Acute and chronic effects by stimulants on behavior and striatal neurotransmission in the rat

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To my family

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Abstract

Nicotine and amphetamines are the most widely abused stimulants. The main aim of the studies in this thesis was to investigate how these two drugs of abuse affect distinct regions of the rat brain involved in development of habitual and compulsive behavior, namely subregions of striatum in the rat. To this end, using a battery of tests including behavior, brain slice electrophysiology, and molecular biology, we have evaluated acute effects by nicotine and amphetamine, as well as progressive changes induced by their chronic use and discontinuation. We show that nicotine acutely depresses synaptic activity in dorsal striatum, an effect that involves multiple receptors. In chronic experiments, we show that a brief exposure to nicotine (15 days) or amphetamine (five days) induces persistent behavioral changes, which sustain over long periods of withdrawal. In addition, we demonstrate that following the drug exposure period, dorsal striatal subregions are engaged in a temporal manner, such that effects in lateral portions only appear after protracted withdrawal, where they sustain for a long time. We also demonstrate that drug-induced effects on behavior and synaptic activity are enhanced in younger animals. In summary, we show acute and long-lasting effects by stimulants on behavior and neurotransmission in striatal subregions, where they also reveal spatiotemporal and age-dependent components.

Keywords: withdrawal, nicotine, amphetamine, striatum, GABA, glutamate, dopamine, sensitization, locomotor activity

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Populärvetenskaplig sammanfattning på svenska

Akuta och kroniska effekter av stimulantia på beteende och striatal neurotransmission i råtta

Missbruk och substansberoende är ett globalt problem som orsakar stort mänskligt lidande och betydande kostnader för samhället. Närmare en halv miljon svenskar är drabbade av beroendesjukdom, men kunskapen om varför vi blir beroende och vad som krävs för att tillfriskna är begränsad. Drogberoende är en kronisk sjukdom som karaktäriseras av kontrollförlust och ett tvångsmässigt drogintag, och klinisk och preklinisk forskning indikerar att många av de beteendemässiga förändringar som associeras med beroende kan sammankopplas med rubbningar i nervcellsaktivitet i specifika hjärnområden. Framför allt påverkar beroendeframkallande substanser integrerade neuronala kretsar i de basala ganglierna, och dessa rubbningar tros ligga till grund för ett eskalerat, okontrollerat drogintag. Striatum är den största kärnan i de basala ganglierna, och olika subregioner av denna kärna anses vara av betydelse vid olika stadier av sjukdomsutvecklingen. Ventrala striatum associeras med belöning och de förstärkande effekterna av droger, medan dorsala striatum kopplats till tvångsmässigt substansintag, och återfall, som förekommer även efter lång tids abstinens.

I denna avhandling har vi kartlagt akuta och kroniska förändringar i striatal neurotransmission och motorik orsakade av de beroendeframkallande substanserna nikotin och amfetamin. Genom elektrofysiologiska mätningar *ex vivo* visar vi att nikotin rekryterar flera olika signaleringsvägar vilket leder till en dämpad neuronala aktivitet i både dorsala (Paper I) och ventrala striatum, och att det finns en åldersberoende komponent till dessa effekter i ventrala striatum (Paper II). En subregions-specifik effekt av nikotin åskådliggörs även genom *in vivo* mikrodialys, där nikotin ökar dopaminnivåerna mer markant i ventrala- jämfört med dorsala striatum (Paper II, III), en effekt som kan vara av betydelse för den belönande känslan av nikotin. Beteendemässigt ser vi att upprepad exponering (15 injektioner över 3 veckors tid) resulterar i en ökad känslighet till den lokomotor-

stimulerande effekten av nikotin (beteendemässig sensitisering). Denna process, samt tolerans mot de aversiva effekterna, sker snabbare i unga jämfört med äldre djur (Paper II). Elektrofysiologiska fältmätningar visar också att endast unga djur får kvarstående neuronala förändringar i ventrala striatum efter upprepad nikotin-administrering (Paper II). Det är möjligt att dessa fynd kan kopplas till den ökade risken för ungdomar att fastna i ett nikotinberoende.

I Paper III studerades progressiva neuroadaptationer orsakade av nikotin i dorsala striatum, den del av striatum som tros vara av betydelse vid etablerad beroendesjukdom. Här ser vi initialt en kvarstående dämpning av neurotransmissionen i den subregion av dorsala striatum som sammankopplats med målinriktade beteenden. Dessa neuroadaptationer, som även involverar förändringar i antalet dendrittaggar samt uttryck av dopamin receptor mRNA, etableras inom fem dagar av upprepad administrering, och kvarstår i upp till en månad efter sista exponeringstillfälle (Paper III). Efter längre tids abstinens ser vi hur liknande neuroadaptationer etableras i den subregion av striatum som sammankopplats med vanebildning och kompulsivt drogintag. Dessa rubbningar i striatal neurotransmission reverseras inom 6 månader efter avslutad drogexponering, men etableras snabbt igen när drogen återinförs. Våra studier av lokomotion visar att nikotinets stimulering av vertikal rörelse, som kan tolkas som ökad exploration, dämpas över tiden, medan ökningen i horisontell lokomotion fortfarande kvarstår efter 6 månaders abstinens. Det är därmed möjligt att dessa beteendemässiga förändringar är livslånga.

För att undersöka om progressiva neuroadaptationer i striatum är fundamentalt för alla typer av beroendesjukdom behandlade vi råttor under fem dagar med amfetamin, en potent psykostimulantia med en annan verkningsmekanism än nikotin. Upprepad administrering av amfetamin orsakade en beteendemässig sensitisering med avseende på både vertikal och horisontell lokomotion som fortfarande kvarstår efter 3 månaders abstinens. Vidare skapade amfetamin en mer ihållande hämning av synaptisk aktivitet, där båda subregionerna av striatum uppvisade dämpad neurotransmission efter 3 månaders abstinens.

Fynden som sammanställs i denna avhandling visar därmed att psykostimulantia som nikotin och amfetamin förändrar neurotransmissionen i striatum både akut och kroniskt, och att denna effekt delvis är åldersberoende. Vidare indikerar våra studier att neurotransmissionen i den striatala hjärnregion som kopplats till målinriktade beteenden initialt rubbas av beroen-

deframkallande substanser, och att dessa rubbningar sedan överförs och etableras i den del av striatum som är av betydelse för vanebildning. Det är möjligt att dessa progressiva förändringar återspeglar en omkoppling av striatala kretsar, och att det finns ett samband mellan striatal omkoppling och de beteendemässiga förändringar som sker när vi går från ett rekreationsmässigt användande av drogen till ett missbruk och beroende. Det kvarstår dock att etablera om det finns ett kausalt samband mellan förändrad neurotransmission i striatala kretsar och beroendesjukdom, samt att fastställa interventioner för att återställa neurotransmissionen efter längre tids drogintag.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Lotfi A***, Licheri V*, Patton MH, Lagström O, Mathur B, Ericson M, Söderpalm B, Adermark L. *Long-lasting inhibition of striatal excitability following nicotine exposure ex vivo*
*shared first author, Manuscript

II. Adermark L, Morud J, **Lotfi A**, Jonsson S, Söderpalm B, Ericson M. *Age-contingent influence over accumbal neurotransmission and the locomotor stimulatory response to acute and repeated administration of nicotine in Wistar rats*
Neuropharmacology. 2015, 97:104-12

III. Adermark L, Morud J, **Lotfi A**, Danielsson K, Ulenius L, Söderpalm B, Ericson M. *Temporal rewiring of striatal circuits initiated by nicotine*
Neuropsychopharmacology. 2016, 41:3051-3059

IV. **Lotfi A**, Licheri V, Lagström O, Söderpalm B, Ericson M, Adermark L. *Temporal and spatial suppression of striatal excitability elicited by amphetamine in Wistar rats*
Manuscript

Contents

Abbreviation

aCSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CB1R	cannabinoid receptor type 1
ChI	cholinergic interneuron
DMSO	dimethyl sulfoxide
fEPSP	field excitatory postsynaptic potentials
FSI	fast-spiking interneuron
D1R	dopamine receptor type 1
D2R	dopamine receptor type 2
DHβE	Dihydro-β-erythroidine hydrobromide
GABA	γ-amino-butyric acid
$GABA_AR$	type A GABA receptors
GP	globus pallidus
mGluR	metabotropic glutamate receptor
mIPSC	miniature inhibitory postsynaptic currents
MLA	methyllycaconitine citrate
mRNA	messenger ribonucleic acid
MSN	medium spiny neuron
nAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptors
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
oIPSC	optically-evoked inhibitory postsynaptic currents
PPR	paired-pulse ratio
PS	population spike
PV	parvalbumin
sEPSC	spontaneous excitatory postsynaptic currents
sIPSC	spontaneous inhibitory postsynaptic currents
SN	substantia nigra
STN	subthalamic nuclei
TAAR	trace amine-associated receptors
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area

1. Introduction

1.1 Abuse, dependence, and addiction

Many people experiment with drugs of abuse. In general, most drugs produce a state of subjective experience of pleasure. This experience of pleasure, in part, increases the probability that the individual uses the drug again and repeatedly. Although not all people who experiment with drugs lose control over their drug intake (Wagner and Anthony, 2002), for some the drug can eventually take control over their behavior and produce a pathological state called addiction. Addiction can cause failure in life roles, and can drive the individual to commit criminal activity in order to obtain the drug. Addiction to drugs of abuse is very costly to the health and wealth of the individual and also of the society. In the United States, 8 to 10% of people 12 years of age or older, i.e. 20 to 22 million people, are addicted to a drug of abuse. Annual drug-related expenses for health care, and judicial and economical costs are estimated to exceed $700 billion (NIDA, 2016).

For a long time, addiction has been viewed as a flaw in personality, lack of moral principle, and a crime in itself (Koppel, 2016). However, with recent advantages in clinical and preclinical research, addiction is now considered a chronic brain disease that is influenced by multiple factors. The factors that influence initiation and extent of addiction range from genetic makeup and family history of drug abuse, to various other socio-economical factors (Tsuang et al., 1998). It is now believed that punishment of addictive behavior or encouraging people to stay off of drugs are not sufficient methods in prevention and treatment of addiction. As an example, the 1980s anti-drug campaign “just say no” to drugs, championed by then US First Lady, Nancy Reagan, has failed to produce adequate results in keeping individuals away from drug use (Lynam et al., 1999). Whereas it is possible for some individuals to control their drug intake and cease drug use on their own, for many others recreational use becomes a chronic and compulsive habit. Although this latter group is often aware of the negative impacts of their drug issue, they have lost control over their habit and are unable to cease drug use. Despite many attempts to quitting, addicted individuals have a high tendency to relapse to drug use after a period of discontinued use. This ten-

dency to relapse often remains long after the last drug episode, when the drug is completely out of the system. Transition from recreational use to compulsive drug intake is a trademark characteristic of addiction disorder.

1.2 Age as a significant factor

One of the factors that influence the extent of addiction is the age of the first exposure to the drug. Often long-term smokers have started earlier in life (Jordan and Andersen, 2017; Kendler et al., 2013). Younger individuals are more susceptible to the rewarding and reinforcing properties of drugs and have a higher rate of experimenting with different drugs and substance use (Warner et al., 1995). In addition, a majority of the population with addictive disorders at later stages in life, have an onset of use as adolescents or young adults (Wagner and Anthony, 2002). These differences might be due to the developing nature of adolescents' brain (especially in areas associated with reward and motivation). Age-dependent effects of drugs of abuse have also been reported in animals. For example, amphetamine produces enhanced effects on locomotor activty and dopamine levels in nAc in younger animals, which indicates an increased sensitivity earlier in life (Crawford and Levine, 1997; Huang et al., 1995). In addition, nicotine's ability to induce a long-lasting enhancement in the activity of midbrain dopaminergic neurons is age-dependent, which could indicate an enhanced rewarding effect in younger animals (Placzek et al., 2009).

1.3 Drugs of abuse and brain reward system

The ability of drugs of abuse to take control of the behavior of the individual appears connected to their influence on the brain reward system. This system is involved in regulation of motivations and the pleasures that the individual receives from natural rewards such as palatable food and sexual activity. The brain reward system ascertains that behaviors aimed at obtaining and taking natural rewards are reinforced, i.e. increases in frequency with experience. However, drugs of abuse have the ability to takeover this system and redirect behaviors towards drug consumption, thus prioritizing drug consumption over natural rewards. Brain reward systems have been conserved evolutionarily, thus providing a model for investigations and manipulations in pre-clinical settings in order to study various molecular, neurological, and behavioral aspects of addiction (Volkow et al., 2016).

One of the first animal studies showing the significance of the brain reward system was published in 1954. In these studies, James Olds and Peter Milner showed that animals choose to self-stimulate regions of the brain associated with the reward system, to an extent where they would stop seeking natural rewards, such as food or water, in favor of electrical stimulation (Olds and Milner, 1954). Further experiments showed that animals would self-administer drugs of abuse directly into ventral tegmental area (Bozarth and Wise, 1981), signifying the role of these regions in drug-related experiences.

Dopamine and reward

The reward system comprises of several regions in the brain, which are linked together by a network of dopaminergic innervations. Some of the reward regions are striatum, ventral tegmental area (VTA), amygdala, and prefrontal cortex, and the medial forebrain bundle (Wise, 1998). The mesolimbic dopamine pathway, one of the dopaminergic pathways in the brain, is a major network in the reward system that projects dopaminergic neurons from VTA to ventral striatum (Figure 1).

Discovered as a neurotransmitter in its own rights in 1958 by Swedish scientists (Carlsson et al., 1958), dopamine is considered the "pleasure molecule" in the brain, and has been implicated in reward-related behaviors. A common feature of drugs of abuse is that they elicit a rewarding response by increasing extracellular levels of dopamine in nucleus accumbens (nAc), located in ventral striatum (Di Chiara and Imperato, 1988; Pidoplichko et al., 1997). The significance of dopaminergic systems in drug use has been verified in lesion experiments, in which animals with lesions in their mesolimbic dopaminergic neurons stop self-administration of the drug (Singer et al., 1982). Dopamine enhancement induced by the drugs can also trigger homeostatic processes and neuroadaptations that, with continued drug intake, shift the balance of neurotransmission in various reward regions in the brain (Kauer and Malenka, 2007; Kourrich et al., 2015). In parallel to these changes, continued drug intake attenuates drug-induced dopamine increase (Volkow et al., 2014; Zhang et al., 2013). This dampened dopamine effect results in a diminished reward experience, either by the drug or by natural rewards, which could drive the individuals to increase their drug intake as addiction advances.

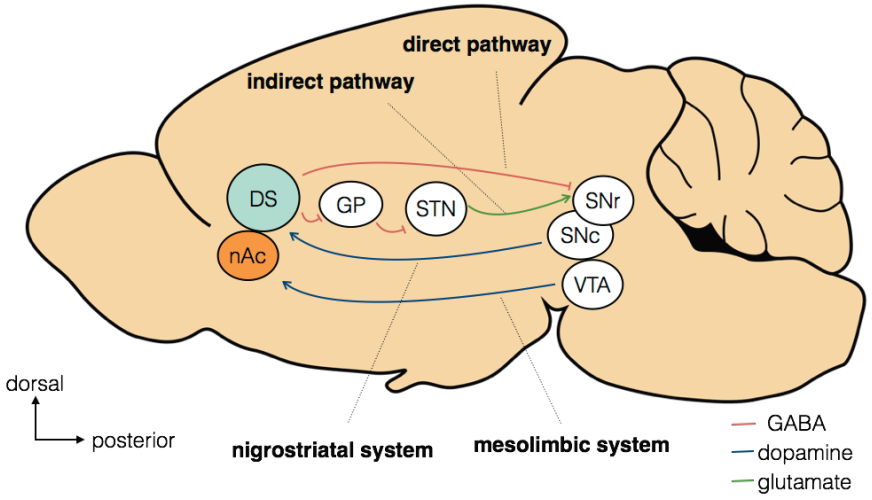

Figure 1 *Schematic drawing of a sagittal section of rat brain showing dopaminergic projections to subregions of striatum. Direct and indirect output pathways of dorsal striatum are depicted. DS=dorsal striatum, nAc=nucleus accumbens, GP=globus pallidus, STN=subthalamic nuclei, SNr=substantia nigra reticulata, SNc=substantia nigra compacta, VTA=ventral tegmental area. Image of the sagittal brain section is adapted from commons.wikimedia.org.*

1.4 Striatum

Basal ganglia comprise an important component of brain reward circuitry and are implicated in controlling functions such as voluntary movements, associative learning, and habit formation (Graybiel, 2005; Yin and Knowlton, 2006). Striatum is a major input nucleus to the basal ganglia circuitry, which integrates inputs from different parts of the cortex and thalamus and conveys them to other nuclei of basal ganglia, ultimately reaching areas of cortex implicated in motor function and executive tasks (Flaherty and Graybiel, 1994; Tepper et al., 2007). Striatum can be anatomically divided alongside a ventral-dorsal axis. These striatal subregions are innervated by specific sets of corticostriatal projections and thus execute different components of behaviors. Ventral striatum, which is the location of nucleus accumbens (nAc), receives its inputs from limbic cortex (Bolam et al., 2000). Dorsal striatum can be further subdivided into dorsomedial striatum (DMS), and dorsolateral striatum (DLS). DMS, which extends ventrally to the limits of nAc, receives its inputs from associative areas of cortex and is

involved in goal-directed behaviors (Eagle and Robbins, 2003; Yin et al., 2005a; Yin et al., 2005b). On the other hand, sensorimotor-related cortical areas project to the DLS, whose activity is implicated in habitual and compulsive behavior (Yin et al., 2004, 2006). It is important to note that striatal subregions are not distinctively separated with defined limits, but cortical inputs, output targets, and neuronal cytoarchitecture of these subregions conform to a ventromedial-dorsolateral gradient that is implicated in the functional overlap between subregions (Voorn et al., 2004) (Figure 2). For example, the rewarding and conditioned reinforcing effects of psychostimulants have been attributed to nAc and its dopmainergic innervations from VTA (Kelley, 2004; McBride et al., 1999; Taylor and Robbins, 1984). However, to some degree, some of these behaviors have been reported to recruit dorsal striatum and nigrostriatal dopaminergic pathway (Dickson et al., 1994; Kelley and Delfs, 1991).

Behavioral shift from goal-directed behavior and habitual performance has been proposed to recruit DMS and DLS in a temporal manner. In fact, the DMS and its inputs from associative cortex is recruited during goal-directed behavior and initial stages of learning of a performance, when the frequency of the behavior is modulated by the value of the outcome. Subsequently, the DLS and its afferents from sensorimotor cortex are implicated in habitual performance, whose frequency is not sensitive to devaluation of the outcome, and is performed in the correct conditions or stimuli (Gremel and Costa, 2013).

Striatal neurotransmission is complicated and is regulated at multiple levels and multiple neuronal populations. Since the focus of the present work is mostly in dorsal striatum, from here on striatum refers to dorsal striatum, unless stated otherwise.

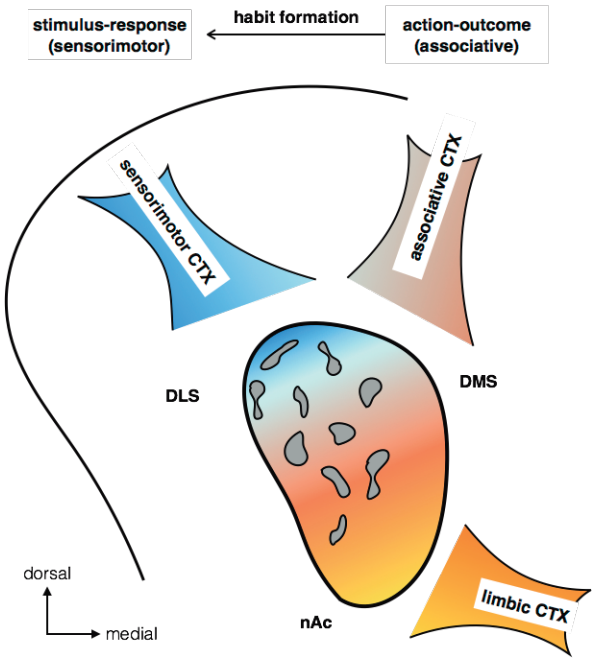

Figure 2 *Descriptive drawing of a coronal section of striatal subregions and their respective inputs from cortex. Habit formation progressively recruits sensorimotor cortex and DLS. Striatal neurons are compartmentalized into striosomes (depicted in gray), surrounded by neurons of matrix. CTX=cortex, DLS=dorsolateral striatum, DMS=dorsomedial striatum, nAc=nucleus accumbens.*

1.4.1 Input-output partitioning in striatum

Dorsal striatum receives dense glutamatergic inputs from cortex (Alexander and Crutcher, 1990; Alexander et al., 1986; Divac et al., 1977; Kita, 1996) and thalamus (Smith et al., 2004), which transport sensory inputs to the dorsal striatum. These glutamatergic inputs form the majority of striatal synapses (up to 80%) and induce phasic firing in principle neurons of striatum (Wilson, 1993, 2007). Glutamatergic projections express muscarinic and nicotinic receptors, and their activity is influenced by a cholinergic tone (Narushima et al., 2006). In addition, dopamine inhibits glutamatergic signaling (Surmeier et al., 2007) through dopamine receptors type 2 (D2R) (Bamford et al., 2004a; Bamford et al., 2004b; Fisher et al., 1994; Sesack et al., 1994; Wang and Pickel, 2002). Dopamine is provided to dorsal striatum by neurons originating from substantia nigra (Anden

et al., 1966) and is essential for habit formation (Faure et al., 2005). Dopamine extensively controls striatal neurotransmission and dopamine receptors are densely expressed in medium spiny projections neurons (MSNs) and interneurons of striatum. Furthermore, nigrostriatal dopaminergic neurons can co-release γ-amino-butyric acid (GABA) (Nelson et al., 2014b). In addition, dorsal striatum receives serotonergic neurons, originating from dorsal raphe nucleus, that regulate neurotransmission in striatal output neurons (Waselus et al., 2006).

The majority (>90%) of neurons in the striatum are MSNs, which are GABAergic neurons and transfer signals to the downstream nuclei. Anatomically, unlike most other brain regions, striatal MSNs do not form a laminar structure and are instead organized into mosaic striosomes that are surrounded by extrastriosomal matrix (Desban et al., 1993) (Figure 2). The striosome-matrix compartmentalization of striatum also represents an input-output partitioning, such that compartments can be characterized by afferents, receptor localization, and the output targets (Crittenden and Graybiel, 2011; Gerfen, 1992; Kincaid and Wilson, 1996). Conforming to this partitioning, dopamine levels are higher in matrix areas (Brimblecombe and Cragg, 2015; Salinas et al., 2016). This compartmentalization is also possibly reflected at the functional level, where e.g. lesions to striosome compartment reduce cocaine-induced stereotypy (Murray et al., 2015).

MSNs are divided into two major pathways, based on their biochemical and receptor profile and output targets, as well as corticostriatal inputs (Flores-Barrera et al., 2010; Guo et al., 2015). MSNs of the direct pathway express excitatory dopamine receptors type 1 (D1R), use substance P as a co-transmitter, and send their axons directly to the substantia nigra reticulata (SNr). On the other hand, the indirect pathway is composed of MSNs that express inhibitory dopamine D2R, use enkephalin as a co-transmitter, and target SNr indirectly via globus pallidus (GP) and subthalamic nuclei (STN) (Bolam et al., 2000). However, recent findings suggest that in a fraction of MSNs co-localization of dopamine D1R and D2R occurs, which might also be of functional relevance (Perreault et al., 2011).

MSNs of the two pathways have differential membrane properties and response to dopamine. While dopamine D2R MSNs are more excitable to current injection than dopamine D1R MSNs in normal conditions, dopamine reverses the excitability in a manner that dopamine D1R MSNs become more responsive and dopamine D2R MSNs are inhibited (Ericsson et al., 2013; Gertler et al., 2008; Planert et al., 2013). It is believed that the two pathways in striatum exert opposite effects on movement initiation (Freeze et al., 2013) and reinforcement

(Kravitz et al., 2012), such that stimulation of the direct pathway increases movement and induces reinforcement, while stimulation of the indirect pathway decreases movement and induces punishment. However, there are findings that the two pathways are activated during task performance and collaborate in action selection (Cui et al., 2013). This could mean that during action initiation, the direct pathway activates the correct set of movements, while activation of the indirect pathway inhibits the movements that can disrupt the correct set of actions.

MSNs elicit a weak feedback inhibitory signal on each other through their long axon collaterals, which regulate excitability of local networks (Lalchandani et al., 2013; Tepper et al., 2004). In addition to GABA, MSNs can also release endocannabinoids, which, through retrograde signaling, induce long-term depression of presynaptic glutamatergic and GABAergic neurotransmission (Adermark and Lovinger, 2007; Adermark et al., 2009; Lovinger and Mathur, 2012). Endocannabinoid signaling also appears vital for habit formation (Hilario et al., 2007), and might thus be an important signaling molecule when studying addiction.

1.4.2 Intrastriatal connections and interneurons

As mentioned earlier, the majority of striatal synapses are formed by corticostriatal glutamatergic neurons. However, the activity of MSNs is not solely dependent on glutamatergic transmission. There are other local neuron types, such as GABAergic and cholinergic interneurons, that are involved in fine-tuning the activity of MSNs (Silberberg and Bolam, 2015).

GABAergic interneurons

GABAergic interneurons produce an inhibitory signal in MSNs. Compared to feedback inhibition induced by MSNs' axon collaterals, the feed-forward inhibition elicited by GABAergic interneurons is powerful and widespread, such that spiking in a single interneuron potentially delays or even blocks the activity in a large number of postsynaptic MSNs (Koos et al., 2004; Tepper et al., 2008). There are at least four characterized subtypes of GABAergic interneurons. Each subtype displays distinct biochemical and electrophysiological properties (Tepper and Bolam, 2004). The most abundant of these GABAergic interneurons are parvalbumin (PV)-expressing fast-spiking interneurons (FSIs), which comprise up to 1% of striatal neurons, that produces strong feed-forward

inhibitory signals in MSNs (Bennett and Bolam, 1994; Koos et al., 2004; Mallet et al., 2005), but very sparsely in other neurons of striatum (Szydlowski et al., 2013). It has been suggested that FSIs have a preference for MSNs of direct pathway (Gittis et al., 2010; Planert et al., 2010). Activity of FSIs is regulated by glutamatergic inputs from cortex (Kita et al., 1990), and also glutamate that is co-released from cholinergic interneurons (Nelson et al., 2014a). In addition, activity of FSIs are differentially controlled by a cholinergic tone, such that while acetylcholine (ACh) activates them through nicotinic receptors, it also inhibits FSIs' influence through inhibitory muscarinic receptors (Bennett and Bolam, 1994; Koos and Tepper, 2002).

Another class of GABAergic interneurons are neuropeptide Y (NPY)-expressing interneurons, which are characterized by low-threshold calcium spikes, and thus termed persistent and low-threshold spike (LTS) neurons (Kawaguchi et al., 1995). The third class of GABAergic interneurons express calretinin, a calcium-binding protein (Bennett and Bolam, 1993). In addition, tyrosine-hydroxylase (TH)-expressing GABAergic interneurons are present in striatum, whose activity might be influenced by dopaminergic projections to the striatum (Ibanez-Sandoval et al., 2015; Kubota et al., 1987a). However, recent studies suggest that other types of GABAergic interneurons might exist in striatum (Munoz-Manchado et al., 2014).

Cholinergic interneurons

Cholinergic interneurons (ChIs) are large aspiny neurons and comprise 1-2% of striatal cells. Their cell bodies can exceed 40 μM in diameter and are tonically active (Tepper and Bolam, 2004). These interneurons fire action potentials in a slow regular pattern, and regulate the activity in striatum by releasing acetylcholine and also glutamate (Higley et al., 2011; Nelson et al., 2014a; Zhou et al., 2002). Their activity increases stimulation of FSIs (Koos and Tepper, 2002) and also drives co-release of GABA from dopaminergic terminals (Nelson et al., 2014b). Although MSNs do not express nAChR, acetylcholine inhibits their activity (especially dopamine D2R MSNs), through muscarinic receptors (Bennett and Wilson, 1999; Wilson et al., 1990). Ultimately, while ChIs can inhibit the activity in MSNs through muscarinic receptors, they can also increase their activity through inhibition of FSIs through nAChR, and thus exert a disinhibiting effect on MSNs. ChIs receive dopaminergic inputs and express both dopamine D5 (D1-type) and dopamine D2R (Bergson et al., 1995; Kubota et al., 1987b). The activity in ChIs is influenced by neighbouring MSNs, but not FSIs

(Chuhma et al., 2011). Overall, ChIs influence the activity in striatal network in a complex manner.

1.5 Re-organization of striatal networks during the progression of addiction

Pathology of striatum has been associated with the development of addiction. In fact, selective engagement of striatal subregions has been associated with the progression of drug use from a recreational activity to a compulsive habit (Everitt and Robbins, 2005, 2013). Administration of most drugs of abuse activate the mesolimbic dopamine pathway, which projects from the ventral tegmental area to the nucleus accumbens. Consequently, there is an enhancement of dopamine levels in response to the drug, which occurs in nucleus accumbens (Di Chiara and Imperato, 1988; Koob and Volkow, 2016). This enhanced dopamine response is believed to be associated with a subjective feeling of pleasure and mediates the hedonic sensations associated with the drug, and induce a state of "liking" (Corrigall, 1999; Di Chiara, 2000). An alternative view is that the increase in dopamine might mediate the incentive saliency of the reward and assign a motivational value to the drug, which could induce a feeling of "wanting" rather than "liking" (Berridge, 2007; Berridge et al., 2009). Therefore, the mesolimbic dopamine pathway is implicated in the initial reward-guided behavior and is significantly important for the establishment of drug self-administration (Singer et al., 1982). On the other hand, the progression from recreational drug use to compulsive and habitual drug intake is associated with recruitment of dorsal striatal subregions (Gerdeman et al., 2003; Ostlund and Balleine, 2008; Volkow et al., 2006). This behavioral shift is one of the hallmarks of addiction and is important for the maintenance of addictive behavior. At earlier stages of cocaine intake, it is believed that drug intake is controlled by goal-directed behaviors, while after prolonged use these behaviors become habitual (Zapata et al., 2010). In line with the roles that the DMS and DLS execute in instrumental conditioning and task performance, it has been suggested that the DMS and DLS have distinct roles in acquisition and performance of drug seeking behaviors. Early acquisition and establishment of instrumental goal-directed drug-seeking behaviors involve DMS (Murray et al., 2012). In addition, drug-induced behavioral sensitization has also been associated with DMS (Durieux et al., 2012). On the other hand, after prolonged drug intake, it is proposed that the locus of behavioral control is gradually transferred to the DLS, whose inactivation after prolonged drug intake decreases cue-controlled drug seeking

(Vanderschuren et al., 2005). In addition, long-term cocaine intake induces progressive emergence of enhanced activity in DLS (Porrino et al., 2004; Willuhn et al., 2012), which appears to be dependent on changes in dopamine signaling of ventral striatum (Belin and Everitt, 2008). Similar subregion-specific recruitment of dorsal striatum has also been shown with alcohol, such that alcohol seeking behaviors that are sensitive to outcome devaluation is disrupted by inactivation of DMS, while following the transition to habitual drug intake (i.e. insensitive to devaluation) is disrupted by inactivation of the DLS (Corbit et al., 2012). Further evidence on subregion-specific influence by drug taking has been shown by the differential effect of amphetamine self-administration on increasing spine density in the DLS as compared to the DMS (Jedynak et al., 2007). Overall, hierarchical recruitment of striatal subregions appears to be associated with the progression from recreational drug-use to a compulsive habit, and addiction.

1.6. Nicotine and its pharmacology

Tobacco leaves (Nicotiana tabacum) are native to South America and have been used for many centuries as a medicinal and recreational drug. The plant is named after Jean Nicot de Villemain, French ambassador in Portugal, who promoted its use in Europe in the 16th century. Nicotine was first extracted from tobacco leaves in 1828. In humans nicotine administration produces a mild pleasurable euphoria, increased arousal, decreased fatigue, and relaxation (Henningfield et al., 1985).

Nicotine is believed to be the main psychoactive ingredient in tobacco that produces the addictive state underlying the sustained use of tobacco (Corrigall, 1999; Di Chiara, 2000). The reinforcing and locomotor-stimulatory properties of nicotine are largely attributed to nicotinic stimulation of mesolimbic dopaminergic pathway, where it causes an enhancement of burst firing dopamine neurons and dopamine release in nAc (Clarke et al., 1988; Corrigall et al., 1992; Panagis et al., 1996). On the other hand, in dorsal striatum, although nicotine has been suggested to increase burst firing of dopaminergic neurons (Grenhoff et al., 1986), it appears that this increase in bursting activity is not associated with robust dopamine enhancement (Chergui et al., 1994; Zhang et al., 2009). However, in dorsal striatum, nicotine appears to have an indirect effect on neurotransmission (Plata et al., 2013), possibly through engaging various interneurons and astrocytes.

Nicotine exerts its effects by activating ligand-gated nicotinic acetylcholine receptors (nAChR) that are composed of various combinations of α-type and β-type subunit compositions (Gotti and Clementi, 2004). There are eight types of α subunits (α2-α7, α9, α10) and three types of β subunits (β2-β4) currently known to be present in the central nervous system (Zoli et al., 2015). Each composition of nicotinic receptors demonstrates distinct pharmacological properties in response to nicotine and endogenous acetylcholine. The most common subtypes are low-affinity α7-containing and high-affinity α4β2-containing nAChR (Dani, 2015). nAChR are present in pre- or postsynaptic terminals and thus regulating neurotransmission in a complex manner. In dorsal striatum, different subtypes of nicotinic receptors are expressed on distinct neural populations. While α6-containing nAChR are primarily expressed on nigrostriatal dopaminergic terminals (Champtiaux et al., 2002; Zoli et al., 2002), α4β2-containing nAChR are expressed on dopaminergic neurons (Champtiaux et al., 2003), GABAergic interneurons (Koos and Tepper, 2002), and ChIs (Azam et al., 2003). α7-containing nAChR are located on glutamatergic terminals (Marchi et al., 2002) and ChIs (Azam et al., 2003). In addition, astrocytes express α4β2-containing and α7-containing nAChR (Delbro et al., 2009; Grybko et al., 2010). As of yet, expression of nicotinic receptors in MSNs of striatum has not been reported. Therefore, nicotine-induced effects in output neurons of striatum are regulated at multiple levels upstream of MSNs.

Acute exposure to nicotine induces a transient activation of nAChR. With continued exposure and as the concentration of nicotine reaches 20-100 nM, nicotine desensitizes nAChR, which inhibits its subsequent stimulation by the agonist (Quick and Lester, 2002). The rate of desensitization is correlated with the affinity of nAChR subtype to nicotine (Brody et al., 2006; Wang and Sun, 2005). Depending on the exposure level to nicotine or the state of receptors, nAChR might become resensitized after a short time, or stay in a desensitized state for a long time (Khiroug et al., 1997). For example, blood concentration level of nicotine in chronic smokers is maintained at a level where the majority of α4β2-containing nAChR are at a constant (or near constant) state of desensitization (Russell et al., 1980). A well-established consequence of long-term nicotine use and constant desensitization of nAChR, in particular α4β2, is up-regulation of nicotinic receptors (Fenster et al., 1999). This upregulation impairs normal cholinergic signaling through these receptors and might contribute to unpleasant withdrawal effects that are experienced upon discontinuation of nicotine use, and thus facilitate relapse (Govind et al., 2009).

1.7 Amphetamine and its pharmacology

Amphetamines are potent psychostimulants that occur naturally in plants of *Ephedra* and the tree *Catha edulis* (aka *khat*). Khat is native to Somalia and Kenya and wildly cultivated in Yemen. Amphetamine's clinical use was reported by 11th century Persian scientist Abu Al-Rihan Bin Ahmed Al-Baironi (Al-Motarreb et al., 2002). Ephedra was used medicinally in ancient China and India. Its active component, ephedrine, was identified in 1887 by Japanese pharmacologist Nagajoshi Nagai. In the same year, Rumanian chemist Lazar Edeleanu produced synthetic amphetamine (Rasmussen, 2015).

At low to moderate doses, amphetamine produces significant euphoria, enhances energy and vigilance, and causes cognitive enhancement and attention (Lees et al., 2015). High doses of amphetamine is associated with possible adverse effects such as anxiety, convulsions, and psychosis (delusions and paranoia) (McCreary et al., 2015). Amphetamine has multiple sites of action (Sulzer et al., 2005). By activating the intracellular trace amine-associated receptor 1 (TAAR1), amphetamine inhibits transporter proteins for dopamine, norepinephrine, and serotonin, and thus promotes action-potential-independent reverse-transport of neurotransmitters into the synaptic cleft (Fleckenstein et al., 2007; Kahlig et al., 2005; Sitte et al., 1998; Sulzer et al., 1995). In addition, amphetamine-induced inhibition of vesicular monoamine transporter 2 (VMAT2) increases the cytosolic content of neuronal catecholamines (such as epinephrine, norepinephrine, and dopamine), and serotonin (Riddle et al., 2002). It has also been shown that amphetamine decreases the metabolism of dopamine by inhibiting the activity of monoamine oxidase, which is responsible for the breakdown of dopamine (Ramsay and Hunter, 2003).

Enhancement of dopamine in nAc, in particular, is thought to be involved in behavioral effects of amphetamine, such as the hedonic state and increased locomotion (Kelly et al., 1975). On the other hand, amphetamine enhances the frequency of stereotypical behavior, which might be associated with its effect in dorsal striatum (Joyce and Iversen, 1984; Staton and Solomon, 1984), where it also promotes dopamine release (Paulson and Robinson, 1995; Steinkellner et al., 2014). Much like other drugs of abuse, long-term use of methamphetamine is associated with a decrease in dopamine D2R in dorsal striatum of humans (a.k.a. caudate-putamen) (Volkow et al., 2001). In addition to the effects on dopamine, amphetamine decreases striatal glutamate acutely (Miele et al., 2000), as well as following repeated administration (Bamford et al., 2008; Wang et al., 2013b). It has also been shown that amphetamine increases striatal GABA levels

(Bustamante et al., 2002; Del Arco et al., 1998), and increases the activity and expression of GABAergic interneurons (Horner et al., 2006; Wiltschko et al., 2010). Amphetamine thus exerts a complex effect on striatal microcircuits that may result in long-lasting neuroadaptations of importance for addictive behavior.

1.8 Behavioral sensitization

One of the behavioral hallmarks of repeated drug intake in rodents is behavioral sensitization. It is defined as an enhancement of behavioral response that is induced by repeated administration of the same dose of the drug. Behavioral sensitization has been modeled extensively in preclinical studies and is widely used as a model of repeated drug intake (Ericson et al., 2010; Olausson et al., 2001; Robinson and Berridge, 1993; Vezina, 2004; Vezina et al., 2007). It is hypothesized that neuroadaptations that underlie behavioral sensitization might increase the saliency of the drug and the associated contextual stimuli by assigning a dysregulated motivational value, and thus increase the sensitivity of the system to further drug administrations (Robinson and Berridge, 2003, 2008). Therefore, drug-induced behavioral sensitization can be used as an indirect measurement of neuroadaptations that occur during repeated administration of drugs of abuse. These neuroadaptations might be implicated in behavioral changes produced by repeated drug intake, including drug craving and susceptibility to relapse (Kalivas et al., 1998; Steketee and Kalivas, 2011).

2. Aims

The overall aim with this thesis was to outline acute effects by stimulants on striatal microcircuits, and define progressive neuromodulations that occur following repeated drug administration. These goals can contribute to increase our understanding of the progression and maintenance of addiction, and can potentially advance the development of novel and more effective treatments for compulsive drug use.

Specific aims of the papers that comprise the present thesis include:

Paper I: To outline acute *ex vivo* effects displayed by nicotine on neurotransmission in the dorsolateral part of the striatum, and to identify key targets mediating these effects.

Paper II: To assess the effects of age on behavioral adaptations and accumbal neurotransmission following acute and intermittent administration of nicotine.

Paper III: To define if a brief period of intermittent nicotine administration followed by protracted withdrawal would be sufficient to produce long-lasting neuroadaptations in dorsal striatal subregions.

Paper IV: To determine if a brief intermittent amphetamine treatment followed by protracted withdrawal induces similar temporal and subregion-specific neuroadaptations in striatum as nicotine.

3. Materials and Methods

In the following section, the methods and techniques that were used in the papers comprising this thesis are briefly described, some theoretical and practical aspects of these techniques are mentioned, and limiting aspects of these methods are discussed.

3.1 Ethics

All the experiments were performed in accordance with the declaration of Helsinki and approved by the Ethics Committee for Animal experiments, Gothenburg, Sweden. Diary numbers: 83/13, 214/14.

3.2 Animals

Animal models have been used in preclinical research extensively throughout the past century. Rodent models are commonly used in preclinical addiction research, due to the conserved nature of the brain reward system between rodents and humans. Rodents also have the benefit of a short gestation time, they produce a relatively high number of offspring, and are fairly easy to handle and care for. In this thesis, Wistar rats were used as the experimental model for chronic and acute studies. The animal facility provides a constant room temperature of 20ºC, relative humidity of 65%, a regular light-dark cycle with lights on at 7:00 a.m. and off at 7:00 p.m., and with *ad libitum* access to food and water. Prior to any procedure, animals were allowed to acclimatize to the environmental conditions. For acute electrophysiological studies (paper I), juvenile rats (in-house breeding at University of Gothenburg, originating from Charles River (Germany), age range 21-35 postnatal days) were used. For paper II and III, rats were purchased from Taconic (Ejby, Denmark) at three different ages (4, 9, or 35 weeks, paper II) or at a weight range of 250-350g (paper III). In paper IV rats weighing 330-360g were purchased from Janvier labs (France). All drug administrations and experiments were performed during the light phase of the light-dark cycle.

3.3 Drugs and solutions

In striatum, various subtypes of nicotinic receptors are expressed on different populations of neurons, which regulate neurotransmission in a complex manner (Figure 3). Therefore, in order to investigate the mechanism underlying the neuromodulatory effects of nicotine, several pharmacological tools were employed in paper I, whose preparation and targets will be discussed in the following. These compounds were stocked in water, unless otherwise stated (dimethyl sulfoxide (DMSO), or 95% ethanol), and diluted in artificial cerebrospinal fluid (aCSF) to the desired concentration right before use. Tocris Bioscience (Bristol, UK): methyllycaconitine citrate (MLA, 40 nM) was used in order to block α7-containing nAChR, which are expressed on glutamatergic afferents projecting to the striatum. Dihydro-β-erythroidine hydrobromide (DhβE, 0.8 μM) was used to antagonize α4β2-containing nAChR, located on GABAergic interneurons, dopaminergic projections, and possibly astrocytes. α-conotoxin PIA (10 nM) was used as an antagonist of α6-containing nAChR, located on dopaminergic projections. 3-bromocytisine (500 nM) was used as an agonist of α4β2-containing nAChR and α7-containing nAChR. D-AP5 (50 μM) and LY 341495 (20 nM, stock in DMSO), blocked N-methyl-D-aspartate receptor (NMDAR)- and metabotropic glutamate receptor (mGluR) 2/3-mediated currents located in MSNs. Quinpirole hydrochloride (5 μM) was used as an agonist of the dopamine D2R family, located on MSNs of the indirect pathway and also on multiple other neurons in the striatum (Clarke and Adermark, 2015). Sigma-Aldrich Sweden AB (Stockholm, Sweden): nicotine hydrogen tartrate salt (0.1, 1, or 10 μM, fresh in aCSF before use), mecamylamine hydrochloride (10 μM) was used as a non-selective antagonist of nAChR family. SCH23390 hydrochloride (0.5 μM) blocked dopamine D1R family, which are located in MSNs of the direct pathway and on ChIs and other GABAergic interneurons in striatum (Clarke and Adermark, 2015). Sulpiride (5 μM, stock in ethanol) was applied to block dopamine D2R. AM251 (2 μM, stock in DMSO) was used to block cannabinoid receptor type 1 (CB1R), which are located on presynaptic glutamatergic and GABAergic terminals. Bicuculline methchloride (20 μM) was used to block $GABA_AR$, which are highly expressed in striatum. CNQX disodium salt hydrate (10 μM) was used as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, when recording inhibitory currents during whole-cell configuration.

To prepare the drug solutions for administration *in vivo* (papers II-IV), nicotine hydrogen tartrate salt (0.36 mg/kg, nicotine base) or d-amphetamine sulphate

(2.0 or 0.5 mg/kg, salt, Apoteket, Stockholm, Sweden) were dissolved in 0.9% NaCl.

The compounds and doses that were used were chosen based on previous literature and also the IC50 reported for the compound. However, there is a possibility of interactions with non-specific targets. As an example of this nonspecific interaction, bicuculline have been shown to possibly interact with nicotinic receptors (Seutin et al., 1997), however, since there was no blockade of nicotine's effect by bicuculline, this interaction was not deemed significant in our setup.

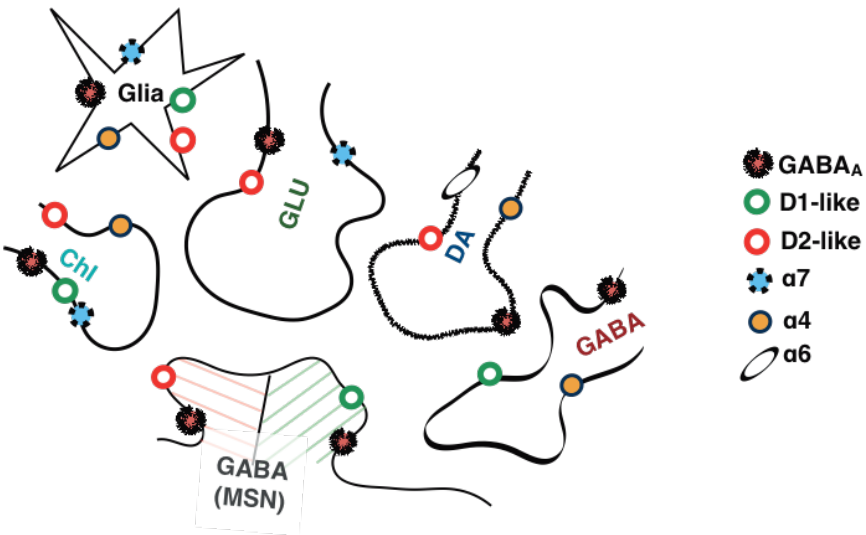

Figure 3 *Schematic simplified drawing of striatal neurons. Some of the receptors that we have manipulated pharmacologically are also depicted. Direct and indirect MSNs are signified with green and red lines, respectively.*

3.4 Behavioral sensitization and measurement of locomotor activity

A behavioral sensitization model was employed in papers II, III, and IV in order to measure the potential of a drug to enhance the locomotor-stimulatory effects following repeated administration. Since the enhancement of the behavioral ef-

fects might reflect neuroadaptations induced by repeated drug administrations (Robinson and Berridge, 1993), this model was used for investigating underpinning neurological correlates of repeated drug administration.

In order to induce behavioral sensitization to nicotine, papers II and III, 15 injections of nicotine were administered over three weeks, and the locomotor response was measured. In addition, to assess the extent of behavioral sensitization in paper III, rats were maintained for six months, during which time the expression of behavioral sensitization to nicotine was measured every six weeks. In paper IV, amphetamine (2.0 mg/kg) was administered for five days (induction phase) and locomotion was measured after the first and last injections. Long-term expression of behavioral effects was assessed at 1 or 10 weeks post-induction with a challenge dose of amphetamine (0.5 mg/kg). The time-course for repeated drug treatment and withdrawal, and additional details for each study are shown in Figure 4.

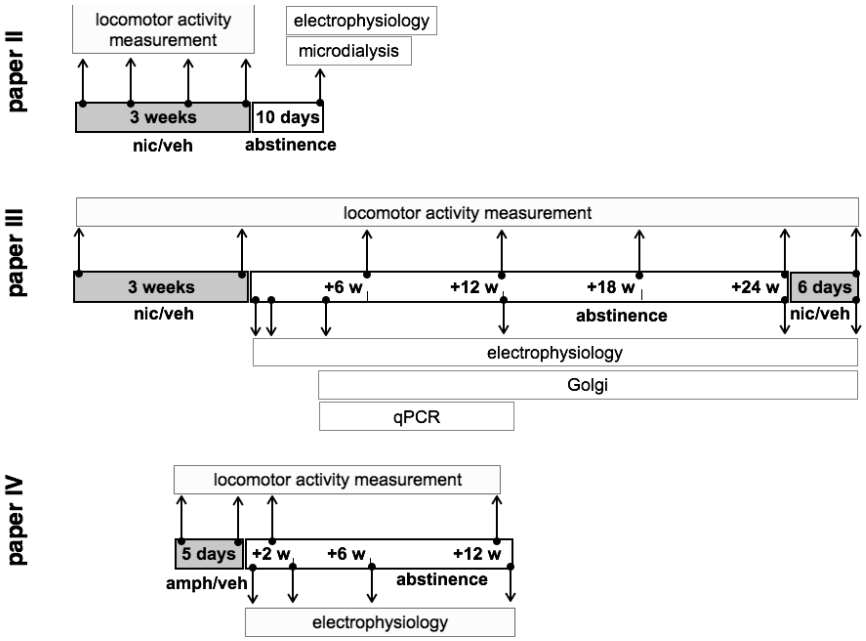

Figure 4 *Time-course of repeated drug regimen and withdrawal periods in papers II-IV. In these papers, we have employed repeated drug administration protocol, followed by withdrawal periods, where we performed various experiments. Outline of the experiments performed at various time-points for each study are depicted. w=weeks*

Measurement of animals' spontaneous activity is a common method for evaluating behavioral effects and development of behavioral sensitization induced by addictive drugs. Therefore, locomotor-stimulatory effects of the drug were assessed by measuring horizontal and vertical activities in an open-field arena (Figure 5). The arena consists of a weakly lit testing box that was equipped with two-layer networks of infrared beams. Beam breaks caused by the animal's movements register locomotor activity in three axes (x, y, and z). The animals were allowed to habituate to the testing box for 30 minutes, in order to dissociate the drug-induced behavioral effects from the movements associated with exploration of the novel environment. After habituation, the subjects received an injection of saline or drug (nicotine or amphetamine), and the activity was recorded for an additional 30 minutes. The activity was analyzed with regards to locomotion (horizontal beam breaks), rearing activity (vertical beam breaks) and, in paper III, time spent in corners or center of the arena.

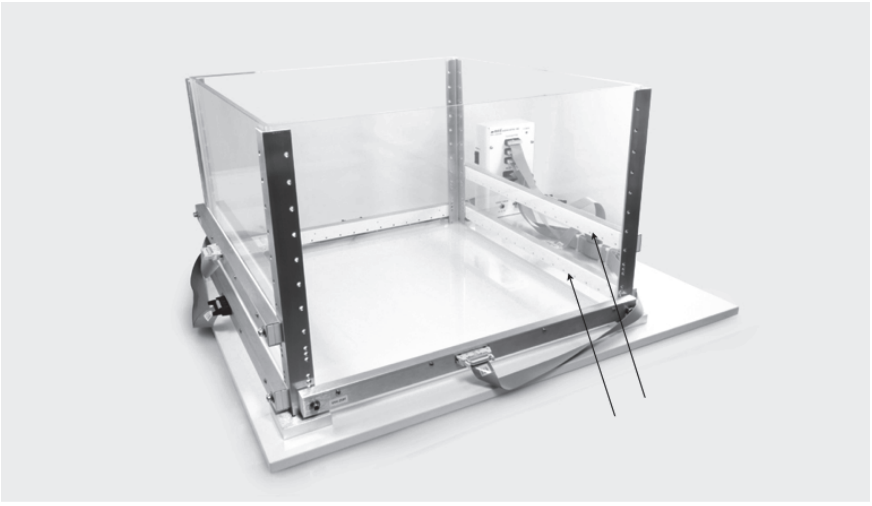

Figure 5 *Picture of an open-field arena that was used for measurements of locomotor activity in our studies. Two layers of infrared beams that detect horizontal (lower rack) and vertical (upper rack) activity are marked. Image adapted with permission from www.med-associates.com.*

Behavioral sensitization has been reported to be a valid model to investigate the neuronal underpinnings and adaptations induced by drugs of abuse (Steketee and Kalivas, 2011). Since neuroadaptations following repeated exposure to addictive drugs were suggested to be implicated in drug craving and induction of relapse

(Kalivas et al., 1998), behavioral sensitization can be used as a model to ensure that drug-induced adaptations are initiated. However, it is important to note that this model relies on non-volitional drug administration, excluding the influence of voluntary drug intake on neuroadaptations associated with the development of addiction.

3.5 Electrophysiology

Brain slice electrophysiology is a common technique that is used in order to measure electrical activity in a brain slice preparation. This *ex vivo* preparation preserves the local neural architecture within the slice, while allowing for a great degree of manipulation for the researcher. In the absence of blood brain barrier, the extracellular environment of the neurons can be easily manipulated by introducing drugs and other chemicals to the perfusion medium. Throughout this thesis, field potential and whole-cell electrophysiology recordings were used to assess the acute effects by a pharmacological compound, or to measure modulations in neurotransmission that were induced by repeated drug administration.

Slice preparation for electrophysiology

Rats were anaesthetized with Forene isoflurane (abbVie AB, Solna, Sweden), decapitated using a guillotine, and the brain was extracted and submerged in cold cutting solution (modified aCSF). Coronal brain slices, containing striatum and the encompassing cortex, were prepared using a Leica VT 1200S vibrotome (Leica Microsystems AB, Bromma, Sweden), at 250 or 350 μm, depending on the age of the animal and the protocol of electrophysiological recordings. Brain slices were transferred to 30 °C-tempered aCSF for 30 minutes, and were then allowed to equilibrate at room temperature for at least another 30 minutes. All solutions were continuously oxygenated with a gas mixture of 95% O2/ 5% CO2.

It is important to note that during brain sectioning the mere procedure of cutting causes mechanical stress and is damaging to the tissue itself, several afferents and efferents of the striatum are also lost in the process. Furthermore, brain slices are kept in aCSF, excluding the effects by various endogenous neuromodulators present *in vivo* which could also be a confounding factor. Thus, the recordings might only reflect local events in the neural architecture within the slice and the effects cannot be directly translated into the functions of an intact brain or *in vivo* recordings. However, removing some of the complexity of the

intact brain allows for in depth studies of neural transmission at the local level, providing important scientific understanding.

Field potential electrophysiology

In order to study neurotransmission in local microcircuits in striatum, field potential electrophysiology was used (Figure 6). Slice recording was initially developed for studying neurotransmission in hippocampus (Skrede and Westgaard, 1971). Unlike lamellar neural organization in hippocampus, dendrites of striatal MSNs are not separated between subregions and dendritic arborizations are not uniformly oriented. Thus, the dipole caused by synaptic current flow is not sufficient to produce measurable field potentials and stimulation of presynaptic glutamatergic projections is required to evoke synchronous excitatory inputs to MSNs. Therefore, the amplitude of the evoked population spike (PS) consistently reflects the efficacy of excitatory synaptic input (Adermark et al., 2011; Misgeld et al., 1979), and is comparable with the field excitatory postsynaptic potential (fEPSP), which is reported for hippocampal recording.

During the recordings, a stimulating electrode (monopolar tungsten electrode, World Precision Instruments, FL, USA, type TM33B) was placed at the border of the subcortical white matter and the DLS, intrastriatal in DMS, or shell region of nAc (Paper II), which activated mainly the presynaptic glutamatergic afferents (frequency of 0.05 Hz). The negative shift in potential caused by the response of postsynaptic neurons resulted in a negative shift in field potential. The negative shift was measured by a recording electrode, which was prepared from borosilicate glass micropipettes (World Precision Instruments, FL, USA) using a Flaming Brown micropipette puller (Sutter instruments, Novato, CA, USA). The stimulus intensity was set to induce a PS amplitude half of the maximal response, in order to provide the necessary margin for increase or decrease throughout the recording. In order to evaluate the effects of repeated drug injections on synaptic efficacy and excitability, an input/output curve was established by stepwise increasing the stimulation intensity (papers II, III, and IV). In addition, to further characterize the cause of putative changes in PS amplitude, we used a paired-pulse stimulation protocol, and analysis of PPR (paired-pulse ratio) was utilized to indicate whether the observed changes are located in presynaptic neurons (e.g. decrease in neurotransmitter release), or in the postsynaptic terminal (e.g. changes in receptor numbers or binding). In order to block the observed effects by nicotine in paper I, pharmacological tools that target the receptors in the striatum (described in section 3.3) were applied in the bath before perfusion of nicotine.

Although stimulation of presynaptic neurons are required to evoke a response in MSNs, the downside of the stimulation protocol and evaluating an evoked response is that it might not represent, or only partly represent, the true *in vivo* pattern of activity in glutamatergic projections and other striatal neurons.

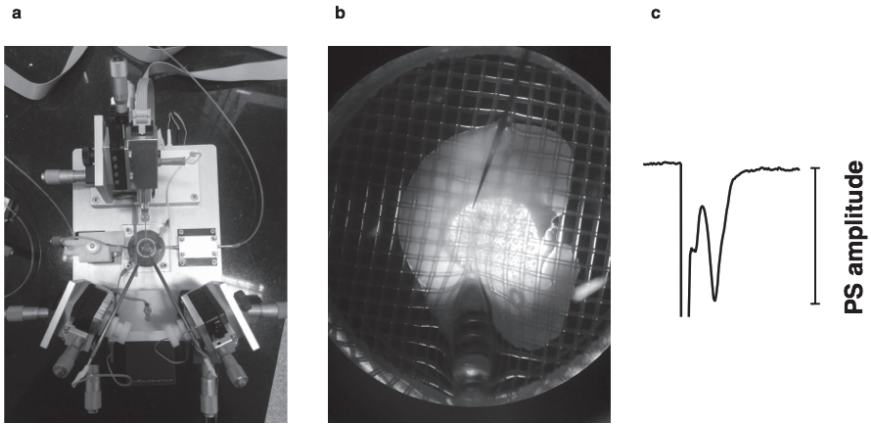

Figure 6 *Field potential electrophysiology a) electrophysiology setup and chamber for placement of brain slices b) striatal coronal slice in the chamber, stimulating (above) and recording (below) electrodes for recording in the DLS are shown. c) Representative trace showing the amplitude of a population spike.*

Whole-cell recordings

In field potential recordings, response of a large population of cells is recorded. This can be beneficial in investigating the net output from a local microcircuit, while events at the level of an individual neuron are not understood. Therefore, in order to provide a deeper level of investigation with a higher spatial resolution, whole-cell recordings were performed from individual neurons (papers I and IV). The whole-cell electrophysiological technique is used for recordings of currents or voltages that are caused by the movement of ions across the membrane of a single neuron. In this preparation, slices were placed in a recording chamber and the region of interest was identified with a 10x/0.30 objective attached to a Nikon Eclipse FN-1 microscope, while using a 40x/0.80 water-immersion objective to localize MSNs for whole-cell recordings. Recording electrodes were prepared from borosilicate glass micropipettes (outer diameter 1.5 µm, resistance ranged from 2.5 to 4.5 MΩ), using micropipette puller and filled with internal solution, according to the experiment. In patch-clamp config-

uration, in order to record sIPSC, 50 μM AP5 and 10 μM CNQX were added to the aCSF to block NMDA and AMPA receptor-mediated currents, while 20 μM bicuculline was added to block $GABA_AR$-mediated currents when recording sEPSC. To measure nicotine-induced changes in membrane potential and action potential frequency (paper I), we used the patch-clamp configuration, where current injections with stepwise increasing of the intensity were applied and membrane potential and action potential frequency were measured in response to hyperpolarization and depolarization of the patched MSNs.

One potentially limiting factor in our experiments was that the recorded MSNs were from both direct and indirect pathways, since they were chosen in random. Thus possible pathway-specific effects are not considered. Another issue is that the volume of internal solution in the recording electrode is considerably larger than that of the cytosol. Therefore, intracellular components that are involved in maintaining the homeostasis in the cytosol might be "washed-out" during the process. This might potentially cause run-down effects over time, especially during lengthy recordings, which might influence the results.

3.6 Optogenetics

To further define the specific role of GABAergic interneurons in the effect produced by nicotine in dorsal striatum (paper I), we used optogenetics combined with whole-cell recording, which was performed in collaboration with Brian Mathur and colleagues (University of Maryland, School of Medicine, Baltimore, MDm USA). Since FSIs in striatum comprise the largest population of GABAergic interneurons, they were targeted in these experiments. In order to express channelrhodopsin-2 in FSIs, *PV-cre* transgenic mice were used. PV is a specific marker for FSIs, and was used for viral-mediated expression of channelrhodopsin-2 in FSIs. After injection of the viral vector construct into the DLS (AP +0.6 mm, ML±2.25 mm, DV −2.4 mm from bregma), the animals were allowed to recover for at least three weeks. Following recovery, brain slices were prepared and recordings were performed. During recordings, FSIs were activated with a pulse of blue light, while oIPSC were recorded in MSNs in voltage-clamp setup. Nicotine was perfused in the bath and nicotine-induced changes in evoked oIPSC were investigated in this condition.

It is important to note that, at the time of the experiment, transgenic mice were the only source of commercially available *PV-Cre* animal models, and thus possible differences between physiology of rats and mice should be considered.

3.7 Gene expression

To measure nicotine-induced modulations of the striatal dopaminergic system at a genetic level (paper III), we used the quantitative polymerase chain reaction (qPCR) technique to record possible alteration of dopamine receptor mRNAs following repeated nicotine administration. At different time-points following the nicotine treatment protocol, striatal subregions (dorsolateral and dorsomedial striatum) were dissected and homogenized in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using a TissueLyser LT (Qiagen). Pool of mRNA from lysed tissue was extracted using Qiagen's RNeasy Lipid Tissue Kit. The quality and concentrations of mRNA were measured with Nanodrop2000 (Thermo Scientific, Waltham,MA) and a total of 1 μg mRNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). qPCRs were carried out with Quantifast SYBR Green Master Mix (Qiagen) in LightCycler 480 Real-time PCR (Roche Applied Science, Indianapolis, IN, USA). Expression levels of target genes (dopamine D1R and D2R) were normalized against reference genes RPL19 (DMS), or GAPDH and SDHA (DLS).

qPCR is used extensively for detecting changes in gene expression. It provides a very high degree of specificity towards the region of interest in the cDNA library, as guaranteed by Qiagen. Also, at the end of each qPCR analysis, melting curve calculation provides information on the purity of the amplicon. It should be noted that, while qPCR provides valuable information on gene expression levels, the level of translation of mRNA to protein and active state of the putative protein could not be elucidated. This should be considered when drawing conclusions on the functional relevance of the results.

3.8 *In vivo* microdialysis

The *in vivo* microdialysis technique allows for detection of neurotransmitters, peptides and other small molecules in the extracellular environment, in awake and freely moving animals. It also enables local administration of a substance to a specific region of interest, a process known as reversed microdialysis. In papers II and III, this technique was employed to measure nicotine-induced alterations of extracellular levels of dopamine in vivo. Two days before the experiments, animals underwent surgery and a custom-made microdialysis probe, equipped with a semipermeable dialysis membrane (molecular weight cut-off 20 kDa), was implanted unilaterally in the region of interest (nAc (coordinates: AP: +1.85 mm; ML: − 1.4 mm relative to bregma; DV: − 7.8 mm rela-

tive to brain surface), DMS (+1.2, − 2.0, − 5.5) or DLS (+1.2, − 3.5, − 5.5)) (Paxinos and Watson, 2007). The area of the dialysis probe that is assigned for passive diffusion of molecules, often referred to as active space, was 2 mm. For surgery and probe implantation, rats were anaesthetized with isoflurane and mounted onto a stereotaxic instrument (David Kopf Instruments, AgnTho's, Lidingö, Sweden). After drilling holes through the skull bone for the probe and anchoring screws, the probe was inserted and fixated onto the scull with Harvard cement (DAB dental AB, Gothenburg, Sweden). On the day of the experiment, probes were connected to a microperfusion pump (U-864 Syringe Pump, AgnTho's, Lidingö, Sweden), and the perfusion rate was set to 2 µl/min. Following two hours of equilibration period, samples were collected every 20 minutes. Four stable baseline samples were analyzed (fluctuation of more than ±10% was not accepted) before administration of nicotine. High performance liquid chromatography (HPLC, Thermoscientific, Waltham, MA, USA) with electrochemical detection was used for measurement of dopamine in the dialysate (Lido et al., 2009). After the experiment had ended, the animals were sacrificed and the brains were extracted and fixated for confirmation of probe placements.

The method of microdialysis is commonly used to monitor neurotransmitter levels *in vivo* and, for mechanistic purposes, following administration of pharmacological tools in conscious and freely moving animals. However, due to a rather low temporal resolution this method does not allow for detection of fast synaptic events such as burst firing of dopamine neurons, but rather monitors changes in volume transmission. In our setup, the sampling rate was set to 20 minutes with the potential disadvantage that some real-time changes in dopamine levels occur at a millisecond scale, and thus might not have been detected in this system.

3.9 Histochemistry

In paper III, we used the histochemical protocol of Golgi staining in slices to explore nicotine-induced temporal modulations in spine density in subregions of dorsal striatum. Golgi dye causes formation of microcrystals within a random subset of neurons, which visualizes them and allows for studying fine neural branching and dendritic spines. Nicotine or vehicle treated rats were perfused intracardiovascularly with Tyrode's solution and 4% paraformaldehyde. Brains were removed and impregnated in a Golgi-Cox Fast Kit (FD NeuroTechnologies, Columbia, MD, USA) for three weeks. Subsequently the brains were sectioned into 60 µm slices with a microtome (Zeiss, Jena, Germany), and stained according to the protocol provided by the company. A Zeiss lsm 700 inverted

confocal microscope (Zeiss, Jena, Germany) with a x63 oil objective (NA 1.4), and an Olympus BX60 microscope (Olympus Corp., Center Valley, PA, USA) equipped with a x100 oil objective (NA 1.3) with cellSens image analysis software were used to visualize the dendrites. Spine counting was carried out blind to treatment on at least two separate occasions/treatments. As described by Li and colleagues, the inclusion criteria were that the extent of the dendrite tree had to be well stained and not obscured by artifacts, such as blood vessels, astrocytes, or other cells (Li et al., 2003).

It is not well known why only a fraction of cells are stained with silver chromate microcrystals. This might potentially reflect a selection bias in the stained cells such that they might not be true representatives of the entire cellular population. On the other hand, since only a fraction of the cells was stained, there is a clear background, which makes it possible to study the entire dendritic tree using thicker sections (60-80 μm).

3.10 Statistical analysis

Data from field potential electrophysiology was analyzed with Clampex 10.3.2 (Molecular Devices, Sunnyvale, CA, USA). sIPSC from whole-cell recordings were analyzed with Minianalysis 6.0 software (Synaptosoft, Decatur, GA, USA). The qPCR data were analyzed in LightCycler 480 software. Gaussian distribution was tested with D'Agostino and Pearson's omnibus normality test. Locomotor activities were analyzed with paired and unpaired t-test and one-way analysis of variance (ANOVA). Field potential recordings and microdialysis experiments involved multiple treatment groups observed over several time points and thus were analyzed with two-way ANOVA. PPR and sIPSC data were analyzed with unpaired t-test. qPCR data were analyzed using Mann-Whitney *U*-test. All analyses were performed with Graph Pad Prism 6 (Graphpad Software, Inc., San Diego, CA, USA).

4. Results and discussions

4.1 Paper I

Dorsal striatum is implicated in habit formation and is a major regulator of the initiation and inhibition of movements (Balleine et al., 2007). Striatal afferents and interneurons express multiple subtypes of nAChR, and nicotine may thus affect the balance between excitation and inhibition in dorsal striatal circuits in a complex manner (Dani, 2015). Even though dorsal striatum primarily has been implicated to play a role following protracted drug intake, defining signaling pathways recruited during acute drug treatment will add important information to elucidate neurobiological underpinnings of nicotine addiction. Thus, the aim of this paper was to investigate the acute effects displayed by nicotine on dorsal striatal neurotransmission in nicotine naïve rodents, with special emphasis on the balance between excitation and inhibition.

To determine the net effect displayed by acute nicotine exposure on striatal output, evoked field potentials were monitored in the DLS during continuous perfusion of nicotine (0.1, 1, or 10 μM). Nicotine significantly depressed striatal output, as did the nicotine receptor agonist 3-bromocystisine (500 nM). Considering that MSNs projecting from the DLS tonically inhibit dopaminergic neurons in the substantia nigra, a decrease in striatal output caused by nicotine could putatively contribute to the locomotor-stimulatory properties of nicotine. However, it should be noted that the effects of nicotine on specific output pathways of the striatum was not studied, and thus it is not known whether the effects are specifically occurring in the direct pathway or the indirect pathway, which have opposite effects on movement (Do et al., 2012) and distinct roles in drug reinforcement (Kravitz et al., 2012).

In order to define the mechanism behind nicotine-induced depression of dorsal striatal output, we hypothesized that nicotine suppressed striatal output by increasing GABAergic neurotransmission. However, although nicotine previously has been indicated to modulate the activity of striatal GABAergic interneurons (Koos and Tepper, 2002; Luo et al., 2013), our battery of electrophysiological recordings, which included optogenetics, whole cell recordings, and field poten-

tial recordings, did not support a role for GABAergic neurotransmission in mediating the net decrease in PS amplitude.

Although the majority of striatal neurons are GABAergic, glutamatergic afferents to dorsal striatum give rise to around 80% of the synapses (Tepper et al., 2007). Field potential recordings also primarily reflect excitatory activity. We thus assessed the effect by nicotine on sEPSC in the DLS. Bath perfusion of nicotine (1 μM) decreased the frequency, but not the amplitude, of sEPSC in the DLS. Thus, nicotine appears to modulate striatal output by decreasing the release of glutamate. In contrast to field potential recordings, the frequency of recorded sEPSC was restored upon drug washout. This in turn indicates that the stimulation applied during field potential recordings may interact with nicotine to produce longer-lasting effects on glutamatergic neurotransmission (Adermark and Lovinger, 2007, 2009).

Somewhat surprisingly, a decrease in field potentials was also seen during administration of antagonists targeting nAChR. Considering that nicotine, which theoretically should increase excitatory neurotransmission (Mansvelder and McGehee, 2000), also may cause desensitization of nAChR (Papke et al., 2009), it is possible that nicotine, at the concentrations applied, acts rather as an antagonist than an agonist in this system setup. α7-containing nAChR are the most common form of nAChR on glutamatergic terminals, and these receptors are rapidly desensitized in the presence of nicotine (Dani et al., 2000). However, the α7-subtype-specific antagonist MLA did not prevent or mimic the depression induced by nicotine, indicating that several receptor subtypes and neurotransmitter systems act in parallel to mediate a net decrease in PS amplitude. Considering the high expression of nAChR on dopaminergic terminals, nicotine-induced modulation of dopamine release might be a prospective candidate for mediating these complex effects (Exley and Cragg, 2008; Wonnacott et al., 2000). Supporting this hypothesis, we found that the dopamine D2R antagonist sulpiride inhibited nicotine-induced suppression of PS amplitude, while the dopamine D2R agonist quinpirole occluded the effect by nicotine. Dopamine receptors are expressed by more or less all striatal neurons and play a key role in regulating striatal neurotransmission (Clarke and Adermark, 2015). Nicotine might thus alter synaptic output directly via nAChR as well as indirectly by altering dopaminergic neurotransmission. It should be noted, however, that nicotine only marginally elevated striatal dopamine levels *in vivo* (Paper III). It is thus possible that, by modulating dopamine D2R signaling, we have rather affected the activity of key cell types involved in mediating these effects, and thereby simply reduced the responsiveness to nicotine. Future studies will look deeper into this, and our

primary target to manipulate will be ChIs. There is a complex interaction between cholinergic and dopaminergic signaling (Exley and Cragg, 2008), and considering that nAChR antagonists depressed PS amplitude, ChIs are most likely tonically active in our system setup. The cholinergic input to the striatum arises solely from ChIs, and our next approach is thus to perform recordings following local chemotoxic lesion of ChIs in the DLS. Considering that nicotine exposure also has been associated with a decrease in firing of ChIs (Storey et al., 2016), this approach will be valuable to further elucidate the signaling pathways and microcircuits modulated by nicotine.

The majority of striatal MSNs belongs to either the so-called "direct" or "indirect" pathway (Alexander and Crutcher, 1990; Freeze et al., 2013). The two pathways inhibit the activity of each other (Lalchandani et al., 2013) and exert opposite effects on initiation of movement sequences (Parent and Hazrati, 1995) and on response to drugs of abuse (Lobo and Nestler, 2011). The acute effects of nicotine presented in paper I reflect the net outcome of the target local microcircuitry in field potential recordings. In addition, the whole-cell recordings were performed "blind", with no indication of the recorded MSNs belonging to the direct or indirect pathway. However, the reported effects could be pathway-specific, in which case they might have opposing effects on behavior. Therefore, in the future it would be of great interest to apply transgenic animals and fluorescent dyes to investigate the tentative pathway-specificity of the acute effects of nicotine in the DLS.

Taken together, the data presented in paper I suggest that nicotine suppresses striatal output, and does so by decreasing the probability for transmitter release from glutamatergic terminals. This effect is most likely partially connected to a direct interaction with α7-containing nAChR on glutamatergic terminals, but also involves other nAChR subtypes and neurotransmitter systems that are directly or indirectly recruited during acute administration of nicotine. Nicotine-induced suppression of striatal output could play a role in the neuronal circuits underlying the locomotor-stimulatory properties of nicotine, but may putatively also play a role when establishing the habit to smoke.

4.2 Paper II

Age of first nicotine exposure greatly influences the severity of nicotine dependence (Breslau and Peterson, 1996), such that even at lower doses, nicotine produces withdrawal symptoms in adolescents (Colby et al., 2000), which might

contribute to a higher rate of relapse when nicotine use commences at a younger age. In animal models, age influences the degree of behavioral and neurochemical effects to nicotine, demonstrated as a more robust conditioned-place preference and a more rapid development of tolerance in younger animals (Belluzzi et al., 2004; Torres et al., 2008). In addition, nicotine metabolism is higher in adolescent rats, which could affect physiological responses differently in different age groups. Therefore in paper II, we investigated the influence of age on nicotine-induced behavioral effects, neurotransmission, and dopamine release in the nAc shell.

In order to assess the influence of age on nicotine's behavioral effect, rats of three different age groups (5, 10, and 36 weeks) were treated with a daily administration of nicotine (0.36 mg/kg) for 15 days. Nicotine's behavioral effect and the development of behavioral sensitization were monitored during the injection regimen, and we observed that behavioral sensitization occurred faster in juvenile and adult rats compared to the old rats.

Assessment of rearing activity revealed that initial administration of nicotine to drug-naïve rats decreased rearing activity, which is in line with previous reports (Clarke and Kumar, 1983; Ksir, 1994). With continued nicotine administration to juvenile and adult rats, nicotine-induced depression of rearing activity resolved rapidly, while in the old rats rearing remained depressed after the first two weeks. Rearing behavior is viewed as an indication of exploratory and information-gathering behavior, and aversive feelings could decrease the frequency of this behavior (Lever et al., 2006). Initial administration of nicotine produces aversive feelings such as dizziness, tension, and nausea (Perkins et al., 2001), which could decrease rearing behavior. Resolution of the rearing-depressant effect of nicotine that occurs with repeated administration may indicate development of tolerance to the aversive effects. In our hands, tolerance to the rearing-depressant effect of nicotine developed much earlier in the younger groups. This suggests that adaptations to nicotine occur faster at a younger age.

In order to investigate potential nicotine-induced modulations in excitability of nAc neurons, we performed field potential recordings on the rats of three age groups. Ten days following the nicotine regimen, neuronal excitability was depressed only in juvenile rats, whereas no modulation in excitability of nAc neurotransmission was detected in adult and old rats. In addition, our data showed that there is an age-dependent effect in how nAc slices from naïve animals respond to nicotine, such that nicotine depressed PS amplitude only in nAc of ju-

venile rats. These differences might arise from age-dependent alterations in expression of nicotinic receptors, especially α4β2 (Doura et al., 2008).

Since dopamine in nAc is associated with drug-induced enhancement of locomotion, nicotine-induced effects on dopamine levels were assessed by *in vivo* microdialysis. Nicotine induced similar increases in dopamine levels in all age groups. However, the dopamine increase in the old age group sustained for a prolonged period, as compared to juvenile and adult animals. This might be related to age-dependent decline of dopamine D2R (Kurotani et al., 2003), which could dampen negative feedback loops (Crawford and Levine, 1997), or down-regulation of dopamine transporters (Hebert et al., 1999). In addition, a decreased rate of nicotine metabolism that occurs with age might contribute to the enhanced dopamine elevation in nAc shell.

Overall, the results of paper II reveal that nicotine-induced sensitization to the locomotor-stimulatory properties of nicotine, as well as the tolerance to the rearing-depressant effect of nicotine, were faster in younger animals. These effects might be due to differential, age-contingent, responsiveness to acute nicotine, with regards to nAc neurotransmission and dopamine release. These age-dependent effects on enhanced locomotion and the development of tolerance might contribute to the higher prevalence of smoking and the susceptibility to relapse in younger individuals.

4.3 Paper III

Repeated nicotine produces progressive behavioral and neurochemical modulations, which sustain even after cessation of drug intake (Vezina et al., 2007). Neuroadaptations that occur as a result of repeated nicotine render the system susceptible to further drug administrations, and might contribute to the development and maintenance of addictive behavior. Temporal engagement of dorsal striatal subregions has been associated with the progression of cocaine use (Murray et al., 2012). It is not well known whether a brief exposure to nicotine and subsequent withdrawal induces similar temporal modulations in neurotransmission of dorsal striatal subregions. Therefore, the goal of paper III was to investigate subregion-specific engagement of dorsal striatum at different timepoints following a 15-day regimen of systemic nicotine administration.

Using open-field activity measurement, we observed that repeated nicotine administration induces behavioral sensitization that sustained even after protracted

withdrawal. The locomotor-enhancing effects of nicotine on horizontal activity sustained for up to 24 weeks following nicotine regimen, whereas the nicotine-induced increase in rearing activity lasted for up to six weeks (paper III, fig. 1). In order to evaluate possible spatiotemporal alterations in subregions of dorsal striatum in response to nicotine, field-potential electrophysiology and qPCR were performed at different time-points after nicotine exposure. Field-potential recordings showed that neuronal excitability in dorsal striatum was initially depressed in DMS and resolved after two months, whereas depression of the DLS occurred three months after nicotine regimen (paper III, fig. 1). In parallel, nicotine-induced changes in dopamine receptor levels were measured by qPCR. The results showed that levels of dopamine D1R and D2R mRNA in the DMS were increased after one month and reversed after three months of withdrawal in nicotine-treated rats. No modulations were observed in the levels of dopamine receptors in the DLS (paper III, fig. 1). These temporal and subregion-specific modulations are in line with a body of evidence implicating temporal engagement of striatal subregions during progression of drug intake (Everitt and Robbins, 2013). The DLS (roughly corresponding to the putamen in humans) has been shown to gradually underlie well-established and habitual drug seeking (Belin et al., 2009; Volkow et al., 2006) and inactivation of DLS inhibits relapse to drug-seeking behavior (Fuchs et al., 2006; Gabriele and See, 2011; See et al., 2007). On the other hand, administration of a dopamine antagonist in DMS impairs acquisition of drug-seeking behavior in its early stages, when the behavior is goal-directed, but does not affect drug-seeking after extended training (Murray et al., 2012). Similarly, in our hands nicotine-induced depression of the DMS excitability was established within five days of repeated nicotine exposure, while depression of the DLS occurs after protracted withdrawal, when depression of the DMS is resolved.

Although we observed parallel alterations in neuronal excitability of the DMS and dopamine receptor levels, modulations in other neurotransmitter systems might also contribute to the observed effects in neuronal excitability. PPR analysis from field potential recordings suggested that there was a nicotine-induced decrease in probability of glutamate release from presynaptic terminals in DMS. This could be in line with previous observations of reduced glutamate tissue levels from dorsal striatum of nicotine-treated rats (Jastrzebska et al., 2014). In addition, in paper I, we observed a depression of glutamate release by acute perfusion of nicotine, albeit in the DLS (paper I, fig. 3). This mechanism may also occur in DMS and contribute to the decreased probability of release that was reported in paper III. Furthermore, nicotine-induced depression of neuronal excitability might also be related to enhancement of inhibitory GABAergic input to

dorsal striatum, induced by repeated nicotine (Miura et al., 2006). These mechanisms can also partially contribute to the changes observed in excitability of the DLS.

Long-lasting nicotine-induced modulations in the DLS might be important for drug-induced relapse and reinstatement of drug-seeking behavior. Following six months after nicotine exposure, the depression in the DLS was seemingly resolved. However, only six injections of nicotine were sufficient to immediately restore the depression in DLS of rats that were nicotine-treated six months before. This is an indication that previous exposure to nicotine has initiated long-lasting processes that render the DLS hypersensitive even after protracted withdrawal. These processes might form a "drug memory" in the DLS that governs the rapid restoration of nicotine-induced depression of neuronal excitability (Everitt, 2014). Since the DLS is implicated in habitual drug use and susceptibility to relapse, this rapid restoration in the DLS excitability might mean that previous drug exposure facilitates transition to compulsive drug use, even after long periods of withdrawal.

In this study nicotine was administered in a passive manner and animals did not perform a task in order to obtain the drug, as in self-administration paradigms (Neugebauer et al., 2014). Learning to perform a task has been attributed to dorsal striatum (Thorn et al., 2010), such that early-stage acquisition of a behavior involves the DMS, while the DLS gradually takes over the control of behaviors (Balleine et al., 2009). However, we have shown that nicotine-induced temporal modulations in dorsal striatum were purely drug-induced and occurred independent of action learning in a non-voluntary drug exposure regimen. Although, it is to be determined whether the transitions and neuroadaptations we have observed would sustain, or even augment, if nicotine administration was paired with action learning. Furthermore, it is important to understand that it is not known whether these neuroadaptations are caused by the nicotine administrations themselves, or by withdrawal following the nicotine regimen.

In conclusion, the results of paper III indicate that a brief period of non-volitional nicotine administration induced progressive neuroadaptations in dorsal striatal subregions, even after long-term withdrawal. Engagement of the DLS, which occurs well into withdrawal, might be important for cue-induced drug seeking and habitual drug intake.

4.4 Paper IV

Similar to many other addictive substances, repeated amphetamine administration is also known to induce behavioral sensitization (Robinson and Becker, 1986). In paper IV, we have extended our findings from paper III to investigate the main hypothesis that temporal and spatial neuroadaptations in the striatum is important for the development of addiction, and therefore occurs during protracted exposure also to other drugs of abuse. We tested this hypothesis by administering amphetamine, which has another mechanism of action than nicotine.

In our hands, systemic amphetamine administration for five days produced an enhanced locomotor response, as assessed by locomotion and vertical movements in open-field activity measurements. Furthermore, persistence of behavioral sensitization was assessed by locomotor response to a challenge dose of amphetamine after one and ten weeks of withdrawal. It was shown that amphetamine-induced locomotion and rearing was still augmented in animals that were previously exposed to amphetamine, even after 10 weeks of withdrawal (paper IV, fig. 2). The increase in locomotion has been attributed to the amphetamine-induced increase of dopamine in nAc (Kelly and Iversen, 1976), while enhancement of rearing is possibly due to the effects on dorsal striatum (al-Khatib et al., 1995; Iversen et al., 1975; Kelly et al., 1975; Laplante et al., 2011), and in particular DMS (Abedi Mukutenga et al., 2012). If compared to the results of paper III, amphetamine-induced enhanced rearing activity was concurrent with a depression of neuronal excitability in the DMS, both sustained for the whole length of the experiment (12 weeks) (paper IV, fig. 2). On the other hand, nicotine-induced depression of DMS excitability and enhancement of rearing activity resolved after 6 weeks (paper III, fig. 1). These variations might be related to different molecular targets and mechanisms of actions between the two drugs. For example, while we did not observe an enhancement of dopamine release by nicotine treatment (paper III, fig. 3), it has been reported that amphetamine induces sensitization of dopamine release in DMS (Patrick et al., 1991). This variation in sensitization of dopamine release, could putatively recruit DMS in a different manner (Wickens et al., 2003). Engagement of DMS following amphetamine-sensitization has also been shown in humans, where human subjects sensitized to amphetamine show an increase in activity of DMS when anticipating a reward. This is an indication that exposure to amphetamine enhances the susceptibility of DMS to further rewards (O'Daly et al., 2014).

Although neuronal excitability was depressed in DMS one day following the amphetamine regimen, it was transiently resolved at the two-week time point, at

which time neuronal excitability in the DLS was depressed, which sustained for the whole period of experiment. Amphetamine has been previously reported to induce differential modulations in spine density of striatal subregions, such that after protracted withdrawal from amphetamine, dendritic spine density is only increased in the DLS (Jedynak et al., 2007). This could indicate an enhanced recruitment of the DLS in action control after protracted withdrawal from amphetamine. In addition, as discussed in paper III, the transition from DMS to DLS and the temporal engagement of each subregion, have been implicated in the progression of drug intake, and might be associated with a behavioral shift to habitual compulsive drug use.

Amphetamine administration has been linked to manipulations in striatal GABAergic neurotransmission. One study shows an increase in activity of GABAergic FSIs following amphetamine administration (Wiltschko et al., 2010). Furthermore, amphetamine enhances striatal GABA levels (Bustamante et al., 2002; Del Arco et al., 1998), and increases the number of NPY-expressing GABAergic interneurons (although at a higher dose and a different paradigm than ours) (Horner et al., 2006). Therefore, in order to investigate whether depression of neuronal excitability involves enhanced GABAergic neurotransmission, we used whole-cell recordings to evaluate possible amphetamine-induced modulations in sIPSC. However, no treatment effects by amphetamine were observed on sIPSC recorded from MSNs of the DMS or DLS at any of the time-points (paper IV, fig. 4). Absence of amphetamine-induced effects on sIPSC might be relevant to behavioral experiments. During the habituation phase, without the drug onboard, no effect by previous exposure to amphetamine was observed on locomotion and rearing activity and the effects by previous amphetamine treatment on behavior was only revealed after administration of the challenge dose of amphetamine. In addition, the increase in PPR observed in field-potential recordings, which primarily reflects excitatory transmission, points to a decrease in neurotransmitter release, which also would be in line with previous studies (Bamford et al., 2008). However, another possibility is that an action-potential-mediated component of sIPSC "mask" the effects on GABAergic neurotransmission. In this case, treatment effects on inhibitory neurotransmission could possibly be revealed in quantal neurotransmitter release, which can be measured by mIPSC.

In conclusion, in paper IV we have shown that a brief period of amphetamine exposure results in temporal engagement of subregions in dorsal striatum. These modulations, which are initiated in the DMS and implicates the DLS in later time-points, might be important in the context of behavioral shift from recrea-

tional to compulsive drug intake. However, it is worth noting that even though synaptic output from striatal subregions is depressed in a time-dependent manner during withdrawal from nicotine or amphetamine, temporal and spatial resolution appear to be different.

5. General discussion and future perspective

Nicotine and amphetamine are common psychostimulants, and repeated use of them is associated with a considerable risk for developing substance use disorders. Given that the pharmacological options currently available for treatment of dependence to nicotine (e.g. varenicline, bupropion, nicotine replacement) (Casella et al., 2010) or amphetamine (e.g. naltrexone, bupropion) (Karila et al., 2010) have limited success rates especially in heavy users, the demand for developing new treatments is significant.

One of the hallmarks of drug addiction is the transition from recreational to compulsive and habitual drug intake, which develops over time in a proportion of individuals (Wagner and Anthony, 2002). It has been postulated that drug-induced neuroadaptations and recruitment of striatal circuits significantly contribute to the development of addiction. Therefore, in this thesis, we have investigated acute and long-lasting effects by nicotine or amphetamine on behavior and neurotransmission in dorsal striatum.

In paper I, *ex vivo* models for investigating nicotine's effects were employed, and it is important to verify these findings *in vivo* as well. Not only do investigations *ex vivo* in slices disregard the influence of various neuronal connections present *in vivo*, but also in the field potential model, artificial concerted neuronal stimulation is employed. One example of these potential complications is that although nicotine stimulates both basal and burst activity of important dopaminergic afferents to the dorsal striatum (Grenhoff et al., 1986), its effect on dopamine release appears to be frequency-dependent and influenced by the basal level of activity (Goutier et al., 2016). Moreover, the net effects of sensory inputs associated with contexts and cues might alter the effect produced by nicotine (Zaniewska et al., 2015). Thus future verification of these findings *in vivo* is important. The present *ex vivo* findings of acute effects by nicotine on neurotransmission in the DLS are nevertheless interesting since this brain region is implicated in habitual behavior and compulsive drug seeking. Thus, acute effects produced by nicotine in the DLS, presumably on glutamatergic neurotransmis-

sion, could initiate processes that, with repeated administration, might be implicated in habitual nicotine use.

In paper II, age-dependent influences by nicotine on behavior and nAc neurotransmission and dopamine levels were investigated. It was shown that nicotine-induced behavioral sensitization and tolerance to the rearing-depressant effects by nicotine develop faster in younger rats. The faster development of behavioral effects in the youngest age group was concomitant with a depression of nAc synaptic excitability. Age-dependent effect by nicotine was also observed after acute exposure to nicotine in slice recordings. Nicotine produced a depression of synaptic output in nAc of young animals. Together these data suggest that younger individuals are more susceptible to nicotine's effects, which might have implications for the higher consumption of nicotine earlier in life (Levin et al., 2007). In addition, the faster development of behavioral adaptations might be due to a higher sensitivity and plasticity of the system at younger age (Adriani and Laviola, 2004; Zhang et al., 2015), which indeed has been suggested to be fundamental for development and learning, and, ultimately, survival of young individuals (Spear, 2000). In line with this reasoning it has been proposed that early onset drug exposure facilitates the transition to drug dependence (Anthony and Petronis, 1995) and that postponing of the debut of substance use reduces the risk for development of substance use disorders (Jordan and Andersen, 2017). However, further research is needed to extend the results of paper II to dorsal subregions of striatum, which are implicated in habitual drug intake, and, again, verification of the *ex vivo* findings *in vivo* would be important.

In paper III, a brief period of repeated exposure to nicotine was shown to induce neuroadaptations that initially engage DMS and subsequently appear to be transferred to the DLS, where they remain for months after the last drug exposure. These neuroadaptations occur in a non-volitional drug administration paradigm, and are therefore independent of action learning that is associated with volitional drug intake. We speculate that these phenomena, which may reflect a rewiring of dorsal striatal circuits, are of particular importance for understanding neural correlates that occur during development and expression of nicotine-induced behavioral sensitization. In addition, the transfer from the DMS to the DLS might be important in the development of addiction to a compulsive habit (Everitt and Robbins, 2016).

Also in paper III, we have signified the importance of the DLS after prolonged withdrawal period, where a brief nicotine re-exposure to rats that received nicotine treatment six months earlier, immediately restores modulations in the DLS,

which initially took months to develop. In humans, the importance of the DLS in the compulsive aspects of drug seeking have been suggested in a recent case report, where an accidental lesion of dorsal striatum resulted in attenuation of nicotine intake (Muskens et al., 2012). Also, cocaine-dependent individuals have been reported to have an enlarged putamen (corresponding to the DLS in rodents) (Ersche et al., 2012). Drug-induced neuroadaptations in DLS might thus be implicated in saliency of the drug and the paired stimuli associated with drug intake and long-lasting drug craving. Therefore, we believe our findings are important for understanding the high susceptibility of relapse to compulsive drug intake, even following protracted withdrawal.

Analyses of PPR in our experiments in paper III suggest that one month after the repeated nicotine regimen the probability of neurotransmitter release, possibly glutamate, from presynaptic terminals is decreased in the DMS. If this effect applies also to the human situation, it might be of relevance for the high prevalence of smoking in individuals with schizophrenia (Sagud et al., 2009). It has been reported that patients with schizophrenia have a high content of glutamate in their caudate nucleus (corresponding to the DMS in rodents) (de la Fuente-Sandoval et al., 2011), and thus nicotine-induced decrease of glutamate release might function as a form of self-medication in these patients. Supporting this theory, cigarette smoking has also been reported to reduce negative symptoms of schizophrenia (Smith et al., 2001).

It has been shown that, following withdrawal, effects such as motivation to self-administer amphetamine, as well as cue-induced reinstatement of drug seeking are progressively incubated (Adhikary et al., 2016; Shepard et al., 2004). Parallel observations have been made in humans, where cue-induced craving, but not baseline craving in the absence of cues, progressively increases and peaks at three months of withdrawal before decreasing subsequently (Wang et al., 2013a). In our experiments in paper IV, treatment effects in the DLS were also sustained up to three months after the amphetamine regimen, which might have implications for e.g. the cue-induced craving after protracted withdrawal. However, whether the effects are still present after longer time-periods requires further investigation. In addition, it will be important to study the tentative behavioral correlates of these findings in the context of drug craving and relapse.

Taken together, the experiments performed in this thesis show drug-induced modulations by nicotine and amphetamine on behavior and striatal neurotransmission in the rat. The neuroadaptations observed *ex vivo* by electrophysiological techniques are associated with behavioral sensitization to the drugs, which

develop over time and might be implicated in transition from recreational drug use to compulsive drug intake, i.e. addiction. Further research is needed in order to understand the specific and comprehensive molecular mechanisms that underlie the depression of neurotransmission in the DMS and DLS following a brief and subchronic drug exposure. Moreover, understanding behavioral correlates related to these changes in the context of maladaptive behaviors, such as cue-induced drug craving and reinstatement of drug seeking after withdrawal requires further studies. Further knowledge of these phenomena will most likely increase the understanding of addiction as a compulsive maladaptive behavior and enhance the possibility of developing new and improved pharmacological means to inhibit, manipulate, or reverse these neuroadaptations and behaviors associated with addiction.

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