

**NEURONAL HISTAMINE AND H3 RECEPTOR  
IN ALCOHOL-RELATED BEHAVIORS -  
FOCUS ON THE INTERACTION WITH  
THE DOPAMINERGIC SYSTEM**

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**ACADEMIC DISSERTATION**

To be presented for public examination with the permission of the  
Faculty of Medicine, University of Helsinki,  
in Lecture Hall 2, Haartman Institute,  
Haartmaninkatu 3,  
on January 23rd 2015  
at 12 o'clock noon

**Helsinki 2015**

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ISBN 978-951-51-0533-2 (paperback)

ISBN 978-951-51-0534-9 (PDF, <http://ethesis.helsinki.fi>)

ISSN 2342-3161 (paperback), ISSN 2342-317X (PDF)

Hansaprint, Helsinki 2014

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## ORIGINAL PUBLICATIONS

This thesis is based on the following publications which are referred to in the text by their Roman numerals

- I. Nuutinen S, **Vanhanen J**, Pigni MC, Panula P (2011): Effects of histamine H3 receptor ligands on the rewarding, stimulant and motor-impairing effects of ethanol in DBA/2J mice. *Neuropharmacology*, vol-60, no.7-8, pp.1193-1199.
- II. Nuutinen S, Lintunen M, **Vanhanen J**, Ojala T, Rozov S, Panula P (2011): Evidence for the role of histamine H3 receptor in alcohol consumption and alcohol reward in mice. *Neuropsychopharmacology*, vol.36, no.10, pp. 2030-2040.
- III. **Vanhanen J**, Nuutinen S, Lintunen M, Mäki T, Rämö J, Karlstedt K, Panula P (2013): Histamine is required for H3 receptor mediated alcohol reward inhibition, but not for alcohol consumption or stimulation. *British Journal of Pharmacology*, vol.170, no. 1, pp.177-187.
- IV. **Vanhanen J**, Kinnunen M, Nuutinen S, Panula P (2014): Histamine H3 receptor antagonist JNJ-39220675 modulates locomotor responses but not place conditioning by dopaminergic drugs. *Psychopharmacology*, Epub ahead of print.
- V. **Vanhanen J**, Nuutinen S, Tuominen M, Panula P (2014): Histamine H3 receptor deficiency impairs sensorimotor gating and modulates dopaminergic signaling. *Submitted manuscript*.

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## Candidate contribution

- I. The candidate performed major part of the experiments including conditioned place preference (CPP), locomotor activity and plasma alcohol concentration studies under the supervision of SN and PP. MCP carried out the rotarod and balance beam experiments. The candidate also participated in the analysis of the results and writing of the manuscript.
- II. The candidate performed part of the behavioral studies including drinking in the dark (DID) with H3R KO mice, locomotor activity, balance beam and rotarod experiments under the supervision of SN and PP. SN conducted the CPP and locomotor activity experiments, ML carried out the two-bottle choice paradigm, TO performed the DID experiment with pharmacological ligands and SR carried out the HPLC analysis. The candidate conducted the plasma alcohol concentration study together with TO and participated in the analysis of the results and writing of the manuscript.
- III. The candidate contributed to the experimental design and performed major part of the experiments including CPP, locomotor activity, *in situ* hybridization, plasma alcohol concentration experiments, supervised JR in the DID study and analyzed the results and wrote the manuscript. ML conducted the two-bottle choice, TM performed part of the locomotor activity experiments and KK partly maintained the mice and assisted with the *in situ* hybridization study.
- IV. The candidate planned the study under the supervision of SN and PP, analyzed the results and wrote the manuscript. MR carried out all experiments under the supervision of the candidate.
- V. The candidate contributed to the experimental design and performed and analyzed major part of the experiments including the locomotor activity, Western blotting and *in situ* hybridization experiments under the supervision of SN and PP. SN and MT conducted the prepulse inhibition study and analyzed the results. The candidate wrote the manuscript together with SN.

## Other publications related to this thesis:

Nuutinen S, **Vanhanen J**, Mäki T, Panula P (2012): Histamine H3 receptor: a novel therapeutic target in alcohol dependence? *Frontiers in Systems Neuroscience*, vol. 6, no. 36, pp 1-7.

## ABSTRACT

Neuronal histamine and its H3 receptor (H3R) regulate several physiological functions and are involved in the pathophysiology of various central nervous system disorders such as Parkinson's disease, Alzheimer's disease, Tourette syndrome and narcolepsy. The first H3R antagonist likely to enter the clinical use is pitolisant (Wakix®) in the treatment of narcolepsy. Studies conducted in experimental animals have also suggested a role for histamine and especially H3R in the effects of drugs of abuse. Furthermore, recent findings have demonstrated that brain histaminergic system might play a role in Tourette syndrome. In this thesis, the main aim was to study how histamine and H3R regulate alcohol-related behaviors and symptoms associated with psychiatric disorders such as Tourette syndrome and schizophrenia. Furthermore, our goal was to investigate the underlying mechanisms in the observed behaviors.

By using both wild type (WT) mice in different background strains and genetically modified mice, we studied whether histamine and H3R regulate the behavioral responses of alcohol. Three different H3R antagonists (ciproxifan, JNJ-10181457 and JNJ-39220675) were used in these studies and it was found that both pharmacological antagonism and genetic knockout of H3R (H3R KO) lead to diminished alcohol consumption and reward. By using histamine deficient histidine decarboxylase knockout (HDC KO) mice, we found that the lack of histamine does not alter alcohol consumption or reward but it is indeed required for the H3R-mediated alcohol reward inhibition. We also found that H3R antagonist JNJ-39220675 inhibited the acute stimulation of amphetamine, but failed to inhibit the rewarding properties of amphetamine. This indicates that although H3R antagonists inhibit alcohol reward, they may not possess the same ability on psychostimulants, such as amphetamine.

Sensorimotor gating is a neurological process in which an irrelevant sensory, cognitive or motor stimulus is suppressed and filtered out in order to focus attention to significant stimulus. It is impaired for instance in Tourette syndrome and schizophrenia and can be studied both in humans and animals by using a prepulse inhibition of the startle reflex (PPI) method. Here, we studied whether histamine, H1 receptor (H1R) and H3R regulate sensorimotor gating in three different gene knockout mice and found that H3R KO but not HDC KO or H1 receptor knockout (H1R KO) mice have impaired PPI indicating a deficiency in sensorimotor gating. The results from all behavioral studies of this work indicated that rather than histamine *per se*, H3R is an important modulator of several brain dysfunctions that are in major part regulated by the brain dopaminergic system.

The findings obtained from the behavioral experiments led us to hypothesize that H3R interacts with the dopaminergic system. This was further studied on a molecular level using both radioactive *in situ* hybridization and semi-

quantitative Western blotting. We found that H3R KO mice displayed lower levels of D1 receptor mRNA in the striatum compared to control mice. In addition, we found that systemic administration of dopamine D1 and D2 receptor agonists, SKF-38393 and quinpirole, respectively, induced an activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in the striatum of the WT mice but failed to do so in H3R KO mice.

Taken together, these findings demonstrate that H3R is an important regulator of both alcohol-related behaviors and sensorimotor gating. The mechanism by which H3R regulates these phenomena might involve the interaction between the striatal H3R and dopamine receptors. The results are important for understanding the role of H3R in the behavioral effects of alcohol and in brain dopaminergic regulation and provide preclinical evidence that H3R antagonists may serve as a novel approach to treat alcohol dependence.

## ABSTRAKTI

### **Aivojen histamiini ja H3-reseptori alkoholikäyttäytymisessä sekä dopaminergisessä säätelyssä**

Aivojen histamiini ja sen H3-reseptori säätelevät useita fysiologisia toimintoja, mutta ovat myös mukana erilaisten keskushermostohäiriöiden patofysiologiassa. Tällaisia ovat muun muassa Parkinsonin tauti, Alzheimerin tauti, Touretten syndrooma ja narkolepsia. Pitolisantti (Wakix®) on ensimmäinen todennäköisesti myös kliniseen käyttöön tuleva H3-reseptorin antagonisti, joka on tarkoitettu narkolepsian oireiden hoitoon. Aikaisemmissa eläinkokeissa on havaittu että histamiini sekä H3-reseptori ovat myös osallisina riippuvuuskäyttäytymisessä. Lisäksi, tuoreessa julkaisussa havaittiin yhteys Touretten syndrooman ja aivojen histaminergisen järjestelmän välillä. Tämän väitöskirjan tavoitteena oli selvittää, miten histamiini ja H3-reseptori säätelevät alkoholikäyttäytymistä sekä muun muassa Touretten syndroomaan liittyvää käyttäytymistä. Lisäksi tavoitteena oli selvittää, mitkä molekyyylitason mekanismit ovat näiden ilmiöiden takana.

Käytimme tässä työssä usean eri taustakannan villityypin sekä geneettisesti muunneltuja hiiriä. Tutkimuksen ensimmäisissä osioissa tutkimme, kuinka H3-reseptori säätelee alkoholikäyttäytymistä. Havaitimme, että kolme H3-reseptorin salpaajaa (siproksifaani, JNJ-10181457 ja JNJ-39220675) sekä H3-reseptorin geenitoiminnan sammuttaminen vähensivät alkoholin kulutusta sekä sen aiheuttamaa mielihyvää. Histamiinin suhteen poistogeenisillä hiirillä ei havaittu eroja alkoholin kulutuksessa tai sen tuottamassa mielihyvässä kontrollihiiriin verrattuna. Kuitenkaan H3-reseptorin farmakologinen salpaus ei estänyt alkoholin tuottamaa mielihyvää histamiinin suhteen poistogeenisillä hiirillä, osoittaen että histamiinilla on estävä vaikutus alkoholin aiheuttamassa mielihyvässä. Havaitimme myös, että H3-reseptorin antagonisti JNJ-39220675 esti amfetamiinin aiheuttamaa liikeaktiivisuuden stimulaatioita, mutta ei mielihyvää. Vaikka H3-reseptorin farmakologinen salpaus estääkin alkoholin tuottamaa mielihyvää, tämä tutkimus osoittaa, että se ei välttämättä estä esimerkiksi psykostimulanttien aiheuttamaa mielihyvää.

Aivojen sensorimotorinen tiedonvälitys on neurologinen tapahtumasarja, jossa epäolennainen sensorinen, kognitiivinen tai motorinen ärsyke suodatetaan ja vaiennetaan, jotta keskittyminen voidaan kohdistaa merkityksellisiin ärsykkeisiin. Sensorimotorinen tiedonvälitys on häiriintynyt esimerkiksi Touretten syndroomassa. Tässä väitöskirjassa tutkimme sensorimotorista tiedonvälitystä histamiinin, H1-reseptorin sekä H3-reseptorin suhteen poistogeenisillä hiirillä. Havaitimme, että sensorimotorinen tiedonvälitys oli häiriintynyt ainoastaan H3-reseptorin suhteen poistogeenisillä hiirillä. Myös tämä käyttäytymistason tutkimus siis vahvisti, että ei niinkään histamiini vaan ainoastaan sen H3-reseptori on tärkeässä asemassa säätelössä erilaisia

käyttäytymistason toimintoja. Aivojen dopaminerginen järjestelmä säätelee näistä kumpaakin, sekä riippuvuuskäyttäytymistä että sensorimotorista tiedonvälitystä.

Yllämainittujen käyttäytymiskokeiden perusteella oletimme, että H3-reseptori on vuorovaikutuksessa aivojen dopaminergisen järjestelmän kanssa, jota seuraavaksi tutkimme solutasolla käyttäen radioaktiivista *in situ* -hybridisaatiota sekä kvantitatiivista geielektroforeesi -menetelmää. Tutkimustuloksemme osoittivat, että H3-reseptorin suhteen poistogeenillä hiirillä ilmenee vähemmän dopamiinin D1-reseptorin lähetti-RNA:ta (engl. messenger RNA, mRNA) striatumin alueella. Havaitimme, että sekä D1-reseptorin että D2-reseptorin farmakologinen aktivaatio aiheutti solunsisäisen ERK1/2 -proteiinikinaasin aktivaatiota ainoastaan kontrollihiirillä, mutta ei H3-reseptorin suhteen poistogeenisillä hiirillä.

Tämä väitöskirjatyö osoittaa että H3-reseptori säätelee niin alkoholikäyttäytymistä sekä sensorimotorista tiedonvälittymistä. Näiden ilmiöiden mekanismiin liittyy H3-reseptorin ja dopamiinireseptoreiden vuorovaikutus aivojen striatumin alueella. Nämä tulokset syventävät tietämystämme alkoholikäyttäytymisestä sekä aivojen dopaminergisen järjestelmän toiminnasta. Lisäksi prekliiniset tutkimustuloksemme osoittavat, että H3-reseptorin farmakologinen salpaus saattaisi olla uusi menetelmä alkoholiriippuvuuden hoidossa.

## ABBREVIATIONS

$\alpha$ -FMH	$\alpha$ -fluoromethylhistidine
AADC	aromatic l-amino acid decarboxylase
AA	Alko Alcohol
AC	adenyl cyclase
ADHD	attention-deficit hyperactivity
AcbC	nucleus accumbens core
AcbSh	nucleus accumbens shell
AKT	protein kinase B
Alcohol-CPP	alcohol-induced conditioned place preference
Amphetamine-CPP	amphetamine-induced conditioned place preference
ANA	Alko Non-Alcohol
ANOVA	analysis of variance
cAMP	cyclic adenosine monophosphate
COMT	catechol O-methyltransferase
CPA	conditioned place aversion
CPu	caudate putamen
CPP	conditioned place preference
CREB	cAMP response element-binding protein
CSF	cerebrospinal fluid
CYP450	cytochrome P 450
DAO	diamine oxidase
DARPP-32	dopamine- and cAMP-regulated phosphoprotein (32kDa)
DAT	dopamine transporter
DID	drinking in the dark
EDS	extensive daytime sleepiness
EMA	European medicines agency
ERK 1/2	extracellular signal-regulated kinase 1/2
GAD	glutamic acid decarboxylase
GABA	gamma-aminobutyric acid
GPCR	G protein-coupled receptor
GSK3	glycogen synthase kinase 3
HDC	histidine decarboxylase
HNMT	histamine N-methyltransferase
H1R	histamine receptor 1
H2R	histamine receptor 2
H3R	histamine receptor 3
H4R	histamine receptor 4
KO	knockout
L-DOPA	L-dihydroxyphenylalanine
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MSN	medium-sized spiny neuron
NMDA	N-methyl-D-aspartic acid
OCD	obsessive-compulsive disorder
PI3K	phosphatidylinositol 3-kinase
PFC	prefrontal cortex
PKA	protein kinase A
PKB	protein kinase B (also termed AKT)
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PP1	protein phosphatase 1
PPI	prepulse inhibition
RM ANOVA	repeated measures analysis of variance
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SSC	saline-sodium citrate
STEP61	striatal-enriched protein tyrosine phosphatase 61
TH	tyrosine hydroxylase
TMN	tuberomamillary nucleus
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
WT	wild type

## **GLOSSARY OF TERMS**

### **Addiction**

Addiction is a chronic, relapsing disorder in which compulsive drug-seeking and drug-taking behaviors persist despite adverse legal, health, economic, and societal consequences. Animal models represent some (e.g. drug dependence) but not all aspects of addiction. In this thesis, however, the term addiction is used in reference of both human and animal studies.

### **Constitutive activity**

Several lines of evidence indicate that G protein-coupled receptors (GPCRs), including H3R, display significant signal transduction stimulation in the absence of activating ligand. This tonic and robust level of stimulation is termed constitutive activity.

### **Inverse agonist**

An inverse agonist is a pharmacological ligand that binds to the same receptor as an agonist but induces a pharmacological response opposite to that of an agonist, i.e. the receptor turns to its inactive state. Inverse agonism occurs only in the constitutively active receptors and decreases their activity below the basal level.

### **Medium-sized spiny neurons (MSNs)**

MSNs also known as spiny projection neurons are a special type of neurons representing the vast majority of the neurons within the striatum of the basal ganglia. MSNs are GABAergic and thus inhibit the neurons they project to. There are at least two types of MSNs in the striatum, one containing D1Rs and projecting to substantia nigra (the direct pathway) and the other containing D2Rs and projecting mainly to globus pallidus (the indirect pathway).

### **Neutral antagonist**

A neutral antagonist is a receptor ligand that binds to its receptor without affecting its state, i.e. it inhibits or suppresses agonist-mediated or inverse agonist-mediated responses. In this thesis, the term antagonist is used for both inverse agonists and neutral antagonist as it is not necessarily known whether the given ligand acts as an inverse agonist or neutral antagonist.

# 1. INTRODUCTION

The modulatory neurotransmitter histamine is a biogenic amine that is synthesized from L-histidine by histidine decarboxylase (HDC) enzyme (Haas and Panula 2003; Panula and Nuutinen 2013). Histaminergic neurons are located in the posterior hypothalamus in the tuberomammillary nucleus from where they send projections to most parts of the brain including striatum, cortex, thalamus, cerebellum and spinal cord. The effects of histamine are mediated in the brain via three G protein-coupled receptors H1R, H2R and H3R. H1R and H2R are postsynaptic receptors whereas H3R is present at both presynaptic and postsynaptic sites (Pillot, *et al* 2002; Haas and Panula 2003). At presynaptic locations H3 autoreceptors regulate the release of histamine. H3R is also expressed on other neuron terminals regulating the release of e.g. glutamate and acetylcholine. In addition, H3R is also localized at the postsynaptic site of the MSNs and in these neurons it has been demonstrated that H3R forms functional heterodimers with both D1R and D2R *in vitro* (Ferrada, *et al* 2008; Ferrada, *et al* 2009).

Histamine in the brain regulates several physiological functions and histamine has been shown to be involved in the pathophysiology of several brain disorders including Parkinson's disease (Rinne, *et al* 2002), Alzheimer's disease (Mazurkiewicz-Kwilecki and Nsonwah 1989; Airaksinen, *et al* 1991), schizophrenia (Prell, *et al* 1995) and narcolepsy (Nishino, *et al* 2001; Kanbayashi, *et al* 2009). Furthermore, altered H3R binding has been associated with schizophrenia (Jin, *et al* 2009). After having been a promising pharmacological target in the treatment of various brain disorders for well over a decade, H3R antagonist pitolisant (BF2.649, tiprolisant) is finally entering the clinical use in the treatment of narcolepsy (EMA 2014).

Studies conducted in rodents have also shown that brain histamine and H3R are involved in reward circuit and addiction (Brabant, *et al* 2010). Interestingly, the data from alcoholic humans suggest that the brain histaminergic system is altered due to high alcohol (ethanol) consumption (Alakarppa, *et al* 2002; Alakarppa, *et al* 2003) and the association between H3R and alcohol consumption was first found using the alcohol-preferring AA rat line (Lintunen, *et al* 2001). Furthermore, preliminary experiments conducted in HDC KO mice also indicated that histamine is involved in the mediation of alcohol-related behaviors (Nuutinen, *et al* 2010). However, these studies left several questions unanswered, which were addressed in this thesis.

Recently, an association between *hdc* gene mutation and Tourette syndrome was found in a family with eight children in two generations all carrying the mutation, indicating a dysfunction of histaminergic system in Tourette syndrome (Ercan-Sencicek, *et al* 2010). The linkage between the symptoms of

Tourette syndrome and the brain histaminergic system has been further supported by studies using HDC knockout mice (Castellan Baldan, *et al* 2014).

Taken together, histamine and H3R have a prominent role in the modulation of various brain dysfunctions. However, histamine and H3R seem to rather modulate these phenomena and is not directly associated with the etiology of the disorders. There is increasing evidence indicating an interaction between the dopaminergic and histaminergic systems (Moreno, *et al* 2011; Moreno, *et al* 2014) possibly explaining histamine's involvement in several brain dysfunctions. Revealing how these aminergic systems interact on both behavioral and mechanistical level is of great importance in order to better understand their functions in both health and disease. In this thesis, the goal was to clarify whether histamine and H3R are involved in the regulation alcohol-related behaviors and symptoms related to e.g. Tourette syndrome and schizophrenia in mice. In addition, the aim was to study by which mechanism histamine and H3R regulate these behaviors.

## **2. REVIEW OF THE LITERATURE**

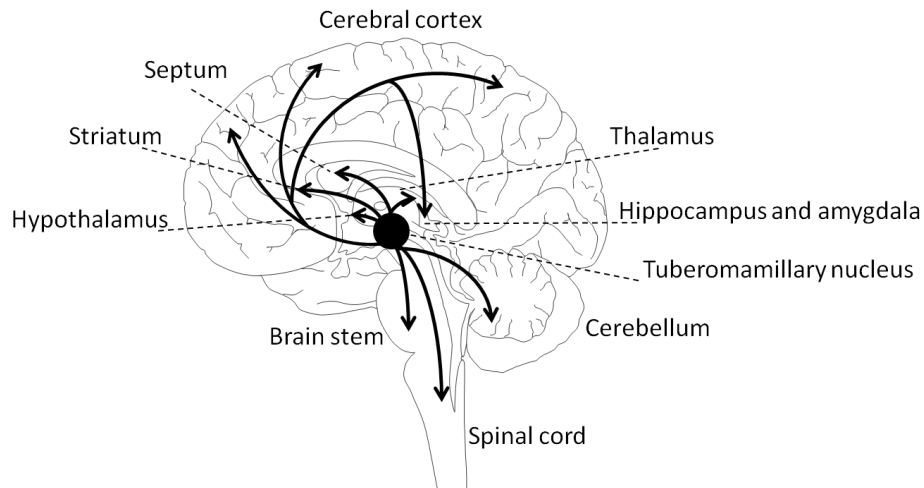
### **2.1 The brain histaminergic system**

The first observation of histamine in the brain was made in the 1940's (Kwiatkowski 1941; Kwiatkowski 1943) but its role as a mammalian neurotransmitter was suggested only several decades later (Schwartz, *et al* 1976). The histaminergic system was generally acknowledged as a distinct anatomically defined neurotransmitter system only in 1984 when it was immunohistochemically demonstrated that histaminergic neurons are located in the posterior hypothalamus from where they project widely throughout the brain (Watanabe, *et al* 1983; Panula, *et al* 1984; Watanabe, *et al* 1984). The sole source of histaminergic cell bodies was later found to be identical with the previously named tuberomamillary nucleus (TMN). The number of histaminergic neurons in the human brain is about 64 000 (Panula, *et al* 1990) whereas in rat 4000 histamine-containing neurons have been observed (Ericson, *et al* 1987).

The histamine neurons are relatively large cells (~25-30  $\mu$ M in diameter) (Panula, *et al* 1984; Watanabe, *et al* 1984). Subpopulations of TMN neurons also express e.g. substance P, galanin, GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) in addition to histamine (Staines, *et al* 1986; Ericson, *et al* 1991; Airaksinen, *et al* 1992). However, it is not currently known whether these are effectively released from the TMN neurons.

#### **2.1.1 Histaminergic projections**

The TMN neurons send out projections almost entirely throughout the brain and some parts of the spinal cord (Panula, *et al* 1984; Watanabe, *et al* 1984) (Figure 1). There are two ascending and one descending histaminergic pathways innervating the brain (Panula, *et al* 1989). The ventral ascending pathway innervates the ventral part of the brain including the hypothalamus and septum, while the dorsal ascending pathway projects to the thalamus, hippocampus, amygdala and rostral forebrain structures. The descending pathway projects to the cerebellum, brainstem and spinal cord.



**Figure 1.** The histaminergic projections in the human brain. The histaminergic neurons originate from the tuberomammillary nucleus located on the posterior hypothalamus from where they send projections throughout the brain.

The rat TMN has been subdivided into five subdivisions (Inagaki, *et al* 1990) but there is little evidence that these five subnuclei are functionally different and they could thus be considered as one functional group (Wada, *et al* 1991). A single neuron from a different TMN subgroup may send divergent projections to different parts of the brain and may even project to both ascending and descending pathways (Kohler, *et al* 1985). However, recent evidence demonstrates that the histaminergic neurons are functionally heterogeneous and organized into distinct circuits (Giannoni, *et al* 2009) but this might more reflect the diversity of the H3R located on the histaminergic neurons and not the histaminergic neurons as such. The functional complexity of the H3R will be reviewed later in this thesis.

### 2.1.2 Basal ganglia and the striatum

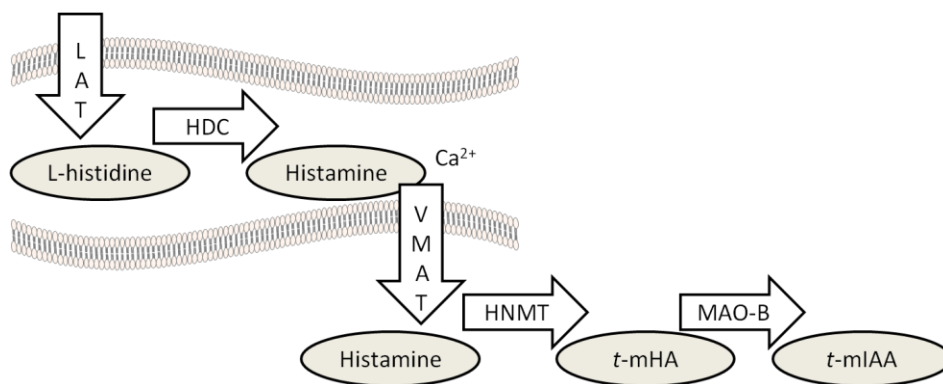
The basal ganglia are a group of subcortical nuclei which comprise the striatum, globus pallidus, substantia nigra and subthalamic nuclei. The principal input structure of the basal ganglia is the striatum, which can be divided into dorsal and ventral striatum (Figure 2). The dorsal part of the striatum is called caudate putamen and is implicated especially in the control of motor functions (Girault 2012). The ventral part of the striatum comprises of nucleus accumbens and olfactory tubercle that contains granular cells called the islands of Calleja. Ventral striatum is crucial in motivated and goal directed behaviors due to its importance in the integration of information from the motor and limbic systems (Morgane, *et al* 2005). The dopaminergic projections from the substantia nigra



basal ganglia. In support of this, HDC activity has been shown to be greater in the striatum compared to hippocampus and cortex (Bischoff and Korf 1978) and histamine turnover increased in the striatum compared to other brain regions, apart from the hypothalamus (Oishi, *et al* 1984). Nevertheless, the effect and function of histamine in the striatum has not been fully elucidated. Interestingly however, HDC deficiency has been linked to TS (Ercan-Sencicek, *et al* 2010) which is a disorder characterized by striatal abnormalities (Peterson, *et al* 2003; Bloch, *et al* 2005).

#### **2.1.4 Histamine synthesis, packaging, release and degradation**

The amount of histamine penetrating the brain from the blood is negligible (Nuutinen and Panula 2010). Histamine in the brain is produced by both mast cells (Martres, *et al* 1975) and neurons (Garbarg, *et al* 1974). Histamine synthesis and metabolism in the brain is illustrated in Figure 3. Histamine precursor L-histidine is the rate-limiting factor for histamine synthesis and it is brought to neurons by L-amino-acid transporter (LAT). Histamine synthesizing enzyme HDC derives L-histidine into histamine (Garbarg, *et al* 1974). The activity of HDC is highest in the TMN but it is also active in histaminergic terminals (Watanabe, *et al* 1983). Histamine is then packed into intracellular vesicles by vesicular monoamine transporter (VMAT) (Merickel and Edwards 1995). Action potential entering the histaminergic nerve terminal induces histamine to be released in calcium ( $\text{Ca}^{2+}$ ) –dependent manner into the synaptic cleft. After its release, histamine is bound to its target receptors and the unbound fraction is inactivated by histamine-N-methyltransferase (HNMT) induced methylation into *tele*-methylhistamine (*t*-mHA) (Brown, *et al* 1959; Reilly and Schayer 1970). Monoamine oxidase B (MAO-B) then converts *tele*-methylhistamine into *tele*-methyl-imidazolaceticacid (*t*-mIAA) by oxidative deamination (Hough and Domino 1979; Hough and Domino 1979). The main histamine metabolizing enzyme in the peripheral tissue is diamine oxidase (DAO) which is not active in the brain under normal conditions (Prell, *et al* 1997).



**Figure 3.** Histamine synthesis and metabolism in the brain. Abbreviations: HDC, histidine decarboxylase; HNMT, histamine-N-methyltransferase; LAT, L-amino-acid transporter; MAO-B, monoamine oxidase B; *t*-mHA, *tele*-methylhistamine; *t*-mIAA, *tele*-methyl-imidazolacetic acid; VMAT, vesicular monoamine transporter.

### 2.1.5 Histamine receptors: H1R, H2R and H4R

In this chapter three of the histamine receptors (H1R, H2R and H4R) are shortly reviewed. H3R will be discussed in more detail in the following chapters as it is the main focus of this thesis. Three of the histamine receptors (H1R, H2R and H3R) are widely expressed in the CNS while H4R is expressed mostly in the bone marrow and leukocytes (Oda, *et al* 2000). Whether H4R is also expressed in the brain is under debate, some studies suggesting very low levels of H4R in different regions of the brain and more prominent expression in the spinal cord (Connelly, *et al* 2009; Strakhova, *et al* 2009). Molecular and functional properties of the four histamine receptors in the brain are listed in Table 1.

The human H1R coding gene is located on the chromosome 3 (Le Coniat, *et al* 1994) and like all histamine receptors, belongs to the 7-transmembrane-spanning G protein-coupled receptor (GPCR) family. More specifically, H1R couples to the  $G\alpha_{q/11}$  protein which activates phospholipase C (PLC) (Traiffort, *et al* 1994). H1R is widely distributed throughout the CNS with the highest expression levels in areas important in arousal, e.g. in the thalamus and cortex (Chang, *et al* 1979; Bouthenet, *et al* 1988). H1R is also highly expressed in the limbic system and the striatum.

H2R gene is located on the human chromosome 5 encoding a  $G\alpha_s$  protein (Traiffort, *et al* 1995). Activation of  $G_s$  protein leads to stimulation of adenylyl cyclase (AC) and enhanced production of cyclic adenosine monophosphate (cAMP) (Baudry, *et al* 1975; Hegstrand, *et al* 1976). H2R is abundant in the

stomach (Diaz, *et al* 1994) where it is responsible for the gastric acid secretion (Hill 1997). H2R is also widespread in the brain and spinal cord (Traiffort, *et al* 1992; Vizuete, *et al* 1997). In the brain, the highest densities of H2R are in the basal ganglia and in the limbic structures including hippocampal formation and the amygdala, but it is also expressed in the cortex. In contrast to H1R, H2R is expressed in low densities in septal areas and hypothalamic and thalamic nuclei. The brain H2R has a role for example in cognition and nociception.

The human H4R gene is on the chromosome 18 (Coge, *et al* 2001) and it shares about 35 % amino acid identity with the H3R and only about 20 % sequence similarity with the H1R and H2R (van Rijn, *et al* 2008). H4R is negatively coupled to pertussis toxin-sensitive  $G\alpha_{i/o}$  proteins which inhibit AC downregulating cAMP-dependent activation of protein kinase A (PKA) and cAMP-response-element binding protein-induced gene transcription (Leurs, *et al* 2009). H4R is expressed e.g. in the bone marrow and leukocytes and is involved e.g. in immune responses and various inflammatory conditions (Oda, *et al* 2000).

**Table 1.** Characteristics of the histamine receptors in the brain. Abbreviations: AC, adenylyl cyclase; CNS, central nervous system; MAPK, mitogen-activated protein kinase; PLC; phospholipase C.

	H1R	H2R	H3R	H4R
<b>Gene locus</b>	Chromosome 3	Chromosome 5	Chromosome 20	Chromosome 18
<b>CNS expression</b>	High	High	High	?
<b>Cellular expression</b>	Postsynaptic	Postsynaptic	Pre- and postsynaptic	Postsynaptic
<b>G protein</b>	$G\alpha_{q/11}$	$G\alpha_s$	$G\alpha_{i/o}$	$G\alpha_{i/o}$
<b>Main signaling pathways</b>	PLC $\uparrow$ Ca <sup>2+</sup> $\uparrow$	AC $\uparrow$	AC $\downarrow$ MAPK $\uparrow$	AC $\downarrow$ MAPK $\uparrow$
<b>CNS function</b>	Arousal, wakefulness	Cognition, nociception	Cognition wakefulness,	?

## 2.2 H3 receptor

### 2.2.1 Physiological functions

Initially H3R was characterized as an autoreceptor in the histaminergic terminals regulating the release and synthesis of histamine (Arrang, *et al* 1983). Thereafter, it has been found to act as a heteroreceptor regulating the release of several other neurotransmitters, including noradrenaline (Schlicker, *et al* 1988; Schlicker, *et al* 1994), acetylcholine (Arrang, *et al* 1995; Blandina, *et al* 1996), GABA (Garcia, *et al* 1997; Yamamoto, *et al* 1997) and glutamate (Molina-Hernandez, *et al* 2001). Interestingly, in the caudate putamen and nucleus accumbens H3R is mainly expressed at the postsynaptic site on the MSNs of both the direct and indirect movement pathways (Pillot, *et al* 2002). The role of the postsynaptic H3Rs has been only quite recently elucidated and is thought to directly regulate the function of the dopaminergic receptors (Ferrada, *et al* 2008; Ferrada, *et al* 2009).

### 2.2.2 Localization and distribution in the brain

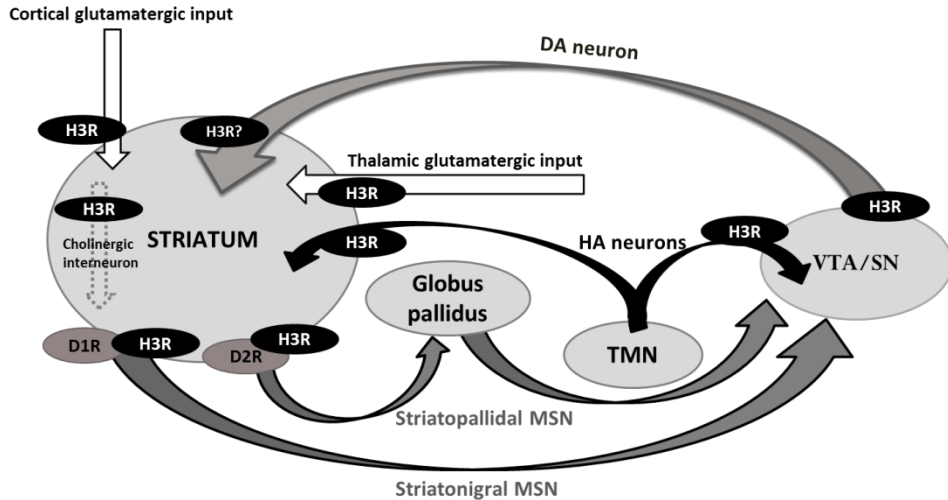
H3R is widely expressed in the brain with the highest expression in the cerebral cortex, the striatum, the olfactory tubercles and the substantia nigra (Pollard, *et al* 1993). The localization of the H3R parallels that of its mRNAs in some areas of the brain, but interestingly there are some discrepancies between the presence of the protein and its mRNA in many brain regions (Pillot, *et al* 2002). In the cerebral cortex for example, H3R mRNA expression is found in all layers and displays a distinct laminar pattern (Pillot, *et al* 2002; Jin and Panula 2005). There is a strong mRNA expression of H3R in the intermediate and deep layers, indicating the presence of H3R on several types of neurons. The H3R binding is also dense in the cortex except in layer V. The very high mRNA expression observed in cortical layer V encodes probably for the presynaptic H3Rs regulating glutamate release from the corticostriatal neurons. In the hippocampus, there is strong mRNA expression but low binding in pyramidal layers of the CA1, indicating that H3Rs are more abundant on the efferent projections of pyramidal cells. Some studies have found no H3R mRNA in the dopaminergic neurons (Anichtchik, *et al* 2001; Drutel, *et al* 2001) whereas Pillot and colleagues found both H3R binding and H3R mRNA expression in the substantia nigra *pars compacta* and suggesting that H3Rs are located upon nigrostriatal afferents (Pillot, *et al* 2002). Interestingly, however, no H3R mRNA was observed in the VTA indicating that not all dopaminergic neurons express H3Rs. In contrast, a recent study demonstrated that H3R mRNA is expressed in the VTA (González-Sepúlveda, *et al* 2013). In the amygdala, there are both high density of receptor and mRNA expression. High expression of H3R mRNA is also observed in the thalamus (Pillot, *et al* 2002; Jin, *et al* 2002; Jin, *et al* 2005) whereas the binding is not that abundant. In the hypothalamus,

the H3R mRNA expression parallels the density of binding sites with the highest being in the TMN.

### **2.2.3 H3R in the basal ganglia and the striatum**

For clarity, the localization of the H3R in the basal ganglia is schematically illustrated in Figure 4. Of importance, in both rodent and human basal ganglia, high densities of both H3R mRNA and binding sites have been observed. The highest density of H3R has been observed in both dorsal and ventral striatum (Pillot, *et al* 2002). The striatal H3Rs are mainly located on the GABAergic MSNs projecting to the globus pallidus and substantia nigra, hence the striatal H3Rs are mainly postsynaptic. In the MSNs H3Rs are colocalized with both D1Rs and D2Rs (González-Sepúlveda, *et al* 2013; Moreno, *et al* 2011; Ryu, *et al* 1994). The H3Rs in the D1R-containing striatonigral pathway are functional as it has been shown that H3R activation reduces D1R-dependent GABA release (Arias-Montano, *et al* 2001) and inhibits D1R-mediated cAMP accumulation (Sanchez-Lemus and Arias-Montano 2004). The proenkephalin and D2R-containing striatopallidal MSNs also express H3R mRNA (Pillot, *et al* 2003) and but the functional role of the H3Rs in the striatopallidal neurons has not been fully elucidated.

Functional studies indicate the presence presynaptic H3Rs upon striatal afferents as the H3 autoreceptors modulate histamine release from rat striatal slices (Arrang, *et al* 1985). In addition, there is also some evidence that H3R regulates the release of dopamine from the dopaminergic neurons (Molina-Hernandez, *et al* 2000; González-Sepúlveda, *et al* 2013). Moreover, the presence of presynaptic H3Rs located on the inputs from the striatal cholinergic interneurons has also been observed (Chazot, *et al* 2001; González-Sepúlveda, *et al* 2013). In addition, H3Rs are also located on the corticostriatal glutamatergic terminals, a finding that is also supported indirectly by the H3R-mediated inhibition of striatal glutamate release (Molina-Hernandez, *et al* 2001).



**Figure 4.** H3R in the subcortical structures forming the basal ganglia circuitry. Abbreviations: DA, dopamine; D1R, dopamine receptor 1; D2R, dopamine receptor 2, HA, histamine; H3R, histamine receptor 3; MSN, medium spiny neuron; SN, substantia nigra; TMN, tuberomamillary nucleus; VTA, ventral tegmental area. References to original publications are found in the text.

## 2.2.4 Splice variants

The human H3R was cloned in the late 1990's (Lovenberg, *et al* 1999) and the availability of the human H3R cDNA has not only had a great impact on understanding the diversity of the H3R and but also on drug development. The human H3R gene is located on chromosome 20q13.33 and it encodes the small, 70 kDa peptide consisting of 445 amino acids. The sequence similarity of H3R with other GPCRs is very low and the similarity with both H1Rs and H2Rs is only about 20 % (Leurs, *et al.* 2005). H3R has several different human and rat isoforms that are derived from a single gene by alternative splicing (Lovenberg, *et al* 1999; Drutel, *et al* 2001; Coge, *et al* 2001; Rouleau, *et al* 2004). At least 20 different human H3R isoforms have been described, but as most of the observations of the H3R have been detected by mRNAs, it cannot be determined whether they are also functionally active (Leurs, *et al* 2005). It has been shown, however, that different H3R isoforms differ in their localization and pharmacological properties such as signal transduction efficiencies (Drutel, *et al* 2001; Hancock, *et al* 2003). The heterogeneous distribution of the isoforms suggests that H3R may display isoform-specific regulation of different brain functions. At least six of the human H3Rs; H3R<sub>(453)</sub>, H3R<sub>(445)</sub>, H3R<sub>(409)</sub>, H3R<sub>(373)</sub>, H3R<sub>(329)</sub> have been shown to have functional activity either by ligand-binding or signal transduction experiments (Leurs, *et al* 2005). In the rat, at least four isoforms have been described as functionally active; H3R<sub>(445)</sub>, H3R<sub>(413)</sub>,

H3R<sub>(410)</sub>, H3R<sub>(397)</sub> (Esbenshade, *et al* 2008) and the form H3R<sub>(413)</sub> has been suggested to function as the presynaptic H3 autoreceptor (Gbahou, *et al* 2012). Better understanding of the specific characteristics of the different H3R isoforms in terms of functional properties and localization would help to elucidate the functional complexity of the H3R.

### 2.2.5 Constitutive activity

The high constitutive activity of the H3R was first shown in the rat recombinant H3R by Morisset and colleagues leading to the suggestion that H3R inverse agonist might have therapeutic applications (Morisset, *et al* 2000). Shortly after, it was shown that also the human H3R display high level of activity in the absence of an agonist (Wieland, *et al* 2001; Rouleau, *et al* 2002). The recognition of the *in vivo* constitutive activity of the H3R has allowed more detailed characterization of the pharmacological ligands and has led to the understanding that many of the ligands originally classified as H3R antagonists are in fact inverse agonists (Arrang, *et al* 2007). The constitutive activity of the H3R is not only of importance in the regulation of the activity of pharmacological ligands but it is also likely to have a major modulatory role in the brain.

### 2.2.6 Signaling

Similarly to the H4R, the effects of H3R are mediated via G $\alpha_{i/o}$  protein (Clark and Hill 1996; Lovenberg, *et al* 2000) that is negatively coupled to AC. AC stimulates the production of cAMP, which in turn activates PKA and cAMP-responsive-element binding protein (CREB) modulating gene transcription. Thus, H3R activation reduces the level of cAMP and its downstream targets such as CREB.

H3R may also engage G $\alpha_{q/11}$  through extensive cross-talk with other GPCRs. Activation of G $\alpha_{q/11}$  leads to activation of PLC. PLC, in turn, induces the activation of protein kinase C (PKC). Subsequently, in addition to inhibition of the cAMP pathway, H3R may also activate other effector pathways including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (Drutel, *et al* 2001; Giovannini, *et al* 2003). Activation of MAPK results in the phosphorylation of ERK1/2 whereas activation of PI3K leads to activation of protein kinase B (PKB, also termed AKT) (PKB, also termed AKT) (Bongers, *et al* 2007; Mariottini, *et al* 2009). AKT phosphorylation subsequently inhibits the action of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), which is the major tau kinase in the brain involved for example in the pathophysiology of Alzheimer's disease (Sun, *et al* 2002).

H3R-mediated activation may also lead to activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) inducing the release of arachidonic acid (Rouleau, *et al* 2002), inhibition of sodium/hydrogen (Na<sup>+</sup>/H<sup>+</sup>) exchanger and reduction of the intracellular Ca<sup>2+</sup> levels (Silver, *et al* 2002).

### **2.2.7 H3R antagonists**

The first synthesized H3R selective and potent H3R antagonist was the imidazole-based thioperamide (Arrang, *et al* 1988). Thioperamide has been extensively used in the preclinical research. There are several other imidazole-based H3R antagonists used in *in vitro* or *in vivo* animal studies, but their major drawback is the imidazole group in the compound leading to interference with the metabolizing cytochrome P450 (CYP450) enzymes in the liver and possible pharmacokinetic interactions with other drugs (Yang, *et al* 2002; Berlin, *et al* 2006). Another drawback of an imidazole-containing compound is a poor brain penetration (Schwartz 2011). The discovery that the imidazole residue can be replaced by nitrogen-containing heterocycles has led to the development of several non-imidazole-based H3R antagonists by different pharmacological companies for various indications, focusing on different CNS implications. The first H3R antagonist that was introduced to the clinical trials was the potent, brain-penetrant, highly selective and orally bioavailable pitolisant (Ligneau, *et al* 2007; Schwartz 2011).

## **2.3 The brain histamine and H3R antagonism in health and disease**

Histamine in the brain regulates several physiological functions and has been shown to be involved in the pathophysiology of several brain disorders including Parkinson's disease (Rinne, *et al* 2002), Alzheimer's disease (Mazurkiewicz-Kwilecki and Nsonwah 1989; Airaksinen, *et al* 1991), schizophrenia (Prell, *et al* 1995) and narcolepsy (Nishino, *et al* 2001; Kanbayashi, *et al* 2009). Histamine release is also a sensitive indicator of stress (Verdiere, *et al* 1977; Westerink, *et al* 2002). In addition, increased H3R radioligand binding has been observed in the prefrontal cortex (PFC) of schizophrenic and bipolar subjects with psychotic symptoms (Jin, *et al* 2009).

The research on the role of the brain histaminergic system in brain disorders has largely focused on H3R-mediated modulation of the dysfunctions and the first histaminergic drug implicated for CNS disorder is likely to be H3R antagonist pitolisant in the treatment of narcolepsy (Dauvilliers, *et al* 2013). The literature about the histaminergic system and especially H3R in health and disease are reviewed in the next chapters.

### 2.3.1 Sleep, arousal and wakefulness

Indirect pharmacological evidence of brain histamine as a regulator of sleep arose from the sedative adverse effect of the classic antihistamines acting as H<sub>1</sub>R antagonists used in the treatment of allergies (Monnier, *et al* 1967). The critical role of the histaminergic neurons in the maintenance of wakefulness was suggested a decade later (Schwartz 1977). Histamine is released in a circadian rhythm from the TMN which parallels the alterations in the firing rate of the histaminergic neurons throughout the sleep-wake cycle (Mochizuki, *et al* 1992). Neuronal histaminergic neurons act as pacemaker cells that are intrinsically able to generate rhythmic bursting activity and display spontaneous firing with low frequency (1-4 Hz) (Haas and Panula 2003). The activity of the histaminergic neurons is high during waking and low or absent during sleep (Saper, *et al* 2005) and the activity of histaminergic TMN neurons is important in the maintenance of the high level of vigilance necessary for cognitive processes (Takahashi, *et al* 2006). Mice lacking endogenous histamine (HDC KO) are less vigilant and fall asleep faster than control mice in a novel environment (Parmentier, *et al* 2002). In human cerebrospinal fluid (CSF), histamine concentration is highest during the day (Kiviranta, *et al* 1994). In addition to the circadian rhythmicity, histamine is released rapidly, in an ultradian rhythm correlating with the delta and theta waves in the electroencephalographic recordings (Philippu and Prast 1991).

**Narcolepsy** is a disabling sleep disorder characterized by disturbed nocturnal sleep and excessive daytime sleepiness (EDS) (Dauvilliers, *et al* 2007). Narcolepsy is often accompanied with cataplexy; the sudden loss of muscle tone. In humans, narcolepsy is caused by deficient neurotransmission of orexin (hypocretin) (Peyron, *et al* 2000). Orexin is an excitatory peptide originating from the lateral hypothalamus and it has widespread projections in the brain. Of importance, the orexinergic system mainly projects to aminergic neurons including the histaminergic neurons. Interestingly, there is also evidence that the activation of histaminergic neurotransmission is required for the waking action of orexin (Huang, *et al* 2001). Lower levels of CSF histamine have been observed in subjects suffering from idiopathic insomnia or narcolepsy (Kanbayashi, *et al* 2009; Nishino, *et al* 2009). The immense preclinical evaluation of the efficacy of H<sub>3</sub>R antagonists in animal models of narcolepsy has resulted in clinical drug development (Tiligada, *et al* 2011). Several H<sub>3</sub>R antagonists have been studied in clinical trials in narcoleptic patients suffering from EDS. The most advanced compound in the H<sub>3</sub>R antagonist drug development is the pitolisant that has a proven efficacy in a phase III study (Lin, *et al* 2008; Dauvilliers, *et al* 2013). Bioprojet Pharma has recently submitted an application to the European Medicines Agency (EMA) for pitolisant (Wakix®) as a new human medicine (EMA 2014).

### 2.3.2 Cognitive deficits

In animal models, H3R antagonists improve cognitive performance probably by increasing the release of histamine and enhancing histamine-mediated attention and vigilance (Cownt, *et al* 2005; Medhurst, *et al* 2007; Chauveau, *et al* 2014). As H3R also regulates the release of other neurotransmitters important in cognitive processes, e.g. acetylcholine and glutamate, it is plausible that H3R antagonism improves cognitive performance via increasing the release of these neurotransmitters.

**Alzheimer's disease** is the most common form of dementia associated with cognitive dysfunctions (Mattson 2004). *Post mortem* studies have revealed that there are deficits in the brain histaminergic system in Alzheimer's disease patients (Mazurkiewicz-Kwilecki and Nsonwah 1989; Panula, *et al* 1998). Histamine levels are lower in individuals with Alzheimer's disease compared to healthy controls e.g. in cortical areas, hippocampus and hypothalamus. Furthermore, there are neurofibrillary tangles in the TMN neurons of Alzheimer's disease patients (Airaksinen, *et al* 1991). Several H3R antagonists have been or are currently being investigated in clinical phase II trials in the treatment of cognitive dysfunctions in Alzheimer's disease, attention-deficit/hyperactivity disorder (ADHD) and schizophrenia (Tiligada, *et al* 2011). MK-3134 alone improved the cognitive impairment associated with scopolamine in healthy humans (Cho, *et al* 2011). The compound also had an additive positive effect on the scopolamine-induced cognition impairment when administered together with a cholinesterase inhibitor donepezil. However, another H3R antagonist MK-0249 over four weeks was not effective in improving cognitive function in Alzheimer's disease patients (Egan, *et al* 2012). GSK239512 displayed positive effects on attention and memory in Alzheimer's disease patients, but unsatisfactory level of tolerability (Nathan, *et al* 2013). However, by altering the dosing regimen, the tolerability might be improved. Another H3R antagonist ABT-288 has been evaluated for safety and tolerability in both young and elderly subjects (Othman, *et al* 2013) but the phase II results in Alzheimer's disease patients have not yet been published.

In addition to Alzheimer's disease, there are cognitive deficits in other disorders including ADHD. ADHD is a neuropsychiatric disorder that is characterized by inappropriate and persistent symptoms of inattention, impulsivity and/or hyperactivity, and motor restlessness. The published clinical evidence has not supported the effectiveness of H3R antagonists in the treatment of ADHD. MK-0249 was not effective in treatment of adult ADHD although it has been shown to have alertness-promoting affect in humans (Herring, *et al* 2012). Neither did another, highly selective, wakefulness-promoting H3R antagonist bavisant display significant clinical effects in the treatment of adults with ADHD (Weisler, *et al* 2012). However, a preliminary finding with pitolisant suggests that it might result in progressive improvement in adults with ADHD (Schwartz 2011).

**Schizophrenia** is a chronic and disabling mental brain disorder characterized by a wide range of psychological symptoms including delusions and altered social behavior. In addition, higher cognitive functions are often disturbed in schizophrenia. It has been demonstrated that the histaminergic system is altered in patients with schizophrenia. The level of *tele*-methylhistamine in the CSF, representing the released and metabolized histamine, has been reported to be increased in individuals with schizophrenia (Prell, *et al* 1995). In addition, H3R binding was increased in the dorsolateral PFC in patients with schizophrenia (Jin, *et al* 2009). Interestingly, ABT-288 is tolerated at significantly higher doses in subjects with schizophrenia than in healthy volunteers (Othman, *et al* 2014). However, ABT-288 has been associated with an increased incidence of psychosis- and sleep-related adverse events in a phase II trial (Haig, *et al* 2014). Furthermore, neither ABT-288 nor MK-0249 resulted in cognitive improvement in adults with schizophrenia (Egan, *et al* 2013; Haig, *et al* 2014).

Taken together, H3R antagonists may have cognition improving properties in disorders including Alzheimer's disease, ADHD and schizophrenia. Nevertheless, to date, the clinical data available indicates that the therapeutic potential of H3R antagonists in cognitive deficits is rather weak. However, although the effects of H3R antagonists alone might be inadequate as cognition enhancers, they may potentially have additive effects when used in combination with other drugs.

### 2.3.3 Motor dysfunctions

**Tourette syndrome** is a neurological disorder characterized by repetitive, stereotyped, involuntary movements and vocalizations termed motor and vocal tics, respectively. Individuals with Tourette syndrome also display deficits in sensorimotor gating (Swerdlow 2013). Genetic evidence has indicated that a dysfunction of the histaminergic system may be important in the pathophysiology of Tourette syndrome. The occurrence of Tourette syndrome in two generations of a family with eight children was associated with a highly penetrant, rare but functional mutation in a gene coding for HDC (*hdc*) (Ercan-Sencicek, *et al* 2010). Later studies conducted in patients with Tourette syndrome and HDC KO mice have thereafter supported the role of the brain histaminergic system in Tourette syndrome (Fernandez, *et al* 2012; Karagiannidis, *et al* 2013; Castellan Baldan, *et al* 2014). These studies suggested that tics could possibly be treated by increasing histaminergic activity. A case study reported that EDS was decreased by pitolisant in a patient suffering from both Tourette syndrome and narcolepsy, but there was no clear decrease in tics (Hartmann, *et al* 2012).

**Parkinson's disease** is a progressive disorder characterized by motor disturbances including bradykinesia, hypokinesia and impaired bilateral coordination as a result from the degeneration of the dopaminergic neurons in the substantia nigra leading to a depletion of dopamine in the striatum. Parkinson's disease has also been associated with increased histamine levels in the basal ganglia (Rinne, *et al* 2002). Furthermore, the morphology of the histaminergic fibers is altered and the density of histaminergic fibers in the substantia nigra is increased in Parkinson's disease (Anichtchik, *et al* 2000). The increased histamine levels and histaminergic innervation might reflect compensatory events due to dopamine deficiency (Panula and Nuutinen 2013). Individuals with Parkinson's disease often suffer from insomnia which may be an outcome of the increased histaminergic tone. Interestingly, the TMN neurons are excited by the commonly used anti-parkinsonian treatment L-DOPA and are able to take up and decarboxylate L-DOPA to dopamine (Yanovsky, *et al* 2011). Thus, the histaminergic neurons can co-release dopamine and histamine from their widely distributed projections. There is also an increase of H3R binding density in the substantia nigra of individuals with Parkinson's disease (Anichtchik, *et al* 2001) while there is no difference in H3R binding in other parts of basal ganglia, including the striatum and globus pallidus between healthy controls and patients with Parkinson's disease (Goodchild, *et al* 1999). In rodent models of Parkinson's disease, it has been shown that H3R ligands have effects on motor coordination and apomorphine-induced stereotyped behaviors (Anichtchik, *et al* 2000; Gomez-Ramirez, *et al* 2006; Nowak, *et al* 2008; Nowak, *et al* 2009). However, there is no clinical data supporting the use of H3R antagonists for motor disturbances in Parkinson's disease. Nevertheless, pitolisant has been shown to reduce sleepiness in patients with Parkinson's disease, while the motor symptoms remained unaffected (Schwartz 2011).

**Huntington's disease** is an inherited neurodegenerative disorder affecting muscle coordination and cognitive functions associated with the loss of striatal and cortical projection neurons (Chen, *et al* 2013). The imbalance in brain dopaminergic functions contribute to abnormal movements and cognitive deficits in Huntington's disease. In Huntington's disease patients, TMN volume and neuronal number is unaltered, whereas HDC mRNA is increased and striatal H3R mRNA decreased (van Wamelen, *et al* 2011). In addition, H3R binding is lower in Huntington's disease patients compared to healthy controls in the caudate putamen and globus pallidus (Goodchild, *et al* 1999). However, there are no published studies on the possible effects of H3R antagonists in the treatment of Huntington's disease.

### 2.3.4 Drug addiction

It was found already in the 1970's that histamine modulates reinforcement in experimental animals (Cohn, *et al* 1973). These studies formed the basis for the hypothesis that histamine plays an inhibitory role in reward and opposes the effect of dopamine in reward and reinforcement. Histamine injected to the lateral hypothalamus of rats inhibited electrical self-stimulation at the injection site without affecting self-stimulation in the contralateral lateral hypothalamus. This effect was blocked by prior treatment with H1R antagonist pyrilamine. Self-stimulation is also increased in TMN lesioned rats, suggesting that destruction of the histaminergic neurons increases addictive behaviors (Wagner, *et al* 1993; Wagner, *et al* 1993).

In support of the inhibitory role of histamine in reward, it has been demonstrated that the histamine precursor, L-histidine, attenuates morphine-induced place preference, while an HDC inhibitor,  $\alpha$ -fluoromethylhistidine ( $\alpha$ -FMH), potentiates morphine reward (Suzuki, *et al* 1995). HDC KO mice also display stronger morphine-induced (Gong, *et al* 2010) and alcohol-induced CPP (Nuutinen, *et al* 2010) compared to control mice. However, contradictory findings to the reward inhibitory role of histamine have also been published. Surprisingly,  $\alpha$ -FMH infused to the lateral hypothalamus decreased, rather than increased self-stimulation (Zimmermann, *et al* 1997). Furthermore, in oppose to the inhibitory function of histamine in reward, HDC KO mice have been shown to be less stimulated by cocaine whereas no differences were observed between HDC KO and control mice in cocaine-induced CPP (Brabant, *et al* 2007).

Systemically injected acute alcohol has no effect on the histamine concentration in the mouse hypothalamus, whereas the level of *tele*-methylhistamine is increased after high doses of alcohol, suggesting that alcohol affects histamine turnover in the brain (Itoh, *et al* 1985; Zimatkin and Anichtchik 1999). In rats, it has been shown that alcohol-induced motor impairment and alcohol sensitivity, as measured by the tilting plane test, can be increased by lowering the brain histamine content with  $\alpha$ -FMH (Lintunen, *et al* 2002). However, no differences were observed between HDC KO and WT mice in the accelerating rotarod test, another measure for alcohol-induced motor impairment (Nuutinen, *et al* 2010).

The association between H3R and alcohol consumption was first found using alcohol-preferring AA rat line (Lintunen, *et al* 2001; Lintunen, *et al* 2002). These studies demonstrated that the histamine levels of the AA rats were elevated compared to those in alcohol-avoiding ANA rats. H3R antagonists thioperamide and clobenpropit also decreased, whereas an H3R agonist R- $\alpha$ -methylhistamine increased, alcohol consumption. Clobenpropit has also been shown to inhibit morphine-induced CPP in a dose dependent manner (Wang, *et al* 2009) and morphine increases the turnover of neuronal histamine (Nishibori, *et al* 1985).

The results of the role of histamine and H3R antagonism on the effects of psychostimulants deviate from those obtained with alcohol and morphine. Acute cocaine has been shown to increase the striatal histamine and HNMT activity (Ito, *et al* 1997). Interestingly however, thioperamide induces place preference with a low dose of cocaine, which is inactive *per se* (Brabant, *et al* 2005) and increases cocaine-induced locomotor stimulation (Brabant, *et al* 2009). Repeated administration of methamphetamine also increases HDC activity in the striatum and cortex and the release of histamine in the cortex (Ito, *et al* 1996; Dai, *et al* 2004). H3R antagonists clobenpropit and thioperamide also potentiate methamphetamine self-administration and methamphetamine-induced accumbal dopamine release (Munzar, *et al* 2004). The contradictory results for the role of H3R in alcohol and morphine reward in comparison to results obtained with psychostimulants might therefore arise from a pharmacokinetic interaction (Panula and Nuutinen 2013). This hypothesis is supported by a study demonstrating that cocaine concentrations in the plasma are elevated by coadministration of thioperamide (Brabant, *et al* 2009). Importantly, both methamphetamine and cocaine are metabolized by liver microsomal CYP450 enzymes which are a common pathway of metabolism for both psychostimulants and imidazole-based compounds such as clobenpropit and thioperamide. As the common liability of an imidazole-containing compound is the inhibition of CYP450 enzymes, the interaction may lead to higher concentration of the psychostimulants in the blood.

There are only few human studies on the role of histamine in addiction. In methamphetamine dependent subjects, H1R antagonist hydroxyzine had no effect over placebo in reducing methamphetamine use, retaining patients in treatment or reducing methamphetamine craving (Ling, *et al* 2012). *Postmortem* studies from alcoholic subjects have suggested that the brain histamine turnover is altered due to high alcohol consumption, with alcoholics having elevated histamine content in the grey matter (Alakarppa, *et al* 2002). Additionally, another study demonstrated increased concentrations of histamine and *tele*-methylhistamine and down-regulation of H3R binding in the brains of patients with liver cirrhosis (Lozeva, *et al* 2003). The majority of the cirrhotic patients were alcoholics. Interestingly, the histaminergic system has also been associated with alcohol addiction via genetic polymorphism (Oroszi, *et al* 2005; Reuter, *et al* 2007). A common and functionally significant polymorphism in the HNMT gene results in a Thr105Ile substitution of the protein encoded, leading to approximately 2-fold higher enzyme activity. These studies on the polymorphism of the HNMT have revealed an association between alterations in the brain histamine and vulnerability to alcoholism.

Although the human data on the role of histamine, H3R and addiction is very limited, the preclinical data obtained in this thesis suggests that H3R antagonists might be an interesting novel approach to treat alcohol addiction and this hypothesis should be tested in humans.

## 2.4 The brain dopaminergic system

There are five abundant biogenic amines in the human body: dopamine, noradrenaline, adrenaline, serotonin and histamine. Of these, noradrenaline, adrenaline and dopamine are catecholamines with dopamine being most abundant catecholamine neurotransmitter in the brain, constituting about 80 % of the catecholamine content in the brain. Dopamine was initially identified as the metabolic precursor of noradrenaline but in the 1950s it was recognized as a neurotransmitter in the mammalian brain (Carlsson, *et al* 1957; Carlsson, *et al* 1958).

Like other catecholamines, dopamine is derived from L-tyrosine. In the first enzymatic reaction tyrosine hydroxylase (TH) catalyses L-3,4-dihydroxyphenylalanine (L-DOPA) from L-tyrosine (Nagatsu, *et al* 1964). TH is the rate-limiting enzyme of this pathway. L-DOPA is then converted to dopamine by aromatic L-amino acid decarboxylase (AADC) (Lovenberg, *et al* 1962). Following its synthesis, dopamine is packed into synaptic vesicles via VMAT2. Once released from the presynaptic nerve terminals, the action of dopamine is terminated by reuptake by sodium ( $\text{Na}^{2+}$ )-dependent dopamine transporter (DAT) (Nirenberg, *et al* 1997). The two major enzymes involved in dopamine metabolism are monoamine oxidase (MAO) and catechol O-methyltransferase (COMT) (Napolitano, *et al* 1995).

Dopaminergic projections originate from the mesencephalic neurons from the substantia nigra and VTA and the dopaminergic neurons innervate several regions of the brain. The dopaminergic neurons project mainly to striatum, PFC and amygdala, of which the two first ones are implicated in the pathophysiology of numerous neurological diseases including addiction (Swanson 1982; Lammel, *et al* 2014). More specifically, the cell bodies of the dopamine neurons innervating the caudate putamen are located in the substantia nigra (nigrostriatal pathway), while the dopaminergic neurons projecting to the nucleus accumbens and limbic structures are located more medially in the VTA (mesolimbic pathway) (Girault and Greengard 2004).

Dopaminergic neurons are pacemakers and spontaneously active at low frequencies (around 5 Hz) providing a basal dopaminergic tone to their target neurons that may be rapidly adjusted by either phasic bursts or transient pauses of activity (Tritsch and Sabatini 2012). Dopamine cannot be characterized as neither excitatory nor inhibitory neurotransmitter, but functions rather as a neuromodulator altering the responses of its target neurons (Girault and Greengard 2004). The dopaminergic system is involved in several CNS disorders including Parkinson's disease, Tourette syndrome and addiction.

### 2.4.1 Receptors

Dopamine activates multiple GPCR subtypes that are heterogeneously distributed and engage different intracellular cascades (Tritsch and Sabatini 2012). Dopamine receptors D1R-D5R are distinct but closely related and can be divided into two major classes based on their structural, pharmacological and signaling characteristics. These families are termed D1-like and D2-like receptors. D1R and D5R belong to the D1-like family whereas D2R, D3R and D4R belong to the D2-like family. Receptors share a high level of homology inside the D1-like and D2-like receptor families at a protein level and pharmacological ligands may readily distinguish between the receptor families. However, ligand specificity between individual receptor subtypes within a receptor family is often lacking (Beaulieu and Gainetdinov 2011). Dopamine has higher affinity for the D2-like receptors, and the affinity is especially high for the short splice variant  $D_{2s}$ -receptor, which is mainly located presynaptically (Usiello, *et al* 2000).

D1R and D2R stimulate and inhibit AC, respectively. However, even though D1R and D2R have opposite effects on the molecular level, they often have a synergistic function when more complex outputs are considered (Girault and Greengard 2004). D1Rs and D2Rs are most prominently expressed in the caudate putamen, nucleus accumbens and olfactory tubercle (Vallone, *et al* 2000). In the striatum, as already described earlier, the direct striatonigral pathway contains D1Rs, whereas the indirect striatopallidal pathway expresses D2Rs. Although D1Rs and D2Rs are predominantly expressed in different MSN subpopulations, recent studies have demonstrated that they can also coexist within the same MSNs expressing both dynorphin and enkephalin (Perreault, *et al* 2010). However, it has been estimated that the number of cells containing both D1R and D2R is less than 5 % (Matamalas, *et al* 2009). These D1R/D2R coexpressing neurons are likely to contribute to the maintenance of the homeostatic balance between the direct and indirect pathways.

### 2.4.2 Signaling

Dopaminergic receptors activate numerous signal transduction pathways; nevertheless the best-characterized ones include the dopamine-mediated activation or inhibition of the cAMP pathways and the modulation of  $Ca^{2+}$ -signaling.

D1R is positively coupled to AC through  $G_{\alpha_s}$  proteins. AC activates cytosolic cAMP production which in turn leads to activation of PKA and phosphorylation of various intracellular downstream targets (Herve, *et al* 1995). In contrast, D2R is negatively coupled to AC through  $G_{\alpha_{i/o}}$  proteins. The activation of D2R therefore leads to inhibition of AC and reduction of cytosolic cAMP production (Stoof and Keibian 1984). D2Rs also modulate the intracellular  $Ca^{2+}$  levels by

acting on ion channels or by triggering the release of intracellular  $\text{Ca}^{2+}$  storages (Beaulieu, *et al* 2007). Interestingly, recent findings demonstrate that neurons that contain both D1Rs and D2R are able to form D1R-D2R heteromers that couple to the  $\text{G}\alpha_q$  protein (Rashid, *et al* 2007). This enables dopamine to have various effects on second messengers, depending whether the given neuron contains D1Rs, D2Rs or D1R-D2R heteromers.

There are three major signaling cascades involved in the regulation of signal transduction in response to dopamine: PKA-DARPP-32, ERK1/2 and AKT-GSK3 pathways. These cascades are shortly described next and illustrated in Figure 5.

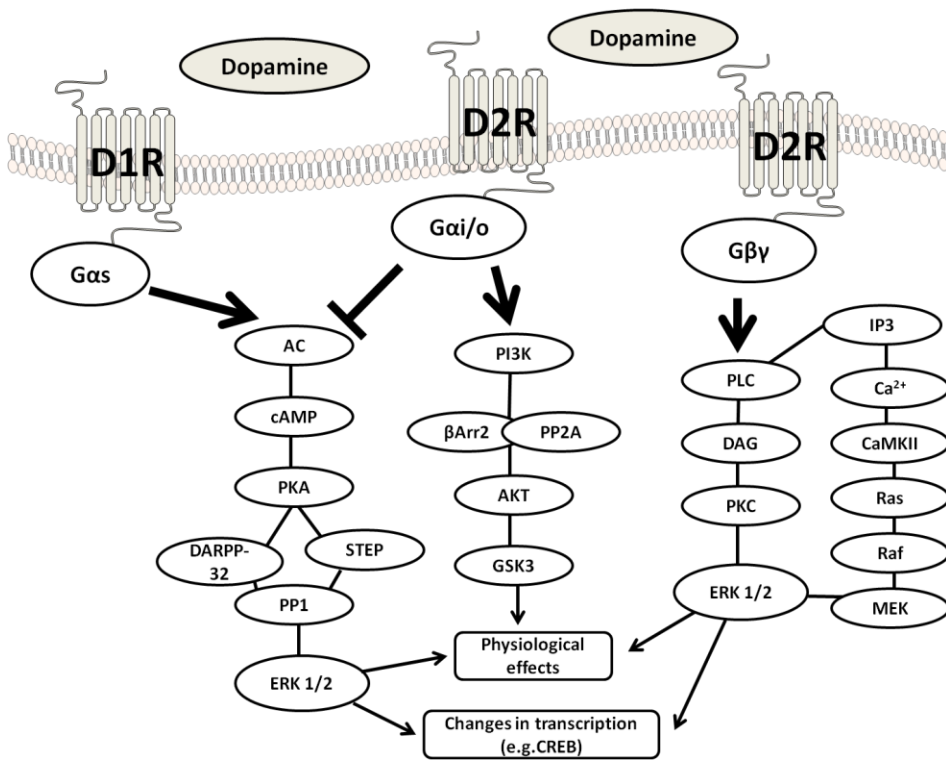
**Dopamine- and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32)** is the best-characterized substrate of dopaminergic signal transduction. DARPP-32 is highly enriched in the striatum in both striatonigral and striatopallidal MSNs (Ehrlich, *et al* 1990; Ouimet, *et al* 1998). DARPP-32 can be phosphorylated in several different amino acid residues, with threonine-34 ( $\text{Thr}^{34}$ ) and threonine-75 ( $\text{Thr}^{75}$ ) being the most common targets of phosphorylation (Girault 2012). In the striatonigral MSNs, DARPP-32 is phosphorylated on  $\text{Thr}^{34}$  upon stimulation of D1Rs, whereas in striatopallidal MSNs the same response is triggered by D2R antagonism (Bateup, *et al* 2008). Activation of D1R and the following PKA-mediated phosphorylation of DARPP-32 at  $\text{Thr}^{34}$  leads to inhibition of protein phosphatase 1 (PP1) (Walaas, *et al* 1983; Hemmings, *et al* 1984). PP1 is important in a wide range of biological processes, from muscle contraction to memory formation (Choy, *et al* 2012). In contrast, D2R activation-mediated inhibition of AC leads to dephosphorylation of DARPP-32 at  $\text{Thr}^{75}$  (Nishi, *et al* 1997). Importantly, when DARPP-32 is phosphorylated at  $\text{Thr}^{75}$  it becomes an inhibitor of PKA (Bibb, *et al* 1999). Thus, regulation of DARPP-32 phosphorylation at either  $\text{Thr}^{34}$  or  $\text{Thr}^{75}$  appears to function in a bidirectionally and modulate dopaminergic signal transduction in a complementary manner (Bateup, *et al* 2008). Importantly, this complex positive feedback loop potentiates dopaminergic signaling. There is increasing evidence of DARPP-32 being the mediator of the effects of different drugs of abuse (Nairn, *et al* 2004; Girault 2012). For example, alcohol, amphetamine, opioids and cocaine induce phosphorylation of DARPP-32 at  $\text{Thr}^{34}$  and dephosphorylation at  $\text{Thr}^{75}$ . In addition to dopamine, DARPP-32 is regulated by e.g. serotonin and glutamate, which is why different drugs of abuse may have different effects on DARPP-32 phosphorylation.

**The extracellular signal-regulated kinase (ERK1/2)**, which belongs to the MAPK-family, is another important mediator of striatal signal transduction downstream from cAMP. ERK1/2 regulates several important cellular functions in the brain, including cell differentiation and synaptic plasticity (Thomas and Huganir 2004). ERK1/2 is also implicated in drug addiction. In striatal MSNs ERK1/2 is activated by many drugs of abuse including cocaine, amphetamine and morphine (Valjent, *et al* 2000; Valjent, *et al* 2004; Valjent, *et al* 2006).

Although these drugs have different mechanisms of action, they all share the property of increasing dopamine release. The regulation of ERK1/2 by drugs of abuse involves the cross-talk between the D1R and NMDA glutamate receptors (Valjent, *et al* 2005; Cahill, *et al* 2014). ERK1/2 phosphorylation can be prevented by both D1R antagonists or by knockout of D1R but also by an NMDA antagonist. This indicates that ERK1/2 activation requires the stimulation of both D1Rs and NMDA receptors. Interestingly, however, although ERK1/2 activation is mainly controlled by D1Rs and NMDA receptors, there are several other important pathways involved which are, to date, far from being fully elucidated. There is for instance evidence, that mGluR5 receptors may also activate ERK1/2 (Mao, *et al* 2005; Voulalas, *et al* 2005) and interestingly, D2R activation also phosphorylates ERK1/2 (Yan, *et al* 1999; Cai, *et al* 2000; Wang, *et al* 2005), via cAMP-independent mechanisms. D2R signaling responses may also be mediated by the G $\beta\gamma$ -subunits, which in turn activate PLC and increase cytoplasmic Ca<sup>2+</sup> concentrations in the MSNs (Hernandez-Lopez, *et al* 2000). Ca<sup>2+</sup> has complex effects on cell signaling, one of them being activation of Ca<sup>2+</sup>/calmodulin - dependent protein kinase II (CaMKII) (Beaulieu and Gainetdinov 2011). CaMKII can activate ERK1/2 via the Ras-Raf-MEK cascade (Fasano, *et al* 2009; Baik 2013). Additionally, PLC regulates diacylglycerol (DAG)-mediated activation of PKC. PKC, in turn, is involved in the activation of ERK1/2 cascade.

As described above, dopamine receptors regulate cAMP-PKA and Ca<sup>2+</sup> - dependent pathways through G protein-mediated signaling. However, recent research on the field has revealed that dopamine receptors exert their effects also through cAMP-independent mechanisms. One of the other pathways utilized by at least by D2Rs is termed the **AKT-GSK3** signaling cascade (Beaulieu, *et al* 2007). The activation of AKT is initiated via GPCRs and plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation and transcription. AKT is a serine/threonine-specific protein kinase that is regulated through P13K-mediated signaling (Scheid and Woodgett 2001). The dopaminergic activation of AKT-GSK3 signaling pathway is independent of cAMP but requires the recruitment of the multifunctional adaptor proteins termed arrestins (Beaulieu and Gainetdinov 2011).  $\beta$ -arrestin 2 ( $\beta$ -Arr2) has been shown to act as a signaling intermediate in the AKT-GSK3-pathway. Upon D2R activation  $\beta$ -Arr2 forms a protein complex with protein phosphatase 2A (PP2A). Activation of AKT inhibits glycogen synthase kinases-3 $\alpha$  (GSK3 $\alpha$ ) and -3 $\beta$  (GSK3 $\beta$ ). GSK3 $\alpha$  and GSK3 $\beta$  are closely related kinases which were originally associated with the regulation of glycogen synthesis in response to insulin (Frame and Cohen 2001). However, more recently their functions in e.g. cell proliferation, cell differentiation and apoptosis have been acknowledged.

Originally, the role of AKT-mediated responses in dopaminergic cell signaling was observed in mice lacking DAT (DAT KO mice) (Beaulieu, *et al* 2004; Beaulieu, *et al* 2006). It was found that the persistently elevated extracellular dopamine in DAT KO mice leads to reduction of AKT phosphorylation and concomitant activation of both GSK3 $\alpha$  and GSK3 $\beta$  in the striatum. In addition, D2R antagonist haloperidol induces AKT phosphorylation and inhibition of GSK-3 and individuals with schizophrenia have lower AKT levels in the frontal cortex (Emamian, *et al* 2004). Additionally, AKT KO mice display increased sensitivity to the sensorimotor gating -disruptive effect of amphetamine, further supporting the role of AKT in the symptoms related to e.g. schizophrenia.



**Figure 5.** Signaling pathways mediated via D1Rs and D2Rs. Abbreviations: AC, adenylyl cyclase; AKT, protein kinase B;  $\beta$ Arr2,  $\beta$ -arrestin 2; CaMKII, Ca<sup>2+</sup>/calmodulin -dependent protein kinase II; cAMP, cyclic AMP; CREB, cAMP response element-binding protein; DAG, diacylglycerol; DARPP-32, dopamine-and cAMP-regulated phosphoprotein; ERK 1/2, extracellular signal-regulated kinase 1/2; GSK3, glycogen synthase kinase 3; IP3, inositol trisphosphate 3; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; STEP, striatal-enriched protein tyrosine phosphatase 61. For a detailed description of these pathways see: Baik 2013, Beaulieu, *et al* 2007, Beaulieu and Gainetdinov 2011, Girault 2012.

### 2.4.3 Drug addiction

It was first found already in 1950's that electrical stimulation in the septal area (medial olfactory area) of the rat was rewarding (Olds and Milner 1954). Later, it became apparent that dopamine has a crucial role in the intracranial self-administration in the ventral tegmentum, nucleus accumbens and medial PFC (Phillips and Fibiger 1978; Fibiger and Phillips 1988). These groundbreaking studies have paved the way for understanding the reward circuit in the brain, although it is still far from being understood with all its complexities. However, dopamine is recognized as one of the most important neurotransmitters in the field of addiction due to its crucial role in motivational control (Wise and Rompre 1989). In spite the fact that different drugs of abuse act through different mechanisms, virtually all abused drug share the property of increasing dopaminergic activity acutely (Di Chiara and Imperato 1985; Di Chiara and Imperato 1986; Imperato, *et al* 1986). In line with this, dopamine antagonists block the maintenance of self-administration of drugs of abuse (Yokel and Wise 1976; Bergman, *et al* 1990).

The mesolimbic dopamine system is the best-characterized reward circuit in the brain (Wise 1998; Wise 2002). However, the reward circuitry includes many other neurotransmitter systems in addition to dopamine. For example, the striatum receives histaminergic innervation from the TMN and dense glutamatergic innervation from the PFC, amygdala and hippocampus. The functional output from these brain regions is also regulated by both GABAergic and cholinergic interneurons. In addition, all these structures receive both serotonergic and noradrenergic input from the raphe nuclei and locus coeruleus, respectively.

The dopaminergic neurons have two distinct modes; the tonic and phasic dopaminergic transmission (Grace 1991). In the tonic mode, dopamine neurons have a steady, basal activity (2-10 Hz) that is vital for the function of their downstream neural structures (Schultz 2007). In the phasic mode, the dopaminergic neurons rapidly increase or decrease their firing for 100-500 ms that induces substantial changes in the dopamine concentrations of the downstream structures (Schultz 1998; Schultz 2007). The dopaminergic burst spiking phasic mode can lead up to 15-30 Hz spiking and is triggered by for example by rewards and reward-related sensory cues (Schultz, *et al* 1997; Wise 2004; Schultz 2007). The phasic dopaminergic responses are therefore thought to play an important role in dopamine-driven motivational control and reinforcement.

Chronic use or administration of drugs of abuse leads to long-term adaptive changes in the neurochemistry of the brain, including alterations in the gene expression (Freeman, *et al* 2008; Schmidt, *et al* 2012). Additionally, chronic use may induce changes in the anatomy of the brain, especially in the morphology of the dendritic spines (Robinson, *et al* 2001; Robinson, *et al* 2002;

Crombag, *et al* 2005). These long-lasting alterations may underlie some of the persistent psychomotor, cognitive and motivational consequences of chronic drug exposure.

## **2.5 The interaction between the H3R and the dopaminergic system**

Interestingly, both histaminergic and dopaminergic systems are involved in the pathophysiology of several neurological and psychiatric disorders, including Parkinson's disease, Tourette syndrome and addiction (Panula and Nuutinen 2013). Clarifying histamine-dopamine interactions in the striatum may shed new light on understanding the function of these circuits and may provide a more comprehensive view on the pathophysiology of several disorders. The evidence for histamine-dopamine interaction involves mainly H3Rs and dopamine receptors, which is not surprising, considering that these receptors are all highly expressed in the striatum and the majority of the striatal D1R- or D2R-containing MSNs coexpress H3Rs (González-Sepúlveda, *et al* 2013).

### **2.5.1 H3R-mediated dopamine and GABA release**

The first evidence of H3R-dopamine interaction arose from a study where it was shown that histamine inhibits dopamine release in the mouse striatum via presynaptic H3Rs (Schlicker, *et al* 1993) suggesting that H3R would be located on the dopaminergic nigrostriatal nerve terminals. Furthermore, H3R activation by immapip inhibits dopamine synthesis in rat striatum (Molina-Hernandez, *et al* 2000). Both histamine and H3R agonist immapip have also been shown to inhibit D1R-dependent [3H]-GABA release from rat striatal slices and the inhibitory effects of both histamine and immapip can be reversed by the H3R antagonist thioperamide (Arias-Montano, *et al* 2001). In addition, immapip inhibits, in a concentration-dependent manner, D1R agonist SKF-81297-induced cAMP accumulation in striatal slices (Sanchez-Lemus and Arias-Montano 2004).

An interesting feature of H3R antagonists is their ability to increase the release of dopamine in the PFC and other cortical areas (Schlicker, *et al* 1994; Fox, *et al* 2005; Medhurst, *et al* 2007; Southam, *et al* 2009), but not to affect the striatal dopamine release (Fox, *et al* 2005; Giannoni, *et al* 2010; Galici, *et al* 2011; Schwartz 2011). Considering that the striatal H3Rs are mainly located postsynaptically on the MSNs, this is not surprising. As already described earlier, the vast majority of both D1R (95 %) and D2R (89 %) containing striatal neurons also contain H3Rs (Moreno, *et al* 2011; González-Sepúlveda, *et al* 2013).

On the other hand, both D2Rs and H3Rs are coupled to  $G\alpha_{i/o}$  protein in the indirect MSN pathway, which might also lead to co-operation of these two receptors, whereas in the direct pathway D1Rs is coupled to  $G\alpha_s$  protein, possibly leading to opposing effects compared to those of H3Rs. In support of this, both D2R and H3R agonist decreased striatal GABA release, whereas D1R agonist increased it (Arias-Montano, *et al* 2001). The study was performed in dopamine-depleted, reserpinized rats, thus indicating that the demonstrated effects arose from the postsynaptic site. Thus, the striatal H3R potentiates the D2R-mediated inhibition of the indirect pathway and hinders the D1R-mediated excitation of the direct pathway.

### 2.5.2 H3R-containing heterodimers

The interaction between the H3R and dopamine receptors is much more complex than described above, as recent evidence have demonstrated that both D2Rs (Ferrada, *et al* 2008) and D1Rs (Ferrada, *et al* 2009) may form functional heterodimers with H3Rs. The existence of H3R-D2R heterodimers was demonstrated *in vitro* in co-transfected cells and further supported by membrane competition experiments, where it was shown that several H3R agonists significantly decreased the affinity of D2R agonist (Ferrada, *et al* 2008). Indirect functional evidence from behavioral studies also support the presence of D2R-H3R heterodimers *in vivo* (Pillot, *et al* 2002; Ferrada, *et al* 2008).

The existence of D1R-H3R heterodimers was also first demonstrated on co-transfected cells *in vitro* (Ferrada, *et al* 2009). It was shown that H3R activation leads to diminished D1R agonist binding and to a significant change in the affinity of the D1R. Interestingly, it was also found that when H3R dimerizes with D1R, there is a switch to  $G\alpha_{i/o}$ -coupling, whereas under normal conditions D1R is coupled to AC via  $G\alpha_s$  protein. The consequence is that whereas normally dopamine activates the direct pathway via D1Rs, in the presence of D1R-H3R heterodimers it inhibits this pathway. Thus, an antagonist of one of the receptor of the D1R-H3R heterodimer can induce conformational adaptations and inhibit the specific signals. The existence of D1R-H3R heterodimers has also been demonstrated in striatal slices suggesting that these heterodimers are also present *in vivo* (Moreno, *et al* 2011). Moreover, in striatal slices H3R activation by imetit leads to activation of ERK1/2 only when the D1R is expressed, but not in D1R deficient mice. This indicates that in the striatum ERK1/2 phosphorylation induced by H3R activation is mediated by D1R-H3R heterodimers. However, the prevalence and significance of either D2R-H3R or D1R-H3R striatal heterodimers *in vivo* is currently not known, as is not known to what extent these heterodimers contribute to the dopaminergic regulation in general.

### **3. AIMS OF THE STUDY**

Previous studies have demonstrated that neuronal histamine and H3R might be involved in the behavioral effects of drugs of abuse. The major goal of this thesis was to clarify the role of histamine and especially H3R in alcohol-related behaviors and to investigate the interaction between the histaminergic and dopaminergic systems.

The specific topics that were studied in this thesis were the following:

1. The role of H3R in alcohol consumption and in the motor-impairing and rewarding effects of alcohol
2. The role of histamine in alcohol consumption and in the H3R-mediated inhibition of alcohol reward
3. The role of H3R in the place conditioning by dopaminergic drugs and in their motor effects
4. The role of histaminergic system in sensorimotor gating
5. The interaction between the histaminergic and dopaminergic systems on both transcriptional and signal transduction level

## 4. MATERIALS AND METHODS

The materials and methods of this thesis work are described in greater detail in the original publications (I-V). Only the methods that the candidate has personally conducted are shortly described in the following chapter. For the supplier information of materials including reagents, drugs and equipment the reader is referred to the original publications.

### 4.1 Experimental animals

The principles of the Finnish Act on the Use of Animals for Experimental Purposes were followed in conducting these studies and the protocols were approved by the National Animal Experiment Board of Finland. For ethical use of laboratory animals, the Russell and Burch's principle of the 3Rs (replacement, reduction and refinement) was applied (Russel and Burch, 1959).

#### 4.1.1 Genetically modified mouse strains

Both female and male mice were used in the studies where genetically modified mice were used. The genotypes of the mice were verified by DNA purification followed by PCR amplification before all experiments. Inbred WT and HDC KO mice in 129/Sv background were used in CPP, two-bottle choice test, locomotor stimulation, radioactive *in situ* hybridization and in plasma alcohol concentration measurements. The generation of HDC gene deletion has been described in detail (Ohtsu, *et al* 2001). Briefly, linearized targeting construct was electroporated into R1 embryonic stem cells (ES cells) that were originally derived from 129/Sv mouse embryo (Nagy, *et al* 1993). The chimeric mice, generated with the confirmed embryonic stem cells, were then crossed with 129/Sv mice to obtain the inbred heterozygous (+/-) mice. HDC KO mice were a gift from Prof. Hiroshi Ohtsu and were bred in heterozygous crosses in these experiments. After being backcrossed to the C57BL/6J background strain in our laboratory, HDC KO and WT mice were used in the DID paradigm as this protocol is developed for C57BL/6J mice due to their high natural alcohol drinking preference (Rhodes, *et al* 2005). Single nucleotide polymorphism analysis was used to analyze that the strains had at least 99.5 % identity with C57BL/6J. This background strain was also used in the PPI study.

H1R KO mice were a gift from Prof. Takeshi Watanabe and they were generated in the Riken Research Center for Allergy and Immunology by homologous recombination (Inoue, *et al* 1996). Briefly, linearized targeting vector was electroporated in E14 ES cells and ES cells carrying the H1R mutation were injected into blastocysts from C57BL/6 mice and the resulting male chimeras were mated with C57BL/6 mice.

Male H3R KO mice were originally supplied by Janssen Research & Development, LLC (La Jolla, CA, USA). They were generated on a background of 129/Ola and C57BL/6J mice (Toyota, *et al* 2002). Selective backcrossing was conducted in Janssen over 10 generations leading to at least 99.5 % genetic identity to C57BL/6J mice.

#### **4.1.2 Commercially available mouse strains**

In the pharmacological studies where exclusively commercial, inbred WT mice were used, only male mice were studied. Inbred WT JAX®DBA/2J mice were delivered from Charles River and C57BL/6J mice were delivered from Harlan Laboratories at the age of 4-8 weeks. Mice were let to habituate with the change of the environment and new facility at least two weeks before starting the experiments.

#### **4.1.3 Drug treatments**

Drugs including alcohol were diluted with sterile saline (0.9 % NaCl) and injections were given intraperitoneally (i.p.) with an injection volume of 10 ml/kg. All drug doses correspond to the free bases of the drugs. Drinking solutions were prepared in cold tap water.

### **4.2 Behavioral studies**

#### **4.2.1 Locomotor activity assessment**

The locomotor activity was assessed using an empty cage, where the animal could freely move throughout the experiment. The activity of the mice was monitored using a video camera and Ethovision® software. Animals were brought to the experiment room 30 minutes prior to the start of the experiment. They were then placed individually to the empty plastic cages (40 x 26 x 20 cm) and habituated to the novel environment for 30-90 minutes depending on the experiment. After the habituation period animals received the studied drugs and were returned to the test cages for further analysis of locomotor activity.

### **4.2.2 Balance beam**

The balance beam experiment was used to study alcohol-induced motor impairment. Mice were trained to walk along a wooden beam (100 cm long, Ø 1.5 cm) placed at 80 cm height from the floor. Training was done three times during two consecutive days. On the experiment day the baseline performance of the mice was tested. H3R ligands were injected 25 minutes prior to the alcohol treatment. The possible effect of the pretreatment on the balance beam performance was tested before alcohol injection. The performance on the balance beam was tested again 10 minutes after alcohol injection (1.2 g/kg). The time to cross the beam was manually recorded with a timer. The mouse behavior during the test was recorded by a digital video camera and the number of foot slips was later measured from the video.

### **4.2.3 Rotarod**

Alcohol-induced motor impairment was also assessed using the accelerating rotarod method. Mice were trained daily (three training sessions in the morning, three training sessions in the afternoon) to run on an accelerating (5-30 rpm, Ø 3 cm) rotarod for three minutes until all animals learned the task. The training period lasted for seven consecutive days. The latency to fall from the rotating rod was manually recorded, and the daily average performance was calculated for each animal. On the experiment day, the baseline performance on the rotarod was first tested and the mice were then given pretreatment with the H3R ligand. The possible effect of pretreatment on rotarod performance was tested 30 minutes later. Thereafter, alcohol (1.5 g/kg) was administered and five minutes later the performance on rotarod was tested again. Immediately after, animals received a second alcohol injection (0.5 g/kg) and the motor performance was tested again after five minutes.

### **4.2.4 Place conditioning**

The CPP paradigm was used as a model for reward and reinforcement. It followed the principles of an unbiased, counterbalanced conditioning (Cunningham, *et al* 2006). Metal grid and plastic mat were used as tactile conditioning cues on the cage floors. The preference for the conditioning cues was tested prior to the experiments and no bias towards either of the floor materials was observed. The activity of the mice was recorded in each phase using a video camera attached to the Ethovision® software. Briefly, on the first day the mice were habituated with the procedure. Mice were weighted, given a saline injection according to their weight and placed into an empty conditioning cage for 5-30 minutes (five minutes in alcohol-CPP, 30 minutes in quinpirole-CPA and amphetamine-CPP). The conditioning sessions were conducted on the

following days. Mice were randomly assigned to the conditioning subgroups (metal or plastic cue). Mice in the metal cue -subgroup received the test drug (alcohol, quinpirole or amphetamine) paired with metal cue and saline paired with the plastic cue on alternating days. Mice in the plastic cue -subgroup received the test drug paired with the plastic cue and saline paired with the metal cue. H3R antagonists were administered 30 minutes prior to the test drug. Each mouse went through four to six 5-30 minutes long conditioning trials of both types (test drug and saline) on alternating days. The place preference test was carried out 24 hours after the last conditioning session. Immediately after saline injection mice were placed in the center of the cage in which they had free access to both metal and plastic cues. Time spent on different compartments of the cage during 15-30 minutes and the total distance moved were analyzed from the recorded data. Time spent on the metal compartment was used as a primary dependent variable in the data analysis.

#### **4.2.5 Drinking in the dark**

Alcohol consumption of H3R KO, HDC KO and WT mice (C57BL/6 background) was studied using the DID procedure with minor modifications (Rhodes, *et al* 2005). The DID paradigm can be used to study binge-like alcohol drinking in non-dependent mice. The light-dark cycle was reversed two weeks prior the experiment and the mice were single-housed for one to two weeks before the beginning of the experiment. In brief, two to three hours after the beginning of the dark period, water bottles were replaced with a graduated tube containing 20 % (v/v) alcohol and left in place for four hours. Control animals received 3-10 % (w/v) sucrose. The volume of alcohol and sucrose consumed was recorded two and four hours after the beginning of each drinking session. The alcohol consumption was converted to g/kg taking into account the density of alcohol (0.7894 g/cm<sup>3</sup>) and the body weight of each animal.

### **4.3 Biochemical studies**

#### **4.3.1 Plasma alcohol concentration measurement**

The plasma alcohol concentrations were studied in DBA/2J mice after pretreatment with H3R ligands followed by alcohol administration. Pretreatment was given 30 minutes prior to alcohol. Animals were euthanized by carbon dioxide (CO<sub>2</sub>) 10, 20, 100 or 150 minutes after alcohol injection and blood samples were collected via heart puncture. Samples were also collected from H3R KO and WT mice five minutes after alcohol injection and after the last DID session. Additionally, samples were collected from HDC KO and WT mice 10, 20, 100 and 150 minutes after alcohol injection. Blood was collected to cold lithium-heparin tubes and centrifuged at 20 000g for two minutes.

Plasma samples were then transferred to clean tubes and kept at -80°C until analyzed. A commercial enzyme assay was carried out to measure the alcohol concentrations of the plasma samples.

### 4.3.2 Radioactive *in situ* hybridization

The mRNA expression of different dopaminergic markers were analyzed by radioactive *in situ* hybridization. The detailed description of the radioactive *in situ* hybridization has been described previously (Lintunen, *et al* 1998). 16-20 µm cryosections were cut from unfixed, freshly frozen H3R KO, HDC and WT mouse brains and kept in a freezer (-80°C) until hybridization. The hybridization was carried out using selective and specific oligonucleotide probes which were designed using the Eprimer3 software and tested prior to experiments. The length of the oligonucleotide probes was 43 bases and the nucleotide sequences are presented in Table 2. Each probe was labeled with radioactive deoxyATP by using terminal deoxynucleotidyl transferase and purified with Sephadex G-50 columns. Sections were covered with the hybridization mixture containing 10 000 000 cpm/ml of the labeled, radioactive probe. Hybridization was carried out at +45°C for 16 hours. After hybridization, the slides were washed with 1 x saline-sodium citrate (SSC) at +55°C for 2 hours. Next, the sections were dehydrated in a series of alcohol. Dried sections were then exposed to films and developed after five to seven days. The expression of mRNAs was quantified with MCID4 Image Analysis Software. Radiocarbon <sup>14</sup>C-standard curve was used in the quantification. The results analyst was blinded to the genotype of the mouse. Cresyl violet staining was carried out in order to select matching striatal sections (Striatum +0.98 mm, VTA -3.52 mm from Bregma).

**Table 2.** Nucleotide sequences of the radioactive *in situ* hybridization probes designed by Eprimer3 software.

mRNA	Oligonucleotide sequence
D1 receptor	ATGGACTGCTGCCCTCTCAAAGCTGAGATGCGCCGGATTTGC
D2 receptor	GCTTTCTTCTCCTTCTGCTGGGAGAGCTTCTGCGGCTCATCG
TH	GGTAGGTTTGATCTTGGTAGGGCTGCACGGCTGCTGTGTCTGG
DAT	TCAGCCACGCTACAGTCTGCAGAGCCAACAGGCCTGCATTTCC
STEP61	AGGTATTCATGGGCTGACTCCTCTCGTGGGGACACCAGGTAGC
DARPP-32 (2 probes combined)	CCACACTCACTGGCGATCCCCGGATGTCAACTTCTGTCAGACC and GCTGGCTCCTTGGGAATCCAGTGGTAGCATGTGGGCTGAAAGG

### 4.3.3 Semi-quantitative Western blotting

Dopaminergic cell signaling was studied in H3R KO and WT mice by Western blotting. Mice were injected with saline, D1R agonist SKF-38393 or D2R agonist quinpirole and decapitated 20 minutes after drug injections. Prefrontal cortices (~1.5-3.5 mm from Bregma) and striata (~0.5-1.5 mm from Bregma) were dissected on ice, immediately frozen and kept in a freezer (-80°C) until analyzed. Samples were weighted and homogenized in lysis buffer by sonication. Homogenates were centrifuged (five minutes at 20 000g), and the protein concentration of the supernatant determined by Bio-Rad Protein assay. Samples (40-50 µg of total protein/well) were separated based on protein size by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane with eBlot® protein transfer system. Phosphorylated forms of ERK1/2 and AKT were detected with the mouse anti-phospho-ERK1/2 and rabbit anti-phospho-AKT primary antibodies. The unphosphorylated forms of the corresponding proteins were detected with rabbit anti-ERK1/2 and rabbit anti-AKT primary antibodies. All primary antibodies were manufactured by Cell Signaling and used at dilutions 1:1000. The signal was detected using infrared-labeled secondary antibodies at dilutions 1:10 000-1:30 000. For monoclonal primary antibodies goat anti-mouse IgG1 and for polyclonal primary antibodies goat anti-rabbit were used. The signal was detected using Odyssey® Imaging System and quantified with the LI-COR Image Studio software. The values were corrected by dividing the signal intensity of the phosphorylated protein with that of the corresponding unphosphorylated protein.

## 4.4 Data analysis

Statistical analyses were performed using GraphPad Prism 4 software. The Student's t-test was applied in comparison between two groups. Data containing more than two groups with only one variable was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's or Newman-Keuls post-hoc tests. Data with two variables was analyzed by two-way ANOVA followed by Bonferroni's post-hoc test.  $P < 0.05$  was considered significant. The results in graphs are expressed as mean  $\pm$  standard error of the mean (SEM).

## **5. RESULTS**

For more detailed description of results and statistical analysis of the data, the reader is referred to the original publications (I-V). The main behavioral results of this thesis work are combined in Tables 3, 4 and 5.

### **5.1 H3R in alcohol-related behaviors**

#### **5.1.1 H3R in CPP (I-III)**

Ciproxifan (3 mg/kg), JNJ-10181457 (5 mg/kg) and JNJ-39220675 (0.3 mg/kg) inhibited the development of alcohol-induced place preference (alcohol-CPP, 2 g/kg) in DBA/2J mice (Figure 1B in publication I, Figure 1A in III). Surprisingly, higher doses of JNJ-10181457 (10 mg/kg) and JNJ-39220675 (3 mg/kg and 10 mg/kg) had no effect on the development of alcohol-CPP (Figure 1A in publication III), indicating that only low doses of H3R antagonist are effective in blocking the rewarding properties of alcohol. H3R KO (in C57BL/6J background) did not develop alcohol-CPP (Figure 4B in publication II).

#### **5.1.2 H3R in alcohol consumption (II)**

In the two-bottle choice paradigm H3R KO mice consumed less alcohol (10 and 20 %) than the control mice (Figure 1A in publication II). There were no differences between the genotypes in the consumption of 3 or 6 % alcohol. In addition, in the DID paradigm H3R KO mice consumed less alcohol (20 %) (Figure 2A,B and C). Additionally, H3R antagonist ciproxifan (0.3, 1, 3 mg/kg) decreased alcohol (20 %) consumption in the DID model ((Figure 3A), whereas H3R agonist immepip (30 mg/kg) increased alcohol drinking in C57BL/6J mice (Figure 3C). Taken together, H3R KO mice consumed less alcohol in both voluntary and binge-like drinking paradigms compared to WT mice. In the binge-like drinking paradigm

#### **5.1.3 H3R in alcohol-induced motor functions (I-III)**

Ciproxifan (3 mg/kg) had no effect on the alcohol-induced locomotor stimulation when alcohol was injected at a dose 1.5 g/kg in either DBA/2J (Figure 2A and B in publication I) or in 129/Sv mice (Figure 4A in publication III). However, ciproxifan increased and extended alcohol stimulation, when a lower dose of alcohol (1 g/kg) was given to DBA/2J mice (Figure 2C and D in publication I). Ciproxifan did not alter the motor-impairing effects of alcohol in

neither balance beam nor rotarod, but H3R agonist immapip (10 mg/kg) further impaired the performance on the balance beam after alcohol (1.2 g/kg) as measured by the number of foot slips (Figure 3 in publication I). H3R KO mice were less stimulated by alcohol (1.5 g/kg) indicated by the lower distance moved compared to controls (Figure 5A and B in publication II) and less sensitive to the motor-impairing effects of alcohol, as measured by the number of foot slips on the balance beam (Figure 5F in publication II). JNJ-39220675 had no effect on alcohol-induced (1 or 1.5 g/kg) stimulation in DBA/2J (Figure 1C in publication III) or 129/Sv mice (Figure 4C in publication III). In DBA/2J background strain all three pretreatment doses of JNJ-39220675 (0.3, 3 and 10 mg/kg) were tested, whereas in 129/Sv we tested two doses of JNJ-39220675 (0.3 mg/kg and 10 mg/kg).

## **5.2 Histamine in alcohol consumption, stimulation and reward (III)**

HDC KO mice consumed as much alcohol as the WT control mice in both drinking paradigms used in these studies: the two-bottle choice and the DID. HDC KO and their WT control mice in the two-bottle choice paradigm mice were in the 129/Sv background strain. There was no differences in the alcohol consumption in any of the given alcohol concentrations (3, 6, 10 or 20 %)(Figure 3A in publication III). After backcrossing the HDC KO mice from 129/Sv to C57BL/6J background, the mice were used in the DID paradigm, which was able to replicate the finding; the lack of endogenous histamine does not affect alcohol consumption (Figure 1C and E). In addition, HDC KO mice were equally stimulated by alcohol as compared to the WT mice (Figure 4). Similarly to WT mice (in 129/Sv background), neither ciproxifan (3 mg/kg) nor JNJ-39220675 (0.3 or 10 mg/kg) altered the alcohol-induced stimulation in HDC KO mice as measured by the distance moved. Interestingly, in HDC KO mice neither ciproxifan (3 mg/kg), JNJ-10181457 (1 and 5 mg/kg) nor JNJ-39220675 (0.3 mg/kg and 10 mg/kg) inhibited alcohol-CPP (Figure 2A).

**Table 3.** Summary of the behavioral responses of HDC KO mice compared to WT mice.

<b>Background</b>					
<b>strain</b>	<b>Pretreatment</b>	<b>Drug</b>	<b>Paradigm</b>	<b>Result</b>	<b>Ref.</b>
129/Sv	–	Alcohol	LMA	Stimulation –	III
129/Sv	–	Alcohol	TBC	Consumption –	III
129/Sv	Ciproxifan	Alcohol	CPP	CPP –	III
129/Sv	JNJ-10181457	Alcohol	CPP	CPP –	III
129/Sv	JNJ-39220675	Alcohol	CPP	CPP –	III
129/Sv	Ciproxifan	Alcohol	LMA	LMA –	III
129/Sv	JNJ-39220675	Alcohol	LMA	LMA –	III
C57BL/6J	–	Alcohol	DID	Consumption –	III
C57BL/6J	–	–	PPI	PPI –	V

Abbreviations: CPP, conditioned place preference; DID, drinking in the dark; LMA, locomotor activity; PPI, prepulse inhibition; Ref., reference; TBC, two-bottle choice. Symbol: – no effect.

### **5.3 H3R in the behavioral effects of dopaminergic drugs**

#### **5.3.1 H3R in the behavioral effects of amphetamine (IV)**

Acutely administrated pretreatment with JNJ-39220675 (1 mg/kg and 10 mg/kg) inhibited locomotor activation induced by amphetamine (2 mg/kg) in C57BL/6J mice (Figure 1A in publication IV). Interestingly, when both drugs were injected subchronically over four consecutive days, JNJ-39220675 lost its inhibitory effect on the amphetamine-induced stimulation (Figure 1B). In other words, on the fourth day of conditioning, both doses of JNJ-39220675 pretreatment combined with amphetamine induced stimulation comparable to that of saline-pretreated amphetamine-group. There was a significant development of place preference by amphetamine (Figure 1D). JNJ-39220675 had no effect on place conditioning by amphetamine on either of the doses tested (1 mg/kg and 10 mg/kg).

### 5.3.2 H3R in the behavioral effects of quinpirole (IV)

D2R agonist quinpirole (0.5 mg/kg) -treated C57BL/6J mice spent significantly more time on the compartment associated with the saline treatment, indicating a development of conditioned place aversion (CPA) (Figure 2B in publication IV). We next studied, whether the pretreatment with JNJ-39220375 has an effect on the development of quinpirole-induced CPA and found that JNJ-39220675 (10 mg/kg) did not alter the aversive effect of quinpirole.

Quinpirole decreased the mobility of the mice as measured by the distance moved, velocity and immobility on the first day of the subchronic treatment (Figure 4A,B and C). JNJ-39220675 pretreatment had no effect on the quinpirole-induced hypokinesia on the first day. However, the hypolocomotion induced by quinpirole was diminished after repeated treatment with quinpirole and on the fifth day of administration, the quinpirole-induced activity was no longer decreased compared to saline-treated mice. This indicated that mice had developed desensitization to the hypokinetic effect of quinpirole. However, JNJ-39220675 was able to inhibit the quinpirole-evoked desensitization as indicated by the significant difference between the saline + saline -treated and JNJ-39220675 + quinpirole -treated mice on the fifth day of administration. JNJ-39220675-mediated inhibition of quinpirole-evoked desensitization was observed in all parameters analyzed (distance moved, velocity and immobility).

**Table 4.** Summary of H3R antagonists-mediated behavioral responses in different background of WT mice.

Background					
strain	Pretreatment	Drug	Paradigm	Result	Ref.
129/Sv	Ciproxifan	Alcohol	LMA	Stimulation –	III
129/Sv	JNJ-39220675	Alcohol	LMA	Stimulation –	III
DBA/2J	JNJ-39220675	Alcohol	LMA	Stimulation –	III
DBA/2J	JNJ-39220675	Alcohol	CPP	CPP ↓	III
DBA/2J	JNJ-10181457	Alcohol	CPP	CPP ↓	I
DBA/2J	Ciproxifan	Alcohol	CPP	CPP ↓	I
DBA/2J	Ciproxifan	Alcohol	LMA	Stimulation ↑	I
DBA/2J	Ciproxifan	Alcohol	BB	Foot slips –	I
DBA/2J	Ciproxifan	Alcohol	Rotarod	Latency to fall –	I
C57BL/6J	Ciproxifan	Alcohol	DID	Consumption ↓	II
C57BL/6J	JNJ-39220675	Amphetamine	CPP	CPP –	IV
C57BL/6J	JNJ-39220675	Amphetamine	LMA	Stimulation ↓	IV
C57BL/6J	JNJ-39220675	Amphetamine	R-LMA	Stimulation –	IV
C57BL/6J	JNJ-39220675	Quinpirole	CPA	CPA –	IV
C57BL/6J	JNJ-39220675	Quinpirole	R-LMA	Desensitization ↓	IV

Abbreviations: BB, balance beam; CPA, conditioned place aversion; CPP, conditioned place preference; DID, drinking in the dark; LMA, locomotor activity; PPI, prepulse inhibition; Ref., reference; R-LMA, locomotor activity after repeated administration. Symbols: – no effect; ↑ increased effect; ↓ decreased effect.

### 5.3.3 H3R in D1R and D2R agonist-induced stimulation (V)

The basal locomotor activity did not differ between H3R KO and WT mice. Surprisingly however, a high dose of amphetamine (5 mg/kg) induced a stronger stimulation in the H3R KO mice as compared to the controls (Figure 2A and B in manuscript V). D1R agonist SKF-38393 (10 mg/kg) induced a long-lasting (about two hours) and equal stimulation in both H3R KO and WT mice. (Figure 3E and F). We observed that the effect of quinpirole (0.5 mg/kg) on locomotion has two phases, and the biphasic effect of quinpirole on locomotor activity has also been previously published (Eilam and Szechtman 1989; Van Hartesveldt 1997). The first hypolocomotor phase lasts about two hours and is followed by the second phase, a moderate stimulation in WT mice. Here we observed that the stimulatory effect of quinpirole did not occur in the

absence of H3R as no biphasic effect of quinpirole was observed in the H3R KO mice (Figure 2C and D). This was observed in both parameters analyzed: the distance moved and the velocity.

#### **5.4 Histamine, H1R and H3R in sensorimotor gating (V)**

Before the actual PPI experiment, the startle responses were examined by exposing the animals to sounds of different intensity (dB). We observed no differences in the startle amplitudes between the genotypes in HDC KO and WT (Figure 1E in manuscript V) and H3R and WT mice (Figure 1A). However, the startle responses were significantly lower in the H1R KO compared to the control mice (Figure 1G). Interestingly, we found that H3R KO displayed impaired sensorimotor gating as measured by the PPI ((Figure 1B). To further investigate the mechanism, we selected two pharmacological ligands D2R antagonist and NMDA antagonist, to enhance and decrease the PPI responses. D2R antagonist haloperidol (0.75 mg/kg) was able to restore the impaired PPI to the normal level in the H3R KO mice ((Figure 1C), whereas the NMDA antagonist MK-801 (1 mg/kg) decreased the PPI responses in both H3R KO and WT mice (Figure 1D). No significant differences in the PPI responses were observed between HDC KO and WT (Figure 1F) and H1R KO and WT mice (Figure 1H). As there were no differences between the genotypes, no pharmacological treatments were further tested in these mouse strains.

**Table 5.** Summary of the behavioral responses of H3R KO compared to WT mice (in C57BL/6J background strain).

Drug	Paradigm	Result	Ref.
Alcohol	CPP	CPP ↓	II
Alcohol	LMA	Stimulation ↓	II
Alcohol	DID	Consumption ↓	II
Alcohol	TBC	Consumption ↓	II
Alcohol	BB	Foot slips ↓	II
Alcohol	Rotarod	Latency to fall –	II
Amphetamine	LMA	Stimulation ↑	V
Quinpirole	LMA	Stimulation ↓	V
SKF-38393	LMA	Stimulation –	V
–	PPI	PPI ↓	V
Haloperidol	PPI	PPI ↑	V
MK-801	PPI	PPI –	V

Abbreviations: BB, balance beam; CPP, conditioned place preference; DID, drinking in the dark; LMA, locomotor activity; PPI, prepulse inhibition; Ref., reference; Rotarod, rotating rod; TBC, two-bottle choice. Symbols: – no effect; ↑ increased effect; ↓ decreased effect.

## 5.5 Biochemical effects of histamine and H3R

### 5.5.1 Plasma alcohol concentrations (I-III)

Neither imidazole-based ciproxifan (3 mg/kg) nor imipip (30 mg/kg) pretreatment had an effect on plasma alcohol concentration 15 minutes after alcohol (1.5 g/kg) was administered (in text in publication I). Neither did the non-imidazole based JNJ-39220675 (0.3, 3 or 10 mg/kg) have an effect on plasma alcohol concentration at any measured time point (10, 20,100 and 150 minutes after 2 g/kg alcohol injection) (Figure 1C in publication III). There was no difference between plasma alcohol concentrations five minutes after alcohol injection in H3R KO mice compared to WT controls (in text in publication II). Neither was there a difference in plasma alcohol concentrations in HDC KO mice compared to WT animals (10, 20,100 or 150 minutes after 2 g/kg alcohol injection) (Figure 2B in publication III).

### **5.5.2 Expression of dopaminergic markers in HDC KO mice (III)**

The quantitative radioactive *in situ* hybridization was used in order to study different dopaminergic signaling components in HDC KO and WT mice striatum (+0.98 mm from Bregma). The striatum was subdivided into five divisions: the dorsolateral caudate putamen (DL CPu), dorsomedial caudate putamen (DM CPu), ventral caudate putamen (V CPu), nucleus accumbens core (AcbC) and nucleus accumbens shell (AcbSh). Of these the DL CPu, DM CPu and V CPu represent the dorsal striatum whereas the AcbC and AcbSh represent the ventral striatum. No differences were observed in the mRNA expression levels of D1R, D2R, striatal-enriched protein tyrosine phosphatase 61 (STEP61) or DARPP-32 between HDC KO and WT mice in any of the striatal subdivisions (Table 1 in publication III).

### **5.5.3 Expression of dopaminergic markers in H3R KO mice (V)**

The expression of D1R and D2R mRNA analyzed from the striatal subdivisions described in the previous chapter (+0.98 from Bregma). TH and DAT were analyzed from the VTA (-3.52 from Bregma). We found no differences between the genotypes in the expression on D2R, TH or DAT mRNA expression (Figure 5B in manuscript V). However, there was a significant overall difference in the mRNA expression of D1R in the striatum, supporting the interaction between the H3Rs and D1Rs in the striatum.

### **5.5.4 Dopaminergic signal transduction in H3R KO mice (V)**

To assess dopaminergic signal transduction in H3R KO and WT mice were injected *in vivo* either with quinpirole or SKF-38393. Dopaminergic signaling was investigated by semi-quantitative Western blotting and phosphorylated forms of ERK1/2 and AKT were selected as markers of dopaminergic activation. Neither quinpirole (Figure 3C and D in manuscript V) nor SKF-38393 (Figure 4C and D) activated ERK1/2 or AKT in the PFC in either of the genotypes. However, in the striatum, both dopaminergic ligands activated ERK1/2 in the WT mice (Figure 3A and 4A). Importantly, no striatal activation of ERK1/2 was observed in mice lacking H3R (Figure 3B and 4B), indicating that H3R is required for both D1R- and D2R-mediated signaling.

## 6. DISCUSSION

### 6.1 H3R in alcohol-related behaviors (I-III)

One of the main aims of this thesis was to study the involvement of the brain histaminergic system, especially H3R in alcohol-related behaviors. To investigate this, different behaviors were assessed using both pharmacological tools and genetically modified mouse models. The CPP paradigm was selected as indicator of reward and positive reinforcement. It is a form of Pavlovian conditioning and is extensively used to measure the rewarding and motivational effects of objects or experiences such as abused drugs (Cunningham, *et al* 2006). In this thesis, the mice were conditioned to associate the drug and a tactile stimulus and if the drug had a motivational significance, mice spent significantly more time in proximity to the drug-associated cue. This was considered to represent drug-driven reward and reinforcement as there was no bias toward the tactile cues prior to conditioning. We found that pretreatment with H3R antagonists ciproxifan and JNJ-39220675 clearly inhibited the development of alcohol-CPP in DBA/2J mice, indicative of lack of reward and reinforcement by alcohol. Additionally, JNJ-10181457 inhibited alcohol-CPP, although its effect was rather weak, although statistically significant. H3R agonist immpip did not alter alcohol-CPP. Previous studies have shown that H3R antagonists are motivationally neutral and are not rewarding or aversive themselves (Munzar, *et al* 2004; Nuutinen, *et al* 2010; Uguen, *et al* 2013). This is of great relevance in the drug development and also indicates that the inhibitory effect of alcohol-CPP does not arise from aversive response to H3R antagonist pretreatment. Nevertheless, the observed inhibition of alcohol-CPP by JNJ-10181457 and JNJ-39220675 demonstrated an idiosyncratic dose-dependence. Surprisingly, only low doses of the antagonists inhibited the development of alcohol-CPP whereas the higher doses had no effect. The underlying mechanisms remain unclear. Pharmacokinetic interactions are unlikely to explain this phenomenon since JNJ-39220675 had no effect on the pharmacokinetic profile of alcohol. The possible explanations for the lack of dose-dependence are discussed next.

Both JNJ-10181457 and JNJ-39220675 penetrate easily through the blood-brain barrier in rat studies carried out in rats. JNJ-39220675 *ex vivo* H3R occupancy reaches the maximal level quickly at both 3 and 10 mg/kg doses in the rat striatum, whereas at a dose 0.3 mg/kg the receptor occupancy is about 75-80 % and stays on that level for about four hours (Galici, *et al* 2011). The H3R has several different isoforms derived by alternative splicing (Lovenberg, *et al* 1999; Drutel, *et al* 2001; Coge, *et al* 2001; Rouleau, *et al* 2004). These different H3R splice variants differ in their localization and pharmacological properties such as signal transduction efficiencies (Drutel, *et al* 2001; Hancock, *et al* 2003). As the different isoforms of the H3R are heterogeneously distributed in

the brain and have different functional properties, it may be that the lower doses of H3R antagonists display isoform-specific regulation of different brain functions. This isoform-specific regulation might thus partly contribute to the findings on this thesis. In the striatum, H3R is located on both presynaptic and postsynaptic sites, which might provide another plausible explanation for the inhibition of alcohol-CPP only at low doses of H3R. H3R antagonists acting at the presynaptic H3R autoreceptors increase histamine release (Arrang, *et al* 1983) allowing more histamine to act on the postsynaptic H3Rs located on the inhibitory GABAergic MSNs projecting to the globus pallidus and substantia nigra regulating the dopaminergic neurons (Pillot, *et al* 2002). Thus the increased release of histamine by H3R antagonists may indirectly inhibit the dopaminergic neurons. However, as both histamine and H3R antagonists bind to the postsynaptic H3Rs, the outcome is complex. Nevertheless, the diversity and complexity of the H3R system provides another plausible explanation for the responses seen with different doses of H3R antagonists.

Further supporting the importance of H3R in the regulation of alcohol reward, we found that H3R KO mice did not develop alcohol-CPP. Additionally, H3R KO were not as stimulated by alcohol as the WT controls and the lack of H3R resulted in mild tolerance to the motor-impairing effects of alcohol, as indicated by the lower number of foot slips in the balance beam experiment. However, the lower motor-impairing effect of alcohol in H3R KO was not observed in the accelerating rotarod. We also observed that H3R KO mice consumed less alcohol than WT mice (C57BL/6J) in both voluntary two-bottle choice test and in the DID model of alcohol drinking. Additionally, ciproxifan was found to decrease and imnepip to increase alcohol drinking in the DID paradigm. Previous studies have also shown that H3R antagonists thioperamide and clobenpropit inhibited alcohol drinking in AA rats with a genetic preference for alcohol (Lintunen, *et al* 2001). Our findings were later confirmed in a study conducted in rats, where it was shown that JNJ-39220675 decreased alcohol consumption in another alcohol-preferring rat (P) line (Galici, *et al* 2011). In mice, a recent study conducted with a novel H3R antagonist ST1283 supports the inhibitory role of H3R antagonism on alcohol consumption and reward (Bahi, *et al* 2013).

All abused drugs including alcohol share the property of targeting the midbrain dopamine neurons via either indirect or direct actions, leading to an increase in the activity of the dopaminergic system. The direct action, by which alcohol increases dopaminergic transmission, is not fully known (Sulzer 2011). There is some evidence that alcohol might directly excite dopaminergic neurons in the VTA (Brodie, *et al* 1999; Okamoto, *et al* 2006), but alcohol might also exert its effects via disinhibition of dopaminergic neurons as the effects of alcohol are partially mediated by GABA receptors (Mereu and Gessa 1985). Regardless of the mechanism of increased dopaminergic transmission by alcohol, we hypothesized that H3Rs inhibit alcohol-reward and consumption via mechanism involving an interaction with the dopaminergic system. This was further studied

using both behavioral and mechanistical experiments and will be discussed in the following chapters.

## 6.2 Histamine in alcohol-related behaviors (III)

Interestingly, we found that none of the tested H3R antagonists (ciproxifan, JNJ-10181457 or JNJ-3922067) were able to inhibit alcohol reward in any tested doses in HDC KO mice. It thus seems that H3R antagonists inhibit alcohol reward in a histamine dependent manner. In other words, histamine is likely to have an inhibitory role in alcohol reward. The major drawback of this study, however, is the use of different mouse strains. It is commonly known that the background strain has a significant impact on the behavioral responses of the mice (Wolfer, *et al* 2002; Eisener-Dorman, *et al* 2009). The alcohol-CPP on HDC KO mice was conducted before the selective backcrossing to C57BL/6J mouse strain, and the mice were thus in 129/Sv background. In the studies we found that ciproxifan, JNJ-10181457 or JNJ-3922067 inhibited alcohol-CPP the DBA/2J mouse strain was used, that is the background strain to which the alcohol-CPP paradigm was also originally developed (Cunningham, *et al* 2006). In an earlier study conducted in 129/Sv background WT mice it was found that ciproxifan increased, rather than decreased, alcohol-CPP (Nuutinen, *et al* 2010). In that study HDC KO mice also displayed enhanced alcohol-CPP. Due to different background strains used in these studies, the interpretation of the results is difficult.

However, the foremost important finding from the experiments conducted HDC KO mice in this thesis arose from the alcohol drinking studies. Considering, that histamine is likely to have an inhibitory role in alcohol-CPP, it was surprisingly observed in two different background strains that the lack of endogenous histamine has no effect on alcohol consumption. If histamine is inhibitory in alcohol reward, mice lacking histamine could be expected to consume more alcohol than the WT mice. This contradiction may possibly be due to a ceiling effect; especially in the binge-like DID alcohol drinking paradigm the alcohol consumption is already remarkably high in the control mice and may not be further increased. Nevertheless, in the voluntary two-bottle choice drinking paradigm the mice were in 129/Sv background strain and no differences were observed between the genotypes in alcohol consumption or alcohol preference over water in any of the alcohol concentrations studied. Additionally, HDC KO mice in C57BL/6J background were used after selective backcrossing in another alcohol drinking paradigm. DID paradigm has been developed in the C57BL/6J background due to the high alcohol preference of this strain (Rhodes, *et al* 2005). Also this study revealed that HDC KO mice consume alcohol similarly to the WT mice. These findings together with the studies conducted in H3R KO mice, support the hypothesis, that rather than histamine *per se* H3R is an important mediator of alcohol drinking behavior. Additionally, this suggests

that H3R has a crucial role in reward and reinforcement induced by alcohol and this is likely to involve an interaction with the mesolimbic dopamine system.

### **6.3 H3R and behavioral responses of dopaminergic drugs (IV-V)**

To extend our current knowledge of the role of H3R in addiction, we also examined the effects of H3R in the behavioral responses of amphetamine. Amphetamine has a large variety of actions on the dopaminergic system, including inhibition of DAT, VMAT and MAO (Sulzer 2011). Amphetamine has many derivatives including methamphetamine, and these two have similar properties in terms of e.g. changes in the dopamine release in the dorsal striatum (Melega, *et al* 1995) and the drugs are not distinguished in human discrimination studies (Lamb and Henningfield 1994).

Surprisingly, we found that a high dose of amphetamine (5 mg/kg) induced a stimulation of locomotor activity that was higher in H3R KO mice compared to control mice. This observation is in contrast with other studies where it has been shown that H3R KO mice are less stimulated by methamphetamine (Toyota, *et al* 2002; Okuda, *et al* 2009). However, the dose of methamphetamine used in those studies is relatively low (1 mg/kg) compared to the dose used in our locomotor activation experiment (5 mg/kg). Nonetheless, the contradictory results may be explained by the original characterization of the H3R KO mice where it was observed that mice lacking H3R are less sensitive to methamphetamine-induced stereotypies (Toyota, *et al* 2002). Thus, it is possible that amphetamine induced more stereotypies in WT animals which made them move less than the H3R KO mice.

We next studied whether H3R antagonism affects amphetamine-induced locomotor activation and found that JNJ-39220675 inhibited amphetamine-induced stimulation acutely but not after repeated administrations. The acute inhibition of amphetamine-induced hyperactivity has also been previously shown using other H3R antagonists, including thioperamide (Akhtar, *et al* 2006), ciproxifan and clobenpropit (Mahmood, *et al* 2012). However, another novel non-imidazole H3R antagonist GSK207040 had no effect on amphetamine-induced stimulation in rats (Southam, *et al* 2009). JNJ-39220675 does not affect dopamine release in the nucleus accumbens (Galici, *et al* 2011) and thus the inhibitory effect of JNJ-39220675 on the amphetamine-induced stimulation is most likely not mediated via H3R-mediated decreased release of dopamine. We hypothesize, that JNJ-39220675 inhibits amphetamine stimulation by binding to H3Rs on GABAergic MSNs and thereby affects the postsynaptic dopaminergic signaling following dopamine release evoked by amphetamine. This is further supported by the findings that both D1Rs and D2Rs may form

functional heterodimers with H3R on the postsynaptic site by which the receptors modulate one another's actions (Ferrada, *et al* 2008; Ferrada, *et al* 2009; Moreno, *et al* 2011). The interaction between H3R and dopamine receptors, however, is extremely complex and far from being fully understood. Nevertheless, this study suggests that H3R antagonist is able to inhibit the behavioral effects of dopaminergic activation acutely, possibly by increasing D2R affinity and thereby strengthening the  $G_{\alpha_{i/o}}$  protein-mediated inhibitory cAMP-signaling of the indirect pathway.

Interestingly, we found that after repeated administrations of the drugs, JNJ-39220675 lost its inhibitory effect on amphetamine stimulation. On the fourth day of the experiment, JNJ-39220675 pretreatment had no effect on amphetamine-induced hyperlocomotion. The possible explanations for the lack of effect of JNJ-39220675 in repeated administration with amphetamine might lie on the upregulation of dopaminergic transmission in response to repeated administration of amphetamine. Subchronic amphetamine or methamphetamine administration has been shown to induce an increase in D1Rs in the striatum and substantia nigra whereas D2R binding has remained unaltered after amphetamine treatment (Ujike, *et al* 1991; Bonhomme, *et al* 1995; Tomić and Joksimović 2000). As the D1R-mediated signaling is increased after repeated administration of amphetamine, the role of D2R-mediated signaling is presumably decreased, at least partly explaining why H3R antagonism is not able to reduce amphetamine stimulation over repeated administration. In addition, subchronic administration of amphetamine may also upregulate DAT and VMAT2 (Lu and Wolf 1997; Shilling, *et al* 1997). Thus, the upregulation of essential components in the dopaminergic regulation may explain why JNJ-39220675 lost its inhibitory effect on amphetamine-evoked stimulation over repeated administrations.

Because the effects of novel non-imidazole H3R antagonists on amphetamine-CPP have not been studied, in this thesis we investigated the effect of JNJ-39220675 on amphetamine reward. Neither of the tested doses (1 and 10 mg/kg) of JNJ-39220675 had an effect on the development of amphetamine-CPP. In agreement with this, methamphetamine-induced CPP was not altered in H3R KO mice (Okuda, *et al* 2009). However, these studies are in contrast with other studies where H3R antagonists have been shown to potentiate psychostimulant-induced behaviors. Thioperamide has been shown to potentiate cocaine-induced CPP (Brabant, *et al* 2005) and both clobenpropit and thioperamide potentiate methamphetamine self-administration (Munzar, *et al* 2004). However, it has been proposed that the potentiation of reward and self-administration are due to pharmacokinetic interaction leading to decreased metabolism of psychostimulants followed by potentiated behavioral responses (Panula and Nuutinen 2013). Nevertheless, as already discussed above, amphetamine has a wide range of effects on the presynaptic dopamine transmission (Sulzer 2011) and it is likely that H3R antagonism is not enough to modulate all these effects and cannot thus inhibit the rewarding properties of

amphetamine. However, it would be interesting to study whether H3R antagonism plays a role in amphetamine-induced withdrawal or relapse.

In order to study whether H3R regulates the locomotor responses of specifically dopamine receptor targeting drugs, we used both D1R-like agonist SKF-38393 and D2R-like agonist quinpirole. The stimulatory response of SKF-38393 has been reported earlier in C57BL/6J mice (Tirelli and Terry 1993) and in this study it was found to be similar in both H3R KO and WT mice. Quinpirole, however, has been reported to have variable effects on locomotion and other behaviors depending on the species and strains used and these differences might reflect distinguishable characteristics in the functional properties of the dopaminergic systems between species and strains (Shannon, *et al* 1991; Halberda, *et al* 1997). In agreement with our findings, however, the biphasic effect of quinpirole on locomotor activity has been observed previously. In the first phase there is a distinct decrease of locomotion which is later followed by hyperlocomotion after about 60-80 minutes of drug administration (Eilam and Szechtman 1989; 1994; Van Hartesveldt 1997). In this thesis we found that the latter activation of quinpirole was significantly lower in H3R KO compared to WT mice. It is not known what causes the biphasic effect but it is possible that the latter effect of quinpirole, the locomotor stimulation, is a response of quinpirole acting on the postsynaptic D2Rs or perhaps binding of quinpirole on the autoreceptors begins to decrease, leading to a release of dopamine. On reserpinized mice it has been shown that quinpirole induces hyperlocomotion already during the first hour after drug administration (Ferrada, *et al* 2008) suggesting that depletion of endogenous dopamine together with postsynaptic D2R activation induce locomotor stimulation. This suggests that the hypolocomotion seen in normal WT mice immediately after quinpirole treatment is an outcome of the presynaptic activation of D2Rs and decreased release of dopamine. The cumulative activity during 30 minutes was also decreased in WT control mice after quinpirole administration as in the present study (Anzalone, *et al* 2012). However, the locomotor activity was increased by quinpirole in conditional D2 autoreceptor knockout mice and decreased more in conditional postsynaptic D2R knockout mice than in WT mice. Taken together, these studies suggest that quinpirole-induced hypolocomotion is an outcome of the activation of the D2 autoreceptors and the latter hyperactivation results from the increased release of dopamine and the activation of the postsynaptic dopamine receptors. This, in turn, suggests that the observed lack of stimulation in the H3R KO mice is regulated via mechanism involving the postsynaptic rather than the presynaptic receptors. As the quinpirole-induced inhibition of dopamine release diminishes in time, dopamine is subsequently released again in the second phase of its effect on locomotion. Thus, in the absence of D2R-H3R heterodimer, it is assumed that the D2R affinity is increased which leads to potentiation of the inhibitory indirect dopaminergic pathway, further supporting the hypothesis that the complex D2R-H3R interaction occurs on the postsynaptic MSNs.

The same conditioning paradigm that was described earlier can be used to study CPA and the aversive effects of drugs. If the drug has negative motivational significance, mice will spend significantly less time in proximity to the drug-associated cue, and can thus be considered aversive (Cunningham, *et al* 2006). In this thesis, we studied the effects of quinpirole on place conditioning in mice. The previous observations of quinpirole in place conditioning are controversial, ranging from no effect (Hoffman, *et al* 1988; Kivastik, *et al* 1996; Graham, *et al* 2007) to significant place preference (Hoffman, *et al* 1988; White, *et al* 1991). However, we found that quinpirole induced a prominent place aversion in C57BL/6J mice. The contradictory results are possibly due to different doses of quinpirole used and methodological differences. In addition, the above-mentioned studies were conducted using rats whereas in our experiments mice were used. Furthermore, as the quinpirole-induced hypolocomotion is likely due to inhibition of dopamine release via D2 autoreceptors (Bello, *et al* 2011) and the conditioning session lasted for 30 minutes, it is not surprising that quinpirole induced CPA rather than CPP. It would be interesting to test whether quinpirole induces CPP if mice were exposed to the conditioning environment during the hyperactive phase of quinpirole (after two hours of injection). Of importance, pretreatment with JNJ-39220675 neither increased nor decreased the aversive effect of quinpirole.

In rats, repeated administration of quinpirole induces abrupt and long-lasting increase in the D2R sensitivity in a process called receptor priming (Einat and Szechtman 1993; Kostrzewa, *et al* 2004). D2R sensitization can be behaviorally detected as e.g. increased locomotor activity. Interestingly, increased D2R affinity and D2R sensitization have been suggested to play a role in the pathophysiology of psychiatric disorders such as schizophrenia and OCD (Seeman, *et al* 2006; Ducasse, *et al* 2014). In rat, quinpirole sensitization has also been used as a model for OCD (Szechtman, *et al* 1998; Perreault, *et al* 2010; Tucci, *et al* 2014). We thus studied the locomotor responses of mice subjected to repeated administration of quinpirole. Furthermore, we investigated whether JNJ-39220675 has an effect on the behavioral outcome. After single administration, JNJ-39220675 did not modify the suppression of locomotion by quinpirole. The locomotor activity was followed for 30 minutes, and thus we could only detect the hypokinetic phase induced by quinpirole. Quinpirole was then administered on five consecutive days with or without the pretreatment with JNJ-39220675. Interestingly, it was found that repeatedly administered quinpirole induced a pronounced tolerance to the acute hypolocomotion. The plausible explanations for this effect involve either sensitized postsynaptic D1Rs and/or D2Rs or desensitized D2 autoreceptors leading to increased release of dopamine. Moreover, JNJ-39220675 inhibited the tolerance that developed to quinpirole-evoked hypolocomotion, suggesting that H3R antagonism inhibits the behavioral responses of desensitized D2 autoreceptors or sensitized postsynaptic D2Rs. In other words, H3R antagonism seems to change the affinity of D2Rs as suggested previously. This gives further evidence of the interaction between H3Rs and D2Rs, which involves the

negative cross-talk between the postsynaptic H3Rs and D2Rs in the GABAergic MSNs leading to altered dopaminergic responsiveness.

#### **6.4 Histamine, H1R and H3R in sensorimotor gating (V)**

PPI is an operational measure of sensorimotor gating, in which the motor response to a sudden, intense stimulus is attenuated by a preceding, weaker, non-startling sensory stimulus (prepulse) (Graham 1975; Hoffman and Ison 1980). PPI is considered to be a protective mechanism to buffer sensory processing and to prevent excessive information. In humans the protecting and facilitating effect of PPI on central information processing has been experimentally demonstrated (Foss, *et al* 1989; Foss, *et al* 1989). PPI is deficient in several psychiatric disorders including schizophrenia, OCD, HD and TS (Kohl, *et al* 2013; Swerdlow 2013). As the brain histaminergic system has been implicated in schizophrenia, HD and TS (Prell, *et al* 1995; Goodchild, *et al* 1999; Ercan-Sencicek, *et al* 2010), in this thesis we studied the PPI in different knockout mouse strains: HDC KO, H1R KO and H3R KO mice.

In this thesis, we observed that H3R KO mice displayed normal responses to the startle stimulus, but interestingly the PPI responses were impaired. In HDC KO mice both startle and PPI responses were normal indicating that the altered PPI response in H3R KO mice occurs independently of histamine. The impairment of PPI in the H3R KO was restored by D2R antagonist haloperidol in both genotypes whereas the NMDA antagonist MK-801 impaired PPI in both genotypes. The higher response of H3R KO mice to haloperidol treatment suggests that H3R gene mutation has led to hypersensitivity of D2Rs. H3R KO mice were found to display similar acoustic startle responses as compared to the WT. In contrast, a previous study reported up to 2.5 times higher startle responses in H3R KO mice than in WT mice (Rizk, *et al* 2004). The controversy with the findings of the present study probably lies on the differences in the methodology. Rizk and colleagues kept the mice in isolation, did not habituate them to the startle measurement and played the acoustic stimuli in ascending order. In our experiments however, mice were group-housed, habituated to the testing chamber and the startle stimuli were tested in a random order. Thus, the mice were possibly more anxious in the experiment by Rizk and colleagues (Rizk, *et al* 2004). The effects of H3R antagonists on PPI have also been tested in several previous studies, and the results have been controversial, probably due to differences both in methodology and mouse strains used. Ciproxifan, BF2.649, and ABT-239 did not improve the PPI impairment produced by apomorphine in rats (Burban, *et al* 2010). However, in DBA/2J mice ABT-239 significantly improved PPI in a dose-dependent manner. Furthermore, another study demonstrated that both thioperamide and ciproxifan enhanced PPI in DBA/2J mice, whereas in C57BL/6 mice the compound had no effect on PPI (Browman, *et al* 2004). These pharmacological models have suggested that H3R has a role in the modulation of PPI, but the

underlying mechanism is not known. As the neural circuits involved in the regulation of PPI are complex, involving e.g. the glutamatergic and dopaminergic systems (Li, *et al* 2009) it is not surprising that different H3R ligands display different behavioral responses, possibly due to different affinities to different H3R isoforms expressed heterogeneously in different brain regions.

## **6.5 Plausible mechanisms underlying the observed behaviors (I-V)**

Changes in alcohol metabolism and kinetics are unlikely to explain any of the findings observed in this thesis, as we found no differences in the plasma alcohol concentrations in between H3R antagonist vs. saline pretreated mice, H3R KO vs. WT mice or HDC KO vs. WT mice in response to alcohol treatment. Furthermore, no significant changes were detected in the dopamine and noradrenaline concentrations in the striatum and PFC between H3R KO and WT mice after 12 days access to alcohol. In agreement with our results, in drug-naive H3R KO mice, the cortical levels of dopamine, noradrenaline and serotonin and their metabolites are comparable to those observed in WT mice (Toyota, *et al* 2002). However, significantly lower level of cortical histamine was observed in the H3R KO mice. This is unlikely to explain the results obtained in this thesis, and the possible mechanisms are discussed next.

As expected from the behavioral experiments indicating that the lack of histamine does not result in major abnormalities in dopaminergic neurotransmission, no alterations in the expression of different dopaminergic signaling components in the striatum were observed in the HDC KO mice. However, H3R KO mice displayed lower levels of D1 receptor mRNA in the striatum whereas TH and DAT mRNA levels were similar in the VTA between control and H3R KO mice. This is in line with the hypothesis that H3R dependent modulation of dopaminergic functions involves an interaction with the dopamine receptors.

Both D1R and D2R activation lead to phosphorylation of ERK1/2 and activation of its target substrates but also drugs of abuse regulate ERK1/2 cascade in the MSNs of the striatum (Baik 2013). Our hypothesis was that both D1Rs and D2Rs interact with the H3Rs *in vivo*, and this might lead to diminished dopaminergic signaling, which in turn may alter behavioral responses such as alcohol reward and sensorimotor gating. The striatum and PFC were selected as target areas as they are involved in the circuits studied in this thesis. The importance of ERK1/2 and AKT in drug addiction including alcohol addiction have been studied extensively (Chen, *et al* 2009; Girault 2012; Girault 2012; Baik 2013; Cahill, *et al* 2014) and were thus selected as

markers of dopaminergic activation. To specifically activate the dopaminergic system, we used D1R agonists SKF-38393 and D2R agonist quinpirole. Previous studies have shown that D2R activation by quinpirole activates ERK1/2 in D2R transfected cells (Wang, *et al* 2005), on brain slices (Yan, *et al* 1999) and in striatal neurons (Cai, *et al* 2000; Brami-Cherrier, *et al* 2002). D1R activation by SKF-38393 has also been shown to induce phosphorylation of ERK1/2 in D1R transfected cells (Chen, *et al* 2004; Ferrada, *et al* 2009) and in rat striatal slices (Moreno, *et al* 2011).

The existence of D1R-H3R heterodimers has been shown in mouse striatal slices, giving indirect evidence of their *in vivo* existence (Moreno, *et al* 2011). In the experiments of this thesis we took another approach as we wanted to study whether the dopaminergic signaling is also modulated by H3R when drugs are administered *in vivo*. Interestingly, we found that the dopaminergic signal transduction is altered in H3R KO mice after *in vivo* exposure to both SKF-38393 and quinpirole. This was observed by the semi-quantitative Western blotting in which we found that in H3R deficient mice neither SKF-38393 nor quinpirole activated ERK1/2 in the striatal tissue, although in the WT mice both compounds were found to phosphorylate ERK1/2 in the striatum. These ligands had no effect on ERK1/2 phosphorylation in the PFC, suggesting that the main location of action of these drugs is in the striatum. Taken together, these results support the previous findings obtained either with transfected cells or striatal sections. Of importance, our studies demonstrate that H3R is involved in the ERK1/2 signaling cascade *in vivo* and that both D1R and D2R signal transduction in the striatum is abnormal in the absence of H3Rs.

## 7. SUMMARY AND CONCLUSIONS

The main findings of the present thesis and the conclusions that can be drawn from the results are the following:

1. H3R KO mice are less sensitive to the stimulatory and motor-impairing effect of alcohol. In addition, both pharmacological blockade and genetic knockout of H3R lead to diminished alcohol consumption and reward.
2. The lack of histamine *per se* does not alter alcohol consumption or reward. However, histamine is required for the H3R-mediated alcohol reward inhibition, which suggests that histamine has an inhibitory role in alcohol reward.
3. Non-imidazole based H3R antagonist inhibits amphetamine-induced locomotor stimulation acutely and modifies D2R activation-mediated behavioral desensitization by quinpirole. However, H3R blockade does not modulate the rewarding effect of amphetamine or the aversive effect of quinpirole.
4. The lack of H3R but not lack of histamine leads to impaired PPI indicating a deficiency in sensorimotor gating in H3R KO mice. This suggests that H3R might play a role in e.g. Tourette syndrome.
5. The mechanism by which H3R regulates the above-described behavioral phenomena involves possibly the interaction between the striatal H3R and dopamine receptors. The interaction might occur on transcriptional level, suggested by the lower D1R mRNA expression in H3R KO mice, and on a signal transduction level, suggested by the lack of ERK1/2 activation in response to dopaminergic activation in H3R KO mice.

## 8. FUTURE PERSPECTIVES

The behavioral data obtained in regard to alcohol-related behaviors indicated repeatedly and consistently that blockade of H3R leads to decreased alcohol consumption and diminishes alcohol reward in mice. One of the tested H3R antagonists used here, JNJ-39220675, has been clinically proven to be safe in humans. The preclinical results from this thesis and the concurrent work conducted in other research groups, encourages H3R antagonists to be tested in human alcoholics. Therefore, both preclinical and clinical studies on alcohol withdrawal and relapse should be conducted. If the results from those studies would be in line with the results obtained in this thesis, H3R antagonism might potentially be a novel approach to treat alcohol addiction.

Here, we also studied whether H3R antagonism inhibits the rewarding properties of amphetamine, and contradictory to what we would in alcohol studies, found that neither of the tested doses of JNJ-39220675 had an effect on amphetamine reward. However, only one dose of amphetamine was tested which is why the results have to be interpreted carefully. In order to clarify the role of H3R antagonism in amphetamine reward other non-imidazole H3R antagonist and different doses of amphetamine should be tested. Additionally, both withdrawal and relapse studies should be conducted using H3R antagonists and amphetamine.

These studies also raise the question whether H3R antagonist could be effective in diminishing the reward evoked by other drugs of abuse. As the majority of the role of H3R antagonist in drug addiction have been obtained using experimental, imidazole-based H3R ligands, their interpretation is difficult, if not impossible. Therefore, novel ligands should be tested in order to establish whether H3R antagonism inhibits self-administration and reward by other drugs of abuse such as cocaine, opioids and nicotine.

Moreover, as the first H3R antagonist is soon entering the clinical use in the treatment of narcolepsy, it is tempting to consider other possible implications for H3R antagonists. We found here, that H3R antagonist modified the locomotor response of repeatedly administered quinpirole. Quinpirole-induced receptor sensitization and the following persistent compulsions have been used to model OCD. On this basis, it would be interesting to study whether H3R plays a role in animal models of OCD.

We also showed that H3R KO mice display impaired sensorimotor gating, whereas contradictory to what others have published, the lack of histamine had no effect on sensorimotor gating. Thus, the role of histamine in sensorimotor gating remains unclear and should be further studied. Interestingly, this study also suggested that the D2R-mediated responses of the H3R KO are stronger

than those of control mice. Additionally, repeated administration of both JNJ-39220675 and D2R agonist quinpirole led the altered response compared to that obtained with only repeatedly administering quinpirole. Although these studies indicate that D2R-mediated behavioral responses are altered both in H3R KO mice and after H3R antagonist treatment, the mechanism remains to be studied. Furthermore, amphetamine-induced locomotor stimulation was increased in H3R KO mice and the stimulatory response evoked by repeated administration of JNJ-39220675 together with amphetamine was more sensitized than that obtained with amphetamine alone. This gives further support that H3R is an important regulator of dopaminergic functions. Investigations on cellular mechanisms, including protein-protein interaction, binding and signal transduction studies, would shed the light on the cross-talk between the H3Rs and dopamine receptors.

## 9. ACKNOWLEDGEMENTS

This work was carried out at the Neuroscience Center and the Institute of Biomedicine, University of Helsinki during the years 2010-2014.

I wish to express my sincere gratitude to my supervisors professor Pertti Panula and Docent Saara Nuutinen. Working in Professor Panula's laboratory with its excellent research facilities has been a privilege. I appreciate his scientific enthusiasm and inspiring guidance. I am sincerely grateful for Saara not only for her endless support but also for being a dear friend. I truly appreciate her help, guidance and never ending encouragement throughout this project.

I acknowledge the reviewers of this thesis; Professor Kalervo Kiiänmaa and Docent Petteri Piepponen for their contribution and constructive comments. I would also like to thank Dr. Beatrice Passani for accepting my invitation to act as my opponent.

I am grateful for Professor Esa Korpi and Docent Petri Hyttiä for being my thesis committee members. I acknowledge the co-authors for their contribution; Tiia Mäki (*nee* Ojala), Marja Kinnunen, Mervi Tuominen, Stanislav Rozov, Maria Chiara Pigni, Joel Rämö, Kaj Karlstedt and Minnamaija Lintunen. I also want to thank students Mira Tissari, Alessandra Nostro and Jasmiini Lemma for their contribution to this project.

I wish to thank all past and present members of PP-group for the friendly work environment. Weekly meetings with the group have provided a great support throughout this project. Especially I wish to thank Maria, Suski and Madhu for their friendship. Reeta, Svetlana and Shamsi are thanked for their help and interesting discussions. It has also been great to share the lab with Jari Rossi and his group members. I also wish to thank Alise for her friendship and great company in and outside work.

I warmly acknowledge The Finnish Graduate School of Neuroscience (FGSN) and doctoral program brain and mind (B&M), The Research Foundation of the University of Helsinki, The Finnish Society of Sciences and Letters, The Medical Society of Finland (Finska Läkaresällskapet) and The Academy of Finland for funding this project. I also thank The Finnish Foundation for Alcohol Studies for awarding my Master's thesis in 2010, which greatly motivated me to continue with research. Furthermore, I would like to thank the European Histamine Research Society for awarding me the first prize in the Young Investigator Award in May 2014.

I wish to thank all my dear friends. I am very grateful for having so many wonderful people in my life. Special thanks belong to Tuija for her never

ending care. I also want to thank Aada's godparents Hanna and Tommi for their friendship. My mother-in-law Raisa and farther-in-law Bjarne are warmly thanked for their love and support.

I especially wish to thank my parents Ritva and Veikko and my fabulous sister Hanna for their encouragement, support and love. You have always had faith in me. I am grateful for Aada for bearing with me and enriching my busy days with the most beautiful smile in the world. My foremost deepest gratitude belongs to my loving husband without whom I wouldn't be the woman I am today. I love you.

Thank you all,

A handwritten signature in black ink, appearing to be the name 'Jou' or 'Jouu', written in a cursive style.

Espoo  
December 2014

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