Nicotine sensitization and the effects of extended withdrawal

- behavioral, neurochemical and electrophysiological studies in the rat

Julia Morud Lekholm

Department of Psychiatry and Neurochemistry
Institute of Neuroscience and Physiology
The Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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ABSTRACT

Tobacco use is one of the primary factors for global burden of disease and often results in life-long nicotine addiction, only a small percentage users are able to maintain cessation. The life-long addiction together with a high relapse risk might be connected to drug-induced altered neural circuits. However, there is still uncertainty concerning the mechanisms involved in the progressive changes of neuronal function induced by repeated nicotine exposure. The rewarding effects of nicotine have been attributed to increased dopamine (DA) levels in the nucleus accumbens, (nAc) after stimulation of nicotinic acetylcholine receptors (nAChRs) in the ventral tegmental area (VTA). To explore long-term and age-dependent effects by nicotine, Wistar rats were exposed to nicotine daily for three weeks, followed by different withdrawal periods after which locomotor stimulatory effects and behavioral disinhibition were assessed by means of activity boxes and the elevated plus-maze, respectively. In addition, neurotransmission was studied in brain slices from the nAc utilizing field potential recordings and whole-cell patch clamp. Histological procedures were also used for estimation of dendritic spine density. The results show that nicotine-induced locomotor sensitization is sustained for up to seven months, concomitant with decreased synaptic

function in nAc and increased response to the dopamine D2 receptor agonist quinpirole in nAc shell. We demonstrate that young animals display a faster response to nicotine and rapid tolerance development to the rearing depressing effect of nicotine. In addition, young animals exhibited lowered accumbal synaptic activity ten days into withdrawal. Moreover, nicotine induces behavioral disinhibition that is not fully developed until three months into withdrawal. These behavioral effects develop in parallel with changes in accumbal synaptic activity, GABAergic transmission and spine density. In addition, gene expression of $GABA_A$ receptor subunits is altered at this time point. Finally, we show that local manipulation of GABAergic transmission in the nAc in drug naïve rats results in disinhibitory behavior. In conclusion, limited exposure to nicotine causes long lasting to chronic alterations in behavior and accumbal neurotransmission. In particular the response to dopaminergic and GABAergic acting drugs is not fully developed until after extended abstinence.

Keywords: Abstinence, Dopamine, Elevated plus-maze, GABA, Inhibitory control, Nicotine, Nucleus Accumbens, Sensitization

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SAMMANFATTNING PÅ SVENSKA

”Nikotinsensibilisering och effekterna av långvarig abstinens - studier av beteende, neurokemi samt elektrofysiologiska förändringar i råtta”

Beroendesjukdomar, till exempel nikotinberoende, är kroniska tillstånd som orsakar mycket fysisk och psykisk ohälsa, och samhällskostnaderna för tobaksrelaterade sjukdomar i Sverige är enorma. Detta beror till stor del på tobakens beroendeframkallande ingrediens, nikotin, som försvårar konsumtionsstopp. Trots att mycket forskning bedrivits kring nikotinets beroendeframkallande effekter har de idag tillgängliga behandlingarna mot nikotinberoende en begränsad effekt, och en stor majoritet återfaller i missbruk. Nikotin är en mycket potent drog med snabb toleransutveckling till drogens aversiva egenskaper, såsom illamående och takykardi. Men även de stimulativa egenskaperna hos drogen, som ökad vakenhet och koncentrationsförmåga, försämras med upprepad konsumtion. Efter ett långvarigt bruk krävs närvaro av drogen för att återställa dessa funktioner till en normal nivå.

Beroendeframkallande droger aktiverar det mesolimbiska dopaminsystemet, vilket resulterar i frisättning av dopamin (DA) i striatala regioner i hjärnan, såsom nucleus accumbens (nAc), som är i fokus för denna avhandling. Dessa nervbanor är del av hjärnans belönings- och motivationssystem, vilka är avgörande för artens fortlevnad, då de förmedlar belönande känslor vid mat- och dryckesintag, samt vid sex. Av denna anledning är dessa nervbanor konserverade genom evolutionen och återfinns även hos lägre stående arter, vilket möjliggör en användning av råtta som modellorganism. I denna avhandling har Wistar råttor exponerats för nikotin dagligen i tre veckor och under dessa veckor har djurens lokomotoriska aktivitet studerats. Detta då många droger orsakar något som kallas sensibilisering, vilket innebär att den lokomotoriskt stimulerande effekten av en given dos av drogen ökar över tiden. Detta är ett välkänt men ännu ej mekanistiskt förklarat fenomen. I avhandlingen studeras bl.a. hur långvarig denna

nikotinsensibilisering är och resultaten visar att nikotin ger upphov till i det närmaste kroniska förändringar, då en tydlig effekt uppmättes i djuren ända upp till sju månader efter senaste nikotinexponering. Detta anses vara en mycket lång tid i en råttas liv, då dessa djur ofta inte lever längre än ca 2 år. Vid samma tillfälle fann vi att det elektrofysiologiska svaret på en DA-receptor-stimulerande drog (quinpirole) hade förstärkts. Vi har också studerat vilken betydelse ålder vid första exponering har för nikotinets effekter. För dessa försök användes djur i tre olika åldrar. Resultaten visar att yngre djur har både snabbare toleransutveckling och sensibilisering än äldre djur. Äldre djur verkar å andra sidan få en mer långvarig DA-frisättning i nAc efter nikotin.

För att förstå mer i detalj vad som händer under abstinensfasen och vilka system i hjärnan som påverkas, förutom det dopaminerga, gjordes försök där vi i tre månader efter avbruten nikotintillförsel följde djurens undersökande beteende i en modell där detta undertrycks av ett aversivt stimulus (en öppen, upphöjd miljö). Vi fann att efter tre månaders abstinens från nikotin var dessa djur mer disinhiberade i sitt beteende jämfört med djur som endast fått kontrollbehandling (koksalt), ett resultat som kan tolkas som ökad impulsivitet, något som även rapporterats hos rökare. Vid samma tidpunkt hade djuren en förändrad neurotransmission i nAc, i synnerhet i det GABAerga systemet, vilket ej var fallet tidigare under abstinensen. GABA är ett hämmande system som ofta beskrivs som bromsen i hjärnan, och vid manipulation av detta system kan man påverka t.ex. impulsivt beteende. Dock brukar nAc ej anses vara en nyckelregion för denna typ av beteende. För att undersöka om förändring av GABAerg transmission i nAc skulle kunna ligga bakom den disinhiberande effekten utfördes försök där en GABAergt verkande substans (bensodiazepinen diazepam) injicerades lokalt i nAc på djuren. Detta ökade kraftigt djurens disinhiberade beteende. Vi kunde också påvisa att tätheten av s.k. ”dendritiska spines” var förändrad vid denna tidpunkt. Spines är små utskott på nervceller där synapser återfinns och en förändrad täthet av dessa tyder också på en förändrad neurotransmission.

I nAc är ca 95 % av neuronen GABAerga och uttrycket av $GABA_A$-receptorer är högt. Dessa reglerar cellaktivitet, och bl.a. dopaminnivåerna lokalt i nAc bestäms av aktiviteten i $GABA_A$-receptorer. Alkohol utövar delar av sina effekter via detta system, liksom bensodiazepiner (lugnande preparat), som också har en missbrukspotential. Sammantaget är det GABAerga systemet ett mycket viktigt system, och små förändringar i dess reglering kan ha stora konsekvenser för övriga regioner med vilka nAc kommunicerar. Slutligen, nikotin initierar förändringar i det GABAerga systemet, som först efter en längre tids drogfrihet är fullt utvecklade. Dessa förändringar kan bidra till ett förändrat svar på andra droger som också verkar via detta system.

Sammanfattningsvis tyder avhandlingens resultat på att en relativt kortvarig exponering för nikotin (tre veckor) leder till mycket långvariga förändringar både i beteende och i neurotransmission, samt att nikotin påverkar synapsfunktion i nAc och att unga individer ter sig mer känsliga för dessa effekter.

LIST OF PAPERS

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

I. **Morud J***, Adermark L*, Perez-Alcazar M, Ericson M, Söderpalm B, *Nicotine produces chronic behavioral sensitization with changes in accumbal neurotransmission and increased sensitivity to re-exposure.* Addiction Biology 2015; 21(2):397-406.

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. **Morud J**, Strandberg J, Andrén A, Ericson M, Söderpalm B, Adermark L. *Extended nicotine withdrawal induces spontaneous disinhibition and alters accumbal neurotransmission.* Submitted 2016.

CONTENT

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
ACh	Acetylcholine
aCSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
cDNA	Complementary DNA
CNS	Central nervous system
DA	Dopamine
DA D1	Dopamine receptor type 1
DA D2	Dopamine receptor type 2
DMS	Dorsomedial striatum
DLS	Dorsolateral striatum
DSM V	Diagnostic and statistical manual for mental disorders
DNA	Deoxyribonucleic acid
EPM	Elevated plus-maze
eEPSCs	Evoked excitatory postsynaptic currents
EPSP	Field excitatory postsynaptic potential
eIPSCs	Evoked inhibitory postsynaptic currents
GABA	γ-aminobutyric acid
GAT-1	GABA transporter 1
HPLC	High performance liquid chromatography
MLA	Methyllycaconitine
mRNA	Messenger RNA
MSNs	Medium spiny neurons
nAc	Nucleus accumbens
nAChRs	Nicotinic acetylcholine receptors
NMDA	*N*-Methyl-D-aspartic acid
PS	Population spike

PPR	Paired pulse ratio
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
sEPSCs	Spontaneous excitatory postsynaptic currents
sIPSCs	Spontaneous inhibitory postsynaptic currents
VTA	Ventral tegmental area

INTRODUCTION

Brief overview of this thesis

This thesis concerns the behavioral and neurophysiological effects of repeated nicotine exposure, extended nicotine abstinence, and the influence of age at first nicotine exposure. The present work is based on three papers, where the first two are published and paper III is a submitted manuscript. These are included as an appendix and referred to in Roman numerals (I-III) in the text

Nicotine addiction and reward pathways

Tobacco (*Nicotiana tabacum*) has been used as a recreational drug in the Americas since long before European explorers introduced it in the old world in mid 16th century (Fletcher, 1941). After introduction in Europe it rapidly gained in popularity, and in the early 17th century tobacco was believed to serve as a panacea, which contributed to making it the first crop to be grown for trade purposes in the 17th century (Wroth and Dickson, 1955).

Nicotine: use and abuse

Nicotine, the active and highly addictive component in tobacco, is a volatile alkaloid that forms salts with most acids. Today, nicotine is one of the most used and abused drugs in the world, with an estimate of 1 billion smokers worldwide (WHO, 2015). Since the 1950s, when reports of the hazardous effects of smoking surfaced, there has been a yearly decline in users. Nevertheless, around 6 million people are still killed yearly in tobacco-related diseases, such as lung cancer and cardiovascular diseases. The annual cost for tobacco-related illness today in Sweden is estimated to 30 billion SEK, and smoking is the foremost preventable cause of illness and premature death in the world (Rostron et al., 2014; SverigesRegering, 2016). Due to the addictive properties of nicotine, few

users are able to maintain nicotine cessation for longer periods of time, even though the negative consequences of smoking are well known.

Addiction

Drug dependence and addiction are chronic and relapsing brain disorders that can be devastating for the afflicted person, with features such as loss of intake control, compulsive drug seeking and incapability to stop taking the drug (Koob and Le Moal, 2008). The molecular principles behind addiction have been intensely researched over the last decades, but even though the knowledge of the disease has substantially increased, the mechanisms by which an addiction is shaped are still largely unknown. These types of diagnoses lack exact physiological measures; instead diagnosis relies on a combination of symptoms that are described in diagnostic manuals (e.g. ICD 10: International Classification od Disease 10th edition, or the DSM-V: Diagnostic and Statistical Manual of Mental Disorders, 5th edition).

The transition from recreational drug use to an addiction can take years to develop, but for some individuals it can be a rapid process. The discrepancy between individuals is both due to genetic and environmental factors (Liu et al., 2004). The choice of drug also has an important role since different drugs have different addictive profiles, with heroin and methamphetamine being very addictive, whereas e.g. ecstasy and LSD have a less addictive profile (Nutt et al., 2007). Tobacco abuse is classified as a substance abuse disorder according to the criteria listed in Table 1, and even tough it lacks some features that are present in other substance abuse disorders, such as substance intoxication, it does share properties such as psychological and physiological withdrawal symptoms.

DSM-5 chriteria for tobacco addiction

1. Tobacco is often taken in larger amount or over a longer period than was intended.
2. There is a persistent desire or unsuccessful efforts to cut down or control tobacco use.
3. A great deal of time is spent in activities necessary to obtain or use tobacco.
4. Craving to use tobacco.
5. Recurrent tobacco use resulting in failure to fulfill major role obligations at work, school or home.
6. Continued tobacco use despite having persistent or recurrent social or interpersonal problems caused by the effects of tobacco (e.g. arguments with others about tobacco use).
7. Important social , occupational or recreational activities are given up or reduced due to tobacco use.
8. Recurrent tobacco use in situations in which it is physically hazardous (e.g. smoking in bed).
9. Tobacco use is continued despite knowledge of having a persistent or recurrent physical or physiological problem that is likely to have been caused or exacerbated by tobacco.
10. Tolerance, as defined by either of the following:
    A: A need for markedly increased amount of tobacco to achieve the desired effect.
    B: A markedly diminished effect with continued use of the same amount of tobacco.
11. Withdrawal, as manifested by either of the following:
    A: The characteristic withdrawal syndrome for tobacco
    B: Tobacco or related substances such as nicotine are consumed for avoidance of withdrawal

Table 1. The DSM-V states that if at least two of the above listed criteria are present during the last 12 months the patient has a substance abuse disorder. The more criteria that are fulfilled, the more sever the addiction is.

Physiological principles for addiction

The brain reward system

An addict's inability to stop consuming the drug is in large parts explained by a drug-induced malfunction of the brain reward system. This system regulates reward and motivation, which are essential components for the survival of species. Intake of food and fluids serves as rewarding agents along with social interactions and sex. Due to its importance for the survival of species, it has been conserved throughout evolution and can be found in more simple species such as rodents and fruit fly, *Drosophila Melanogaster* (Waddell, 2013). This provides model systems to enable preclinical manipulations and characterization of the

system. The brain reward system comprises of several brain regions, such as the medial forebrain bundle, ventral tegmental area, striatal regions, raphe nuclei, septal regions, amygdala and prefrontal cortex areas to mention a few (Wise, 1996) (Fig. 1). These regions were identified in the early 1950's when Olds and Milner discovered that rats learned to prefer locations that had been paired with electrical stimulation of specific brain areas (Olds and Milner, 1954). They continued to demonstrate that rats could learn to self-administer electrical stimulation into septal regions to such an extent that natural rewards, such as feeding and drinking, were abolished. Shortly after this discovery it was also confirmed that animals would self-administer drugs of abuse in the same manner as previously demonstrated with intracranial electrical self-stimulation (Schuster and Thompson, 1969).

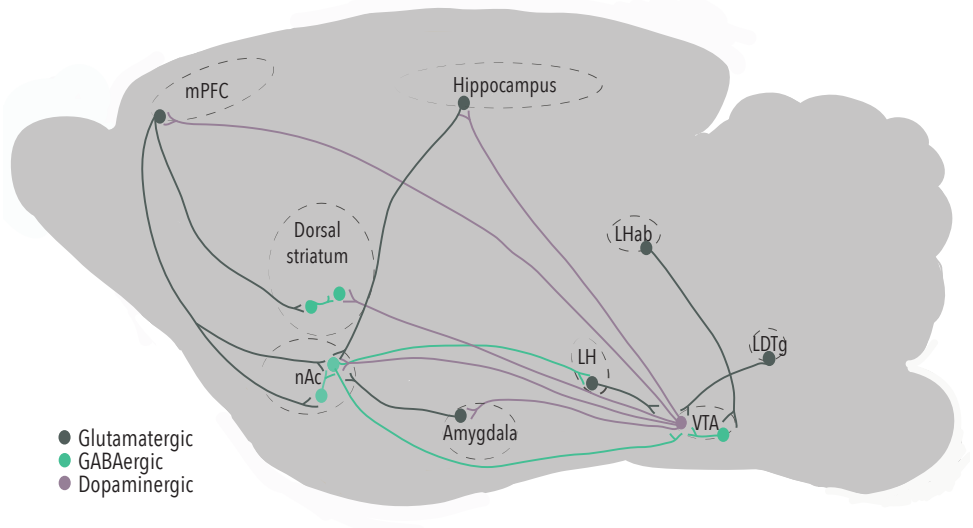

Figure 1. Graphical example of a simplified version of the reward pathways in rats, describing the major glutamatergic (brown), GABAergic (light green) and dopaminergic (purple) pathways. Abbreviations: mPFC: medial prefrontal cortex, nAc: nucleus accumbens, LHab: lateral habenula, LH: lateral hypothalamus, VTA: ventral tegmental area, LDTg: lateral dorsal tegmentum. Image adapted from (Russo and Nestler, 2013).

The mesolimbic dopamine system and nucleus accumbens

In 1958, Carlsson and colleagues discovered that dopamine (DA) is a neurotransmitter in its own right (Carlsson et al., 1958). Subsequently, histological studies demonstrated that DA and other monoamines were located to discrete sets of neurons with their cell bodies in the midbrain (Carlsson et al., 1964; Dahlstrom and Fuxe, 1964). The highest density of dopaminergic cell-bodies were found in the ventral midbrain which encompasses the ventral tegmental area (VTA) and the substantia nigra. Neurons originating in the VTA project to a number of limbic areas and is thus called the mesolimbic DA system. This system has been implicated to be directly or indirectly involved in a wide range of behaviors, including the rewarding and positive reinforcing effects of drugs of abuse, attention and motivation, and motor control (Berridge, 2012; Schultz, 2006; Wise, 1996). The nigrostriatal system, on the other hand, has been more implicated in motor-activating effects (Obeso et al., 2008; Trudeau et al., 2014).

Dopaminergic neurons exhibit two signature firing-patterns: a phasic burst firing and a regular tonic single-spike dependent firing (Grace and Bunney, 1984a, b). The positive reinforcing function is primarily associated with phasic burst-dependent firing, whereas the motor function is more related to stabile tonic firing (Marinelli and McCutcheon, 2014). The DA neurons in VTA have projections that run via the medial forebrain bundle into frontal areas such as the ventral striatum - also known as the nucleus accumbens (nAc), the frontal cortex, septum and the olfactory tubercle, but they also project to other areas such as the hypothalamus, hippocampus and the amygdala (Trudeau et al., 2014). Based on this it can easily be understood that axons of the DA neurons are heavily branched and a single neuron might give rise to 300,000 axon terminals (Arbuthnott and Wickens, 2007), implying that manipulations of DA neurons might produce very complex and multiregional effects.

The nucleus accumbens (nAc) is a relatively small region in the basal ganglia that mainly consists of GABAergic neurons. The majority are

medium spiny neurons (MSNs), which are projecting neurons containing either dopamine receptor 1 (D1) or dopamine receptor 2 (D2) receptors and mediating excitatory and inhibitory neurotransmission, respectively (Vicente et al., 2016). These neurons project to regions such as the lateral habenula and the VTA (Russo and Nestler, 2013). The nAc responds to hedonic, novel and aversive stimuli (Everitt, 1990; Salamone, 1994; Schultz et al., 1997) and can be divided into several subregions. A division into a core and shell region is the most commonly used (Floresco, 2015).

The shell region is part of the extended amygdala whereas the core region has more similarities to the dorsal part of the striatum (Alheid and Heimer, 1988). DA in the nAc core has been suggested to be involved in gaining control over amygdala-dependent appetitive learning, and lesions of the core do not affect Pavlovian conditioning. DA in nAc shell, on the other hand, has been implicated in hippocampal-dependent spatial information processing, and lesions here influence instrumental and Pavlovian learning (Corbit et al., 2001; Ito and Hayen, 2011).

Regulation of the nucleus accumbens

The organization of cells within the nAc is different from most brain regions, since it completely lacks a laminar organization. Instead the MSNs are organized in mosaic patches, these patches also represent different compartments of input-output segregations (Voorn et al., 1989). Interestingly, this organization appears to be represented even on a receptor localization level (Gerfen, 1992). Depending on the receptor expression on the MSNs in nAc they can either be part of the excitatory direct pathway (D1 containing), which directly innervates the VTA, or the inhibitory indirect pathway (D2 containing), that regulates VTA through intervening GABAergic neurons in the ventral pallidum (Russo and Nestler, 2013; Vicente et al., 2016).

There is also a small population of cholinergic and GABAergic interneurons that even though low in numbers have an important regulatory function on accumbal activity. The precise mechanisms by

which cholinergic interneurons regulate accumbal function are not known, but they can regulate striatal output rapidly by controlling GABA release from dopaminergic terminals (Nelson et al., 2014). Fast spiking GABAergic interneurons can inhibit MSNs directly by postsynaptic inhibition whereas the MSNs themselves can inhibit basal ganglia output and form functional synapses through local axonal connections (Tunstall et al., 2002). Together these different pathways form striatal microcircuits, having their own separate functions (Tepper and Plenz, 2006). The nAc also receives a substantial glutamatergic input from areas such as the prefrontal cortex, hippocampus and amygdala (Russo and Nestler, 2013).

Drugs of abuse and accumbal dopamine

Most drugs of abuse produce a large increase in accumbal DA after systemic injections; nicotine is no exception (Koob and Volkow, 2016). Self-administration of drugs will also release DA in nAc (Ikemoto and Bonci, 2014) and this behavior can be attenuated via DA antagonists or by destruction of DA neurons (Corrigall and Coen, 1991; Corrigall et al., 1992), with a few drugs, such as ethanol and opiates, as exceptions (Dworkin et al., 1988; Pettit et al., 1984; Rassnick et al., 1993). It has been suggested that DA is required for the establishment of ethanol self-administration (Ikemoto et al., 1997), whereas once established it may not be critical for maintaining ethanol self-administration (Ikemoto et al., 1997; Rassnick et al., 1993). Interestingly, low doses of DA antagonists increase drug self-administration, which is often interpreted as a compensatory increase in intake due to a blunted drug response (Di Chiara and Bassareo, 2007; Yokel and Wise, 1975).

The mechanisms underlying nicotine-induced DA increases differ from those of many other drugs of abuse, e.g. cocaine and amphetamine that act directly on the dopaminergic transporters located on the neuronal terminals. Nicotine exerts its DA increasing effect by several parallel mechanisms, such as stimulation of nAChRs situated on DA neurons in VTA, or by increasing glutamatergic excitation and altering the influence of GABAergic inhibition on DA neurons (Pidoplichko et al., 2004;

Schilstrom et al., 1998; Tolu et al., 2013). The GABAergic influence over nicotine-induced DA release is very complex since the GABAergic neurons of the VTA are not strictly interneurons. There are also GABAergic neurons projecting to same frontal regions, as do DA neurons (Omelchenko and Sesack, 2009). Cholinergic modulation of GABAergic interneurons in the VTA is also very important for spontaneous baseline DA burst firing (Tolu et al., 2013). Additionally, nicotine effects mediated via nAChRs are receptor subunit dependent since receptors with different subunit configurations behave differently when exposed to nicotine, some desensitize rapidly at low concentrations whereas some are more robust (Pidoplichko et al., 1997; Pidoplichko et al., 2004).

Drug dependence and impulsive behavior

Impulsivity is a multidimensional concept that can be dissected into several components of behavior, such as behavioral disinhibition and impulsive choice, e.g. delayed monetary reward in humans. Overall impulsive behavior can be described as acting prematurely and an inability to constrain inappropriate behaviors. According to the DSM V several criteria for nicotine dependence, or drug dependence in general, include components of impulsive behavior (**Table 1**). There is an increasing body of evidence both in animal studies and human experiments that stimulants, such as nicotine, induce impulsive behavior (Ericson et al., 2000; Fields et al., 2009; Fillmore et al., 2002; Kolokotroni et al., 2011; Olausson et al., 2001b; Paine and Olmstead, 2004). This has implications for the interpretation of the DSM V criteria, since the drug use itself will cause heightened impulsivity and as such, fulfill some of the listed criteria. In addition, individuals who exhibit impulsive traits are overrepresented among drug users, and due to this, impulsivity has been identified as an important risk factor for the development of drug abuse as well as for relapse (Tomko et al., 2016).

In the past, regions such as the prefrontal cortex and the amygdala have been considered to be central for regulating impulsive behaviors, but recent evidence also implicates nAc as an important region for

controlling impulsive behaviors (Feja et al., 2014). Also, DA together with noradrenaline have been considered to be central for impulsive behaviors, but recent data also suggest a role for GABAergic transmission in regulating these types of behaviors (Hayes et al., 2014).

Nicotine and nicotinic acetylcholine receptors

Nicotine was isolated from the leaves of the tobacco plant already in 1828 by the two German chemists Posselt and Reimann, who thought of nicotine as a poison (Posselt, 1828). At the turn of the 20th century new hypotheses on how neurons were stimulated by different substances emerged. The assumption that neuronal active substances acted directly on nerve endings was proven wrong after works by Langley and colleagues (Langley, 1901, 1918). Nicotine was used as the model substance in their work that led up to the identification of "receptive substances" – receptors. The nicotinic acetylcholine receptors (nAChRs) were thereby among the first receptors to be studied.

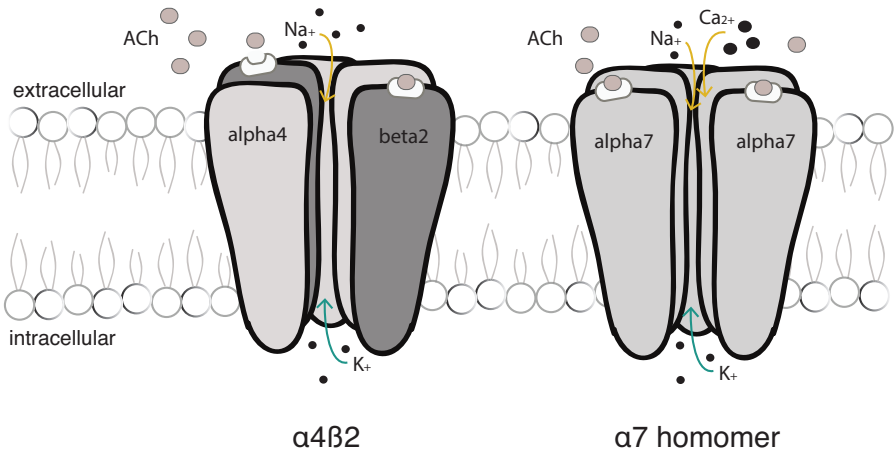

Figure 2. A descriptive image of the heteromeric α4β2 and the homomeric α7 pentameric nicotinic acetylcholine receptor, showing discrete ligand binding-sites and that they have different ion permeability.

The nicotinic acetylcholine receptor and its pharmacology

In the 1980s it was confirmed that nAChRs existed in the CNS, that these were different from previously studied neuromuscular receptors, and that they contributed to the psychoactive properties of nicotine in a subunit-dependent manner (Caulfield and Higgins, 1983; Clarke and Pert, 1985; Clarke et al., 1984). The use of α-bungarotoxin, a neurotoxin that binds irreversibly to nAChRs, was proven useful since it helped in identifying different subpopulations of the receptor (Clarke et al., 1986; Clarke et al., 1985). In CNS, α-bungarotoxin specifically binds homomeric α7, the only homomeric composition of neuronal nAChRs. The nAChRs have a pentameric structure (Fig. 2), which always consists of at least one alpha subunit. The different subunits represented centrally are eight α-type (α2-α7, α9-α10) and three β-type (β2-β4) subunits, which combined create a large variety in structure and function due to the many possible combinations (Zoli et al., 2015). The two most commonly expressed neuronal combinations are the low nicotine-affinity α7 homomer and the high nicotine-affinity α4β2 heteromer (Dani, 2015). The rat midbrain DA neurons are generally believed to express the α3-α7 and the β2-β3 subunits. Different subunit compositions are located either pre- or postsynaptically where the α7 homomer is most commonly, but not exclusively, presynaptic and the α4β2 are often located postsynaptic or somatic (Zhang et al., 2009). Different antagonists are available for the detection of different subunit compositions (e.g. MLA for α7 or α3/α6 containing, and DhβE for α4β2), which have been used for decades to study the diverse function of nAChRs.

The nAChRs are selectively permeable to cations in a subunit-dependent manner, and as such all compositions are permeable for influx of sodium and efflux of potassium, whereas the α7 homomer is also highly permeable for calcium (Albuquerque et al., 2009; Dani, 2001). This is due to the type of amino acids, and the charged residues, that line the inner and outer parts of the receptor pore (Bertrand et al., 1993). Calcium permeability has major impact on neurotransmission since high influx of calcium in to neurons activates intracellular signaling cascades, which

then influences the probability for neurotransmitter vesicle release. Additionally, nAChRs themselves are allosterically modulated by calcium so that high concentrations of calcium increase the probability for opening the channel (Vernino et al., 1992).

Acute nicotine effects and receptor desensitization

Immediately after tobacco is consumed, brain nicotine levels will increase rapidly and already at 20 nM the nAChRs will start to desensitize (Brody et al., 2006). In humans, prominent nAChR desensitization has been reported already after the consumption of one cigarette (Matta et al., 2007). The high-affinity α4β2 nAChR is very sensitive to increased nicotine concentrations and will rather quickly be substantially desensitized, as compared to the low-affinity α7 that requires higher concentrations to be desensitized (Brody et al., 2006). The affinity of nicotine for the α4β2 receptor ranges between 0.5-14 nmol, which translates to 0.01-2.3 ng/ml, and a human smoker often has blood plasma concentrations of 10-50 ng/ml (Benowitz et al., 1990; Sihver et al., 2000). This indicates that in human smokers, the α4β2 nAChRs are most of the time completely saturated. This has consequences for GABAergic neurotransmission, since these receptors are mostly located on GABAergic interneurons, with implications for inhibition on DA neurons in the VTA. Desensitization is a conformational state of the receptor (together with "rest" or "open"), in which the receptor is not available for agonist stimulation (Quick and Lester, 2002). This state has been suggested to be either short-lived or long-lasting, partly depending on the nicotine exposure level (Khiroug et al., 1997) or the phosphorylation state (Khiroug et al., 1998). Long periods of low nicotine exposure will eventually create a very deep desensitization of the receptor that is difficult to reverse, a scenario similar to what happens in human smokers. This has effects on normal ACh activity at cholinergic synapses, since the availability of receptors is decreased - the synaptic response after ACh release will be blunted (Pidoplichko et al., 2004).

Chronic effects and behavioral sensitization

The homeostatic response to nAChRs desensitization is receptor upregulation (Fenster et al., 1999; Picciotto et al., 2008), and it seems as if both chronic nicotine exposure and early withdrawal causes nAChRs upregulation (Staley et al., 2006). Long-term exposure to nicotine will increase the number of nAChRs binding sites on the cell surface. It is not yet known whether this actually is due to an increased number of functional nAChRs, that still can be activated, or if it mostly represents desensitized and inactive nAChRs (Vallejo et al., 2005; Wonnacott, 1990).

Repeated administration of stimulant drugs, such as nicotine, induces the phenomenon of behavioral sensitization, which implicates an increased drug-response over time to the same dose of drug in behaviors such as locomotion (Clarke and Kumar, 1983a). Sensitization can be viewed as the opposite of tolerance development, in which the drug response is decreased over time. The effect on locomotion has been suggested to partly derive from altered responsiveness of DA receptors in the nAc, which results in a hypersensitive receptor (Di Chiara, 2000; Molander and Soderpalm, 2003). This assumption is partly based on the observation that activation of accumbal postsynaptic DA receptors increases locomotion, while the opposite – antagonism or disruption of DA receptors/neurons – will attenuate this behavior (Clarke, 1990; Clarke et al., 1988). Although, increased receptor responsiveness has never been detected in the acute exposure phase, it appears to develop during the early withdrawal phase (Robinson and Berridge, 1993; Zhang et al., 2012).

Age discrepancies

Regular smokers have often had their first experience of nicotine at a young age, and nicotine's rewarding and reinforcing properties appear to be age-dependent (Kendler et al., 2013). Thus, teenagers report stronger and more positive effects from smoking, both in terms of the rewarding feeling and a stronger addiction with a lower chance of cessation (DiFranza et al., 2000). Additionally, teenagers report fewer aversive effects at their first nicotine exposures, as compared to adults (DiFranza et al., 2000). One of the suggested mechanisms underlying these

phenomena is an increased sensitivity during adolescence of midbrain excitatory synapses on DA neurons, where nicotine will increase the AMPA/NMDA ratio of evoked EPSCs in both young and adult rats. Although, young animals require a much lower dose to produce the same magnitude in response as adults (Placzek et al., 2009). Animal studies have revealed that young rats are more sensitive to nicotine than adult rats, so as that young rats form a stronger place preference to nicotine, which has been suggested to be due to an increased nicotine-induced long-term potentiation in younger rats (Placzek et al., 2009). It also appears that small amounts of nicotine during adolescence will affect the response to nicotine later in life, as well as increase nicotine self-administration (Adriani et al., 2003). In addition, early life exposure has been reported to alter nAChR subunit compositions resulting in an increased sensitivity to nicotine (Adriani et al., 2003).

Nicotine as a gateway drug

The gateway drug hypothesis refers to the notion that the use of "light drugs", such as nicotine and alcohol, precedes the use of cannabis, which in turns precedes the use of "heavier drugs", such as cocaine, amphetamine or heroine (Kandel, 1975). Epidemiological studies have supported this model, and nicotine appears to be of particular importance for the gateway process (Yamaguchi and Kandel, 1984). As was reported in the most recent U.S. National Survey on Drug Use and Health, a decline in tobacco use (40.8 % - 30.6 %) was detected in the US between years the 2002-2013; interestingly this was also the case for cocaine use (6.7 % - 4.4 %). While this could be due to sociological aspects, such as changes in drug policies or changes in law enforcement strategies, preclinical reports also support a connection between nicotine and cocaine. Pretreatment with nicotine in young mice increases the response to cocaine later in life, both in the form of increased drug-induced locomotion and a stronger conditioned place-preference (Levine et al., 2011). The mechanism is partly believed to be through altered epigenetics, in which the expression of FosB is altered, resulting in reduced cocaine-induced LTP (long term potentiation) in the nAc. Nicotine has been described to prime the brain for subsequent drug use

by altering LTP (Huang et al., 2013). Interestingly, the effect is unidirectional, as pretreatment with cocaine does not influence nicotine-induced LTP in the same manner (Huang et al., 2013).

GABA and the $GABA_A$ receptor

The GABAergic (γ-aminobutyric acid) system is the main inhibitory neurotransmitter system in mammalian brains, and approximately 20% of all neurons in the brain are GABAergic. These neurons can either be interneurons of different kinds or projecting neurons, such as the before mentioned MSNs of the striatum. The interneurons are often sparsely spiny, whereas the dendrites of the MSNs are dense with spines.

The $GABA_A$ receptor and its pharmacology

The mammalian GABA system is comprised of GABA receptors (A and B) and GABA releasing neurons. GABAergic receptors are very abundant in the brain, and are present on almost all neurons (Mody and Pearce, 2004). The $GABA_A$ receptors are ligand-gated ion channels that are permeable to chloride after activation, which will cause the cell to be hyperpolarized (Semyanov et al., 2003) (Fig. 3). The receptor shares some similarities to the nAChRs, in that they are pentameric structures with several possible subunit compositions (Fig. 3). The $GABA_B$ receptors act very differently, since they are g-protein coupled receptors of a metabotropic nature.

The $GABA_A$ receptors can be located both synaptically and extrasynaptically (Soltesz et al., 1990), which indicates that they participate both in phasic inhibition, by producing inhibitory postsynaptic currents (IPSCs), and tonic inhibition on neurotransmission (Brickley and Mody, 2012). The charge produced by tonically activated $GABA_A$ receptors has been estimated to be up to three times the size of those produced by phasic events (Nusser and Mody, 2002). The difference between tonic and phasic $GABA_A$ receptors is in part due to different receptor subunit compositions, where the δ-unit appears important for tonically active receptors (Nusser et al., 1998). Receptors located

extrasynaptically are also more sensitive to low concentrations of the $GABA_A$ antagonist picrotoxin, which indicates a discrepancy between different classes of $GABA_A$ receptors in their pharmacological profile (Semyanov et al., 2003).

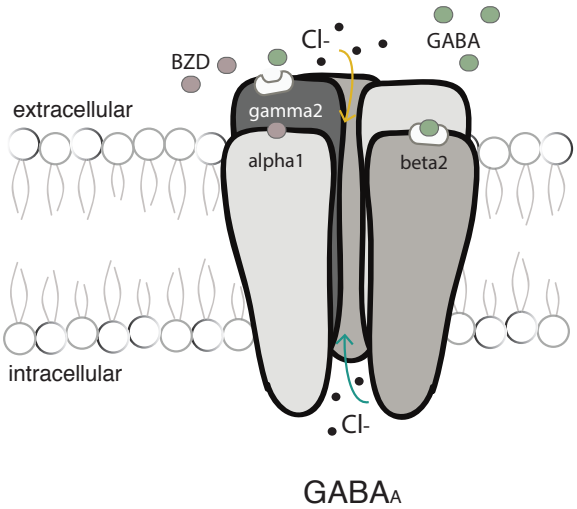

Figure 3. A conceptual image of the heteromeric $GABA_A$ receptor, showing the endogenous ligand binding-site in green, as well as the allosteric binding site for benzodiazepines (BZD) in pink.

The $GABA_A$ receptor has several different binding sites besides the main site for GABA, and GABA-influencing drugs such as benzodiazepines and barbiturates allosterically affect the receptor via interaction with their specific binding sites. More specifically, benzodiazepines influence the probability for the opening of the ion-channel and will thereby increase the influx of chloride in to the cell. The benzodiazepine effect is dependent on the presence of the endogenous ligand as opposed to barbiturates, which can influence the opening time of the channel on its own (Jembrek and Vlainic, 2015).

GABA, anxiolysis and impulsive behavior

GABAergic transmission has for long been implicated in anxiety disorders (Perna et al., 2016). This is in part due to the profound anxiolytic effect produced by $GABA_A$ receptor acting drugs, such as benzodiazepines, which will increase GABAergic inhibition. Benzodiazepines will also produce a sedative and hypnotic effect, and the dissociation between these effects is not yet fully understood, but the involvement of specificity for different subunit compositions has been suggested (Chagraoui et al., 2016).

In animal studies, anxiolytic-like behaviors have been validated using the elevated plus maze (*detailed description in Methods*), in which the animal's activity on open, as opposed to closed, arms can be increased by systemic injections of benzodiazepines. However, this could also reflect a disinhibited, impulsive behavior, since an animal might still be anxious, even though it acts disinhibited towards an aversive stimulus. Consistent with this, GABAergic transmission has lately been suggested to be of importance for regulating also impulsive behaviors (Hayes et al., 2014). This is also true for nicotine, which repeatedly has been demonstrated to induce impulsive behaviors, both in the acute and chronic phase (Kolokotroni et al., 2011; Olausson et al., 1999; Olausson et al., 2001b). Traditionally, brain subregions such as the amygdala and prefrontal cortex have been attributed an important role in these types of behaviors, but recent evidence also imply the involvement of nAc (Feja et al., 2014).

OBJECTIVES

Improved understanding of nicotine's effects on neurotransmission, as well as, progressive neuronal changes that occur during nicotine abstinence may provide critical insight into the mechanisms that underlie addiction and could aid in developing adequate treatments. The overall aim of this thesis is to characterize nicotine-induced alterations in behavior and accumbal neurotransmission, some of which develop during the course of extended withdrawal.

More specifically the objectives of this thesis are:

- To investigate the longevity of nicotine behavioral sensitization and the ensuing alterations in dopaminergic transmission within the nAc (**Paper 1**)
- To increase the knowledge of the importance of the age at first exposure for nicotine and the impact age has on nicotine effects (**Paper 2**)
- To study the effects of extended nicotine withdrawal on accumbal GABAergic transmission, GABA-related behavior, and $GABA_A$ receptor subunit transcripts (**Paper 3**)

RESULTS AND DISCUSSION

Nicotine causes chronic behavioral sensitization (paper I)

Nicotine is known to increase locomotion after repeated administration, but the persistency of these alterations has previously not been investigated for periods longer than 4 weeks (Miller et al., 2001). The behavioral effects have in part been attributed to alterations in the dopaminergic system, which have been reported to be present after one week into abstinence and forward (Le Foll et al., 2003; Molander and Soderpalm, 2003). Also, suggestions to whether there is a regional discrepancy between the effects in the nAc core and shell have been made, where nicotine primarily has been reported to promote DA release in the core region (Benwell and Balfour, 1992; Cadoni and Di Chiara, 2000).

In order to test the longevity of nicotine-sensitization, and the localization of nicotine-induced alterations in dopaminergic transmission after extended withdrawal, rats were exposed to nicotine daily during three weeks. The behavior and accumbal transmission was assessed up to seven months into abstinence. The effects of a relapse episode were mimicked by re-exposure to nicotine seven months after cessation.

Nicotine-induced locomotor sensitization sustains for up to seven months, together with effects in synaptic activity and the response to a DA D2 agonist

We found that nicotine induces long lasting changes in behavioral sensitization (Fig. 4), as the effect could still be detected at the end point of the study - seven months into withdrawal. The maximum locomotor response was detected after nine weeks of abstinence (Fig. 4D), which could be an indication of a drug-incubation effect that previously has been reported for drugs such as cocaine (Grimm et al., 2001).

After seven months of abstinence we also observed a depressant effect in synaptic activity, both in nAc core and shell (Fig. 5A-B), as measured by a stepwise increase of the stimulation strength. Interestingly, the response

to DA D2 receptor acting drugs has previously been reported to be altered in nicotine-sensitized rats (Olausson et al., 2001a). And since this could be part of the explanation for the long-lasting locomotor effect, the electrophysiological response to the DA D2 receptor agonist quinpirole was investigated. Interestingly, in the nAc shell, but not core, a significant increase in quinpirole response was observed (Fig. 5D). This could suggest that the two regions do not respond uniformly to nicotine exposure. The observed effect to quinpirole in the nAc shell could be due to an increased postsynaptic D2 receptor responsiveness in this region.

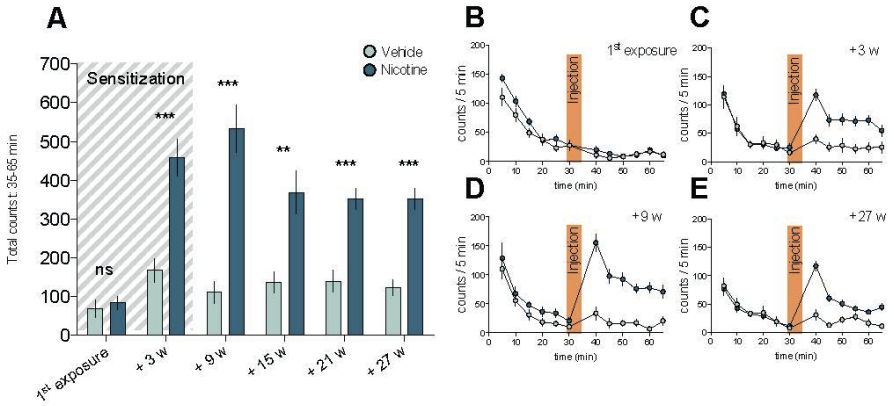

Figure 4. Three weeks of nicotine exposure induces long-lasting effects in nicotine-induced locomotion (A). The maximum response was observed after a brief abstinence period (D). After 15 to 27 weeks of abstinence the locomotor response appeared to have plateaued and no tendency to a decline was found after 27 weeks of abstinence (E).

The present data from nAc core indicates a lack of effect of quinpirole in slices from nicotine-treated animals (Fig. 5E), and since previous reports have demonstrated the importance of nAc core after acute nicotine exposure (Cadoni and Di Chiara, 2000), the results could be suggestive of both a temporal and region-specific development. The dopaminergic alterations might thus be present in the core region in the acute phase and later re-localized to the shell region after an extended withdrawal period.

Moreover, data presented in this paper supports occurrence of specific differences in dopaminergic function within nAc subregions, possibly indicating a pharmacological specificity between shell and core. The lack of effect of quinpirole in nAc core is most likely not due to an overall lowly responsive system, since the synaptic activity was depressed in both subregions. The discrepancies in response between the two regions was also reflected on the probability for transmitter release, where the probability was significantly decreased in slices from nicotine animals in the shell region, whereas a tendency to an increase instead was seen in the core region (Fig. 5C). Together with the reduced synaptic activity, the effect on PPR in nAc shell could thus be related to a reduced expression, or function, of postsynaptic receptors.

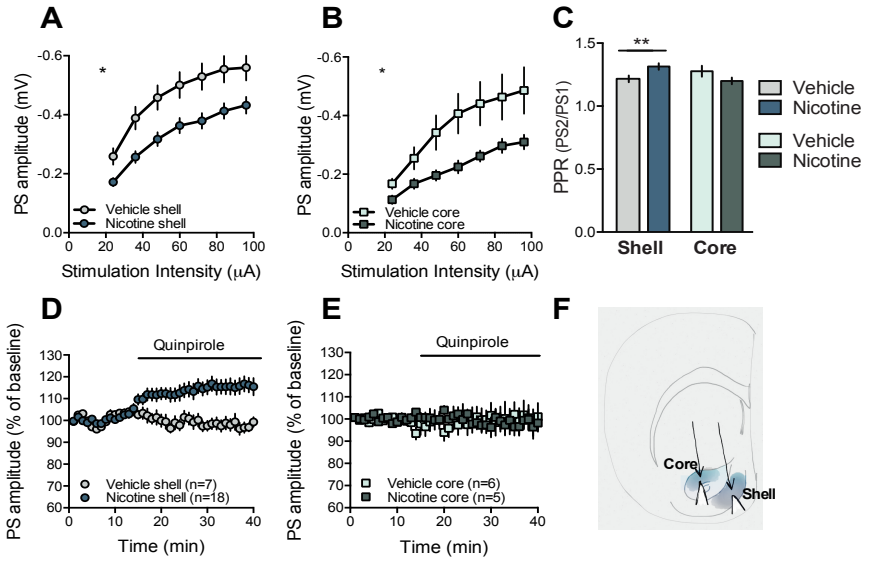

Figure 5. A-B) Depressant effects in synaptic activity were still present, both in nAc core and shell, 7 months after nicotine abstinence. D-E) The response to the DA D2 receptor agonist quinpirole was both treatment- and region-specific, and a significant effect was observed in nAc shell of nicotine treated animals. A decreased probability for transmitter release was also observed in the shell of nicotine treated animals (C). The image describes a graphical representation of how the recording and stimulating electrodes were positioned in the nAc (F).

High receptiveness to re-exposure is reflected in changes of accumbal synaptic activity

In order to mimic the effects of a relapse period we re-exposed animals to nicotine during six days after a seven-month abstinence period. Previously vehicle treated animals were also exposed to nicotine as an age-matched control. An immediate locomotor response was detected after the first nicotine injection in all animals, and after six days of treatment the previously nicotine treated animals responded with a higher level of stimulation than observed during the initial exposure period (*see paper I, fig. 5)*. This was concomitant with a decreased synaptic activity as well as an increased responsiveness to quinpirole in slices from animals previously treated with nicotine (*see paper I, fig. 6)*. This could indicate that the dopaminergic alterations produced by previous nicotine treatment are very robust and also easily boosted at re-exposure to the drug. This is in line with human studies that describe a heightened drug sensitivity after extended withdrawal, due to persistent drug sensitization (Miller et al., 2001), and could be a phenomenon related to the high relapse risk observed after minimal nicotine exposure in previous smokers (Brigham et al., 1990). In addition, a lack of effect was seen on transmitter release in both groups, which could indicate a discrepancy in how nicotine modulates synaptic activity in short and long-term manners. And even though repeated nicotine administration induces effects in synaptic activity, this is not necessarily concomitant with altered transmitter release.

In conclusion, the data obtained suggests that a limited period of nicotine exposure induces very long-lasting alterations, both in locomotion and accumbal transmission in the rat. In addition, the response to the DA D2 receptor agonist quinpirole is specifically altered in the nAc shell region after seven months of abstinence. Moreover, after an extended withdrawal period the nicotine-treated animals are very sensitive to re-exposure, which was displayed as a more rapid drug response - both in behavior and accumbal neurotransmission.

Age at first nicotine exposure influences both stimulatory response and accumbal neurotransmission (paper II)

The risk for developing a more severe nicotine addiction is increased if the first nicotine exposure occurs during adolescence (Breslau and Peterson, 1996). This is partly believed to be due to the differences in the perceived reward sensation and reinforcing effect between adults and adolescents (Shram et al., 2006). Teenagers also report less aversive effects from nicotine than adults (DiFranza et al., 2000). Animal studies have reported an increased sensitivity of excitatory synapses in the midbrain of young animals, in which the nicotine-induced effect on eEPSCs are increased (Placzek et al., 2009). In addition, an increased firing of cholinergic neurons in the lateral tegmental nucleus has been reported in young rats (Christensen et al., 2014). Adolescent rats also display an increased nicotine metabolism (Vieira-Brock et al., 2013), which substantially could influence the effects of the drug.

In light of this we wanted to investigate the influence of age on nicotinic effects, more specifically on behavioral alterations and accumbal neurotransmission. We also tested if nicotine-evoked DA release was influenced by age in nAc of drug naïve animals.

Increased behavioral sensitization and tolerance development in young animals

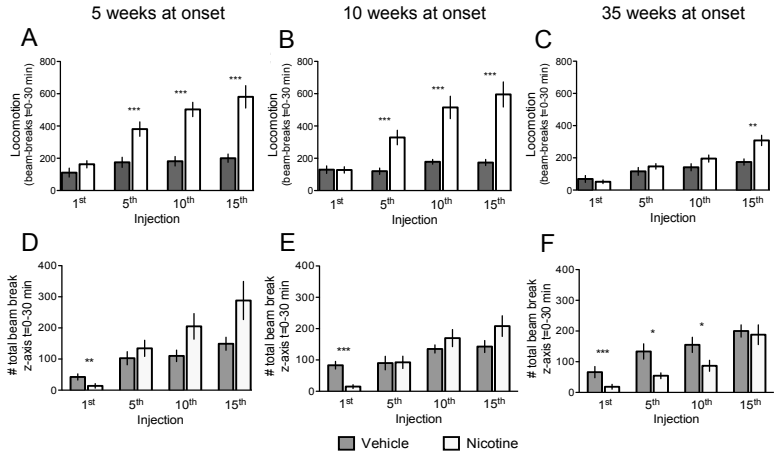

Figure 6. A-C) Nicotine influences locomotion in an age-dependent manner, displayed as faster behavioral sensitization development in young animals. D-E) The nicotine-induced depressant effect on rearing is influenced by age and the development of tolerance to these depressant effects is slower in old animals.

Locomotion and rearing behavior were assessed in Wistar rats during a three-week period with daily nicotine injections. The onset of behavioral sensitization was very rapid in the two younger age groups (Fig. 6A-B), whereas in the older group a significant effect was not established until completion of the three-week treatment period (Fig. 6C). At this time point the total activity in nicotine treated old rats was substantially lower than the activity of the younger groups, although the activity in the control groups were very similar throughout the groups.

An interesting finding regarding the development of tolerance to the aversive effects of nicotine was done looking at rearing behavior. In drug-naïve rats nicotine induces an acute depressant effect on rearing activity. Interestingly, nicotine is known to also induce ataxia in non-tolerant subjects (Clarke and Kumar, 1983b). However, the rearing activity is rapidly regained and after two weeks of treatment often instead increased in nicotine-treated animals. This pattern was observed in the two younger age groups (Fig. 6D-E), but the opposite was found in the old animals where the controls instead were performing significantly more rearing after two weeks of treatment (Fig. 6F). This could indicate that tolerance development, or simply neuronal adaptations, to nicotine develop slower at an older age, which would render young individuals more susceptible to nicotine. Interestingly, rearing activity has also been correlated with local DA levels in nAc, in which high levels of DA have been linked to high rearing activity (Thiel et al., 1999), which could suggest that young animals experience higher levels of accumbal DA after repeated nicotine exposure.

Nicotine influences synaptic activity together with dopamine output in drug-naïve rats in an age-dependent manner

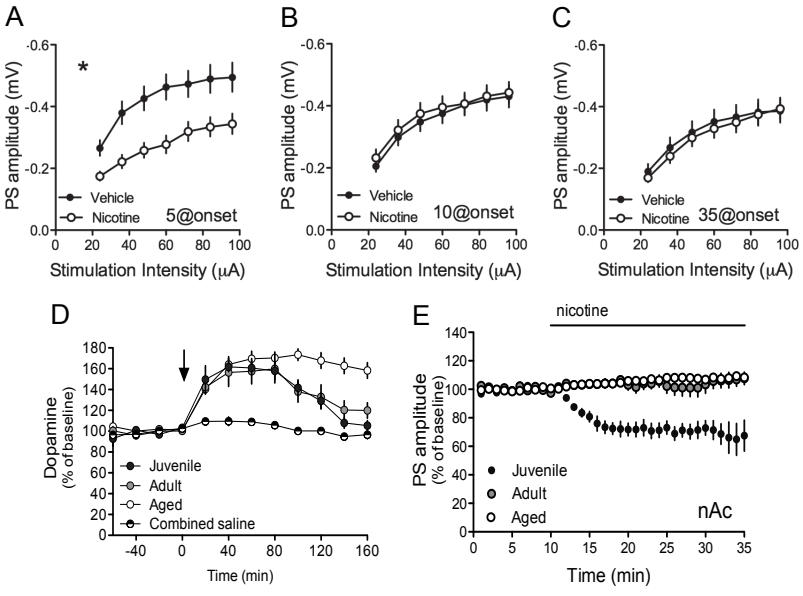

Figure 7. Different aspects of nicotine-induced effects were investigated in both nicotine-treated and nicotine-naïve animals. Three weeks of nicotine treatment specifically depressed synaptic activity in young animals after ten days of nicotine withdrawal (A). In nicotine-naïve animals DA release was estimated by microdialysis, indicating a prolonged DA effect in old animals (D). When measured by field-potential recordings the excitatory response to nicotine was specifically depressed in juvenile rats (E).

Estimation of synaptic efficacy in nAc was assessed after ten days of nicotine withdrawal, following a three-week treatment period. This revealed an age-dependent effect, since a significant synaptic depression was detected specifically in slices from nicotine-treated juvenile rats (Fig. 7A), whereas a lack of effect was seen in the other age groups. Moreover, in slices from nicotine-naïve animals only the juvenile group displayed a depressive effect on excitatory output after acute nicotine exposure (Fig. 7E).

Since the age-dependent effect in locomotion and rearing might be linked to differences in nicotine-evoked accumbal DA, the dopaminergic output was estimated by *in vivo* microdialysis in drug-naïve animals at three different ages. Even though the results show a lack of effect on the maximum response to nicotine, a significantly sustained DA elevation was seen in the oldest age group. This could be suggestive of age-dependent changes in DA receptor or dopaminergic transporter expression (Rahman and McBride, 2002), or a reduced feedback control of mesolimbic DA release from the nAc (Rahman and McBride, 2002). A low receptor expression could influence the progress of both the development of behavioral adaptations and the acute nicotine-induced DA output.

However, since nicotine-evoked DA release was very similar across the age groups, the changes detected in synaptic activity (Fig. 7A) and DA release cannot be directly linked. Rather, the effect seen in field-potential recordings, where only slices from drug-naïve juvenile animals displayed an effect, suggest that neurotransmission in this age group is easily altered and that the neural circuits might be extra sensitive to drug stimuli at this young age.

In conclusion we report an increased sensitivity to nicotine, both with respect to behavior and accumbal transmission, in juvenile and adolescent animals, as compared to older animals. We also observed a decreased pace of tolerance development to some of the aversive effects of nicotine in older animals. The behavioral differences could possibly be attributed to differences in acute nicotine effects on synaptic activity and excitatory transmission in nAc between young and old animals or to altered dopaminergic release in the older animals.

Extended nicotine withdrawal influences GABAergic neurotransmission and GABA-related behaviors (paper III)

The reinforcing and rewarding properties of nicotine have often been attributed to increased levels of DA in the nAc, through activation of

nAChRs in VTA. Stimulation of nAChRs will modulate the activity of projecting DA neurons and thereby also DA levels in selective striatal subregions, such as nAc (De Biasi and Dani, 2011). But the impact nicotine has on target neurons in nAc, i.e. mainly GABAergic neurons, and thereby GABAergic transmission and GABA release, is less well studied. Although, recent studies describe nicotinic influence on GABAergic transmission (Hernandez-Vazquez et al., 2014). Interestingly, nicotine can by stimulation of presynaptic nAChRs on GABAergic terminals directly influence GABA release (Hernandez-Vazquez et al., 2014), and GABA can even be co-released with DA from dopaminergic terminals (Nelson et al., 2014). Thus when considering that GABAergic transmission causes tonic inhibition of dopaminergic transmission, it is plausible that nicotine can influence dopaminergic signaling through altered GABAergic transmission (Adermark et al., 2011). In addition, functional upregulation of nAChRs in mesolimbic pathways after nicotine exposure appears to be limited to midbrain GABAergic neurons (Nashmi et al., 2007; Xiao et al., 2009).

Considering GABAergic involvement in nicotinic mechanism, we wanted to investigate how GABAergic transmission is altered during the course of extended nicotine withdrawal, ranging from acute exposure up to seven months of abstinence. We also investigated the role an altered GABAergic transmission may have on impulsive behavior, such as behavioral disinhibition, as an altered impulsive control could be involved in continued, or future, drug intake.

Protracted nicotine abstinence affects behavioral disinhibition and produces gradual changes of ex vivo sensitivity to $GABA_A$ acting drugs

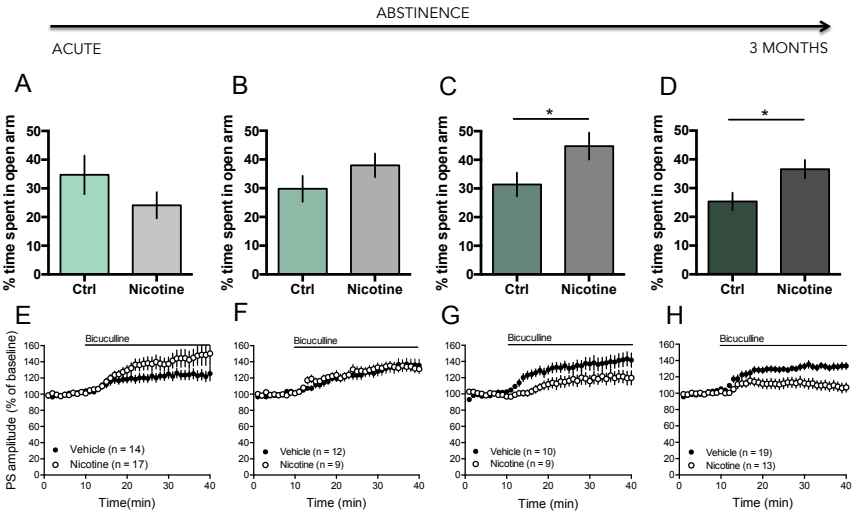

Figure 8. During the course of extended withdrawal the performance on the elevated plus-maze was altered in a temporal manner (A-D), and after two and three months of abstinence the nicotine treated animals displayed increased behavioral disinhibition (C-D). Such temporal development was observed also with respect to the response of the $GABA_A$ receptor antagonist bicuculline, which was significantly depressed in nicotine treated animals after two and three months of withdrawal (G-H).

Nicotine has been reported to employ a bimodal effect on anxiety-related behaviors (File et al., 1998), where lower doses are likely do produce an anxiolytic-like effect and higher doses have been reported to be anxiogenic (File et al., 1998; McGranahan et al., 2011; Ouagazzal et al., 1999). In our hands nicotine abstinence induced an anxiolytic-like effect, or behavioral disinhibition, that developed during the course of extended withdrawal (Fig. 8A-D). This was detected as an increased open arm activity on the elevated plus-maze in previously nicotine treated animals. Interestingly, in the acute withdrawal phase the nicotine exposed animals showed a tendency to a decreased open arm activity (Fig. 8A), which over time progressively developed into the opposite (Fig. 8D). A gradual development was also observed with respect to the electrophysiological response to the $GABA_A$ antagonist bicuculline. Thus, the increased response observed in slices from nicotine treated animals in the acute withdrawal phase (Fig. 8E) was transposed into a decreased response two to three months later (Fig. 8H). This finding could suggest that $GABA_A$

receptor function is increased in the acute abstinence phase and later decreased, possibly due to receptor subunit conversion or decreased gene expression. sIPSCs recordings revealed increased sIPSC amplitudes in the acute phase, as would suggest an enhanced postsynaptic $GABA_A$ response (*see paper III, fig. 2*). In fact, the amplitude was instead significantly reduced following three months of withdrawal, thus being in line with the lack of effect of bicuculline at this time point and suggesting that tonic inhibition in the system was reduced.

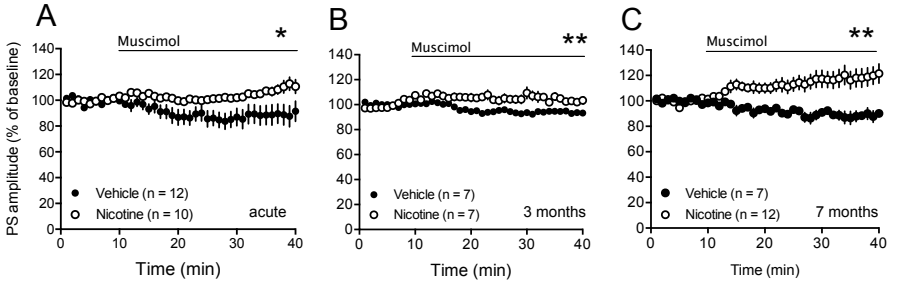

Figure 9. The progressive effect to $GABA_A$ acting drugs was also observed with respect to the response of the agonist muscimol, where the difference increased over time (A-C). Recordings revealed an inverted response to muscimol as compared to the response in controls, and after en extended withdrawal period of seven months the excitatory effect was largely increased in nicotine treated animals (C).

The effect of the $GABA_A$ agonist muscimol was also evaluated during extended withdrawal, and as can be seen in figure 9 the expected decreased excitatory response was observed in slices from controls only. In slices from nicotine-treated animals, an increased excitation was detected instead and the response increased in size during the course of withdrawal (Fig. 9 A vs. C). Several possible mechanisms could underlie this rather unexpected finding, such as a switch in $GABA_A$ signaling properties, from inhibitory to excitatory, which has been described previously for VTA $GABA_A$ receptors after opiate exposure and brief opiate withdrawal (Laviolette et al., 2004; Vargas-Perez et al., 2009).

Extended withdrawal induces progressive changes in accumbal neurotransmission together with altered spine density

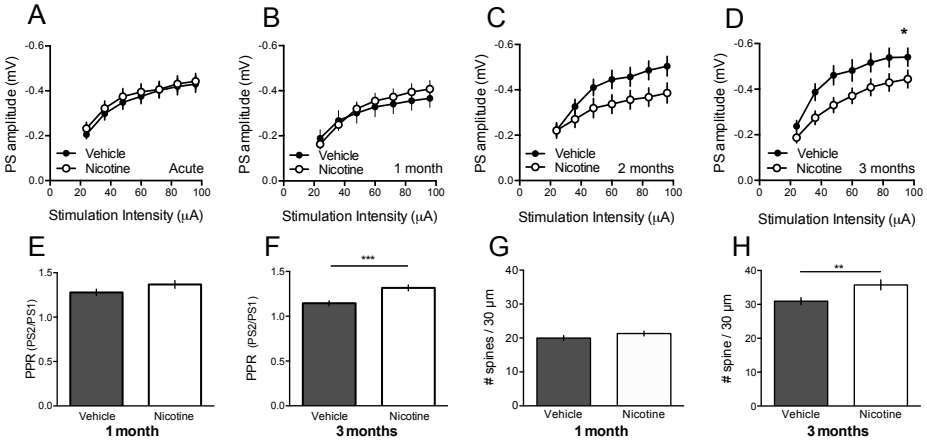

Figure 10. A progressive effect on synaptic efficacy was detected during the course of withdrawal and a significant depression was observed after three months of withdrawal in nicotine treated animals (D). An effect of time was noted also with respect to probability for transmitter release (F), as well as in spine density (H), where a significant effect was seen first after three months of withdrawal.

To further evaluate if extended nicotine withdrawal would influence accumbal neurotransmission and synaptic function, we examined synaptic efficacy, transmitter release and spine density in nAc (Fig. 10). Again a delayed effect was observed with respect to synaptic efficacy, probability for transmitter release and spine density, where the effects were significantly altered first after an extended withdrawal period (Fig. 10D, F, H). Synaptic efficacy and probability for transmitter release were also depressed, whereas spine density was increased. This could suggest a compensatory synaptic effect in nAc due to an increased population of lowly functional synapses or AMPA receptor-silent synapses, which has previously been described after morphine and cocaine exposure (Graziane et al., 2016). Also, evoked inhibitory postsynaptic currents (eIPSCs) have been shown to be augmented in dorsal striatum during the acute abstinence phase, suggesting that a comparable process may occur in several subregions of the striatum (Miura et al., 2006). In addition, recent data indicates that nicotine induces rewiring of striatal circuits in a temporal manner, and these results could thus extend to involve the

recruitment of nAc later in the functional reorganization following that of dorsal parts (Adermark et al., 2016).

In conclusion, we show that nicotine induces progressive changes in GABAergic neurotransmission, displayed as temporal-dependent alterations in the sensitivity to $GABA_A$ acting drugs as well as in spontaneous GABAergic neurotransmission. The temporal effect in accumbal neurotransmission is concomitant with the development of a spontaneous disinhibitory behavior.

GENERAL DISCUSSION

The numerous effect of nicotine described in this thesis point to a complex pattern of neuronal alterations, which may or may not contribute to the severe addictive properties of the substance. Today only a few pharmacological options are available for reducing nicotine use, and these show limited efficacy and low rates of long-term cessation. When considering the ensuing increased risk for severe illness associated with long-term tobacco use, the need for improved therapies is significant. Thus, in order to increase the chances of developing new treatment it is of great importance to understand the processes and alterations underlying nicotine addiction. The purpose of this thesis was to investigate the impact of nicotine exposure and long-term nicotine abstinence on behavior and neuronal circuits in the reward system, as well as to determine how long-lasting these effects are.

The possibility for the induction of chronic alterations after three weeks of nicotine exposure was assessed in paper I, in which we show that the effects of nicotine on locomotion were still present seven months after exposure. This was concomitant with an increased *ex vivo* response to a DA D2 receptor agonist in the nAc shell of nicotine-treated rats. These results are of particular importance for understanding the basic mechanisms of nicotine-induced behavioral sensitization and the neural alterations that contribute to the displayed behavior. Pre- and postsynaptic alterations of the DA system have for long been suggested to be involved in the sensitized response (Molander and Soderpalm, 2003; Vezina, 1996), and here we show for the first time a significantly enhanced electrophysiological response to DA D2 receptor activation specifically in the nAc shell.

We also show that nicotine induces chronic effects on the excitatory response in nAc, as measured by the input/output function, which could be due to a decreased glutamatergic drive or an increased inhibition from GABAergic transmission. Several parameters were also changed after

nicotine re-exposure, suggesting that the system has been reorganized to become highly receptive to the drug. A parallel can be made to observations in humans, where only a small amount of nicotine is sufficient to cause relapse (Brigham et al., 1990). Taken together, our results suggest that relatively modest exposure to nicotine (15 days) is enough to induce longstanding behavioral and neuronal effects that may have implications for the low probability of maintaining cessation, and that nicotine addiction probably should be considered a chronic state.

The possible impact of age at first nicotine exposure was evaluated in paper II. We observed that young rats appeared to display a more rapid behavioral and neurophysiological response to nicotine exposure; this was manifested as faster behavioral sensitization as well as a more rapid tolerance development to the rearing depressing effects of nicotine. Moreover, we observed an age-dependent effect on synaptic activity, where only the youngest animals showed a depressant effect. But more importantly, the two older age groups did not differ at all in synaptic activity. This, together with the behavioral data, would suggest that the younger animals are more susceptible for nicotine, and one could speculate that neuronal changes are more easily established in young animals due to a highly plastic neuronal system with high synaptic density (Huttenlocher, 1979).

We also investigated the response to nicotine in drug-naïve animals, both by examining accumbal DA release and excitatory output in field-potential recordings. Both of these methods revealed an age-dependent response to nicotine, in which field potential recordings detected an increased effect of nicotine in slices from juvenile animals. The DA analysis also revealed an age effect; the aged animals displayed an elongated DA output after a systemic nicotine injection.

Additionally, in paper III we describe progressive effects in GABAergic transmission, induced by extended nicotine withdrawal, in which e.g. sIPSCs amplitudes were significantly decreased after a three-month withdrawal period, while the response to a $GABA_A$ antagonist was blunted at the same time point. In addition, the response to the $GABA_A$

agonist muscimol developed progressively during extended withdrawal, and after seven months the effect surprisingly switched to excitation, similar to what is known to happen after opiate exposure and brief opiate withdrawal (Laviolette et al., 2004). Interestingly, chronic exposure to drugs, and opiates in particular, has been reported to increase levels of brain-derived neurotrophic factor (BDNF) in VTA neurons, and local infusions with BDNF into VTA can alone influence the switch in $GABA_A$ signaling function (Vargas-Perez et al., 2009). Together this could suggest an important role for BDNF regulation in the development of the long-lasting and multifaceted drug-induced changes.

Whether the GABAergic changes seen after seven months of abstinence are related to the enhanced response to the DA D2 receptor agonist observed in paper I, remains to be determined. In striatum quinpirole exerts its effects through presynaptic DA D2 autoreceptors on DA neuronal terminals, and through postsynaptic DA D2 receptors located on MSNs and cholinergic interneurons, but the electrophysiological effects observed in slices are most likely of a postsynaptic nature. Since it appears likely that $GABA_A$ receptors and DA D2 receptors interact on MSNs, one could thus suggest that aspects of the altered dopaminergic function after seven months of withdrawal could be due to a malfunctioning GABAergic inhibition.

We also demonstrate that the behavior on the elevated plus-maze, which was used to assess behavioral disinhibition, is altered in a time-dependent manner in nicotine-treated animals, such that the activity on the open arms is increased after three months of abstinence. Increased open arm activity may be interpreted as an anxiolytic-like effect or as behavioral disinhibition. We further demonstrated that these types of behaviors possibly could be related to GABAergic transmission in nAc, since local injections with diazepam led to increased activity on the open arms. These findings add to recent evidence suggesting that nAc may be more important for anxiolytic or disinhibitory behaviors than previously thought (Feja et al., 2014). The induction of such a behavior could thus

be connected to nicotine-induced transformation of accumbal GABAergic transmission.

Moreover, it seems as if the biphasic effect of $GABA_A$-acting drugs over the course of withdrawal is reflected in gene expression of $GABA_A$ receptor subunits. This was assessed by qPCR, and several $GABA_A$ subunits, as well as the synaptic GABA transporter GAT-1, were downregulated in a parallel with development of a blunted response to $GABA_A$ acting drugs in nicotine treated animals. This could suggest that the blunted response to $GABA_A$-acting drugs after three months of withdrawal is due to fewer or altered receptor targets.

In conclusion, the results presented in this thesis describe longstanding effects from previous nicotine exposure, both on behavior and on neurotransmission in nAc, including GABAergic and dopaminergic systems. These changes are detectable up to seven months into withdrawal. Interestingly, several of the detected alterations appear first after an extended withdrawal period. These findings are relevant for the ongoing discussion regarding nicotine as a potential gateway drug, since the exposure to nicotine induces a cascade of neuronal events that together might increase the possibilities for future drug intake. The results also describe an age-dependent component in the nicotinic effects, where younger animals appear to be more susceptible to changes in accumbal transmission and develop behavioral adaptions to nicotine faster. Further knowledge of the nature of these alterations might increase the understanding of nicotine addiction, and thereby increase the chances of developing improved pharmacotherapies for this devastating disorder.

FUTURE PERSPECTIVES

The pharmacological options to treat nicotine addiction are few and focused on substitution (nicotine replacement therapies; varenicline) or manipulation of the DA system (bupropion; varenicline). Given the limited effects of these treatments, there appears to be a significant need for looking at the disease from new angles. The results of the present thesis indicate a need for considering the role of altered accumbal neurotransmission in a broader sense, i.e. beyond DA, even though DA mechanisms cannot be disregarded.

Development of a low functioning, or after seven months of abstinence possibly even reversed, GABAergic transmission in nAc raises the possibility that the GABAergic system should be considered as a potential drug target for nicotine dependence. One must however remember that these data derive from slice recordings, *ex vivo*. The status of GABAergic transmission in intact animals after seven months of nicotine abstinence remains to be determined, even though the results obtained in the elevated plus-maze after three months of abstinence are compatible with GABA-related behavioral changes. Further, we do not know if the observed GABAergic alterations are limited to nAc or if they extend to other brain regions. It is thus necessary to investigate additional regions, both in brain slice preparations and *in vivo,* before drawing conclusions as to how nicotine exposure and ensuing withdrawal alter GABAergic transmission. In addition, experiments capturing the behavioral features of addiction will be required to connect these changes to the phenomenon of nicotine addiction. Moreover, we do not know if intracellular chloride concentrations are altered, for example due to malfunctioning or reversed function of the $GABA_A$ receptors, or if the amount of GABA in the extracellular space is augmented. These questions remain to be investigated.

Recently published data from the group also describes nicotine-induced rewiring of striatal subregions (Adermark et al., 2016), a dynamic process that in the acute phase involves changes in synaptic activity in DMS and

in later stages in DLS and nAc. At the later time points, the changes earlier detected in DMS are completely abolished and normalized to that of controls. Whether or not the changes in accumbal synaptic activity are propagated to other brain regions connected to the striatum is not known. The nicotine-induced effect in DLS has also been shown to disappear after six months of withdrawal (Adermark et al., 2016), at a time point where it is still present in the nAc. If and when nicotine-induced alterations disappear at a later time point in nAc is not known.

The vast majority of studies looking at nicotine-induced effects, both in terms of behaviors and neurotransmission, utilizes short time periods - both for treatment and in particular for the succeeding abstinence period. Due to this, important aspects of nicotine addiction may have been overlooked, and extended withdrawal should be considered to a greater extent.

Historically, mechanistic studies of nicotine have focused intensely on the VTA, due to the fact that nAChRs are dense on DA neurons in VTA. In contrast, the interest for striatal regions, apart from DA release, has been limited. When looking only at DA release and/or DA receptor function in striatal regions, important consequences of nicotine exposure, such as the induction of silent synapses or the role of BDNF regulation may be missed. It is thus of interest to continue investigating how nicotine alters local accumbal transmission using a more multifaceted approach, and to determine if the observed changes can be prevented, inhibited or reversed by pharmacological pretreatments or treatments.

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ADDITIONAL PUBLICATIONS

Adermark L, **Morud J**, Lotfi A, Danielsson K, Ulenius L, Söderpalm B, Ericson M, *Temporal Rewiring of Striatal Circuits Initiated by Nicotine,* ***Neuropsychopharmacology****,* 2016 Aug 10, doi: 10.1038/npp.2016.118

Jonsson S, **Morud J**, R Stomberg, M Ericson, B Söderpalm, *Involvement of lateral septum in alcohol's dopamine-elevating effect in the rat,* ***Addiction Biology****,* 2015 Sep 14. doi: 10.1111/adb.12297

Pickering C, Alsiö J, **Morud J**, Ericson M, Robbins TW, Söderpalm B, *Ethanol impairment of spontaneous alternation behaviour and associated changes in medial prefrontal glutamatergic gene expression precede putative markers of dependence****, Pharmacol Biochem Behav****, 2015 Mar 2, 2015 Mar 2;132:63-70*

Simpson EH**, Morud J,** Winiger V, Biezonski D, Zhu J, Malleret G, Polan J, Ng-Evans S, Phillips PE, Kellendonk C, Kandel ER, *Genetic Variation in COMT Activity Impacts Learning and Dopamine Release Capacity in the Striatum,* ***Learning and Memory****, 2014, Mar, 17;21(4):205-14*

Morud J, Adermark L, Ericson M, Söderpalm B. *Alteration is ethanol-induced accumbal transmission after acute and long-term zinc depletion,* ***Addiction Biology****, online Sept 2013, 2015 Jan;20(1):170-81*

Jonsson S, **Morud J**, Pickering C, Adermark L, Ericson M, Söderpalm B. *Changes in Glycine receptor subunit expression in forebrain regions of the Wistar rat over development*, ***Brain Research****, 2012, Mar 29;1446:12-21*

APPENDIX: MATERIALS AND METHODS

Below follows a brief introduction and reflections on practical as well as theoretical aspects to the different experimental methodologies used in papers comprising this thesis.

Animals

During the last century, the use of animal models has contributed enormously to the progress in understanding physiological principles. This thesis is based on experiments performed in Wistar rats, a commonly used animal model for studying addiction and neurotransmission. The translation between humans and rats is possible due to the fact that several key brain regions are conserved and there is an approximate genomic overlap of 95%. The validity of each animal model used is briefly discussed in each subchapter below. All experiments presented in this thesis were approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden.

In the nicotine sensitization studies (papers I and III) outbred adult Wistar rats were used, weighing 240-280 g at the start of the experiment (Taconic, Ejby, Denmark). All animals were housed in groups of three. In the age-contingent nicotine study (Paper II) Wistar rats of three different ages were used, 4, 9 or 35 weeks old (Taconic, Ejby, Sweden). All animals were housed at room temperature 21-22°C and with a 12-h light/dark cycle and had free access to rat chow (Lantmännen, Sweden) and tap water.

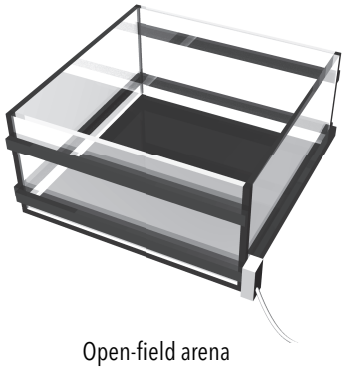
Drugs and Chemicals

Nicotine was dissolved in 0.9% NaCl and the pH was adjusted to 7.2-7.4 with $NaHCO_3$. The animals were given 0.36 mg/kg s.c. (active substance) daily for three weeks. The benzodiazepine diazepam was dissolved in a sham solution consisting of: 10% 95%-ethanol, 40% propylene glycol, 50% Ringer's solution and injected bilaterally into the nAc, 20 μg in total.

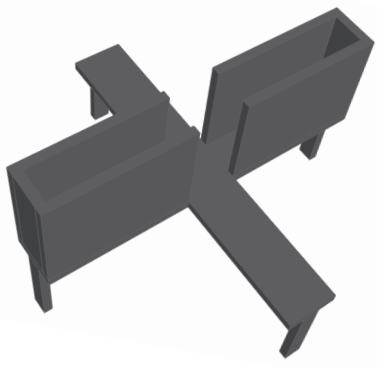
Ringer's solution contained (in mM): 140 NaCl, 1.2 $CaCl_2$, 3.0 KCl, and 1.0 $MgCl_2$. For electrophysiological experiments, nicotine (1 μM), and the D_2/D_3 agonist quinpirole (5 μM) were dissolved in artificial cerebrospinal fluid (aCSF), which consisted of (in mM); 194 sucrose, 30 NaCl, 4.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 10 d-glucose. The $GABA_A$ antagonist bicuculline was dissolved in DMSO and diluted to 20 μM in aCSF and the $GABA_A$ agonist muscimol was dissolved in H_2O to 50 mM and diluted in aCSF to 1 μM. The cutting solution for brain slice preparation contained (in mM): 194 sucrose, 30 NaCl, 4.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 10 D-glucose. For whole-cell patch clamp purposes the aCSF constituted of: (in mM) 1.25 NaH_2PO_4, 124 NaCl, 26 $NaHCO_3$, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 10 D-glucose and the pipette solution contained (in mM): 130 Cs-methanesulfonate, 2 NaCl, 10 HEPES, 0.6 EGTA, 5 Qx-314, 4 Mg-ATP and 0.4 GTP (pH 7.3, adjusted with D-gluconic acid).

Behavior

The studies of animal behavior that are included in this thesis all utilize ethological behavioral principles, as opposed to operant, in manners of investigating naturalistic influences of nicotine.



Open-field arena



Elevated-plus maze

Figure 11. Descriptive figures of the equipment used for behavioral assays described below.

Locomotor measurements

The animals were injected daily with nicotine (0.36 mg/kg, s.c.) to induce behavioral sensitization, a phenomenon when the same dose of drug induces a heightened drug response after repeated exposure (Benwell and Balfour, 1992). The stimulatory effect of the drug was assessed by measuring locomotor activity in an open-field arena (Fig. 11), once weekly during the three weeks drug administration period. The open-field arena was equipped with infrared beam detectors in two layers, which are triggered once the animal disrupts any beam allowing movement patterns to be traced in three dimensions (x, z and y-axis). The rats were allowed to habituate for 30 minutes in the test boxes (paper I and II: box diameter 70 x 70 cm, Kungsbacka Mät- och reglerteknik AB, Kungsbacka, Sweden, paper III: 40 x 40 cm, Med Assoc, Fairfax, VT, USA), in order to separate the drug effect from initial exploration of the novel environment. After habituation the animals were injected with nicotine or 0.9% NaCl and the registration of locomotion continued after a five minutes pause in order to avoid influence of injection-induced hypermotility. Testing continued for a total of 35 minutes after injection and total ambulatory movement, rearing activity and the time spent in corners were recorded throughout the test.

Measures of behavioral disinhibition

To evaluate behavioral disinhibition, the elevated plus-maze (EPM) test was used (Fig. 11). This test exploits the conflict between the urge to explore a novel environment and the simultaneous natural tendency for rodents to avoid open, elevated and bright spaces (Walf and Frye, 2007). Normally the exploration of the open spaces is suppressed or inhibited. Entering into the open spaces thus represents a disinhibited behavior, which in turn could be due to a number of alterations of the animal's internal state, e.g. anxiolysis or increased impulsivity or perhaps even confusion.

The apparatus consisted of black plastic with two closed arms and two open arms measuring 10 x 50 x 1 or 40 cm (Med Associates, St Albans, VT, USA). The maze was elevated 1 m above the floor and the animals were habituated to the testing room during 30 minutes before testing. Before placement on the maze the animals were placed in an unfamiliar open-field arena for 5 minutes, in order to stimulate explorative behavior. Each animal was assessed during 5 minutes on the maze, the time spent in each arm and number of entries made into each arm was recorded.

Local accumbal injections

For paper III, drug naïve animals (n=34), weighing 280-330 g, were injected with a GABAergic agonist diazepam (20 μg) into nAc (Chau et al., 2010), before testing on the elevated plus maze. Briefly, accumbal injections were given via implanted guide cannula (Plastics One Inc, Roanoke, VA, USA), which was positioned bilaterally during isoflurane anesthesia (Baxter, Apoteket AB, Stockholm, Sweden) into the nAc (AP:+1.7, ML:±1.4, DV: -6.0) one week before experiments. The guide cannula was fixated to the skull using acrylic cement (Dentalon, AgnThos, Sweden). Each animal was injected with a total volume of 1 μl/hemisphere (perfusion rate 0.5 μl/min) of either active substance or control solution.

Methodological considerations

Automatic locomotor recording are commonly used for the study of drug-induced stimulatory responses. The initial arena size used in papers I and II was slightly larger than the one in paper III. This larger arena can be utilized for open-field testing, making the measurements of time spent in corners or the center more accurate, as compared to the case in the smaller arena. Therefore, no such measurements were included in paper III. This is somewhat unfortunate as such measurements could have been a good complement to the EPM test. The drug-induced increase of locomotion measured in the two types of arenas should however be comparable, since the drug effect is so prominent that the deviation in size between the two different arenas most likely can be overlooked.

The EPM relies on rodents' tendency to prefer dark and enclosed spaces and to fear bright and open spaces (Walf and Frye, 2007). Impulsive behaviors can be subdivided into different behaviors, such as impulsive choice or behavioral disinhibition; both types of behaviors involve the inability to constrain an unplanned action. Several operant or ethological assays used in order to measure impulsive behaviors uses noxious/negative stimuli (e.g. electric shock, odor presentation), which often produce a conditioned response. The valence of both a positive and negative conditioned stimuli can thus be avoided using the simple model of EPM. This model utilizes spontaneous exploration of two different types of environments, of which one is being a bit more frightening for the rat. Although, the EPM is in fact most commonly used for studying anxiety-like behaviors, since it has high face and construct validity for anxiety due to the induction of anxiolytic-like effects after administration of benzodiazepines or 5-HT_{1A} agonists, which causes anxiolysis in humans. These substances will increase the propensity of animals entering into open arms and spending more time in the open arms (Soderpalm and Engel, 1990), and this is then interpreted as a sign of anxiolysis. However, in the present thesis and in paper III we have avoided to interpret the outcome in EPM in terms of anxiety or anxiolysis and instead used the operational term "behavioral disinhibition", since this is what is observed. A disinhibited, impulsive animal may very well enter the aversive, open arm in spite of being anxious. Thus, as mentioned previously, entering into the open arms of the EPM could be due to increased impulsivity and/or anxiolysis, and/or spatial confusion.

In paper III animals were injected with diazepam locally in nAc before testing of disinhibitory behavior. The placement of the injection needle was examined after the experiments in brain slices from these animals, but this is still a rough estimation of the exact area that was affected by the drug. One needs to account for the drug perfusion rate in the tissue and how far the drug might spread before testing. As diazepam is a lipophilic substance, the expected spread in the tissue is rather high. To minimize this regional bias, we only allowed the drug to incubate for five

minutes before starting the test. But we have not done region-specific controls to completely assure that no other region was affected that might also be involved in the disinhibitory behavior observed after diazepam injection.

In vivo Microdialysis

In vivo brain microdialysis allows measurement of extracellular substances, such as neurotransmitters, in awake and freely moving animals. The technique also enables the administration of substances locally to the surrounding area of the implanted microdialysis probe, so-called reversed dialysis (Fig. 12). In study II this technique was used to measure extracellular levels of DA.

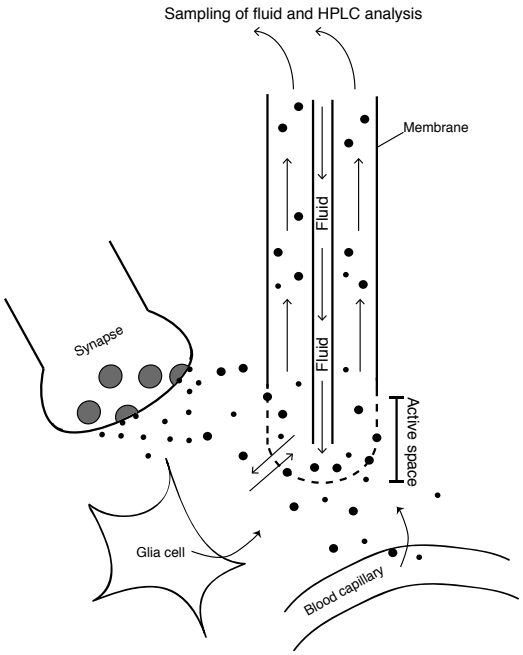


Figure 12. Graphical description of the principle behind *in vivo* microdialysis, in which a semi-permeable membrane allows molecules from the extracellular space to diffuse over and into the probe from which the fluid is then sampled and analyzed by HPLC. The membrane also allows for molecules to diffuse out from the probe and into the extracellular space, enabling local pharmacological treatment.

Microdialysis technique

Two days before running the microdialysis experiment animals were unilaterally implanted with a custom-made I-shaped microdialysis probe, equipped with a semi-permeable dialysis membrane, in to the nAc (AP: +1.65 mm, ML: -1.4 mm relative to bregma, DV: -7.8 mm relative to dura) (Paxinos G, 2007) as described previously in (Morud et al., 2015).

The zone availed for passive diffusion on the probe, often referred to as the active space, was 2 mm Briefly, rats were anesthetized using isoflurane and mounted into a stereotaxic instrument (David Kopf Instruments, AngTho's, Lidingö, Sweden), animals were locally injected with Marcain® (buvipacaine, AstraZeneca, Sweden) for a local anesthetic effect around the incision. The animals were kept on a heating pad throughout the surgery. Two holes for anchoring screws were drilled, and one for insertion of the probe. The probe and the anchoring screws were fixated to the scull using Harvard cement (DAB Dental AB, Gothenburg, Sweden). After the surgery all rats received a 0.9% saline injection to prevent dehydration. On day of experiments probes were connected to a microperfusion pump (U-864 Syringe Pump, AgnTho's, Lidingö, Sweden), via a swivel. Probes were then perfused with Ringer solution for two hours before the sampling started in order to obtain a balanced fluid exchange, samples were then taken every 20th minute following this. Animals were sacrificed immediately after the experiment and the brains were removed and fixated for later probe placement conformation.

Neurochemical assay

High performance liquid chromatography (HPLC), (ThermoScientific, Waltham, MA, USA), with electrochemical detection (system 1: 400 mV system 2: 220 mV, versus the cell) was used for detection of DA in the dialysate. Two systems were used in parallel, system one was equipped with a 2x150 mm ion exchange column (Nucleosil SA, 5 μM, pore size 100 Å, Phenomenex, Sweden) and the mobile phase consisted of in mM: 135 NaOH, 58 citric acid, 0.107 Na-EDTA, 20% methanol. System two utilized a 2x50 mm reversed phase column (silica, 3 μm diameter, pore size 100 Å, Phenomenex, Sweden) and the mobile phase consisted of in mM: 150 NaH_2PO_4, 4.76 citric acid, 3 SDS, 50 μM EDTA, 10 % methanol, 15% acetonitrile. For identification of DA peaks and calculation of DA concentrations, an external DA standard was used (3.25 fmol/μl). Before introducing the drug to be tested, four stable baseline samples were analyzed from each animal (internal difference

more than ±10% was not accepted). The following samples were treated as percentage of baseline.

Methodological considerations

In the experimental setting used in our group for sampling brain micro dialysates, the sampling rate is often set to every 20th minute. The low sampling rate is necessary for the collection of sufficient amounts of fluid needed for the HPLC analysis, which has implications for missing real-time changes in dopaminergic transmission that takes place on a millisecond level. Due to this, *in vivo* microdialysis is mostly suitable for monitoring slower processes such as effects after chronic systemic treatments or aggravated effects on transmission. Thus, it is a powerful tool for studying neural circuits since the use of multiple microdialysis probes enables one brain area to be manipulated *in vivo* at the same time as another is monitored for changes in transmission. Also, the type of microdialysis technique used in paper II does not measure absolute concentrations of extracellular DA and these measurements should then rather be considered to reflect changes in volume transmission.

Another challenge with *in vivo* microdialysis is how to dose your substances for local perfusion through the probe. Both due to the fact that most literature on these substances reflects systemic administration or for use *ex vivo,* on slice preparations that use a different range of concentrations and doses. But most importantly, due to the features of the semi-permeable membrane at the tip of the probe it is hard to estimate how large the fraction is that will enter into the extracellular space. The membrane has an average molecular cut-off at 20 kDa, but this can likely vary due to intrinsic properties of substances e.g. the polarity of the drug and shape, but also factors such as perfusion rate and temperature. Altogether, there are several difficulties to be met when it comes to estimating the amount of active substance that will be available in the tissue, and therefore we often estimate an excovery rate of 10-20%, which means that the actual *in situ* concentration might both be higher and lower than anticipated and therefore is the perfusate concentration always stated in the method and not the actual dialysate

concentration. Additionally, the impact made by the microdialysis probe in the surrounding tissue needs to be accounted for. The probe is implanted two days prior sampling and during these days a substantial amount of gliosis has occurred around the probe (Norton et al., 1992) and the surrounding tissue might also have been obstructed by blood or mechanical damaged from the surgery.

Electrophysiology

Electrophysiological studies in acutely prepared brain slices are well-established experimental models for studying electrical activity and cellular mechanisms. In paper I, II and III field-potential recordings were performed and in paper III whole-cell patch clamp was also utilized.

Brain slice preparations

Animals were deeply anesthetized with isoflurane (Forene®, Baxter, Sweden) before decapitation. The brains were then quickly removed and placed in cold cutting solution. After five minutes of incubation the brains were cut coronally on a vibratome (Zeiss, Jena, Germany) in 400 μm thick slices, including the striatal nucleus and the overlaying cortex. Brain slices were then allowed to equilibrate for at least 1 h at room temperature in regular aCSF solution bubbled with a mixture of 95% O2/5% CO2 gas before transferred to the recording chamber.

Field potential recordings

In brief, synaptic efficacy was estimated by measuring the amplitude of the population spike (PS). Stimulation was yield through a tungsten electrode, which delivered pulses every 20s and placed in the nAc shell or core (paper I) or only shell (paper II, III). The stimulus intensity was set to yield a PS amplitude approximately half the size of the maximal evoked response. After monitoring a stable baseline for 10 min slices were treated with either quinpirole (5 μM) or nicotine (1 μM) (paper I, II), or muscimol (1 μM) and bicuculline (20 μM) (paper III). In a subset of recordings differences in input-output function were evaluated by stepwise increasing afferent stimulation strength. Changes in release

probability were also estimated by calculating the amplitude ratio (second pulse/first pulse) between two paired pulses, PPR, delivered with a 50 ms interpulse interval.

Whole-cell patch clamp

In paper III medium spiny neurons of nAc were investigated using whole-cell patch clamp, in which the spontaneous excitatory or inhibitory postsynaptic currents (sEPSC or sIPSC) of the cell can be measured.

During the experiment the brain slices were continuously perfused with aCSF (2–3 ml/min) and all recordings were performed at 30°C. The neurons were visually identified with an infrared differential interference contrast video microscopy mounted on a Nikon E600FN microscope. Whole-cell patch clamp recordings were performed with a patch clamp amplifier (EPC-9, Heka Elektronik, Lambrecht, Germany), the sampling frequency was 10 kHz and filtered at 2.9 kHz. For recording of sEPSCs the neurons were voltage clamped at -70 mV and at 0 mV to record sIPSCs. Patch pipettes (borosilicate, OD 1.5 mm, ID 0.86 mm) had a resistance of 3.0–6.5 MΩ. Series resistance was monitored using a 10 mV hyperpolarizing pulse and there were no differences in the average series resistance between the different groups.

Methodological considerations

Measuring neuronal signaling by estimation of electrical activity is a common method in neuroscience and it was first initiated in the early 1970's in hippocampal brain slices (Skrede and Westgaard, 1971). Due to several reasons, such as the lamellar organization, electrophysiological protocols used in hippocampus has been the major experimental model for both field-potential recordings and whole-cell patch clamp recordings. When utilizing this technique in striatal slices some aspects needs to be considered, such as the complexity in striatal cytoarchitecture where the dendritic arbors are not separated between subregions and the dendrites of the striatal MSNs are not uniformly oriented. Therefore we report the size of the amplitude population spike over slope of the EPSP (field excitatory post-synaptic potential), since this reflects the efficacy of

excitatory synaptic input through synchronous synaptically driven action potentials.

When preparing the brain slices parts of the neuronal input is lost, since the tissue is cut and several afferent and efferent connections are lost. And as such the recordings only reflect local outcomes and cannot directly be translated into the functions of an intact brain. The field recordings are preferable over patch clamp recording in some matters, such as keeping the intracellular composition of the cells intact, since the patch clamp technique requires that the neuronal membrane to rupture. However, this is also the strength of the patch clamp technique since you get a direct contact with the cell interior, which allows for recordings of electrical currents and potentials. In addition, the cutting procedure itself might cause severe tissue damage and cell death in the outer layers of the slice, with implications for cellular function and immunological responses.

Histochemistry

Slices from nicotine treated or vehicle treated rats were stained using the Golgi staining. This method is based on the formation of silver chromate microcrystals within neurons, which make a random number of neurons visual enabling the study of fine neural structures such as dendritic spines.

Rats were perfused with Ringer's solution and cold 4% *para*-formaldehyde. Brains were removed and post fixated for 90 minutes in room temperature, they were then impregnated in a Golgi-Cox fast kit (FD NeuroTechnologies Inc, Columbia MD, USA) for a total of three weeks (solutions were changed three times) and the brains were cut at 60 μm using a microtome (Zeiss, Jena, Germany) and stained accordingly to the company protocol. A Zeiss lsm 700 inverted confocal microscope (Zeiss, Jena, Germany), or an Olympus BX60 (Olympus Corp. PA, USA) was used to identify the dendrites of medium spiny neurons within the nAc. A 63x oil objective (NA 1.4) with 1.7 digital zoom was used on the Zeiss microscope and a 100x oil objective (NA 1.3) with cellSens image

analysis software was used to capture images on the Olympus setup. A subset of images of dendritic segments was done using the Z-stack function with 0.25 μm steps, these images were used as descriptive micrographs of in paper I. Spine inclusion criterions for dendrites were: the extend of the dendritic tree had to be well stained and visible, not obscured by blood vessels, astrocytes or other cells as described by Li and co-workers (Li et al., 2003).

Methodological considerations

Why only a small fraction of all cells are stained with the silver chromate microcrystals is still largely unknown. This is very beneficial in matters of visibility, since it allows for a clear background with only one intact cell in focus. This creates a clear tissue, which makes the possibility of using thicker sections (60-80 μm) doable, enabling the study of the entire dendritic tree. Therefore we chose to measure spine density starting at the third branch of the dendritic tree and all the way out to the tip of the dendrite. The shortcoming of not knowing the mechanism behind the staining selection is the risk of measuring spine density in what could be a few percentages of cells that deviate in some manners, and it might thus not represent the true cellular population.

Gene expression

Gene expression analysis is a common method for gaining insight in the regulation of genes, as this could have implications for receptor function. In paper III the gene expression of selected $GABA_A$ subunits and the GABA transporter GAT-1 were measured using the technique quantitative polymerase chain reaction (qPCR). The method is based on the possibility to amplify a specific fragment of DNA using multiple temperature cycles, this results in such high quantities of DNA so as it can be reliably measured.

Tissue from nAc was snap frozen after dissection and later homogenized using a phenol and guanidine thiocyanate (QIAzol Lysis Reagent, Qiagen, Hilden, Germany) solution. RNA was extracted using the Lipid Tissue

mini Kit from Qiagen. RNA concentrations were measured in a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and a total of 800 ng of RNA was converted to cDNA using the QuantiTect Reversed Transcription Kit (Qiagen). The expression of target genes (*Gabra1, Gabra2, Gabrb2, Gabrd2, Gabrg2* and *SLC6A1*) was normalized against three reference genes (*RPL-1, RPL-19* and *HPRT*, all primers from Qiagen, QuantiTect Primer assay). qPCR reactions were performed using the QuantiFast SYBR Green Master Mix (Qiagen) in a LightCycler® 480 PCR System (Roche Applied Science, Indianapolis, IN, USA). The data was analyzed in LightCycler® 480 software (version 1.5).

Methodological considerations

The accuracy of PCR is largely dependent on the primer specificity, for the PCR experiments performed in paper III pre-designed primers were used. Although this means that the company supplies you with an approximate location and guarantees a high specificity, but this was in fact never tested from our end. And since the company keeps the exact sequence a secret we cannot control for this. One also needs to keep in mind that the measure of gene expression is not a direct correlate to protein (e.g. receptor) function, since the receptor can be unavailable due to an inactive state or rapid internalization even though highly expressed.

Statistical analysis

All data was tested for Gaussian distribution with the D'Agostino-Pearson omnibus normality test, before being assign a specific statistical test, which resulted in either a parametric Student's t-test or a nonparametric Mann-Whitney U test. Group and treatment effects were tested using a one or two-way ANOVA (for microdialysis data with repeated measures) and a Sidak's test for multiple comparisons. Field potential recordings were analyzed with Clampex 10.1 (Molecular Devices, Foster City, CA) and whole-cell patch clamp data with Mini Analysis Program (version 5.6.28, Synaptosoft Inc., Fort Lee, NJ, USA). All other analysis was done in GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA).

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