in the nucleus accumbens but not in the dorsal striatum (Young et al., 1998). Schultz and co-workers have shown that there is a bursting activity of dopamine neurons when a reward occurs without prediction, but when the reward is predicted by conditioned stimulus the bursting activity occurs during the prediction and not later when the reward actually occurs (Schultz, 1998). Also, when there is a reward prediction but no reward, the initial bursting activity is seen but dopamine neurons are depressed at the time when the reward should have occurred.

During repeated drug administration, neuroadaptive processes occur in the dopaminergic system. Sensitization of the mesolimbic dopaminergic system has been proposed to play an important role in the development of compulsive drug use (Robinson and Berridge, 2003). Withdrawal from a repeated drug use can also cause depression of the dopaminergic systems which might be involved in dysphoria, anxiety and depression (Koob and Le Moal, 2001). Taken together, these data suggest that dopamine is involved in the formation of associations between contextual stimuli and rewarding or aversive events.

2.4.2. Sensitization

During repeated administration of abused drugs, the behavioural effects can increase gradually; thus, the behavioural responses are sensitized in rodents (Ahtee and Attila, 1987; Kalivas and Stewart, 1991; Robinson and Berridge, 2003). In the case of psychostimulants, such as cocaine or amphetamine, effects on locomotor activity can increase dramatically, by three- to four-fold as compared with the first dose, or locomotor activity may turn into stereotyped behaviour. In the case of opioids, the initial sedative or cataleptic effects may turn into enhanced locomotion and stereotyped behaviour during repeated opioid treatment (Ahtee, 1974; Babbini and Davis, 1972). The molecular mechanisms underlying sensitization are not completely understood, but they are likely to include the sensitization of dopamine output, alterations in the sensitivity of dopamine receptors, alterations in the dopamine uptake system or other neurotransmitter systems (Robinson and Berridge, 2003).

There is ample evidence of the involvement of dopaminergic systems in the sensitization phenomenon (Ahtee and Attila, 1987; Di Chiara, 1995; Spanagel and Weiss, 1999). Alcohol-preferring AA rats, which voluntarily consume alcohol, are more easily sensitized to repeated morphine and cocaine than alcohol non-preferring ANA rats, and this phenomenon is accompanied by higher brain dopamine output in the AA rats (Honkanen et al., 1999; Mikkola et al., 2001; Mikkola et al., 2002; Ojanen et al.,

2003). In addition, withdrawal from repeated morphine treatment enhances the sensitivity of dopamine D2-like receptor function (Piepponen et al., 1996).

2.4.3. Fos family of immediate early genes

Striatal dopaminergic systems are involved in the regulation of the *fos* immediate early gene (IEG) family (Berretta et al., 1992; Pennypacker et al., 1995). IEGs are a set of transcription factors that are expressed rapidly after cell stimulation. Transcription factors encoded by IEGs regulate the expression of target genes. Regulation of gene expression is one mechanism that could underlie the long-term behavioural changes seen during repeated administration of abused drugs (Nestler, 2001). Transcription factors of the Fos family form complexes with members of the Jun family. The heterodimers formed are called activator protein-1 (AP-1) complexes, which bind to AP-1 sites present in regulatory regions of many genes (Morgan and Curran, 1995).

The most studied IEG, *c-fos,* has been used to characterize of postsynaptic activation in different brain areas (Chaudhuri, 1997; Sagar et al., 1988). Acute administration of morphine, cocaine, amphetamine and nicotine, as well as acute stress or electrical stimuli have been shown to induce c-Fos rapidly in striatal brain areas (Harlan and Garcia, 1998). The expression of *c-fos* is regulated by dopaminergic and glutamatergic transmission mainly in the GABAergic medium-sized spiny projection neurons in striatal brain areas. Dopamine D1 receptors mediate *c-fos* expression in projection neurons containing substance P and dynorphin while glutamate NMDA-receptors mediate *c-fos* expression in projection neurons expressing substance P and dynorphin as well as in enkephalin expressing projection neurons (Berretta et al., 1992). During repeated treatment with drugs of abuse, interesting differences have been noted in *c-fos* expressing GABAergic projection neuron cell types. c-Fos was present in dynorphin containing neurons when the drug was administered in the home cage, but when the drug was associated with a novel environment, c-Fos was localized in both types of GABAergic projection neurons (Badiani et al., 1999; Uslaner et al., 2001).

Another Fos-family protein, ΔFosB, is accumulated in striatal brain regions after chronic administration of abused drugs (Nestler et al., 2001). Eric Nestler and co-workers have extensively studied the role of ΔFosB in drug addiction as it persists in the striatal brain regions after repeated treatment with cocaine, morphine and electroconvulsive seizures due to its long half-life (Hope et al., 1994a; Hope et al., 1994b; Nye and Nestler, 1996). The ΔFosB protein is a truncated splice variant of full-length FosB, which is encoded by the *fosb* gene. In mice overexpressing ΔFosB in dynorphin containing GABAergic medium-sized spiny projection neurons, the rewarding effects of cocaine and cocaine-induced locomotion were increased (Kelz et al., 1999). ΔFosB is also strongly regulated by dopaminergic transmission. Dopamine D1 receptor antagonists attenuate its cocaine-induced expression (Nye et al., 1995), and in D1 receptor knockout mice, repeated cocaine administration does not increase ΔFosB levels in striatum (Zhang et al., 2002).

2.4.4. Effects of psychostimulants and morphine on dopamine

Cocaine and amphetamine are potent psychostimulants. Both elevate extracellular

dopamine concentrations in the nucleus accumbens and in the caudate/putamen by acting directly on the nerve terminal (Figure 2.4.; Di Chiara and Imperato, 1988; Koob, 1992). Besides dopaminergic systems, cocaine and amphetamine also increase neurotransmitter release in noradrenergic and serotonergic systems. Cocaine increases dopamine release by blocking dopamine uptake, and increases the duration of action of released dopamine. Cocaine has similar effects on noradrenaline and serotonin uptake. Surprisingly, the rewarding effect of cocaine is not mediated by DAT alone as in DAT knockout mice cocaine induces place preference (Sora et al., 1998). Indeed, it has later been shown that none of these transporters alone is crucial for cocaine-induced reward (Sora et al., 1998; Uhl et al., 2002). Amphetamine affects DAT in a different way from cocaine. Inside the nerve terminal amphetamine acts at synaptic vesicles, causing a redistribution of dopamine into the cytoplasm, and dopamine is thought to be released by reversal in the direction of transport mediated by DAT (Sulzer et al., 1995;Kahlig et al., 2005).

The effects of opioids are mediated by μ-, δ- and κ-opioid receptors. They activate brain reward circuitry by dopamine-dependent and -independent mechanisms (Spanagel and Weiss, 1999). Dopamine independent mechanisms are relevant as dopamine antagonists (Ettenberg et al., 1982; Van Ree and Ramsey, 1987) and destruction of dopaminergic nerve terminals in the nucleus accumbens (Dworkin et al., 1988; Pettit et al., 1984) have failed to attenuate opiate self-administration. Dopamine-dependent mechanisms are based on the ability of opioids to modulate activity of ascending dopaminergic neuronal pathways in the brain and release of dopamine in basal ganglia areas, especially in the nucleus accumbens. Direct intraventricular administration of μ- or δ- opioid receptor agonists increase extracellular dopamine concentrations in the nucleus accumbens, but κ- opioid receptor agonists decrease it (Spanagel et al., 1990). Morphine enhances dopamine output by activating μ-opioid receptors on GABAergic interneurons in the VTA and in the SN (Figure 2.4.). The activation of μ-opioid receptors disinhibits GABAergic interneurons in the VTA and thus leads to enhanced release of dopamine (Johnson and North, 1992; Lacey et al., 1989). μ-opioid receptors are widely expressed in the CNS. In the SNpc, SNpr, VTA and caudate/putamen they are localized in the neurites and in the nucleus accumbens, ventral pallidum and globus pallidus they are localized both in the neurites and soma (Mansour et al., 1995). The effects of morphine on A9 dopaminergic cells has been shown to depend on striatonigral feedback pathways (Gysling and Wang, 1983) and direct administration of morphine into globus pallidus increases accumbal dopamine output (Anagnostakis and Spyraki, 1994). Morphine's effect on dopamine output in the nucleus accumbens and caudate/putamen is bell-shaped, increasing the morphine dose increases dopamine output up to certain doses, and after that further dose increases reduce the dopamine response (Maisonneuve et al., 2001). The dual effect on striatal dopamine release might be indirect and linked to findings that intrastriatal administration of the μ-opioid receptor agonists morphine and DAMGO ([D-Ala^2,MePhe^4,glycinol^5]-enkephalin) decreases extracellular striatal dopamine levels (Piepponen et al., 1999). However, accumbal infusions of μ-opioid receptor agonists do not alter dopamine output (Spanagel et al., 1992),

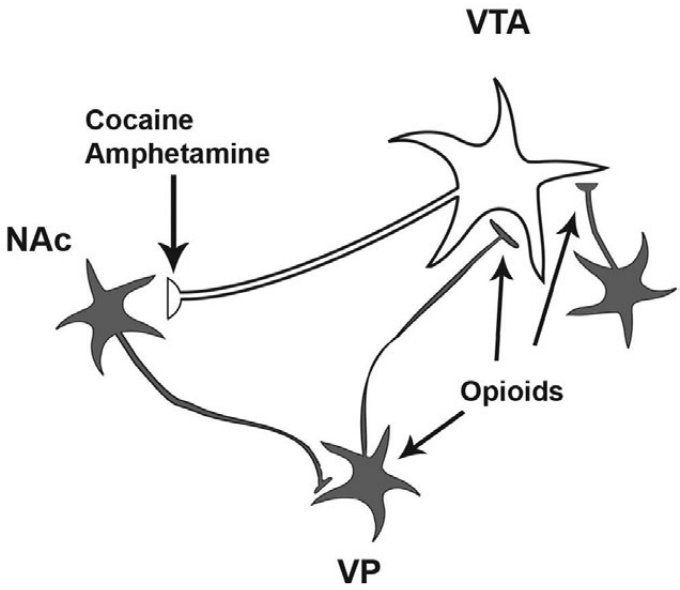

Figure 2.4. The mechanisms of action of acute cocaine, amphetamine and opiate drugs on mesolimbic dopaminergic neurons. NAc = nucleus accumbens, VP = ventral pallidum, VTA = ventral tegmental area, grey neurons represent GABAergic neurons and white neuron represents mesolimbic dopaminergic neuron. Cocaine and amphetamine release dopamine by acting directly on nerve terminals of dopaminergic neuron, whereas opioids, e.g. morphine increase accumbal dopamine release by disinhibiting GABAergic tone that regulates the activity of dopaminergic neurons. Figure combined from Anagnostakis and Spyraki, 1994; Johnson and Napier, 1997; Spanagel and Weiss, 1999.

but administration of morphine into the ventral pallidum increases accumbal dopamine output (Anagnostakis and Spyraki, 1994; Johnson and Napier, 1997). Thus, morphine's effect is inhibitory in the dorsostriatal terminal regions and stimulatory in the somatodendritic regions, ventral pallidum and globus pallidus.

2.4.5. Neurotrophic factors and drugs of abuse

Brain-derived neurotrophic factor (BDNF) and GDNF have been shown to be involved in adaptations during drug addiction. Exogenous GDNF seems to decrease the rewarding effects of abused drugs whereas exogenous BDNF increases them. However, both BDNF and GDNF have been shown to block the molecular and cellular changes induced by cocaine and morphine. BDNF administration into the VTA has been shown to block cocaine- or morphine-induced increased levels of TH and glial fibrillary acidic protein in the VTA (Berhow et al., 1995; Bolanos and Nestler, 2004) and to prevent morphine-induced reduction in the cell-body and dendrite size of dopaminergic neurons in the VTA (Sklair-Tavron et al., 1996). VTA injection of GDNF has been shown to block morphine- and cocaine-induced increased levels of TH and cocaine-induced increase of ΔFosB and the NMDAR1 glutamate receptor

subunit (Messer et al., 2000). In contrast, BDNF infusion into the VTA enhances locomotor activity and CPP to cocaine (Grimm et al., 2003; Horger et al., 1999; Lu et al., 2004), and in BDNF+/- knockout mice, the effects of cocaine are decreased (Hall et al., 2003; Horger et al., 1999). Administration of GDNF into the VTA has been shown to attenuate cocaine-induced place preference and to decrease self-administration of cocaine and alcohol (Green-Sadan et al., 2003; Green-Sadan et al., 2005; He et al., 2005; Messer et al., 2000), and decreased levels of GDNF have been shown to increase rewarding effects of cocaine and sucrose (Griffin et al., 2006; Messer et al., 2000).

Hence, the present hypothesis is that increased GDNF levels in the mesolimbic system attenuate and decreased levels of GDNF augment the molecular and behavioural changes induced by abused dugs. In addition, drugs of abuse have opposing effects on GDNF and BDNF expression, as cocaine has been shown to decrease GDNF mRNA (Green-Sadan et al., 2003) whereas BDNF mRNA expression and protein levels are increased by amphetamine, morphine and cocaine (Grimm et al., 2003; Le Foll et al., 2005; Le Foll et al., 2002; Zhang et al., 2002).

3. AIMS OF THE STUDY

The discovery of neurotrophic factors has led to studies on their role in the development and maintenance of specific neuronal systems. During the past years, there has been increasing research to seek novel treatment strategies based on neurotrophic factor signalling in several diseases including Parkinson's disease, but also for other diseases where brain dopaminergic systems are affected. The brain dopaminergic systems are involved in the mediation of drug reward and functions in the learning process during formation of associations between contextual stimuli and rewarding events. Thus, interactions of the neurotrophic factors with brain dopaminergic systems are of great interest when studying the role of neurotrophic factors in drug addiction. Thus, the specific aims of this study were:

- To clarify the role of endogenous GDNF in the regulation of striatal dopaminergic transmission in heterozygous GDNF knockout mice.

- To study the effects of reduced GDNF levels on cocaine- or morphine-induced striatal dopamine release, locomotor stimulatory effects and place conditioning in mice.

- To study the effects of constitutive RET tyrosine kinase receptor activity on the dopaminergic system and on the locomotor stimulatory effects of cocaine in mice. Further questions asked were how GDNF mediates its effect on dopaminergic neurons *via* RET, and how specific its effects on dopaminergic neurons are.

4. MATERIALS AND MAIN METHODS

4.1. Animals

Heterozygous GDNF+/- knockout mice with deletion of one allele of the GDNF gene, described by Pichel et al. (1996), and their wild-type littermates were used in studies I, II and III. Homozygous and heterozygous transgenic MEN2B mice and their wild-type littermates were used in the study IV. This mouse model for MEN2B was generated by introducing a single point mutation, Met919Thr, corresponding to human Met918Thr, into the mouse *RET* gene (Smith-Hicks et al., 2000). Mice were housed and experiments were conducted under standard laboratory conditions with controlled temperature under a 12:12 h light/dark cycle. The mice had free access to mouse chow and water. All experiments were approved by the committee for Animal Experiments of the University of Helsinki or by the chief veterinarian of the provincial government. 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 from tail DNA by PCR.

Methodological considerations

One way to study the role of GDNF-GFRα-1/RET signalling in regulation of the brain dopaminergic systems is to use gene-manipulated mouse models, where a gene is knocked out or overexpressed, or the function of a protein is altered by a mutation. GDNF, GFRα-1 and RET homozygous knockout mice all express similar phenotypes, and homozygous knockout mice die at birth. On the other hand, heterozygous mice are viable and indistinguisable by eye from their wild-type littermates (Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). Heterozygous GDNF, GFRα-1 and RET knockout mice can be used to study the effects of reduced GDNF-GFRα-1/RET signalling. The heterozygous GDNF knockout mice used in the present experiments are by appearance similar to their wild-type littermates but about 10% of the mice have one kidney smaller than the other one.

GDNF-GFRα-1/RET signalling is increased in mice overexpressing GDNF in the mesencephalic dopaminergic system (Kholodilov et al., 2004). The model for increased signalling used in these studies is the MEN2B mouse model where a point mutation was introduced in the endogenous mouse gene encoding *RET*, resulting in a constitutively active receptor. Heterozygous MEN2B/+ and homozygous MEN2B/MEN2B mice display features of the human MEN2B syndrome, including C-cell hyperplasia, pheochromocytoma and ganglioneuromas (Smith-Hicks et al., 2000). Almost all homozygous MEN2B/MEN2B mice have C cell hyperplasia at the age of 6-10 months, but pheochromocytoma is not seen in heterozygous mutant mice at any age, nor in the homozygous mutants younger than 3 months. Ganglioneuromas of the adrenal glands and sympathetic ganglia are seen only in homozygous mutants at all ages. However, no abnormalities are seen in the enteric nervous system (Smith-Hicks et al., 2000). We used mice aged 10 to 12 weeks, when they are considered adults but the abnormalities are still at minimum. In the MEN2B mice, the mutation might lead to altered substrate specifity of the RET catalytic domain (Santoro et al., 1995; Songyang et al., 1995), and thus, the intracellular signalling might be different as compared with that induced by GDNF-family ligands. However, to date

no substantial qualitative differences are known between the oncogenic and ligand-activated RET signalling pathways (Sariola and Saarma, 2003).

A major limitation in conventional gene manipulated mouse models is that the gene is deleted or mutated from the earliest moment of embryonic development. When the mice are used as adults, the changes observed might reflect developmental compensatory changes rather than an active role in adult plasticity (Chapman, 2002). In the case of GDNF-GFRα-1/RET signalling, known to be critically important for development, this is a relevant limitation.

4.2. Drugs and treatments

Morphine hydrochloride (studies II & III, the University Pharmacy, Helsinki, Finland), cocaine hydrochloride (studies I & IV; the University Pharmacy, Helsinki, Finland) and d-amphetamine hydrochloride (previously unpublished data, the University Pharmacy, Helsinki, Finland) were dissolved in 0.9% NaCl solution (Sal). Morphine and d-amphetamine injections were given subcutaneously and cocaine injections were given intraperitoneally. The doses refer to the base form, and the injection volume of 10 ml/kg was used. Morphine or cocaine was given both acutely and repeatedly. In the repeated morphine paradigm, morphine 30 mg/kg was administered daily on four consecutive days, and after 96 h withdrawal, a challenge dose (5 or 10 mg/kg) was given. In the repeated cocaine administration, cocaine was given on consecutive days. In the repeated amphetamine experiment, mice were given amphetamine 2 mg/kg daily on days 1-3, amphetamine 4 mg/kg daily on days 4-6, and after a six-day withdrawal period, a challenge dose of 2 mg/kg was given.

4.3. Microdialysis in freely moving mice

Detailed descriptions of *in vivo* microdialysis can be found in studies I, II and III. Briefly, a mouse was anaesthetised and a microdialysis guide cannula was aimed at the point above the dorsal striatum or the nucleus accumbens. Five to seven days after the surgery, at about 4 p.m., a microdialysis probe was inserted into the guide cannula. The microdialysis membrane was perfused overnight at low perfusion rate with a physiological solution closely resembling the cerebrospinal fluid but devoid of neurotransmitters and their metabolites. In the morning of the experimental day, the flow rate was increased and the obtained dialysates were separated by high performance liquid chromatography and concentrations of dopamine, DOPAC, HVA and 5-HIAA were analyzed by an electrochemical detector. After four baseline samples, the drug to be studied was administered. Samples were collected continuously every 20 min. The effects of drugs are presented as relative to the baseline (= the average concentration of four consecutive stable samples defined as 100%). The experiments with the MEN2B mice were conducted similarly, but different probes were used (MAB4, Agn Tho's AB, Lidingö, Sweden).

Methodological considerations

Microdialysis mimics the function of a small blood vessel. It is used for exploring the chemistry of a tissue at the extracellular level (Ungerstedt, 1991). With a semi-permeable membrane, substances can be withdrawn from and delivered to a tissue where the probe has been inserted. In neuropharmacological research microdialysis is commonly used for studying the effects of different treatments on extracellular neurotransmitter

concentrations in different brain regions of laboratory animals. The main advantages of *in vivo* microdialysis, as used in the present experiments, are the use of awake and freely moving mice and continuous monitoring of the concentrations of dopamine and its metabolites over a long time. Also, as the stabilization time is long, the basal concentrations are stable. The probes used in the experiment have low recovery, about 5% (CMA/7), and thus, the removal of dopamine and its metabolites minimally interferes with physiological dopamine turnover. The main disadvantages are the relatively large size of the microdialysis probe (o.d. 240 μm) and that insertion of the probe causes damage to the tissue. In addition, with microdialysis a relatively poor time resolution can be achieved (20 min).

4.4. Cerebral monoamine analysis from tissue samples

Detailed descriptions of monoamine analysis can be found in studies II and IV. Briefly, in study II, mice were given saline or morphine 5, 10 or 30 mg/kg s.c. one hour before the mice were killed by decapitation. The brains were rapidly excised, dissected and stored at -80°C until assay. Monoamines and their metabolites were detected using an ESA® CoulArray Electrode Array Detector and chromatograms were processed and concentrations of monoamines calculated using CoulArray® software. Monoamine and metabolite values were calculated as nanograms per gram (ng/g) wet weight of the tissue.

Methodological considerations
Monoamine analysis from tissue samples is a technique to study the chemistry of a tissue on the intracellular level. Its limitations are mainly related to the dissection technique, and the dissection method might cause variability and bias to the analysed brain region. Dissection can be performed either by punching or by pinching from a brain section containing the structures of interest. Punching can be used to collect dorsal and ventral striatum separately, whereas pinching with forceps can be used to collect the entire striatum. In study IV, the SN/VTA was punched, and it is to be noted, that the tissue taken did not cover those brain nuclei totally, but only partially.

4.5. Locomotor activity monitoring

A detailed description of the method used can be found in studies I, III and IV. Briefly, locomotor activity was monitored in transparent plastic cages. The movement of the mice caused interruptions of infrared photobeams, and these were recorded by a computer and afterwards analyzed. The mice were habituated to the test boxes in all other experiments but not in the novelty seeking experiment of the MEN2B mice (study IV).

Methodological considerations
Locomotor activity can be used to study the effects of different drug treatments or the effects of gene manipulations on motor behaviour. Neurotransmitters, such as dopamine, noradrenaline and excitatory amino acids have been shown to be involved in locomotor activity. Behavioural sensitization has been defined as drug-induced enhancement of locomotor activity responses following repeated injections of a drug, and it has been observed with most abused drugs.

4.6. Place conditioning

A detailed description of the method used can be found in study III. Briefly, the tests were conducted in standard conditioned place preference (CPP) boxes. CPP trials were divided into three stages: 1. Habituation & preconditioning 2. Conditioning 3. Postconditioning. A temporal interval of 10 min was used in the pre- and postconditioning tests. About half of the animals were conditioned in the side where they spent less time, while the other half were conditioned in the side where they spent more time, to be paired with morphine. In the mornings, the mice were given saline and assigned to the saline-paired compartment for 60 min. Three to four hours later, the mice were given morphine or saline and assigned to the drug-paired compartment for 60 min. Different protocols were used in morphine 5 mg/kg and 10 mg/kg experiments, as the aim was to study both the development and duration of the place preference (Table 4.1.).

Methodological considerations

The CPP paradigm has proven to be a useful tool in the investigation of rewarding properties of drugs (Hoffman, 1989). CPP reflects a preference for a context due to the contiguous association between the context and a drug stimulus. In CPP experiments, morphine causes incentive learning, eliciting drug-seeking behaviour that is believed to be mediated by nucleus accumbens -related reward circuitry (Bardo and Bevins, 2000; Spanagel and Weiss, 1999; Tzschentke, 1998). In the present experiments, CPP testing was done accordingly to criteria of unbiased principles. The mice were conditioned about equally to the side where they preferentially spent less time and to the side where they spent more time. In the biased method, all mice are conditioned to the side where they spent preferentially less time, but this protocol is subject to interference from the potential antianxiety effect of the test drug.

Table 4.1. Time schedules of CPP experiments.

	Morphine 5 mg/kg	Morphine 10 mg/kg
Day 1	Habituation	Habituation
Day 2	Habituation	Habituation
Day 3	Preconditioning test	Preconditioning test
Day 4	Conditioning	Conditioning
Day 5	Conditioning	Conditioning
Day 6	Postconditioning test	Conditioning
Day 7	Conditioning	Conditioning
Day 8	Conditioning	Postconditioning test
Day 9	Postconditioning test	
Day 15	Postconditioning test	
Day 22		Postconditioning test
Day 29		Postconditioning test
Day 44		Postconditioning test

4.7. Immunological methods

Immunohistochemistry was done as described in studies I and IV. Briefly, the mice were anaesthetized and perfused intracardially with phosphate-buffered saline followed by 4% paraformaldehyde. The brains were removed, postfixed for 4 h and stored in sodium phosphate buffer containing 20% sucrose at 4°C. Coronal sections (30-40 µm) were cut on a cryostat (Leica CM 3050, Leica Microsystems Nussloch GmbH, Nussloch, Germany) throughout the brain. Free-floating sections from selected brain regions were stained using standard immunohistochemical procedures. In study I, polyclonal rabbit antibody to FosB (H-75, sc-7203, Santa Cruz Biotechnology Inc., CA, USA) was used. In study IV, polyclonal rabbit antibody to TH (Chemicon, AB152) and monoclonal rat antibody to the DAT (Chemicon, MAB 369) was used. In experiments with c-Fos polyclonal rabbit antibody (PC38, Calbiochem) was used. The stained sections were mounted on gelatin/chrome-alume coated slides and coverslipped with DePex®. The atlas of Franklin and Paxinos (1997) was used to identify different brain regions. Immunostained nuclei and cells were quantified with a computerized image-analysis system including a camera, a microscope and a computer with either Image-Pro Plus 4.0 or StereoInvestigator software. The countings were conducted so that the observers were blinded to the experimental design.

GDNF concentrations were analyzed from heterozygous GDNF+/- mice by ELISA-assay as described in study I. Briefly, dorsal and ventral striatum were dissected and frozen into microcentrifuge tubes on dry ice to minimize condensation and frozen and stored at -80°C. Tissues were homogenized in 400 µl of lysis buffer (TBS, 1% NP40, 10% glycerol, Complete Protease Inhibitor Cocktail tablet, Roche Diagnostics, Basel, Switzerland). GDNF concentrations were measured using GDNF E_{max}® immunoassay system (Promega, WI, USA), strictly following the manufacturer's instructions. For each GDNF measurement, tissues from 8-9 mice were pooled, and the results presented are means (± SEM) of three separate measurements.

Methodological considerations

Immunological methods are useful in locating and quantifying proteins. They are based on exquisite specificity of antibodies for their target proteins. Antibodies can be either monoclonal or polyclonal. Polyclonal antibodies are heterogeneous mixtures of antibodies, each specific for one of the various epitopes of the antigen. Monoclonal antibodies are produced by a single antibody-producing cell clone and they recognize a specific epitope. In the present experiments, antibodies to FosB, c-Fos and TH were polyclonal and the antibody to DAT was monoclonal. The FosB antibody recognizes both the FosB and ΔFosB isoforms. The main limitations in using immunological methods are the detection methods of the immunostained proteins, which usually are semi-quantitative. In ELISA experiments a limitation is the dissection accuracy, and when analyzed immunostained brain sections a question is whether the whole region of interest is systematically analyzed or only parts of it.

4.8. Statistics

Locomotor activity data were analyzed by one- or two-way analysis of variance (ANOVA) for repeated measurements. The CPP data were

analyzed by two-way ANOVA and by paired *t*-test. Microdialysis data were analyzed by one- or two-way ANOVA for repeated measurements and further analyses were conducted with Tukey/Kramer *post hoc* test. Basal dopamine, DOPAC, HVA, 5-HIAA and GDNF concentration data were analyzed by Student's *t*-test. Immunohistochemistry data were analyzed by one-way ANOVA and Student's *t*-test. All results are given as mean ±SEM, and the results were considered statistically significant at $P<0.05$.

5. RESULTS

5.1 Striatal GDNF concentrations in GDNF+/- mice and in their wild-type littermates (I)

Tissue GDNF concentration in the dorsal striatum was 8.9±0.7 pg/mg tissue in the Wt mice and 5.0±0.5 pg/mg tissue in the GDNF+/- mice. The GDNF concentrations were significantly reduced in the GDNF+/- mice by 44% (P=0.009, Student's *t*-test). In the ventral striatum GDNF concentration in the Wt mice was 5.4±0.4 and in the GDNF+/- mice 4.4±0.3. The difference (18%) was not significant (P=0.085).

5.2. Effects of depletion of one GDNF allele on the brain dopaminergic system and on basal locomotor activity (I, II)

As summarized in Table 5.1, there were no significant differences in the *post-mortem* tissue concentrations of striatal dopamine, DOPAC and HVA between the Wt and the GDNF+/- mice (dopamine: P=0.0759; DOPAC: P=0.3648; HVA: P=0.3284, Student's *t*-test).

As summarized in Table 5.1, the steady-state extracellular concentration of dopamine was higher in the dialysates of the caudate putamen and the nucleus accumbens of the GDNF+/- mice than in those of the Wt mice. The increase was 2.0-fold in the caudate putamen (P<0.001, Student's *t*-test) and 1.6-fold in the nucleus accumbens (P=0.017, Student's *t*-test). There were no significant differences between DOPAC and HVA concentrations in the caudate putamen or in the nucleus accumbens perfusates between GDNF+/- and Wt mice.

As summarized in Table 5.1, the cumulative ambulatory counts in the 24-h locomotor activity assessment were similar.

5.3. c-Fos and FosB/ΔFosB immunostaining in GDNF+/- mice and in their wild-type littermates (I, unpublished)

As summarized in Table 5.2, the number of FosB immunostained nuclei was clearly higher in the striatal brain regions in the GDNF+/- mice than in the Wt mice. The elevation was 2.2-fold in the nucleus accumbens core area (P=0.044, Student's *t*-test), 1.6-fold in the nucleus accumbens shell area (P=0.04, Student's *t*-test), and 5.7-fold in the caudate putamen (P=0.026, Student's *t*-test). In the cingulate cortex, there was no statistically significant difference in the number of FosB immunostained nuclei between the GDNF+/- and Wt mice (P=0.077, Student's *t*-test, unpublished data). Similarly, the number of c-Fos immunostained nuclei was increased in the caudate putamen and cingulated cortex in the GDNF+/- mice (P=0.045, P=0.031, respectively, Student's *t*-test, unpublished data).

5.4. Effects of acute and repeated cocaine in GDNF+/- mice and in their wild-type littermates (I)

As summarized in Table 5.3, the locomotor activities after acute saline did not differ between the GDNF+/- and Wt mice. Acute cocaine, 10 mg/kg, stimulated locomotor activity in the mice of both genotypes (treatment effect: P<0.0001, genotype X treatment interaction: P=0.8276, ANOVA). The stimulatory effect of cocaine on locomotor activity was gradually and

Table 5.1. Dopamine, DOPAC, and HVA concentrations in the *post mortem* striatal tissues and in the steady-state microdialysis samples in the GDNF+/- and Wt mice. ↔ indicates that the difference between GDNF+/- and Wt mice is less than 30%, ↑ indicates 50-70% increase, ↑↑ indicates 80-110% increase, * P<0.05, ***P<0.001.

Tissue concentrations (ng/g) n=23-24			
Brain area and genotype	**Dopamine**	**DOPAC**	**HVA**
CPu			
Wt	12700 ± 50	1020 ± 70	750 ± 50
GDNF+/-	11500 ± 40	930 ± 60	680 ± 50
%-difference	↔	↔	↔
Extracellular concentrations n=12-26			
Brain area and genotype	**Dopamine (nM)**	**DOPAC (µM)**	**HVA (µM)**
CPu n=12-15			
Wt	0.31 ± 0.04	0.13 ± 0.02	0.13 ± 0.02
GDNF+/-	0.63 ± 0.07 ***	0.15 ± 0.01	0.15 ± 0.02
%-difference	↑↑	↔	↔
NAc (n=24-26)			
Wt	0.37 ± 0.04	0.10 ± 0.01	0.07 ± 0.01
GDNF+/-	0.60 ± 0.08 *	0.12 ± 0.01	0.08 ± 0.01
%-difference	↑	↔	↔
24-h locomotor activity (cumulative ambulatory counts, n=11-12)			
Wt	3130±280		
GDNF+/-	2850±270		

significantly increased from the first dose to the fourth dose and locomotor sensitization to cocaine developed similarly in the GDNF+/- and Wt mice (day effect: P<0.0001, day X genotype interaction: P=0.911, ANOVA for repeated measurements).

The effects of acute cocaine, 10 mg/kg, on accumbal dopamine output were similar in the GDNF+/- and Wt mice, when the dopamine output was calculated as percentage from the baseline. After pre-treatment with cocaine 10 mg/kg daily on three consecutive days, the response to cocaine on the fourth day was decreased in the GDNF+/- mice. There was a significant difference between the Wt and GDNF+/- mice (ANOVA for repeated measures, 100-160 min, P=0.046). After pre-treatment with cocaine, the difference in baseline dopamine concentrations between the GDNF+/- and Wt mice was only 14%.

5.5. Effects of repeated amphetamine treatment on locomotor activity in GDNF+/- mice and in their wild-type littermates (unpublished).

As shown in Figure 5.1, locomotor activities after amphetamine 2 mg/kg (s.c.) injections were similar in the GDNF+/- and Wt mice. During the first three days, there were similar daily increases in amphetamine-enhanced locomotion in both genotypes (genotype effect: P=0.3360, day effect: P=0.0217, day X genotype interaction: P=0.9074, ANOVA for repeated measures). After withdrawal,

there was similar and robust locomotor sensitization in the Wt and GDNF+/- mice (day effect between days 3 and 12: P=0.0001; genotype effect: P=0.8969; day X genotype interaction: P=0.6625).

5.6. Effects of acute and repeated morphine on locomotor behaviour in GDNF+/- mice and in their wild-type littermates (III)

When a large dose of morphine (30 mg/kg s.c.) was given daily on four consecutive days, there was a difference between the genotypes in distance travelled on day 4. The first dose of morphine increased distances travelled by the Wt and GDNF+/- mice similarly. However, after the fourth daily morphine dose the GDNF+/- mice travelled less than on day 1 (time 60-120 min, P=0.0458, ANOVA for repeated measures), whereas the distances travelled by the Wt mice were similar after the first and the fourth doses (n=28-40).

The effects of acute morphine (5 and 10 mg/kg) were similar on locomotor activity in the Wt and GDNF+/- mice. However, after 96 h withdrawal from the fourth daily injection of morphine (30 mg/kg), the morphine challenge dose 5 mg/kg stimulated locomotor activity in the GDNF+/- mice, but no such stimulation occurred in correspondingly treated Wt mice. At 15-65 min after administration of the challenge dose, the distance travelled by the morphine pretreated GDNF+/- mice was significantly longer than that of the Wt mice (P=0.024, ANOVA for repeated measures). However, the responses to the morphine 10 mg/kg challenge dose were robustly and similarly sensitized in mice of both genotypes after 96 h withdrawal from the 4-day morphine pre-treatment. The distances travelled were significantly longer (5-8-fold) in morphine pretreated than in saline pretreated mice (pretreatment effect 15-120 min: P=0.0002, ANOVA for repeated measures). When both the morphine challenge doses were analyzed together, there was a tendency for genotype X treatment interaction (P=0.0898), and a statistically significant time X genotype X treatment interaction was found (P=0.0315, time 20-60 min).

Table 5.2. Number of FosB/ΔFosB and c-Fos immunostained nuclei in caudate putamen (CPu), nucleus accumbens core (NAcC), nucleus accumbens shell (NAcSh) and in the cingulate cortex (CG) of the GDNF+/- and Wt mice. ↑ indicates 50-90% increase, ↑↑ indicates 100-130% increase, ↑↑↑ indicates 140-170% increase, ↑↑↑↑ indicates 440-500% increase * P<0.05 as compared with the Wt mice.

Brain region and genotype	FosB/ΔFosB	c-Fos
CPu		
Wt	9 ± 3	7 ± 2
GDNF+/-	49 ± 13	42 ± 13
%-difference	↑↑↑↑ *	↑↑↑↑ *
NAcC		
Wt	105 ± 24	34 ± 10
GDNF+/-	230 ± 37	62 ± 16
%-difference	↑↑ *	↑
NAcSh		
Wt	228 ± 16	73 ± 9
GDNF+/-	365 ± 47	111 ± 26
%-difference	↑ *	↑
CG		
Wt	29± 5	40 ± 12
GDNF+/-	54± 9	100 ± 17
%-difference	↑	↑↑↑ *

5.7. Effects of acute morphine on striatal tissue dopamine, DOPAC and HVA concentrations in GDNF+/- mice and in their wild-type littermates (II)

Acute morphine administration did not alter striatal tissue dopamine concentrations in mice of either genotype (treatment effect:

Table 5.3. The effects of acute (day 2) and repeated cocaine (day 5) on locomotor activity and on accumbal dopamine concentrations. In locomotor activity the fold-change is calculated from the corresponding saline (day 1) treatment. In the lower panel, the maximal dopamine output is from the time point 120 min after cocaine administration (Study I). ↑↑ indicates 120-150% increase, ↑↑↑ indicates that the increase is 210-220%.

Locomotor activity				
	Saline day 1	Cocaine day 2	Cocaine day 5	
Wt	1750±290 cm	2.9-fold	8.9-fold	
GDNF+/-	1720±370 cm	3.2-fold	9.7-fold	
Dopamine concentrations nM				
	Baseline in saline pre-treated	Maximal concentration of cocaine-induced output in saline pre-treated	Baseline in cocaine pre-treated	Maximal concentration of cocaine-induced output in cocaine pre-treated
Wt	0.48 ± 0.10	1.18 ± 0.37 ↑↑	0.47 ± 0.08	1.49 ± 0.39 ↑↑↑
GDNF+/-	0.67 ± 0.19	1.61 ± 0.45 ↑↑	0.54 ± 0.16	1.20 ± 0.40 ↑↑

P=0.0791; treatment X genotype interaction: P=0.4061, ANOVA). As summarized in Table 5.4, morphine increased striatal DOPAC in the Wt and GDNF+/- mice (treatment effect Wt: P<0.0001; GDNF+/-: P=0.001, genotype X treatment interaction: P=0.1922) and HVA in the Wt mice (treatment effect P=0.0192) but not in the GDNF+/- mice (treatment effect: P=0.1281; genotype X treatment interaction: P=0.4461). Morphine 5 mg/kg did not have effect on DOPAC or HVA concentrations, morphine 10 mg/kg elevated DOPAC significantly only in the GDNF+/- mice and morphine 30 mg/kg elevated DOPAC both in the GDNF+/- and Wt mice (P<0.05, Tukey/Kramer *post hoc* test). Morphine 30 mg/kg elevated the metabolites more in the Wt mice (DOPAC: 66%, HVA 35%) than in the GDNF+/- mice (DOPAC: 45%, HVA 23%), when just the effects of morphine 30 mg/kg were analyzed there was a statistically significant genotype effect on DOPAC (P=0.0203) and on HVA (P=0.0421).

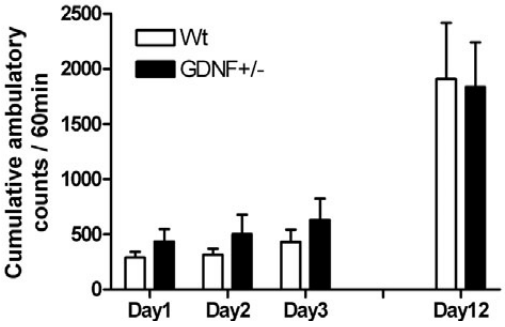

Figure 5.1. The effect of amphetamine 2 mg/kg on locomotor activity. The mice were given amphetamine 2 mg/kg s.c. on days 1-3, amphetamine 4 mg/kg on days 4-6 and six days after withdrawal, on day 12, the mice were given a challenge dose of amphetamine 2 mg/kg, n=12.

5.8. Effects of acute and repeated morphine on accumbal dopamine, DOPAC and HVA output in GDNF+/- mice and in their wild-type littermates (II, III)

Baseline dopamine, DOPAC and HVA concentrations in morphine-withdrawn mice

As shown in Table 5.5, repeated morphine treatment reduced the basal extracellular dopamine concentrations in the GDNF+/- mice but did not alter those in the Wt mice. In morphine-naive GDNF+/- mice the

extracellular dopamine concentration in the nucleus accumbens was 1.6-fold higher than in the Wt mice. However, there were no differences in the steady-state accumbal extracellular dopamine concentrations between the Wt and GDNF+/- mice withdrawn 96 h from 4 day morphine treatment (GDNF+/-: 0.43±0.07 nM, Wt: 0.41±0.09 nM, P=0.8961, Student's t-test, n=13). Neither were there any differences in

Table 5.4. Tissue DOPAC and HVA (ng/g) concentrations in morphine-treated (5, 10 and 30 mg/kg) Wt and GDNF+/- mice as compared with saline treatment. In saline-treated mice DOPAC concentrations were 1020±70 (Wt) and 930±60 (GDNF+/-) and HVA concentrations were 750±50 (Wt) and 680±50 (GDNF+/-). ↑ indicates increase of 20-30 %, ↑↑ indicates increase of 30-60%, ↑↑↑ indicates increase of 60% or more, ↔ indicates that the difference is less than 20%, * indicates P<0.05 as compared with the corresponding saline treatment, ° indicates P<0.05 as compared with corresponding Wt mice, Tukey/Kramer *post hoc* test.

	Morphine 5		Morphine 10		Morphine 30	
DOPAC						
Wt	900±60	↔	1230±100	↑	1690±130	↑↑↑ *
GDNF+/-	1020±70	↔	1170±70	↑ *	1350±80	↑↑ * °
HVA						
Wt	670±80	↔	790±60	↔	1010±70	↑↑
GDNF+/-	710±70	↔	810±40	↔	840±70	↑ °

Table 5.5. The effects of acute and challenge morphine (Mo) on dopamine, DOPAC and HVA output in the nucleus accumbens. The arrows indicate the effect of morphine at 120 min after the injection as compared with the baseline. The baseline values for each group are given in brackets. ↑ indicates increase of 20-40 %, ↑↑ indicates increase of 41-70%, ↑↑↑ indicates increase of 71-100%, ↔ indicates less than 20% increase, * indicates P<0.05 as compared with the saline treatment, ° indicates P<0.05 as compared with effect of morphine 5 mg/kg, # indicates P<0.05 as compared with the corresponding Wt mice.

	Acute Mo5		**Acute Mo10**		**Challenge Mo5**		**Challenge Mo10**	
	Baseline concentration	Concentration at 120 min after injection	Baseline concentration	Concentration at 120 min after injection	Baseline concentration	Concentrationat 120 min after injection	Baseline concentration	Concentration at 120 min after injection
Dopamine (nM)								
Wt	0.36±0.06	0.51±0.13 ↑ *	0.37±0.11	0.58±0.14 ↑↑ * °	0.41±0.09	0.49±0.11 ↑ *	0.43±0.18	0.74±0.21 ↑↑↑ * °
GDNF+/-	0.64±0.15	1.16±0.22 ↑↑↑ * #	0.56±0.16	0.76±0.24 ↑↑ * °	0.42±0.09	0.63±0.15 ↑↑ * #	0.45±0.12	0.63±0.16 ↑ *
DOPAC (µM)								
Wt	0.11±0.02	0.08±0.01 ↔	0.11±0.03	0.17±0.05 ↑↑ * °	0.11±0.02	0.14±0.02 ↑ *	0.15±0.04	0.23±0.04 ↑↑ * °
GDNF+/-	0.11±0.02	0.15±0.02 ↑ *	0.13±0.04	0.16±0.02 ↑ *	0.12±0.02	0.16±0.01 ↑ *	0.08±0.01	0.12±0.02 ↑↑ * °
HVA (µM)								
Wt	0.07±0.01	0.09±0.01 ↑ *	0.07±0.02	0.10±0.03 ↑↑ * °	0.10±0.01	0.14±0.02 ↑ *	0.12±0.02	0.18±0.02 ↑↑ *
GDNF+/-	0.08±0.01	0.11±0.02 ↑ *	0.09±0.02	0.11±0.02 ↑ *	0.10±0.01	0.14±0.01 ↑ *	0.07±0.02	0.10±0.02 ↑↑ *

DOPAC (GDNF+/-: 0.11±0.014 µM, Wt: 0.13±0.017 µM, P=0.3170, Student's *t*-test, n=13) or HVA (GDNF+/-: 0.089±0.003 µM, Wt: 0.109±0.011 µM, P=0.1803, Student's *t*-test, n=13) concentrations between the Wt and GDNF+/- mice withdrawn 96 h from 4 day morphine treatment.

Effects of acute morphine on accumbal dopamine, DOPAC and HVA output

As summarized in Table 5.5, acute morphine elevated accumbal dopamine output differently in the GDNF+/- and Wt mice (treatment effect: P=0.0167, treatment X genotype interaction: P=0.0699, treatment X genotype X time 20-320 min interaction: P<0.0001). Acute morphine dose-dependently increased the accumbal dopamine output in the Wt mice (ANOVA for repeated measures P=0.0379, P<0.05 between saline& morphine 5 mg/kg, saline & morphine 10 mg/kg and morphine 5 mg/kg & morphine 10 mg/kg; Tukey/Kramer *post hoc* test). In the GDNF+/- mice, morphine 5 mg/kg increased the accumbal dopamine levels robustly and clearly more than the dose of 10 mg/kg (ANOVA for repeated measures P=0.0232, P<0.05 between saline & morphine 5 mg/kg, saline & morphine 10 mg/kg and morphine 5 mg/kg & morphine 10 mg/kg; Tukey/Kramer *post hoc* test) or than the 5 mg/kg dose in the Wt mice (genotype effect: P=0.0359). Morphine 10 mg/kg -induced increases were similar in both genotypes; about 80% in the Wt mice and about 60% in the GDNF+/- mice.

As summarized in Table 5.5, the effect of morphine on DOPAC output differs significantly between the genotypes (genotype X treatment interaction 100-320 min: P=0.0287). In the Wt mice, acute morphine increased the accumbal DOPAC output only at the dose of 10 mg/kg (ANOVA for repeated measures, P=0.0228, P<0.05 between saline & morphine 10 mg/kg and morphine 5 mg/kg & morphine 10 mg/kg; Tukey/Kramer *post hoc* test). However, in the GDNF+/- mice, both morphine doses (5 & 10 mg/kg) increased DOPAC output (P=0.0449, P<0.05 between saline & morphine 5 mg/kg and saline & morphine 10 mg/kg, morphine 5 mg/kg and morphine 10mg/kg; Tukey/Kramer *post hoc* test). Morphine increased HVA output statistically significantly in the mice of both genotypes (treatment effect: P=0.0042, treatment X genotype interaction: P=0.9252, 100-320 min, ANOVA for repeated measures). In the Wt mice, this effect was dose-dependent (P<0.05 Tukey/Kramer *post hoc* test), but in the GDNF+/- mice both morphine doses (5 and 10 mg/kg) increased HVA output similarly.

Effects of morphine challenge on accumbal dopamine, DOPAC and HVA output in morphine-withdrawn mice

As summarized in Table 5.5, 96 h after withdrawal from repeated morphine treatment, challenge doses of morphine increased the accumbal dopamine output in both the Wt and GDNF+/- mice (ANOVA for repeated measures, P=0.0048). Interestingly, there was a highly significant difference at the time 100-320 min (time X genotype X treatment interaction: P=0.0159) showing that the time-response curves of morphine differ between the mice of the two genotypes. When just the effects of morphine 5 mg/kg were analyzed, morphine was found to significantly increase dopamine output more in the GDNF+/- mice than in the Wt mice (ANOVA for repeated measures, genotype effect at 180-260 min: P=0.0329). In addition, the effect of morphine was dose-dependent in the Wt mice (treatment effect at 100-260 min: P=0.0140, P<0.05 between saline &

morphine 5 mg/kg, saline & morphine 10 mg/kg and morphine 5mg/kg & morphine 10 mg/kg; Tukey/Kramer *post hoc* test), but in the GDNF+/- mice morphine 5 and 10 mg/kg increased dopamine output similarly (treatment effect at 100-260 min: P=0.0469; P<0.05 between saline & morphine 5 mg/kg and saline & morphine 10 mg/kg, Tukey/ Kramer *post hoc* test).

Challenge doses of morphine similarly and dose-dependently increased the accumbal DOPAC output in the Wt and GDNF+/- mice (treatment effect: P=0.0013, X genotype interaction: P=0.7443, 140-320min, P<0.05 between saline & morphine 5 mg/kg, saline & morphine 10 mg/kg, morphine 5 mg/kg & morphine 10 mg/kg; Tukey/Kramer *post hoc* test). Both challenge doses of morphine increased the accumbal HVA output similarly in the Wt and GDNF+/- mice (treatment effect: P<0.0001, treatment X genotype interaction: P=0.7066, 140-320min).

5.9. Effects of morphine on CPP behaviour in GDNF+/- mice and in their wild-type littermates (III)

The effects of morphine 5 mg/kg on CPP are summarized in Table 5.6. The preconditioning times (s±SEM) before morphine 5 mg/kg or saline treatments were similar in the Wt and GDNF+/- mice (Wt saline: 301±16; Wt mo5: 282±13; GDNF+/- saline: 327±23; GDNF+/- mo5: 316±25). On day 6, after two conditioning sessions, place preference to morphine 5mg/ kg or saline had not developed in either genotype. After four conditioning sessions on day 9, place preference to morphine had developed similarly in the Wt (353±16) and GDNF+/- mice (357±17, treatment effect: P=0.0041; treatment X genotype interaction: P=0.9957, two-way ANOVA). On day 15, 7 days after the last conditioning session, place preference was seen only in the Wt mice (P= 0.0117; paired *t*-test, n=10-20).

The effects of morphine 10 mg/kg on CPP are summarized in Table 5.6. In this experiment the duration of morphine-induced place preference in the Wt and GDNF+/- mice was studied by conditioning the mice with morphine 10 mg/kg on four days and measuring the postconditioning times one day, two weeks, three weeks and five weeks after the conditioning period. The preconditioning times (s±SEM) before morphine treatment were similar in the Wt and GDNF+/- mice (Wt: 255±23; GDNF+/-: 272±18). Morphine-induced place preference developed similarly in both genotypes, as on day 8 the time spent in the drug-paired side was significantly increased both in the Wt mice (P=0.0176, paired *t*-test) and in the GDNF+/- mice (P=0.00315, paired *t*-test). However, 2 weeks (day 22) and 3 weeks (day 29) after the conditioning, retention of the place preference was seen only in the Wt mice (P=0.0357; P=0.0123, paired *t*-test), whereas in the GDNF+/- mice the time spent in the drug-paired side had

Table 5.6. The effects of morphine 5 and 10 mg/kg on CPP, - indicates that there was no place preference, + indicates that there was place preference. Days refer to the length of the experiment so that day 1 is the first habituation day (see table 4.1).

Morphine 5 mg/kg	**Wt**	**GDNF+/-**
Day 6	-	-
Day 9	+	+
Day15	+	-
Morphine 10 mg/kg		
Day 8	+	+
Day 22	+	-
Day 29	+	-
Day 44	-	-

returned to the preconditioning level. Five weeks after conditioning (day 44) place preference was not seen in the mice of either genotype.

5.10. The effects of codon 919 mutation in murine *RET* (to encode threonine rather than methionine) on cerebral tissue dopamine, noradrenaline and serotonin (IV)

As summarized in Table 5.7, dopamine, DOPAC and HVA were significantly increased in both the dorsal striatum (genotype effect for dopamine, DOPAC and HVA: P<0.0001, one-way ANOVA) and in the ventral striatum (genotype effect for dopamine and DOPAC: P < 0.0001, for HVA: P<0.0032 one-way ANOVA) of the MEN2B/+ and MEN2B/MEN2B mice as compared with their wild-type littermates. In the dorsal striatum, dopamine concentrations were increased in the MEN2B/+ mice by 54% and in the MEN2B/MEN2B mice by 94% as compared with the Wt mice. DOPAC concentrations were increased in the MEN2B/+ mice by 102% and in the MEN2B/MEN2B mice by 185%. HVA concentrations were significantly increased by 72% in the MEN2B/+ mice and by 137% in the MEN2B/MEN2B mice. In the dorsal striatum of the MEN2B/MEN2B mice dopamine, DOPAC and HVA concentrations were significantly higher than those in the MEN2B/+ mice (P<0.01, P<0.01, P<0.05, respectively, Tukey/Kramer). In the ventral striatum, dopamine concentrations were about 60%, DOPAC concentrations about 120-130% and HVA concentrations about 80% higher in the MEN2B/+ and MEN2B/MEN2B than those in the Wt mice. Also, it is to be noticed that in contrast to dorsostriatal tissues, there were no differences in the ventrostriatal dopamine, DOPAC or HVA concentrations between the MEN2B/+ and the MEN2B/MEN2B mice.

As summarized in Table 5.8, only dopamine concentrations and neither noradrenaline nor serotonin concentrations were increased in the target areas of their neuronal pathways in the MEN2B mice. Cortical dopamine was increased by about 70% in the MEN2B/+ and MEN2B/MEN2B as compared with the Wt mice. Cortical DOPAC and HVA concentrations were also significantly higher

Table 5.7. Dopamine, DOPAC and HVA concentrations in the *post mortem* dorso- and ventrostriatal tissues in the MEN2B mice. ↑ indicates 50-70 % increase, ↑↑ indicates 70-100% increase, ↑↑↑ indicates 100-140% increase ↑↑↑↑ indicates 180-190% increase, * indicates P<0.05, ** P<0.01 as compared with the Wt mice, °° indicates P<0.01 as compared with the MEN2B/+ mice

	MEN2B/+		MEN2B/MEN2B	
Dorsal striatum				
Dopamine	↑	**	↑↑	** °°
DOPAC	↑↑↑	**	↑↑↑↑	** °°
HVA	↑↑	**	↑↑↑	** °°
Ventral striatum				
Dopamine	↑	**	↑	**
DOPAC	↑↑↑	**	↑↑↑	**
HVA	↑↑	*	↑↑	**

in the MEN2B/+ mice (respectively by 61% and 58%) and MEN2B/MEN2B mice (respectively by 116% and 89%) than in the Wt mice. In the hypothalamus, dopamine, DOPAC and HVA concentrations were significantly elevated only in the MEN2B/MEN2B mice (respectively by 32%, 73% and 92%) as compared with the Wt mice. In the lower brain stem, the concentrations of dopamine and its metabolites were small and similar in mice of the three genotypes. The concentrations of serotonin were similar within the three genotypes in all brain regions studied. The concentrations of noradrenaline did not differ between the mice of the three genotypes except in the lower brainstem, where the concentration was increased by 27% in the MEN2B/MEN2B mice as compared with the Wt mice.

5.11. Extracellular dopamine, DOPAC and HVA concentrations in the dorsal striatum of MEN2B/MEN2B mice and in their wild-type littermates (unpublished)

As summarized in Table 5.9, extracellular dopamine concentrations were similar in the Wt and MEN2B/MEN2B mice in the dorsal striatum. However, the extracellular DOPAC and HVA concentrations were increased significantly by 97% and by 62%, respectively in the mutant mice (DOPAC: P=0.005, HVA: P=0.021, Student's *t*-test).

5.12. Spontaneous and 24-h locomotor activity in knock-in MEN2B mice and in their wild-type littermates (IV)

Spontaneous activity in nonhabituated Wt and in MEN2B/+ and MEN2B/MEN2B mice declined during the 60 min recording period. Both the MEN2B/+ and MEN2B/MEN2B mice moved less than the Wt mice (genotype effect: P= 0.0038, repeated measures ANOVA) and there were no differences between the heterozygous and homozygous mice. When the mice were habituated there were no differences in 24-h locomotor activity between the mice of the three genotypes.

5.13. The effects of acute cocaine on locomotor activity in knock-in MEN2B mice and in their wild-type littermates (IV)

Table 5.10 summarizes the effects of cocaine on locomotor activity in MEN2B mice and

Table 5.8. Dopamine, noradrenaline and serotonin concentrations in the *post mortem* cerebral tissues in the MEN2B mice. ↔ indicates that the difference compared to the Wt mice is less than 20%, ↑ indicates 20-40 % increase, ↑↑ indicates 50-70% increase, ↑↑↑ indicates 80% increase or more, nd indicates not detected, * indicates P<0.05, ** P<0.01 as compared with the Wt mice, ° indicates P<0.05 as compared with the MEN2B/+ mice

	MEN2B/+	MEN2B/MEN2B
Cortex		
Noradrenaline	↔	↔
Serotonin	↔	↔
Dopamine	↑↑ **	↑↑ **
Hippocampus		
Noradrenaline	↔	↔
Serotonin	↔	↔
Dopamine	nd	nd
Hypothalamus		
Noradrenaline	↔	↔
Serotonin	↔	↔
Dopamine	↔	↑ *
Lower brain stem		
Noradrenaline	↔	↑ ** °
Serotonin	↔	↔
Dopamine	↔	↑

in their wild-type littermates. Locomotor activity of saline-treated MEN2B mice and their wild-type littermates did not differ significantly. Cocaine (5, 10 or 20 mg/kg) significantly and dose-dependently increased locomotor activity in mice of all three genotypes. Two-way ANOVA showed that the effects of cocaine differed between the Wt and MEN2B mice (genotype x treatment interaction, P= 0.0055). The effect of cocaine 5 mg/kg was similar in all genotypes (two-way ANOVA, genotype effect, P=0.9811). Cocaine 10 and 20 mg/kg increased locomotor activity significantly more in the MEN2B/+ and MEN2B/MEN2B mice than in the Wt mice (genotype effect for 10 mg/kg: P =0.0024 and for 20 mg/kg: P = 0.0033, genotype x treatment interaction for 10 mg/kg: P = 0.0005 and for 20 mg/kg: P = 0.0017, two-way ANOVA). In addition, cocaine 20 mg/kg increased locomotor activity in MEN2B/MEN2B mice more than in correspondingly treated Wt mice (P<0.01, Tukey/Kramer *post hoc* test).

5.14. Effects of repeated cocaine on locomotor activity in knock-in MEN2B mice and in their wild-type littermates (unpublished)

The locomotion-increasing effects of daily cocaine doses of 5 mg/kg were similarly enhanced on days 2-5 in all genotypes (Figure 5.2, ANOVA for repeated measures, day effect on days 2-5: P=0.002, n=11-22). Thus, the effect of cocaine on locomotion was 75-110% higher on day five than on day two in all genotypes.

The effects of daily injections of cocaine 10 mg/kg were also increased on days 2-5 in all genotypes (Figure 5.2, lower panel, ANOVA for repeated measures, day effect on days 2-5: P<0.001, n=7-13). The first dose of cocaine 10 mg/kg increased locomotor activity in MEN2B/MEN2B mice more than in the Wt mice, as the distance travelled by the homozygous mutants was 160% higher than that of Wt mice (P=0.0298, Student's *t*-test).

Table 5.9. The steady-state extracellular dopamine, DOPAC and HVA concentrations from dorsal striatum in the Wt and MEN2B/MEN2B mice, n=6-7, * indicates P<0.05, ** P<0.01 as compared with the Wt mice.

	Dopamine nM	DOPAC µM	HVA µM
Wt	1.73±0.42	0.31±0.05	0.43±0.05
MEN2B/MEN2B	1.47±0.28	0.61±0.07 **	0.68±0.07*

Table 5.10. The effects of acute cocaine 5, 10 and 20 mg/kg on distance travelled during 30 min. ↔ indicates that the distance travelled as compared with the corresponding saline treatment is increased less than 100%, ↑ indicates that the increase is 100-200%, ↑↑ indicates that the increase is 200-350%, ↑↑↑ indicates that the increase is 350-500%, ↑↑↑↑ indicates that the increase is 500-850%, * indicates statistically significant difference as compared with corresponding saline treatment, ° indicates statistically significant difference as compared with corresponding Wt mice.

Genotype	Cocaine 5 mg/kg	Cocaine 10 mg/kg	Cocaine 20 mg/kg
Wt	↔	↔	↑↑↑ **
MEN2B/+	↔	↑ *	↑↑↑↑ **
MEN2B/MEN2B	↑	↑↑ *	↑↑↑↑ ** °°

When the distances travelled were compared between the fourth and the first dose of cocaine, there are clear differences between the genotypes. In the Wt mice, the increase was about 220%, in the MEN2B/+ 90% and in the MEN2B/MEN2B mice only 58%. However, it is to be noticed that distances travelled after the fourth dose of cocaine 10 mg/kg were similar in all genotypes.

5.15. TH and DAT protein and mRNA expression in the striatum and in the SN/VTA area (IV)

TH protein levels were clearly increased both in the striatum and in the SN/VTA region of MEN2B mutant mice as compared with Wt mice. Optical density measurements in TH-immunostained sections showed that the MEN2B mutation had a dose-dependent effect on striatal TH levels. Optical density from immunostained brain sections was 55% greater in the MEN2B/+ and 75% greater in the MEN2B/MEN2B mice than in the Wt mice. This effect was confirmed by the Western blotting analysis. In the SN/VTA region, TH protein was found to be elevated by 60% in the MEN2B/+ and by 180% in the MEN2B/MEN2B mice, and also the TH mRNA expression was increased by about 60% in the MEN2B/MEN2B mice as compared with the Wt mice.

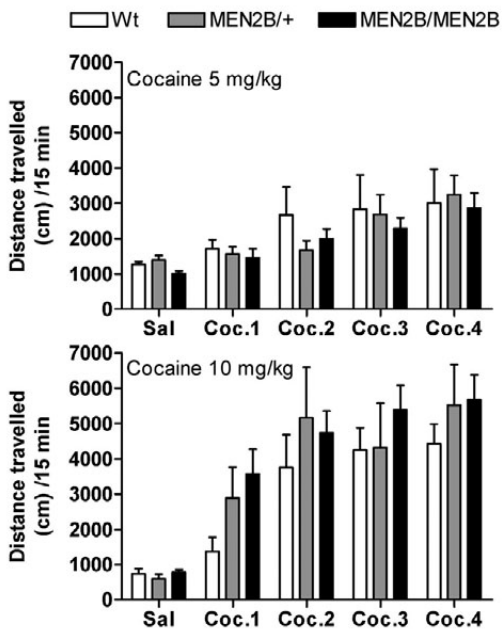

Figure 5.2. The effects of daily cocaine injections on distance travelled (cm) in the Wt, MEN2B/+ and MEN2B/MEN2B mice. The mice were given saline injections on the first day and thereafter daily cocaine 5 mg/kg i.p. (upper panel) or 10 mg/kg i.p. (lower panel).

DAT protein levels were increased in the striatum in MEN2B mice. Optical density measurement revealed about 50% increase of DAT in both MEN2B/+ and MEN2B/MEN2B mice as compared with Wt mice. This effect was confirmed by western blotting analysis. In the SN/VTA area DAT mRNA expression was found to be slightly (30%) but not significantly higher in the MEN2B/MEN2B mice than in the Wt mice.

5.16. TH-positive neurons in substantia nigra pars compacta and ventral tegmental area (IV)

The number of TH-immunostained cells in the SNpc was found to be increased by 26% in the MEN2B/MEN2B mice as compared with the Wt mice (Wt: 354±34, MEN2B/+: 387±31, MEN2B/MEN2B: 448±18, P=0.0246, Student's *t*-test). No differences were found in the number of TH-immunostained cells in the VTA between the genotypes (Wt: 1161±88, MEN2B/+: 1131±50, MEN2B/MEN2B: 1207±121.

6. DISCUSSION

6.1. Striatal dopaminergic system in mice with lowered GDNF concentrations

One of the most unexpected findings in the present experiments was that extracellular dopamine concentrations were increased in both striatal brain regions studied in the heterozygous GDNF+/- mice. The extracellular dopamine concentration increase in GDNF+/- mice, was not accompanied by an increase in metabolite concentrations, which was expected as metabolites have different diffusion properties from those of dopamine, and also as extracellular metabolite concentrations are higher. There was a clear increase in the number of FosB/ΔFosB and c-Fos positive nuclei in the striatal brain regions of these mice. Also Messer et al. (2000) reported that the accumbal levels of ΔFosB are increased in the GDNF+/- mice. The increased number of FosB/ΔFosB and c-Fos immunostained nuclei in the mesolimbic and nigrostriatal projection areas of GDNF+/- mice could be linked to the elevated extracellular dopamine concentrations, as amphetamine or cocaine-induced increases in ΔFosB and c-Fos levels can be blocked by D1-receptor antagonist (Berretta et al., 1992; Nye et al., 1995). However, c-Fos and FosB levels are increased by a variety of drugs administered acutely and by stress (Harlan and Garcia, 1998), whereas ΔFosB accumulates in striatal brain regions during chronic administration of drugs of abuse (Nestler et al., 2001). Thus, increased FosB/ΔFosB levels in the dopaminergic projection areas of the GDNF+/- mice suggest sustained postsynaptic activation.

Previous studies involving the role of endogenous GDNF have shown that there are no major changes in the dopaminergic systems in homozygous GDNF knockout mouse embryos or in heterozygous GDNF+/- knockout mice. In mouse embryos lacking GDNF there are no differences in the number or density of dopaminergic cells in the SNpc or in the striatal dopaminergic innervation as compared with normal mouse embryos (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). In line with previous experiments (Gerlai et al., 2001), in the present studies it was found that the basal tissue concentrations of striatal dopamine and its metabolites are similar in the GDNF+/- and in the Wt mice. However, it has been shown that GDNF has an important role in postnatal development of the brain dopaminergic systems. When foetal neural tissues lacking GDNF are transplanted into the adult mouse brain, the number of dopaminergic neurons in the ventral midbrain is reduced (Granholm et al., 2000). In SN of Parkinson's disease patients, decreased levels of GDNF have been found (Chauhan et al., 2001). Neurodegeneration in Parkinson's disease is thought to be caused by oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammation and by alterations in neurotrophic factor synthesis. As dopamine undergoes oxidative metabolism and can generate cytotoxic free radicals, increased extracellular dopamine levels can be harmful for dopaminergic neurons in the long run. Indeed, very recently it has been reported that the number of dopaminergic neurons originating from the SNpc is decreased more in aged GDNF+/- mice than in aged Wt mice, starting from the age of 12 months (Boger et al., 2006).

There are several mechanisms that may cause the increased extracellular dopamine concentrations in the GDNF+/- mice. Firstly, as GDNF has been shown to enhance outgrowth of dopaminergic neurons and to

increase synapse formation (Bourque and Trudeau, 2000; Granholm et al., 2000), it is possible that synapse formation of the dopaminergic neurons in the GDNF+/- mice differ from those of the Wt mice, and neurons increase dopamine output to maintain normal functioning. Secondly, as exogenously administered GDNF has been shown to increase stimulus evoked dopamine output (Feng et al., 1999; Hebert et al., 1996; Lin et al., 1993; Xu and Dluzen, 2000), which could be related to its ability to increase activity of TH (Salvatore et al., 2004) and to its ability to inhibit transient A-type K^+-channels (Yang et al., 2001), the reduced GDNF levels might affect dopamine output through potassium channels. Another explanation for increased extracellular dopamine concentrations is that there may be direct alterations of the dopaminergic neurons. Most of the synaptically released dopamine is taken up by the DAT and thus, one possibility is that the amount or function of DAT is altered in the GDNF+/- mice. However, it is unlikely that the amount of DAT would be altered, as in the present experiments, acute administration of cocaine stimulated locomotor activity similarly in the GDNF+/- and Wt mice, and cocaine-induced percentage elevations of dopamine output in the nucleus accumbens and basal motor activities were also similar. In addition, our preliminary results from DAT immunostained striatal samples did not show any differences in the optical density measurements. Further studies could be conducted to measure dopamine kinetics in GDNF+/- mice by using *in vivo* voltammetry. There is evidence of an interaction between dopamine D2 receptors and GDNF. Dopamine D2-receptor knockout mice have reduced GDNF levels (Bozzi and Borrelli, 1999), and on the other hand, there are indications that in the GDNF+/- mice the D2 mRNA levels are increased in the striatum at the age of 12 months (Boger *et al*. 2004). As presynaptic dopamine D2 autoreceptor function mediates the feedback control of dopaminergic activity, it might thus be altered in the GDNF+/- mice.

Increased dopaminergic transmission in striatal brain regions of heterozygous GDNF+/- mice might be induced by compensatory alterations in the mesolimbic and nigrostriatal dopaminergic pathways. As GDNF/RET-signalling has been shown to be important for functioning of the dopaminergic systems, the increased dopamine release described above might be a compensatory mechanism to enhance the synthesis of GDNF. It has been demonstrated that dopamine regulates GDNF synthesis through D1-receptors, but only at high concentrations (Bozzi and Borrelli, 1999; Ohta et al., 2003; Ohta et al., 2000). However, we found no differences in the numbers of D1-receptors between the GDNF+/- and the Wt mice in dopamine D1-ligand binding experiments in striatal membrane preparations with ^{3}H-SCH-23390 .

As multiple neuronal systems are known to depend on GDNF for development and maintenance, it is possible that the neurotransmitter systems regulating dopaminergic neurons differ between the GDNF+/- and Wt mice. GABA, glutamate, and opioid peptides, among several other neurotransmitters, are known to regulate dopaminergic transmission. Indeed, our results show that morphine's effects on dopamine output and metabolism differ between the GDNF+/- and Wt mice. Morphine is thought to enhance dopamine output by activating μ-opioid receptors on GABAergic interneurons in the VTA and in the SN. The activation of μ-

opioid receptors reduces the inhibitory effect of GABA, which leads to enhanced release of dopamine (Johnson and North, 1992; Lacey et al., 1989). In the present experiments, acute morphine at doses of 5 and 10 mg/kg increased accumbal dopamine output dose-dependently in the Wt mice, but in the GDNF+/- mice morphine 5 mg/kg enhanced the accumbal dopamine output robustly to a greater extent more than a dose of 10 mg/kg. Also, similar differences were found between the genotypes in the effects of morphine on accumbal extracellular DOPAC output. Also, the morphine-induced elevations in striatal DOPAC and HVA tissue concentrations were dose-dependent in mice of both genotypes, but the effect of 30 mg/kg was significantly smaller in the GDNF+/- mice than in the Wt mice. Moreover, as the GDNF binding co-receptor GFRα1 has been shown to be located both in the dopaminergic and GABAergic neurons in the midbrain (Sarabi et al., 2001), GDNF is likely to regulate their functions. As GABAergic interneurons in the VTA and SN play an essential role in the mechanism of action of morphine, it is possible that the GABAergic regulation of dopaminergic neurons is altered in the GDNF+/- mice as compared with their wild-type littermates.

Maisonneuve et al. (2001) has reported that in rats, the dose response curve of morphine's effect on dopamine output in the nucleus accumbens and caudate/putamen is bell-shaped. Morphine increases dopamine output up to certain dose, and after maximal effects, increasing of the dose reduces the dopamine response. Morphine appears to have a dual effect on striatal dopamine release: an inhibitory effect in the terminal regions and a stimulatory effect in the somatodendritic regions. Our laboratory previously reported that intrastriatal administration of μ-opioid receptor agonists morphine and DAMGO ([D-Ala^2,MePhe^4, glycinol^5]enkephalin) decreases extracellular striatal dopamine (Piepponen et al., 1999). Our results suggest that the bell-shaped dose-response curve of morphine is shifted to the left in the GDNF+/- mice. It is possible that morphine's inhibitory effect on dopamine release in the terminal regions becomes predominant in the GDNF+/- mice at smaller doses than in the Wt mice.

6.2. Effects of repeated administration of cocaine, amphetamine and morphine on locomotor activity in the GDNF+/- mice

Psychomotor sensitization is a long-lasting plastic phenomenon and it may be involved in drug-seeking behaviour and in the development of drug addiction (Robinson and Berridge, 2003). The locomotor responses to repeated cocaine and amphetamine were sensitized similarly in the GDNF+/- and Wt mice. In contrast to the study of Messer *et. al.* (2000), in the present experiments cocaine did not induce more robust behavioural sensitization in the GDNF+/- mice than in the Wt mice. However, our result is in line with a similar observation by Griffin *et al.* (2003).

In the morphine experiment, we found that tolerance to morphine-induced locomotor stimulation developed faster in the GDNF+/- than in the Wt mice. Tolerance is an important component of the actions of opioids. It is thought that development of tolerance and drug addiction are caused by plastic neuroadaptive changes induced by chronic drug administration (Nestler et al., 2001). As neurotrophic factors are also crucial for the plasticity of the CNS, it is likely that they may be involved in long-term responses to drug exposure. Indeed, our results indicate

that endogenous GDNF is involved in the development of tolerance to morphine. After 96 h withdrawal from repeated morphine treatment, the effects of morphine at 5 mg/kg were found to be sensitized only in the GDNF+/- mice; however, increasing the dose induced robust and similar sensitization in mice of both genotypes. This clearly indicates that GDNF+/- mice are more vulnerable to morphine-induced psychomotor sensitization than their wild-type littermates.

The transcription factor ΔFosB has been linked to psychomotor sensitization and drug addiction (Nestler et al., 2001). In the present experiments, basal levels of FosB/ΔFosB were increased in the GDNF+/- mice as compared with the Wt mice. Thus, the increased sensitivity of the GDNF+/- mice to psychomotor sensitization to morphine 5 mg/kg might well be related to increased FosB/ΔFosB levels in brain areas involved in addiction.

6.3. Effects of repeated cocaine and morphine on accumbal dopamine output

Another unexpected finding in these studies was that, after repeated morphine and cocaine treatments, the elevation of basal extracellular dopamine concentrations seen in drug-naive GDNF+/- mice was restored to a similar level as in Wt mice. Repeated morphine treatment has been shown to cause several neuroadaptations in the VTA. Withdrawal from chronic morphine treatment retards striatal dopamine metabolism (Ahtee and Attila, 1987; Ahtee et al., 1989; Attila and Ahtee, 1984) and decreases the size and calibre of dendrites and cell bodies of VTA dopamine neurons (Nestler, 1992). Chronic morphine has been shown to increase the levels of TH in the VTA (Beitner-Johnson and Nestler, 1991; Self et al., 1995), an effect that can be blocked by exogenous GDNF (Messer et al., 2000). In addition, drugs of abuse have been shown to have direct effects on GDNF-GFRα1/RET signalling in the dopaminergic system, as chronic cocaine and morphine decrease the amount of phosphorylated RET in the VTA (Messer et al., 2000) and as chronic cocaine decreases the amount of GDNF mRNA in the striatum (Green-Sadan et al., 2003).

Thus, apparently repeated morphine treatment modifies the plasticity of the dopaminergic system more readily in mice with reduced telencephalic GDNF, which suggestion is also supported by more rapid development of tolerance to the locomotor enhancing effects of morphine discussed above.

There is ample evidence for the involvement of dopaminergic systems in sensitization phenomenon (Ahtee and Attila, 1987; Di Chiara, 1995; Spanagel and Weiss, 1999). In the present experiments, repeated administration of cocaine did not induce sensitization of dopamine release in either the Wt or the GDNF+/- mice. In accordance with the increased psychomotor sensitization to morphine 5 mg/kg, this challenge dose increased accumbal dopamine output slightly more in the GDNF+/- than in the Wt mice after repeated treatment. The robust and similar psychomotor sensitization seen in mice of both genotypes after morphine 10 mg/kg agrees with the finding that, at this challenge dose, morphine enhanced accumbal dopamine output similarly in the Wt and GDNF+/- mice. In the GDNF+/- mice, morphine 10 mg/kg induced a greater locomotor response than the 5 mg/kg dose although both morphine doses increased the accumbal dopamine output similarly. Also,

the cocaine-induced dopamine output was significantly lower in the GDNF+/- mice than in the Wt mice. Furthermore, in the GDNF+/- mice, the effect of a challenge dose of morphine 5 mg/kg on dopamine output after repeated treatment was clearly less than that of an acute 5 mg/kg dose. Thus, it is likely that also other mechanisms besides enhanced accumbal dopamine output after repeated treatment are involved in the psychomotor sensitization.

6.4. Effects of morphine on CPP

The CPP is used to assess the rewarding properties of drugs. Morphine-induced CPP has been shown to persist for a long time (Mueller et al., 2002). Increased GDNF concentrations have been shown to attenuate reward (Green-Sadan et al., 2003; Green-Sadan et al., 2005; He et al., 2005; Messer et al., 2000) and reduced GDNF concentrations to augment it (Griffin et al., 2006; Messer et al., 2000). As accumbal dopamine plays an important role in the drug reward and we found that acute morphine 5 mg/kg increases accumbal dopamine clearly more in the GDNF+/- mice than in the Wt mice, we wanted to investigate the development and duration of morphine-induced CPP in the GDNF+/- mice. We showed that morphine 5 and 10 mg/kg induced CPP initially similarly in the GDNF+/- and Wt mice. However, the morphine-induced CPP lasted longer in the Wt than in the GDNF+/- mice in both the CPP paradigms used. Thus, an augmented response of accumbal dopamine to acute morphine 5 mg/kg in the GDNF+/- mice does not apparently affect the development of place conditioning. Indeed, the duration of place preference in the GDNF+/- mice was relatively short. The shortened duration of morphine-induced CPP in the GDNF+/- mice might be related to their impaired spatial working memory as seen by weakened water maze learning performance (Gerlai et al., 2001) rather than impaired appetitive value of morphine. However, weakened spatial memory is difficult to reconcile with the initially similar development of place preference in the GDNF+/- mice.

6.5. Brain dopaminergic system in mice with constitutively active RET

The present experiments show that constitutive RET activity, induced by a Met919Thr mutation, leads to robust increases in brain tissue dopamine concentrations in mesolimbic, mesocortical and nigrostriatal systems. In addition, the mutation seems to be rather specific for the dopaminergic system as noradrenaline or serotonin concentrations were not altered in the MEN2B mice. There may be two main reasons for increased dopamine concentrations. Firstly, as TH protein levels were increased in the striatal brain areas of the MEN2B mice, it is likely that increased dopamine levels are caused by increased synthesis. Secondly, it is likely that some of the increase in dopamine concentrations is due to the increased cell number, since in the SNpc of homozygous MEN2B mice the number of TH-positive cells was found to be increased by 26%.

Knockout studies have shown that GDNF-RET signalling is not important during embryonic development for midbrain dopaminergic neurons (Enomoto et al., 1998; Marcos and Pachnis, 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). However, it has been shown that the number of dopaminergic

neurons is regulated during early postnatal development. This development has been shown to be dependent upon GDNF (Kholodilov et al., 2004; Oo and Burke, 1997; Oo et al., 2003). Our finding of increased numbers of TH-positive neurons in the MEN2B mice is thus more likely to be due to neurotrophic support during postnatal apoptotic phases. However, it is also possible but less likely that increased TH-positive cell numbers in the SNpc are due to increased RET activity during embryonic development, possibly related to more neurons acquiring a dopaminergic phenotype.

The most prominent elevations of tissue dopamine levels were found in the dorsal striatum of the homozygous MEN2B mice, where the effect on dopamine concentrations was mutation-dependent. However, in the ventral striatum and in the cortex, dopamine was similarly elevated in the heterozygous and homozygous mutants, suggesting that in these brain areas dopamine concentrations are maximally elevated by even a lower RET activity. The extracellular dopamine concentrations in the dorsal striatum did not differ between the Wt and MEN2B/MEN2B mice, indicating that dopamine in the MEN2B mice is increased only intracellularly. The normal extracellular dopamine concentration in the MEN2B/MEN2B mice is in accord with the finding that their striatal DAT level is increased, and thus, there is more rapid uptake for released dopamine. The normal extracellular dopamine concentration in MEN2B/MEN2B mice is also in agreement with the finding that 24-h locomotor activities of MEN2B mice did not differ from those of the Wt mice. However, reason for the reduced exploratory activity in the mice of both MEN2B genotypes remains unclear. It might be due to effects of RET on motoneurons or sympathetic ganglia, but it is also temping to suggest that presynaptic D2 receptors function is altered in these mice. Activation of presynaptic dopamine D2 receptors is known to reduce locomotion, and these autoreceptors are known to be activated by a smaller dopamine concentration than the postsynaptic receptors (Carlsson, 1977).

As the tissue concentrations of dopamine metabolites were increased in the MEN2B mice, it is likely that increased tissue dopamine is not caused by decreased metabolism in the MEN2B mice. DOPAC and HVA concentrations were also found to be increased extracellularly, which indicates that additional intracellular dopamine, which is not stored into vesicles, is metabolized. DOPAC is mainly an intracellularly produced metabolite (Roffler-Tarlov et al., 1971) and HVA is formed outside of the dopaminergic neuron (Wood and Altar, 1988).

The finding that TH is up-regulated due to constitutive RET activity is rather surprising. Although, GDNF has been shown to increase the expression and stability of TH mRNA (Xiao et al., 2002) and to increase dopamine levels and turnover in the striatum and SN (Hudson et al., 1995; Martin et al., 1996), chronic and even acute GDNF treatments have been shown to down regulate TH expression (Georgievska et al., 2004; Salvatore et al., 2004). The GDNF-induced TH down regulation is thought to be a compensatory alteration maintaining the functional stability of the dopaminergic system. However, the present findings indicate that in the MEN2B mice, increased tissue dopamine is evidently compensated by other mechanisms, such as increased DAT levels.

6.6. Effects of cocaine in the MEN2B mice

The finding that the MEN2B mice are more sensitive to the stimulatory effect of acute cocaine is in line with increased striatal dopamine concentrations and up-regulation of DAT in these mice. As dopaminergic nerve endings of MEN2B mice are loaded with dopamine, it is likely that cocaine increases dopamine output more in these mice and induces greater locomotor activity than in the Wt mice.

When cocaine was given repeatedly there were no differences in the effects of cocaine at 5 mg/kg between the genotypes. However, the effects of cocaine 10 mg/kg differed, so that in the MEN2B/MEN2B the relative increase of the stimulatory effect by the daily cocaine injections was clearly smaller than that in the Wt mice. Nevertheless, this experiment does not confirm the hypothesis that GDNF/RET signalling reduces drug reward or protects against drug addiction.

6.7. Role of neurotrophic factors in the development of drug addiction

As reviewed in section 2.4.5, the present hypothesis on the role of GDNF in the effects of abused drugs is that increased GDNF levels in the mesolimbic system attenuate and decreased levels of GDNF augment molecular or behavioural changes induced by abused drugs. This hypothesis is also supported by the present findings that in GDNF+/- mice the development of tolerance to morphine's locomotor enhancing effect is faster, and that in mice with reduced GDNF levels there is increased sensitivity to morphine-induced psychomotor sensitization. Furthermore, morphine robustly increased dopamine output when given acutely as a small dose. However, in contrast to the above hypothesis, we found that the rewarding effects of morphine are initially similar in the GDNF+/- mice and in their wild-type littermates. In addition, although increased RET activation increases the stimulatory effects of cocaine on locomotion acutely, the locomotor sensitization to cocaine is smaller in the mice with constitutive RET activity.

7. CONCLUSIONS

- The present findings stress the importance of endogenous GDNF in the regulation of brain dopaminergic systems. An important finding in the present experiments was that extracellular dopamine concentration is increased in both mesolimbic and nigrostriatal brain areas of the heterozygous GDNF mice. This finding is further supported by the increase in the number of FosB/ΔFosB and c-Fos positive nuclei in the postsynaptic neurons in striatal brain areas of these mice. Thus, the results indicate that neuronal activity in dopaminergic brain areas of heterozygous GDNF+/- mice, with reduced striatal concentrations of GDNF protein, is increased.

- The present findings show that accumbal dopamine output in the heterozygous GDNF+/- mice is more sensitive to a small dose of morphine than in their wild-type littermates, and the apparent bell-shaped dose-response curve of morphine is shifted to the left. Another important finding in the present experiments was that after repeated morphine and cocaine treatments the elevation of basal extracellular dopamine concentrations seen in drug-naive GDNF+/- mice was restored to a similar level as in the Wt mice. Although, development of psychomotor sensitization to cocaine, amphetamine and morphine at 10 mg/kg was similar between GDNF+/- and Wt mice, these findings suggest that reduced GDNF levels increase sensitivity to psychomotor sensitization to a 5 mg/kg morphine dose, and that GDNF is involved in the development of tolerance to locomotor enhancing effects of morphine. Thus, our findings suggest that reduced brain GDNF levels alter the response of dopaminergic systems to morphine. The initial rewarding effects of morphine as measured by CPP appear to be similar in the GDNF+/- and the wild-type mice but the duration of preference lasts longer in the wild-type mice, suggesting that endogenous GDNF has a crucial role also in drug-associated motivational memory.

- The present results show that sustained, increased activity of RET dramatically increases telencephalic concentrations of dopamine, mainly by increasing dopamine synthesis. Furthermore, these results strongly argue for the role of RET tyrosine kinase as a signalling receptor of GDNF in the dopaminergic system. In addition, the increased number of TH-positive cells in the SNpc of homozygous MEN2B mice clearly shows *in vivo* that RET signalling is involved in the regulation of the number nigrostriatal neurons. However, neither the extracellular dopamine concentration nor the 24-h locomotor activity was elevated in the MEN2B mice, indicating the dopaminergic systems' ability to compensate in order to maintain normal functioning.

- Taken together, the present experiments emphasize the role of GDNF/RET-signalling in the regulation of brain dopaminergic systems and drug addiction.

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