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THE ROLE OF THE DOPAMINE SYSTEM IN THE ABILITY OF (-)-OSU6162 TO REDUCE VOLUNTARY ALCOHOL DRINKING AND BINGE-EATING IN THE RAT

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THE ROLE OF THE DOPAMINE SYSTEM IN THE ABILITY OF (-)-OSU6162 TO REDUCE VOLUNTARY ALCOHOL DRINKING AND BINGE-EATING IN THE RAT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Vicente

The greatest enemy of knowledge is not ignorance, it is the illusion of knowledge.

Daniel J. Boorstin

ABSTRACT

BACKGROUND AND AIMS: The dopamine system is involved in the reinforcing effects of both food and alcohol and is thus a potential treatment target for alcohol use disorder (AUD) and binge-eating disorder (BED).

Alcohol use disorder is characterized by difficulties to control alcohol drinking and by drinking despite adverse consequences. Few pharmacological treatments are available and their efficacies are limited. The monoamine-stabilizer (-)-OSU6162 has been identified as a potential novel treatment for AUD by showing that it reduces alcohol drinking, alcohol seeking, relapse to seeking and withdrawal symptoms in long-term drinking rats (Steensland *et al*, 2012). In the present thesis, the possible underlying mechanisms in the ability of (-)-OSU6162 to reduce alcohol drinking were investigated.

Binge eating disorder is characterized by episodes of eating large amounts of foods in a relatively short amount of time, the difficulty to control binge-eating and feelings of shame and guilt. Recently, lisdexamfetamine was approved for the treatment of BED. Here, the potential of (-)-OSU6162 as a treatment for BED was investigated.

METHODS: Rats were drinking in the two-bottle choice intermittent-access to 20% ethanol (IA20E) paradigm for three to ten months. The effects of long-term voluntary alcohol drinking on dopamine D2 receptor (D2R) expression (paper I) and dopamine output (paper II) in the nucleus accumbens (NAc) were investigated using qPCR and proximity ligation assay microdialysis. The effects of (-)-OSU6162 on dopamine in the NAc in long-term drinking rats were investigated using microdialysis (paper II). Pharmacological antagonists were used to study the role of the D2R and the serotonin 5-HT2A receptor in the ability of (-)-OSU6162 to reduce alcohol drinking (paper III). Using a model of binge-eating and the second-order schedule of reinforcement, the effects of (-)-OSU6162 on binge-like eating and cue-controlled seeking for chocolate-flavored sucrose were evaluated (paper IV).

RESULTS: Long-term voluntary alcohol drinking downregulated dopamine levels, D2R expression levels and D2R-D2R homoreceptor complexes, and increased adenosine A2AR-D2R heteroreceptor complexes in the NAc. Moreover, alcohol drinking reduced the dopamine response to an alcohol challenge. (-)-OSU6162 increased dopamine levels in the NAc and normalized the dopamine response to an alcohol challenge. Pre-treatment with a 5-HT2A antagonist, but not a D2 antagonist, prevented the ability of (-)-OSU6162 to reduce voluntary alcohol intake. (-)-OSU6162 reduced binge-like eating and seeking (second-order schedule of reinforcement) of palatable food. Furthermore, (-)-OSU6162 infused into the NAc core reduced food seeking.

CONCLUSIONS: (-)-OSU6162 counteracted the dopaminergic downregulations induced by long-term alcohol intake. This effect, together with partial agonism at the 5-HT2A receptor might be involved in the effects of (-)-OSU6162 to reduce voluntary alcohol intake.

(-)-OSU6162 affected behaviors relevant to BED, indicating the potential of (-)-OSU6162 as a novel treatment for BED.

LIST OF SCIENTIFIC PAPERS

I. **Feltmann K**, Borroto-Escuela DO, Rüegg J, Pinton L, de Oliveira Sergio T, Narváez M, Jimenez-Beristain A, Ekström T, Fuxe K and Steensland P: Effects of Long-Term Alcohol Drinking on the Dopamine D2 receptor: Gene expression and Heteroreceptor complexes in the Striatum in Rats (under revision in Alcoholism Clinical Experimental Research)

II. **Feltmann K*,** Fredriksson I*, Wirf M, Schilström B, Steensland P. (2016) The monoamine stabilizer (-)-OSU6162 counteracts downregulated dopamine output in the nucleus accumbens of long-term drinking Wistar rats. *Addict Biol* 21(2):438-49

*These authors contributed equally

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Egecioglu E, Steensland P, Fredriksson I, **Feltmann K**, Engel JA, Jerlhag E. (2013) The glucagon-like peptide 1 analogue Exendin-4 attenuates alcohol mediated behaviors in rodents. *Psychoneuroendocrinology* 38(8):1259-70

Malmlöf T, **Feltmann K**, Konradsson-Geuken Å, Schneider F, Alken RG, Svensson TH, Schilström B. (2015) Deuterium-substituted L-DOPA displays increased behavioral potency and dopamine output in an animal model of Parkinson's disease: comparison with the effects produced by L-DOPA and an MAO-B inhibitor. *J Neural Transm* 122(2):259-72

Feltmann K, Konradsson-Geuken Å, De Bundel D, Lindskog M, Schilström B. (2015) Antidepressant drugs specifically inhibiting noradrenaline reuptake enhance recognition memory in rats. *Behav Neurosci* 129(6):701-8

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LIST OF ABBREVIATIONS

A2AR	Adenosine receptor 2A
AUD	Alcohol use disorder
BED	Binge-eating disorder
CS	Conditioned stimulus
DAT	Dopamine transporter
D1R	Dopamine receptor D1
D2R	Dopamine receptor D2
DOPAC	Dihydroxyphenylacetic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
EMA	European Medical Agency
FDA	US Food and Drug Administration
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal axis
HVA	Homovanillic acid
HPLC	High performance liquid chromotagraphy
IA20E	Intermittent-access to 20% ethanol
L-DOPA	L-3,4-dihydroxyphenylalanine
MSN	Medium spinal neuron
MDMA	3-4-Methylenedioxymethamphetamine
NAc	Nucleus accumbens
NIH	US National Institute of Health
PLA	Proximity ligation assay
PPIA	Peptidylprolyl isofmerase A
qPCR	Quantitative polymerase chain reaction
VTA	Ventral tegmental area
WHO	World Health Organization

1 INTRODUCTION

1.1 DOPAMINE TRANSMISSION

The neurotransmitter dopamine (Carlsson, 1959; Carlsson *et al*, 1957) is involved in motor behavior, reward, motivation and memory. Dopamine neurons originate in the midbrain and project to various brain areas (Dahlström and Fuxe, 1964; Ungerstedt, 1971) resulting in four dopaminergic pathways (Fig. 1). These pathways are dysregulated in several psychiatric and neurological disorders, such as schizophrenia, mood disorders and Parkinson's disease. Especially, the mesocortical and mesolimbic pathway, or mesocorticolimbic pathway, is important in reward and addiction (Diana, 2011; Pierce and Kumaresan, 2006; Wise, 2006). This pathway projects from the ventral tegmental area (VTA) to the cortex (e.g. prefrontal cortex), the ventral striatum (where the nucleus accumbens (NAc) is located), the amygdala and the hippocampus.

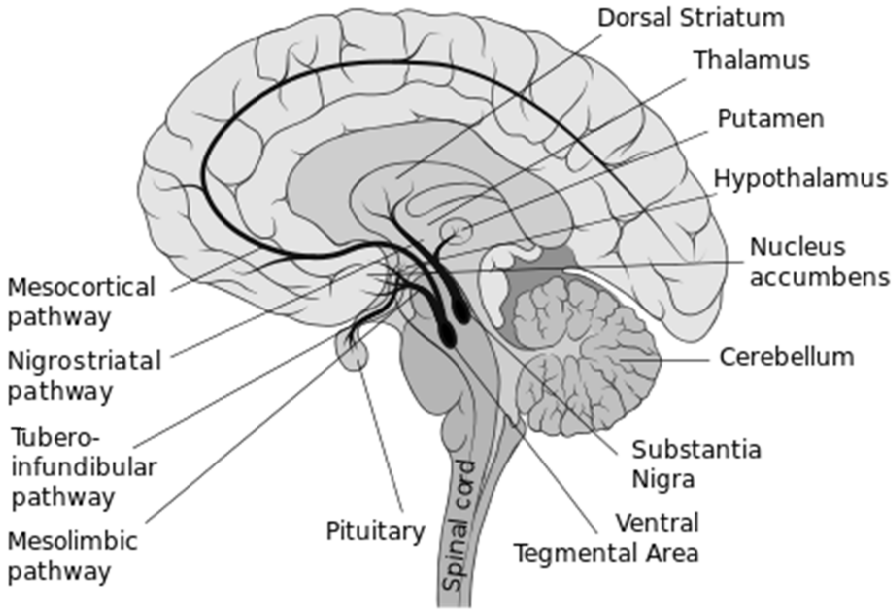

Figure 1. Dopaminergic pathways
The four distinct dopaminergic pathways from cell body to axon terminal are illustrated: mesocortical pathway (VTA to the cortex, mainly prefrontal cortex), mesolimbic pathway (VTA to the ventral striatum, amygdala, hippocampus), nigrostriatal pathway (substantia nigra to dorsal striatum), tuberoinfundibular pathway (hypothalamus to the pituitary gland).
The mesocortical and mesolimbic pathway is involved in reward, motivation, emotion, memory and cognition. The nigrostriatal pathway regulates motor control and the tuberoinfundibular pathway controls hormone secretion, mainly prolactin.
Note, that these pathways are based on rodent studies (Dahlström and Fuxe, 1964; Ungerstedt, 1971). In primates, an additional pathway projecting from different areas to the thalamus has been found (Sanchez-Gonzalez *et al*, 2005).

The picture was originally in color and the figure legend has been rewritten.

The firing of dopamine neurons in the VTA causes dopamine release in the projection areas, such as the NAc. Dopamine neurons can fire action potentials in either a tonic single spike or a burst firing pattern (Bunney *et al*, 1973; Grace and Bunney, 1984a, b). Whereas tonic firing causes stable, low concentrations of dopamine (endogenous dopaminergic tone), burst firing causes large, transient increases in dopamine levels (Gonon and Buda, 1985; Venton *et al*, 2003).

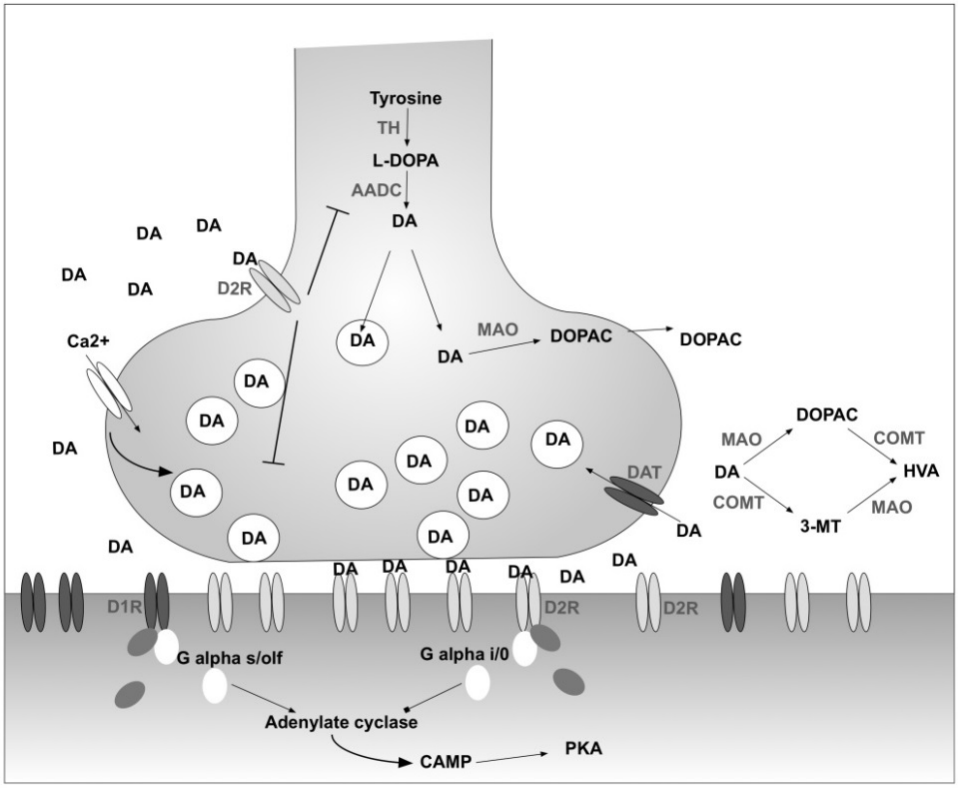

Figure 2. The dopamine synapse
Release: Arrival of an action potential at the axon terminal stimulates calcium entry, which in turn, increases dopamine (DA) synthesis and exocytosis-mediated dopamine release. Released dopamine is taken up by the dopamine transporter (DAT).
Synthesis: Dopamine is synthesized from tyrosine. Tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) via tyrosine hydroxylase (TH), which is the rate-limiting step. L-DOPA is converted to dopamine via aromatic L- amino acid decarboxylase (AADC). Dopamine is stored in vesicles.
Metabolism: Free-available intracellular dopamine is metabolized to dihydroxyphenylacetic acid (DOPAC) via monoamine oxidase (MAO) and aldehyde dehydrogenase (ALDH). Extracellular dopamine is metabolized to 3-Methoxytyramine (3-MT) by catechol-O-methyltransferase (COMT). DOPAC and 3-MT can be further metabolized to homovanillic acid (HVA) via COMT and MAO plus ADLH, respectively.
Signaling: Whereas, D1 receptors (D1R) stimulate adenylate cyclase via Gαs/olf, D2 receptors (D2R) inhibit adenylate cyclase via Gαi/o. Adenylate cyclase produces cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA). PKA phosphorylates numerous cytoplasmic and nuclear proteins, regulating cell metabolism and ion channel function.

Dopamine diffuses from the synaptic cleft and is rapidly taken up by the dopamine transporter (DAT) into the axon terminal (Cragg and Rice, 2004; Pickel *et al*, 1996) or, at a slower rate, metabolized (Michael *et al*, 1985) (Fig. 2). A significant amount of dopamine

diffuses into the extrasynaptic space (Garris *et al*, 1994), indicating a role of extrasynaptic transmission.

Dopamine binds and stimulates dopamine receptors, which are G-protein-coupled receptors of distinct gene sequences. Dopamine receptors can be divided into type 1 (D1 receptors: D1R, D5R) and type 2 (D2 receptors: D2R, D3R, D4R) based on their associated localization, cell signaling (Fig. 2) and affinity for pharmacological compounds. D1 and D2 agonists/antagonists can modulate the activity of each receptor type and differ several 100 folds in their affinity for D1 versus the D2 receptors, but not in their affinity for receptors within each type (Vallone *et al*, 2000).

Whereas the D1R and D5R are found postsynaptically, the D2R and D3R are found both post- and presynaptically (Vallone *et al*, 2000). Dopamine binding to presynaptic D2 receptors (autoreceptors, Fig. 2), located on axon terminals or soma of dopamine neurons, inhibits dopamine synthesis (Kehr *et al*, 1972), release (Gonon and Buda, 1985) and cell firing (Bunney *et al*, 1973), contributing to a negative feedback loop.

The D2R is highly expressed in the striatum, the substantia nigra and the VTA (Beaulieu and Gainetdinov, 2011; Khan *et al*, 2000; Vallone *et al*, 2000). Through alternative splicing the D2R exists in a long and short isoform, differing by an intracellular loop of 29 amino acids (Dal Toso *et al*, 1989). The short and long isoform are associated with different cellular signaling (Lindgren *et al*, 2003; Senogles, 1994) and thought to be mainly expressed pre- and postsynaptic, respectively (Khan *et al*, 1998).

Dopamine receptors form homoreceptor complexes (e.g. D2R-D2R) or heteroreceptor complexes with various other receptors. Heteroreceptor complexes can be associated with different cell signaling than homoreceptor complexes. For instance, the D1R-D2R heteroreceptor complex can activate Gαq-Phospholipase C signaling (Beaulieu and Gainetdinov, 2011). Furthermore, receptors within the complex can reciprocally agonize or antagonize each other and thereby, modulate signaling (Fuxe *et al*, 2014b). For example, within the A2AR-D2R heteroreceptor complex, activation of one receptor attenuates the affinity of the interacting receptor for its own ligand. This allosteric antagonism between receptors within the A2AR-D2R receptor complex reduces $G\alpha_{i/o}$ mediated signaling and increases β-Arrestin-mediated signaling, favoring receptor internalization (Fuxe *et al*, 2014b).

1.2 THE ROLE OF DOPAMINE IN REWARD AND ADDICTION

Addiction is conceptualized as a three-stage cycle that affected individuals repeatedly go through: binge intoxication, withdrawal and drug anticipation. During the development of addiction, the rewarding effects of the drugs of abuse are reduced, the withdrawal symptoms worsen and a powerful craving for the drug occurs during drug anticipation. Initial studies showing that rats self-administer electrical stimulation in the septal area (Olds and Milner, 1954) and the ventral tegmental area (VTA) (Corbett and Wise, 1979; Routtenberg and Malsbury, 1969) and that drugs of abuse release dopamine in the NAc (Di Chiara and Imperato, 1988) indicated a role of the mesocorticolimbic dopamine system in binge intoxication. This system is now thought to play a role in all stages of addiction (Diana, 2011; George *et al*, 2012; Koob and Le Moal, 2001; Koob and Volkow, 2010). Below, three main theories of the role of the dopamine system in reward and addiction are discussed.

One theory of the role of dopamine in reward is the one of reward prediction error (Schultz *et al*, 1993; Schultz *et al*, 1997). Schultz and co-workers have shown that a food reward can increase the number of dopamine neurons in the VTA showing a phasic response. However, during training, the reward becomes predictable causing no increase in phasic dopamine response any longer. Instead, neurons respond to the predicting cue (conditioned stimulus). In contrast, if an expected reward is omitted dopamine neurons stop phasic firing. Thus, dopamine is suggested to signal when a received reward mismatches expectations (reward prediction error). According to this theory, the large increase in phasic dopamine firing induced by drugs of abuse causes a strong association to predicting stimuli (Schultz, 2002).

Another theory is that of associate learning (Di Chiara, 2002; Di Chiara *et al*, 1999). This theory states that the dopamine release caused by drugs of abuse (Di Chiara and Imperato, 1988) is involved in the learning of associating environmental stimuli with drug taking (conditioned stimuli). Furthermore, drug-induced dopamine release is larger compared to dopamine release following exposure to natural rewards, such as food (Hernandez and Hoebel, 1988) or sex (Pfaus *et al*, 1990). Hence, although dopamine can be involved in learning of predictive stimuli of natural rewards, the underlying processes are enhanced in drugs of abuse. In fact, a conditioned stimulus can reinstate previously extinguished drug seeking in animal models of relapse. In humans, drug-associated cues can induce cravings for drugs in substance-dependent patients (Garavan *et al*, 2000).

The theory of incentive salience (Berridge, 2007; Berridge and Robinson, 1998) argues that dopamine is important for attribution of incentive salience to reward-related stimuli. According to this theory, the reward can be divided into two discrete components, namely ‘wanting’ and ‘liking’. While dopamine is not necessary to elicit hedonic ‘liking’ reactions in

rodents, it is vital for establishing 'wanting' or drug-seeking behavior (Berridge, 2007). However, although human imaging studies could show a correlation between striatal dopamine release and subjective reward (self-reported high) after methylphenidate administration (Volkow *et al*, 2002), researcher promoting this theory argue against a primary rewarding effect mediated through dopamine. Instead, they suggest that most studies measuring subjective reward either insufficiently separate wanting from liking or that study subjects want to be consistent in their response (Berridge, 2007).

The theories presented above do not necessarily exclude each other. Although Dr. Schultz and Dr. Berridge provide arguments against each other theories (Berridge, 2007; Schultz, 2013), some authors have also tried to combine both theories (McClure *et al*, 2003).

In addition, there are three other behavioral concepts of addiction, in all of which the dopamine system seems to be involved. The first concept is a suggested shift from impulsive to compulsive drug taking (Dalley *et al*, 2011). Impulsivity, the tendency to act on an impulse without regarding the consequences, is a risk factor for initial drug taking but is also increased by drugs of abuse (de Wit, 2009). With long-term drug use, drug taking becomes compulsive, continues despite adverse consequences and becomes very hard to control (even when there is a desire to stop drug taking). Serotonin and dopamine play an important role in impulsivity and compulsivity (Dalley *et al*, 2011; Fineberg *et al*, 2010).

The second concept is a shift from positive to negative reinforcement (Wise and Koob, 2014). Drugs are initially taken for their pharmacological, rewarding effects (positive reinforcement). However, over time neuroadaptations cause a tolerance to the rewarding effects of drugs, so that a higher concentration of the drug is needed to obtain the same effects. Furthermore, long-term drug use causes physical and psychological withdrawal symptoms and drugs are taken to omit these withdrawal symptoms (negative reinforcement). The dopamine system plays a role in both the positive reinforcing effects, as well as the negative reinforcing effects (Wise and Koob, 2014).

The third concept suggests a shift from goal-directed drug taking to habitual drug taking (Everitt, 2014). Initially, an individual is seeking the drug with the aim to benefit from its pharmacological effects. If this expected outcome is devalued, drug seeking is diminished and thus, is goal-directed. Long-term drug use makes the act of drug seeking habitual, insensitive to devaluation, and controlled by conditioned stimuli. The dopamine system, especially a shift from the ventral to the dorsal striatum, has been shown to be involved in this concept (Everitt, 2014). Importantly, these concepts are not mutually exclusive and all elucidate different aspects of the complex disorder of addiction.

1.3 GLOBAL ALCOHOL USE

In Europe, approximately 70-80% of adults are drinking alcohol, a significantly higher amount than the global average of about 40% (Rehm *et al*, 2013; Shield *et al*, 2013; WHO, 2014). In South and Southeast Asia, as well as North Africa/Middle East more than 90% of people are life-time abstainers from alcohol. Whereas Western Europe and North America have an average yearly consumption of 15 L and 14 L of pure alcohol per drinker, respectively, the highest consumption is found in Southern Africa (34 L per drinker) and Eastern Europe (26 L per drinker) (Shield *et al*, 2013). In Sweden, the total alcohol consumption per person is lower, whereas the prevalence of heavy episodic drinking and alcohol dependence (DSM-IV) is higher than the European average (Ramstedt M., 2014; Shield *et al*, 2013; WHO, 2014).

Alcohol use is associated with an increased risk for over 200 health conditions, such as cardiovascular diseases, infectious diseases, gastrointestinal diseases, injuries and cancer (WHO, 2014). Moreover, alcohol use can also cause neuropsychiatric conditions, such as epilepsy, anxiety, depression and alcohol use disorder (AUD) (WHO 2014, Amsterdam). A recent American study estimated the 12-month and lifetime prevalence of AUD to be 14% and 29%, respectively (Grant *et al*, 2015). In addition, alcohol causes harm to children and spouses of the person drinking, as well as to victims of alcohol-related violence and traffic accidents caused by driving under influence. Together, these consequences of alcohol drinking form a huge economic and public health burden on society (van Amsterdam and van den Brink, 2013). The WHO estimates that 5% of the global health burden of disease and injury are related to alcohol drinking (WHO, 2014). Moreover, alcohol consumption causes 1 in 7 deaths in men and 1 in 13 deaths in women and 71% of this burden is caused by alcohol dependence (Rehm *et al*, 2013).

1.4 ALCOHOL USE DISORDER

1.4.1 Diagnosis

Alcohol use disorder is a chronic psychiatric disorder caused by long-term alcohol drinking. AUD is characterized by lack of control over alcohol drinking despite negative health and social consequences. Moreover, in AUD, tolerance to the intoxicating or rewarding effects of alcohol can develop over time and physical and psychological withdrawal symptoms can occur, driving further alcohol intake. During abstinence, persons suffering from AUD can experience intense cravings for alcohol, which can initiate drinking and thereby, contribute to relapse (Schneekloth *et al*, 2012). The Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) defines AUD as “a problematic pattern of alcohol use leading to

clinically significant impairment or distress, as manifested by at least two of 11 symptoms, occurring within a 12-month period" (See Table 1).

Previous to DSM-5 (2013), in the DSM-IV, alcohol abuse was distinguished from alcohol dependence (DSM-IV). In brief, alcohol abuse could be characterized by alcohol-related social, occupational and legal problems or alcohol use in physically hazardous situations and alcohol dependence was characterized by tolerance and withdrawal symptoms, loss of control over alcohol intake and a central role of alcohol drinking in the person's life. In the DSM-5, all these were combined into a single construct entitled AUD. The criterion of alcohol-related legal problems was omitted, the criterion of craving was added and the severity of AUD was based on the number of criteria fulfilled. Since this change in diagnosis was fairly recent, most publications cited in this dissertation are based on the DSM-IV diagnosis. Therefore, in the present thesis, the term alcohol-dependent patients has been used when citing human studies that used the DSM-IV criteria and AUD was used when generally referring to the disorder.

Table 1. Diagnostic Criteria for Alcohol use disorder according to DSM-5

1. Alcohol is often taken in larger amounts or over a longer period than was intended.
2. There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.
3. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
4. Craving, or a strong desire or urge to use alcohol.
5. Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
7. Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
8. Recurrent alcohol use in situations in which it is physically hazardous.
9. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
10. Tolerance, as defined by either of the following:
    1. A need for markedly increased amounts of alcohol to achieve intoxication or desired effect.
    2. A markedly diminished effect with continued use of the same amount of alcohol.
11. Withdrawal, as manifested by either of the following:
    1. The characteristic withdrawal syndrome for alcohol (refer to Criteria A and B of the criteria set for alcohol withdrawal).
    2. Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms.

The occurrence of at least two symptoms within a 12-month period is required for a diagnosis of alcohol use disorder. These criteria can be used to determine the severity of the disorder (2-3 symptoms = mild, 4-5 symptoms = moderate, 6 or more symptoms = severe).

1.4.2 Risk factors

The risk to develop AUD is influenced by individual factors, as well as factors within the family and the social environment. Especially, a higher alcohol consumption during adolescence is associated with alcohol dependence in adulthood (McCambridge *et al*, 2011).

Individual risk factors include impulsivity, conduct problems and negative affectivity during adolescence (Chartier *et al*, 2010). Moreover, there is a significant genetic vulnerability, indicated by a family history of alcohol dependence being a strong risk factor for the development of AUD (Cotton, 1979). Adoption and twin studies estimate that 50-60% of the vulnerability to develop AUD might be explained by genetic factors (Cloninger *et al*, 1981; Goodwin *et al*, 1974; Heath *et al*, 2011; Kendler and Baker, 2007; Verhulst *et al*, 2015). Although a large number of genetic variants have been found, the strength of association for each variant with AUD is weak (Heath *et al*, 2011). Thus, similar to other psychiatric disorders, their contribution to AUD is not well understood, but there appears to be a cumulative effect.

Moreover, there is a great amount of co-morbidity with other psychiatric disorders, such as major depressive, bipolar and other substance use disorders (Grant *et al*, 2015; Kessler *et al*, 2005). Alcohol might be consumed excessively to alleviate symptoms of these disorders. However, the development of AUD is not necessarily a reaction to other psychiatric conditions or symptoms. Instead, common genetic and environmental factors could render the individual vulnerable to the development of both AUD and other psychiatric disorders.

The childhood environment can also contain important risk factors, such as physical, emotional and sexual abuse and emotional neglect (Fenton *et al*, 2013). Furthermore, other important factors that put a child at increased risk for the development of AUD is to grow up with parental substance misuse (e.g. heavy drinking) and parental psychopathology (Peleg-Oren and Teichman, 2006). Apart from being risk factors for AUD development, early adversity in childhood is a general risk factor for psychiatric disorders (Green *et al*, 2010).

Finally, the social environment, including peer- and social relations (e.g. peer pressure), as well as alcohol availability, influences alcohol drinking and can increase the risk to develop AUD (Chartier *et al*, 2010).

1.4.3 Treatment

Alcohol use disorder is frequent in adults below 30 years of age but less frequent in older adults (Grant *et al*, 2015) and some people manage to self-remit from AUD without any treatment (Walters, 2000). However, many people with AUD have a life-long struggle with

this disorder and are in need of treatment (Connor and Hall, 2015). Although evidence-based treatments are available, less than 10% of people with AUD are in treatment (numbers for the EU: (Alonso *et al*, 2004)) and below 20% of people with lifetime AUD had ever received treatment (numbers for the US: (Cohen *et al*, 2007; Grant *et al*, 2015)), making AUD one of the most under-treated psychiatric disorders (Connor and Hall, 2015).

Detoxification programs can aid to safely discontinue heavy alcohol drinking and alleviate symptoms of the alcohol withdrawal syndrome (Mattick and Hall, 1996). The alcohol withdrawal syndrome occurs more frequently in severely alcohol-dependent people, a few hours after they stop drinking and can last for some days. This syndrome can include sweating, tachycardia, tremor, insomnia, nausea and vomiting, hallucination, anxiety and seizures. Benzodiazepines can be used to treat alcohol withdrawal syndrome (Soyka *et al*, 2008). However, detoxification programs are not effective as long-term treatments, since most patients relapse into excessive alcohol drinking (Mattick and Hall, 1996).

There are a variety of psychological treatments available for AUD, such as 12-step facilitation (peer support by Alcoholics Anonymous), cognitive behavioral therapy, motivational enhancement therapy and cue exposure behavioral therapy (for review, see (Connor and Hall, 2015)). These treatments can help to reduce hazardous drinking, as well as maintain abstinence.

Pharmacological treatments available and approved for AUD by both the European Medical Agency (EMA) and the US Food and Drug Administration (FDA) are disulfiram, acamprosate and naltrexone. In 2013, the EMA approved nalmefene as a treatment for AUD. Disulfiram and acamprosate might be better suited when alcohol abstinence is the treatment goal. Naltrexone and nalmefene might be better choices when the aim to reduce heavy drinking (Jonas *et al*, 2014; Mason and Lehert, 2012; Rosner *et al*, 2010a; Rosner *et al*, 2010b).

Disulfiram irreversibly inhibits the liver enzyme aldehyde dehydrogenase, leading to the accumulation of aldehyde when alcohol is taken, which causes nausea, flushing, vomiting, sweating and tachycardia (Soyka *et al*, 2008). The psychological threat of this unpleasant reaction is thought to maintain abstinence (Skinner *et al*, 2014). However, dosing needs to be supervised as compliance is otherwise low. A recent meta-analysis indicated that open-label, but not blinded, studies proved disulfiram to be efficacious in maintaining abstinence, which was explained by the proposed mechanism of the psychological threat (Skinner *et al*, 2014). Although disulfiram is associated with a number of side-effects, the meta-analysis reported

no increased incidences of deaths or serious adverse side-effects requiring hospitalization following disulfiram treatment compared to placebo (Skinner *et al*, 2014).

The mechanism of action of acamprosate is not fully understood, but it is thought to normalize NMDA transmission, thereby reducing hyperexcitability during withdrawal. Furthermore, it has been suggested that acamprosate can maintain abstinence by attenuating cue-induced conditioned reactions and reduce drinking by attenuating alcohol reward (Rosner *et al*, 2010a). Acamprosate treatment decreases the risk of returning to drinking and increases abstinence duration, although effects are moderate. The main reported side-effect from acamprosate treatment is diarrhea (Rosner *et al*, 2010a).

Naltrexone is a competitive antagonist at the μ-opioid receptor, which is thought to block the rewarding effects of alcohol, as well as craving for alcohol (Rosner *et al*, 2010b). One meta-analysis (Rosner *et al*, 2010b) showed that naltrexone reduces the risk of heavy drinking with moderate efficacy and slightly decreases the number of drinking days, but does not significantly affect return to any drinking. However, a more recent meta-analysis showed that naltrexone reduces the return to drinking and was not different from acamprosate (Jonas *et al*, 2014). Naltrexone causes gastrointestinal and mild psychiatric side-effects that rarely cause discontinuation of treatment (Rosner *et al*, 2010b).

Nalmefene antagonizes μ-opioid and δ-opioid receptors and partially agonizes κ-opioid receptors. Similar to naltrexone, nalmefene is thought to reduce the alcohol's rewarding effects, as well as reduce craving for alcohol (Rosner *et al*, 2010b). The recommendation is to take nalmefene as an "as-needed" medication, i.e. 1-2 h before an event where the patient feels at risk of drinking (Jonas *et al*, 2014). Based on the limited evidence available, nalmefene reduces the number of heavy drinking days per month and the number of drinks per drinking day (Jonas *et al*, 2014).

1.5 THE EFFECTS OF ALCOHOL ON NEUROTRANSMITTER SYSTEMS

Acute alcohol intoxication affects a variety of neurotransmitter systems inducing both stimulating and sedative effects (Fig. 3, left) (Vengeliene *et al*, 2008). Mainly, alcohol increases transmission of the inhibitory transmitter γ-aminobutyric acid (GABA) and decreases transmission of the excitatory transmitter glutamate, which results in a depressant effect on the central nervous system. Moreover, an increase in dopamine levels is contributing to alcohol's reinforcing effects.

Chronic alcohol intake induces several neuroadaptations that are thought to underlie various withdrawal symptoms (Fig. 3, right) (Becker and Mulholland, 2014; Korpi *et al*, 2015). Especially, a shift to upregulated glutamate transmission and downregulated GABA

transmission causes hyperexcitability of the central nervous system (e.g. seizures) during alcohol withdrawal. Moreover, long-term alcohol intake also affects several other neurotransmitter systems, which are associated with withdrawal symptoms and can contribute to the maintenance of alcohol drinking.

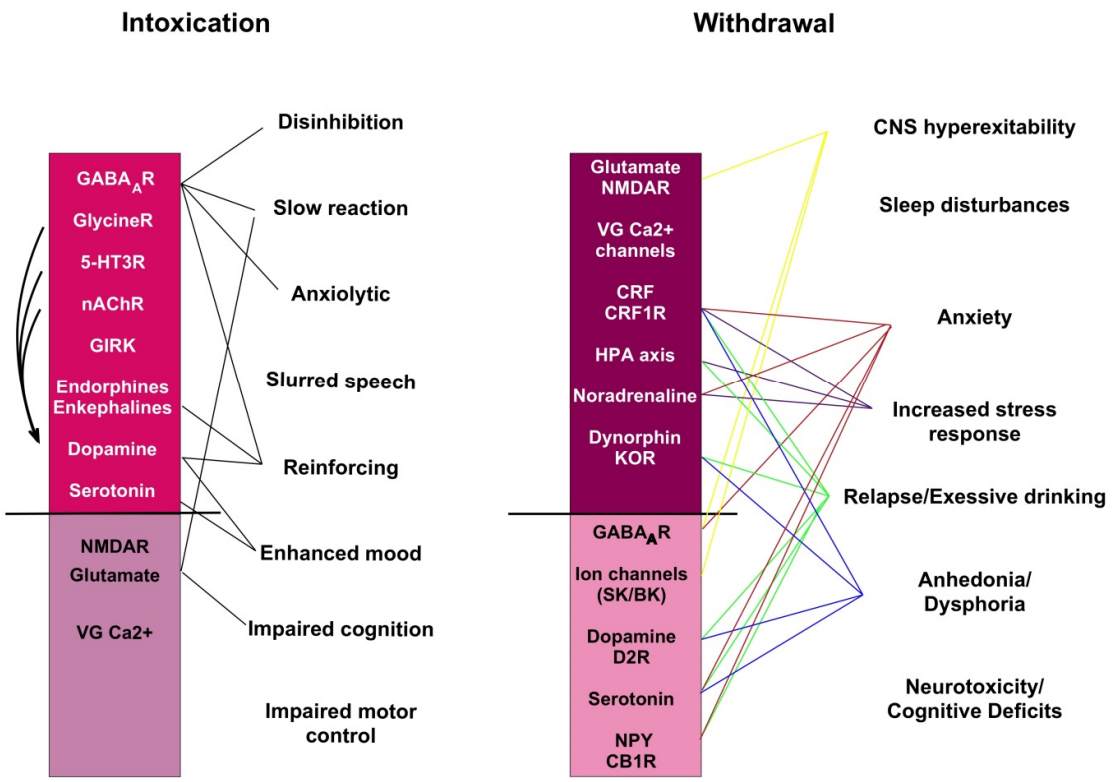

Figure 3. Neurochemical effects of acute and chronic alcohol use
Left: Acute alcohol intoxication increases and decreases concentrations of various neurotransmitters and stimulates and inhibits various receptors, which are related to a variety of behaviors. Alcohol releases dopamine indirectly (arrows).
Right: Chronic alcohol intake upregulates and downregulates several neurotransmitter systems, which is related to various withdrawal symptoms.
These information has largely been obtained from animal studies (Becker and Mulholland, 2014; Korpi *et al*, 2015; Vengeliene *et al*, 2008) but human imaging studies have confirmed a number of these effects in alcohol-dependent patients (Ravan *et al*, 2014; Volkow *et al*, 2017).
For sake of clarity, the lines connecting the neurobiological effects with behavior are selected examples of found associations. γ-aminobutyric acid (GABA), N-methyl-D-aspartate receptor (NMDAR), G-protein-activated inwardly rectifying K+ channels (GIRK), Voltage-gated calcium channels (VG Ca2+), nicotinic acetylcholine receptor (nAChR), serotonergic receptor 3 (5-HT3R), corticotropin releasing factor (CRF), Hypothalamic-pituitary-adrenal axis (HPA), κ-opioid receptor (KOR), small conductance (SK), large conductance (BK).

Based on its role in reward the mesocorticolimbic dopamine system has been extensively studied and is believed to be involved in the development and maintenance of AUD (for review, see (Engel and Jerlhag, 2014; Jayaram-Lindström *et al*, 2016; Ma and Zhu, 2014; Soderpalm and Ericson, 2013; Tupala and Tiihonen, 2004)). However, in contrast to central stimulants (e.g. cocaine, amphetamine), alcohol increases dopamine release indirectly through its actions on other neurotransmitters (Fig. 3). Furthermore, this increase in dopamine release is rather moderate compared to other drugs of abuse (Di Chiara and Imperato, 1988; Ericson *et al*, 1998), which might underlie the fact that alcohol is not a very powerful

reinforcer in rodents, which usually do not drink to intoxication. Therefore, it has been difficult to study AUD in animal models. Moreover, dopamine does not seem crucial for alcohol reinforcement. This suggestion is indicated by studies showing that dopaminergic lesions do not affect alcohol self-administration in rats (Fahlke *et al*, 1994; Lyness and Smith, 1992; Rassnick *et al*, 1993). However, D2R knockout mice have a reduced alcohol preference (Phillips *et al*, 1998), indicating a role of D2R transmission in the reinforcement of alcohol.

The general lack of validated rodent alcohol models in many studies, as well as the complexity of the neurochemical effects of alcohol, makes it difficult to understand the importance of dopamine in the development and maintenance of AUD. Nevertheless, the effects of acute alcohol intoxication on the dopamine system, as well as dopaminergic adaptations after chronic alcohol use are presented in the following sections, in order to provide a concise view of a possible role of the dopamine system in AUD.

1.5.1 Effects of acute alcohol on striatal dopamine release

Acute alcohol administration (oral or injected) increases dopamine release in the NAc in animals (Di Chiara and Imperato, 1988; Doyon *et al*, 2005; Ericson *et al*, 1998; Larsson *et al*, 2005; Molander and Soderpalm, 2005; Weiss *et al*, 1996). In humans (healthy volunteers), oral alcohol intake was initially shown to change [11C]raclopride binding in the NAc, an indirect measurement of dopamine release (Boileau *et al*, 2003). However, this change in binding potential did not correlate to the subjective self-reported level of intoxication (Boileau *et al*, 2003).

Intravenous alcohol administration in healthy volunteers did not consistently produce striatal dopamine release, (Pfeifer *et al*, 2017; Yoder *et al*, 2007; Yoder *et al*, 2005), questioning the true dose-response relationship of blood alcohol and striatal dopamine release. However, the same studies also indicate that dopaminergic measures, such as D2R binding in the left NAc (Yoder *et al*, 2005) and in several frontal cortex regions (Pfeifer *et al*, 2017) correlate with the level of intoxication and liking, respectively.

A recent study in male heavy drinkers showed that beer flavor (conditioned stimulus) increased right NAc dopamine release and intravenous alcohol (unconditioned stimulus) increased left NAc dopamine release, which was significantly correlated to self-reported levels of intoxication (Oberlin *et al*, 2015). In contrast, Yoder and co-workers recently showed that alcohol administered intravenously significantly increases striatal dopamine release in the right ventral striatum in alcohol-dependent individuals, but not in social drinkers (Yoder *et al*, 2016).

Finally, one study has reported that only in men, but not in women, correlates the alcohol-induced dopamine release in the ventral striatum with self-reported subjective activation by alcohol (Urban *et al*, 2010). Together, these studies indicate that at least in male heavy drinkers alcohol releases striatal dopamine, correlating with the level of intoxication.

1.5.2 Effects of chronic alcohol on the dopamine system

In detoxified alcohol-dependent humans, reduced striatal D2R binding (Hietala *et al*, 1994; Volkow *et al*, 1996) and reduced central stimulant-induced striatal dopamine release (Martinez *et al*, 2005; Volkow *et al*, 2007), compared to healthy control, has been repeatedly demonstrated. Moreover, reduced striatal D2R levels have been associated with alcohol craving and relapse (Heinz *et al*, 2010; Heinz *et al*, 1995; Heinz *et al*, 2004). However, it has been suggested that reduced striatal D2R binding could reflect a prevailing vulnerability factor (Volkow *et al*, 2006).

Animal studies are needed to determine the role of long-term alcohol consumption on levels of striatal dopamine and D2Rs. However, the literature regarding this issue has been inconsistent. Repeated alcohol injections reduce extracellular dopamine levels in the ventral striatum and inhibit VTA dopamine neuron firing during alcohol withdrawal (Diana *et al*, 1993; Rossetti *et al*, 1993). Similarly, three to five weeks of continuous involuntary alcohol access to liquid diet reduces NAc dopamine levels during alcohol withdrawal and rats self-administer just enough alcohol to restore dopamine levels back to baseline (Weiss *et al*, 1996). However, increases in striatal dopamine levels in the NAc after repeated alcohol injections (Smith and Weiss, 1999) have also been reported. Finally, ten weeks of drinking in the two-bottle choice intermittent-access to 20% ethanol paradigm (the model of voluntary alcohol drinking used in this thesis) confirmed a downregulation of dopamine levels during withdrawal (Barak *et al*, 2011).

The effect of alcohol consumption on striatal D2R levels has been inconsistent in animal studies. For example, several radioligand studies reported a downregulation of striatal D2R densities by chronic involuntary alcohol intake (Muller *et al*, 1980; Rommelspacher *et al*, 1992; Syvalahti *et al*, 1988). However, other binding studies of chronic involuntary alcohol intake reported increased (Hruska, 1988; Lai *et al*, 1980) or no changes (Hietala *et al*, 1990), as well as decreases followed by increases (Hamdi and Prasad, 1992) in striatal D2R densities. Using voluntary alcohol access of 14 weeks, one study showed increased D2R radioligand binding in the NAc in alcohol-preferring rats (Sari *et al*, 2006). Both increases (Kim *et al*, 1997) and decreases (Jonsson *et al*, 2014) of D2R gene expression have been found, using involuntary and voluntary alcohol intake models, respectively. In addition to

effects on D2R expression, both forced (Rothblat *et al*, 2001) and voluntary (Kashem *et al*, 2012) long-term alcohol intake induce changes of various proteins in the striatum, involved in dopamine synthesis and signaling.

1.6 DOPAMINE TRANSMISSION AS A POTENTIAL TREATMENT TARGET FOR AUD

As reviewed in the previous sections, dopamine plays a role in alcohol's reinforcement and there are indications for a hypo-functioning dopamine system in AUD, suggesting the dopamine system as a potential treatment target for AUD. However, although many dopaminergic manipulations in animals decrease voluntary alcohol intake in animals, dopaminergic medications have shown inconsistent results in human studies of AUD.

In animals, systemic and intra-NAc administration of dopamine antagonist have shown conflicting results on voluntary alcohol intake (Dyr *et al*, 1993), but consistently reduce alcohol seeking (Czachowski *et al*, 2001; Hodge *et al*, 1997; Pfeffer and Samson, 1988; Rassnick *et al*, 1992). In humans, typical antipsychotics (D2 antagonists) have shown to reduce alcohol craving in alcohol-dependent patients (Modell *et al*, 1993; Swift, 2010). However, the D2 antagonist flupenthixol increased the rate of relapse in recently detoxified alcohol-dependent patients (Wiesbeck *et al*, 2001). Furthermore, typical antipsychotics are associated with severe side-effects, such as extrapyramidal side-effects. Extrapyramidal side-effects are less common with atypical antipsychotics, which target dopamine receptors but also several other receptors. Atypical antipsychotics, such as tiapride, olanzapine and quetiapine, have shown efficacious in reducing craving and drinking, and increasing length of abstinence (Hutchison *et al*, 2001; Swift, 2010).

Animal studies have shown that increasing dopamine output in the NAc (Bass *et al*, 2013; Feduccia *et al*, 2014; Pohorecky and Sweeny, 2012; Samson *et al*, 1991), as well as increasing D1 or D2 transmission (Cheng *et al*, 2017; Dyr *et al*, 1993; Thanos *et al*, 2004; Thanos *et al*, 2001) reduces voluntary alcohol intake. However, clinically the use of dopamine agonists, such as bromocriptine, has not been effective in the treatment of alcohol-dependent patients (Swift, 2010). Nevertheless, one study showed that Modafinil (atypical DAT inhibitor but also affecting glutamate) increases the time to relapse and number of abstinent days in alcohol-dependent patients with high impulsivity at baseline, but had the opposite effect in patients with low impulsivity at baseline (Joos *et al*, 2013a). Moreover, Modafinil improves working memory, impulse inhibition (Schmaal *et al*, 2013) and impulsive decision making (Schmaal *et al*, 2014) in alcohol-dependent patients (Joos *et al*, 2013b).

Finally, the partial D2 agonist aripiprazole has been shown to reduce alcohol intake in alcohol-dependent patients (Martinotti *et al*, 2009; Martinotti *et al*, 2007; Voronin *et al*, 2008), although not in the largest study performed so far (Anton *et al*, 2008). Furthermore, in alcohol-preferring rats, aripiprazole only reduced voluntary alcohol intake at doses that also suppressed locomotor activity (Ingman *et al*, 2006).

Taken together, although dopamine seems to be involved in acute and chronic alcohol intake, traditional dopamine agonists and antagonists cannot be used for the treatment of AUD. However, compounds that target the dopamine system using different mechanisms of action might become promising novel treatments of AUD.

1.7 BINGE-EATING DISORDER

1.7.1 Diagnosis

In DSM-5 , binge-eating disorder (BED) is recognized as a specific eating disorder diagnosis. It is defined as recurring episodes (≥once/week for at least three months) of consuming large amounts of food in a short time (food-bingeing), together with a feeling of loss of control over eating during the episode. Furthermore, BED is characterized by at least three of the following: fast eating, eating until uncomfortably full, binge-eating when not being hungry, eating alone due to embarrassment and feeling disgusted, depressed or guilty after binge-eating. In addition, there is a feeling of distress about the binge-eating and there is no compensatory behavior, such as purging or extreme exercising, which are characteristics of bulimia nervosa.

1.7.2 Similarities to addiction

Binge-eating disorder is characterized by loss of control over food intake, food cravings, as well as a heightened cue-responsivity (Curtis and Davis, 2014; Schienle *et al*, 2009; Sobik *et al*, 2005). These characteristics of BED resemble substance use disorders (Schreiber *et al*, 2013). In fact, Cassin and Ranson (2007) conducted interviews with 79 women with BED and reported that over 90% fulfill the DSM-IV criteria for substance dependence when the word ‘substance’ is substituted for ‘binge-eating’. Another study of 81 people with BED identified that 57% meet the criteria for ‘food addiction’ using the ‘Yale food addiction scale’ (YFAS) (Gearhardt *et al*, 2012). The YFAS is a 25-item self-report measure that is closely related to the DSM-IV criteria for substance use disorder but where the word ‘substance’ has been replaced with ‘food’. Nevertheless, some researchers question the concept of ‘food addiction’ (Ziauddeen and Fletcher, 2013), whereas others argue that it should not be rejected prematurely (Avena *et al*, 2012).

1.7.3 Dopamine transmission as a potential treatment target

Dopamine has been suggested as a potential treatment target for BED (Berner *et al*, 2011). This suggestion is supported by several animal and human studies. The mesocorticolimbic dopamine system plays an important role in food reward (Wise, 2006). For example, intake of palatable food, such as sucrose, its taste or cues predicting this food release dopamine in the NAc (Avena *et al*, 2006; Bassareo and Di Chiara, 1999; du Hoffmann and Nicola, 2014; Rada *et al*, 2005). In addition, chronic binge-like intake of palatable food induces dopaminergic changes in the NAc, such as reduced D2R binding, as well as addictive-like behaviors (Adams *et al*, 2015; Alsio *et al*, 2010; Avena, 2007, 2010; Avena *et al*, 2008; Colantuoni *et al*, 2001; Robinson *et al*, 2015).

There are associations of certain polymorphisms of the D2R and the DAT with BED (Bello and Hajnal, 2010). Moreover, the presentation of food-associated cues increases striatal dopamine release to a higher extent in obese patients with BED compared to obese patients without BED, although this study consisted of very few subjects and thus, needs to be replicated (Wang *et al*, 2011). Nevertheless, several reviews have recognized a potential role of dopamine in BED (Bello and Hajnal, 2010; Carlier *et al*, 2015; Kessler *et al*, 2016).

Several studies indicate that modulating dopamine transmission might be a treatment strategy for BED. Increasing dopamine release in the NAc through optogenetics decreases voluntary sucrose intake (Mikhailova *et al*, 2016). The dopamine-releasing compound methylphenidate reduces food consumption in rodents and humans (Davis *et al*, 2012; Thanos *et al*, 2015). Finally, the monoamine-releasing compound lisdexamfetamine is the only FDA-approved drug for the treatment of BED (McElroy *et al*, 2016) but is also associated with significant abuse liability.

Considering the role of dopamine in food reward and BED, targeting dopamine transmission might be a promising treatment strategy in BED.

1.8 THE MONOAMINE STABILIZER (-)-OSU6162

The monoamine stabilizer (-)-OSU6162 was developed by Nobel laureate Dr. Arvid Carlsson and co-workers in an attempt to develop antipsychotic medications that do not induce extrapyramidal side-effects (for review, see (Carlsson and Carlsson, 2006)). One of these compounds was the partial D2 agonist (-)-3PPP, which has been shown to decrease motor activity in the presence of high dopamine activity and increases motor activity in the presence of low dopamine activity. This compound seemed to stabilize motor activity to the level of its intrinsic activity. However, since the clinical antipsychotic effect was lost after one week, it

has been hypothesized that its intrinsic activity might be too high and thereby, leading to autoreceptor desensitization. (-)-OSU6162 is structurally similar to (-)-3PPP (Fig. 4) and has a similar stabilizing pattern of psychomotor activation, i.e. it increases motor activity in arena-habituated rats and prevents amphetamine-induced hyperlocomotion (Rung *et al*, 2008; Sonesson *et al*, 1994). However, (-)-OSU6162 seems to have no intrinsic activity and is thus, a D2 antagonist (for review, see (Carlsson and Carlsson, 2006)).

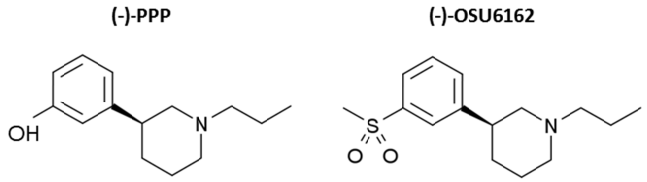

Figure 4. Chemical structures of the dopamine stabilizers (-)-PPP and (-)-OSU6162
For more information about chemical synthesis and pharmacological evaluation of these compounds, see (Sonesson *et al*, 1995; Sonesson *et al*, 1994).

Despite having a low D2R affinity *in vitro*, (-)-OSU6162 possesses characteristics of autoreceptor D2R antagonism *in vivo*, such as increased dopamine release and turnover (Sonesson *et al*, 1994; Steensland *et al*, 2012). Indeed, (-)-OSU6162 has a high D2R occupancy in non-human primates (Ekesbo *et al*, 1999; Neu *et al*, 1997). Moreover, (-)-OSU6162 stabilizes L-[11C]DOPA influx rate depending on the prevailing dopaminergic tone in primates (Tedroff *et al*, 1998) and prevents levodopa-induced dyskinesia in a monkey model of Parkinson's disease without causing akinesia (Ekesbo *et al*, 1997). Furthermore, in contrast to the typical antipsychotic and D2 antagonist haloperidol, (-)-OSU6162 does not induce catalepsy in rats (measure for extrapyramidal side-effects) even at high D2R occupancies (Natesan *et al*, 2006).

Although *in vitro* studies have suggested a certain partial agonism (Burstein *et al*, 2011; Kara *et al*, 2010; Seeman and Guan, 2007), *in vivo* studies indicated no partial agonism. These *in vivo* studies showed that (-)-OSU6162 does not induce contralateral rotations in a 6-OHDA lesion model (dopaminergic lesion in one-hemisphere) (Nichols *et al*, 2002), does not enhance locomotion and stimulates (instead of inhibits) DOPA accumulation in reserpinized rats (monoamine-depleted) (Natesan *et al*, 2006; Sonesson *et al*, 1994), as well as induces plasma prolactin levels (Natesan *et al*, 2006). (-)-OSU6162's behavioral stabilizing effect is thought to underlie a preferential antagonism at extrasynaptic D2R receptors (Carlsson and Carlsson, 2006).

(-)-OSU6162 is a partial agonist at the serotonin 5-HT2A receptor, which has also been shown to play a role in its motor-stabilizing effects (Burstein *et al*, 2011; Carlsson *et al*,

2011). For example, in contrast to the study in rats (Sonesson *et al*, 1994), (-)-OSU6162 (at high doses) was able to induce motor activity in reserpinized-mice, which could be blocked by a 5-HT2A antagonist but not a D2 antagonist. Thus, it has been renamed from its original classification as a "dopamine stabilizer" to a "monoamine stabilizer". Finally, (-)-OSU6162 is clinically safe with a mild side-effect profile (Johansson *et al*, 2012; Khemiri *et al*, 2015; Kloberg *et al*, 2014; Tedroff *et al*, 1999) and has been shown to improve symptoms of Huntington's disease (Kloberg *et al*, 2014; Tedroff *et al*, 1999) and mental fatigue following stroke (Johansson *et al*, 2012).

In 2009, it was demonstrated that (-)-OSU6162 increases the threshold of electrical stimulation required for maintenance of self-administration into the lateral hypothalamus, indicating an attenuation of reward (Benaliouad *et al*, 2009). In 2011, my thesis supervisor Dr. Steensland together with my colleague Dr. Fredriksson started to investigate the potential of (-)-OSU6162 as a treatment for AUD (Steensland *et al*, 2012). They showed that (-)-OSU6162 attenuates voluntary alcohol drinking, motivation to obtain alcohol (e.g. progressive ratio), cue-induced reinstatement (model of relapse) and withdrawal symptoms. Before the beginning of my PhD studies, Dr. Schilström and I started to collaborate with Dr. Steensland. We investigated the effects of (-)-OSU6162 on dopamine output in the NAc in alcohol-naïve rats. We confirmed that (-)-OSU6162 significantly increased dopamine release and turnover and showed that (-)-OSU6162 attenuates the alcohol-induced dopamine output, which we hypothesized to be the underlying mechanism for the effects on alcohol-mediated behaviors (Steensland *et al*, 2012).

In summary, the monoamine-stabilizer (-)-OSU6162 is a promising potential novel treatment for AUD, but very little is known about the mechanisms involved in its effects on alcohol-mediated behaviors.

2 AIMS

The main aim of this thesis was to understand the role of the dopamine system in the previously found ability of (-)-OSU6162 to reduce voluntary alcohol drinking. A secondary aim of this thesis was to evaluate if (-)-OSU6162 could be a potential treatment for BED. Specifically, the following hypotheses were tested:

1. Long-term alcohol drinking downregulates striatal dopamine output and D2 receptor levels (paper I, II)
2. OSU6162 reduces voluntary alcohol intake via its effects on dopamine output, the D2 and 5-HT2A receptor (paper II, III)
3. OSU6162 reduces sugar binge-eating and seeking (paper IV)
4. OSU6162 reduces sugar seeking via its effects in the striatum (paper IV)

3 METHODOLOGICAL CONSIDERATIONS

3.1 ANIMALS

Male Rcc Wistar Han rats were used in the alcohol studies (paper I-III) and male Long-Evans rats were used in the sucrose study (paper IV).

Importantly, rats were never food- or water-deprived or restricted in any study, with the exception of a 2-hour food-deprivation in the binge-eating model and food- and water deprivation during dialysis (final hours of the animals' life).

3.1.1 Superiority of males

Only male rats were used in this thesis, since voluntary alcohol intake in females varies during their estrous cycles (Ford *et al*, 2002; Forger and Morin, 1982). Therefore, it is difficult to use females to investigate the effects of pharmacological compounds using Latin-square designs. However, there is a substantial a sex bias in biomedical research, especially in neuroscience (Beery and Zucker, 2011). Emerging evidence demonstrates crucial neurobiological sex differences (Cahill, 2006). For example, a study using female rats revealed important sex-differences regarding the effects of maternal separation and restraint stress on voluntary alcohol intake (Roman *et al*, 2004) by comparing the effects to previous studies that had only used male rats. In fact, at the national institute of health (NIH, US) it is now a requirement to include both sexes in all studies. In future studies both sexes should be included to be able to detect sex-differences early on.

3.1.2 Outbred rats

Outbred rats, i.e. rats not specifically bred for alcohol preference, were chosen to obtain results of translational value to a general population without any genetic predisposition. Wistar rats drink more than Sprague Dawley rats (Wise, 1975). More specifically, Rcc Han Wistar rats (Harlan Laboratories, The Netherlands) were chosen since they have shown higher voluntary alcohol intake and alcohol preference than other Wistar rats (Palm *et al*, 2011) or than other Wistar Han rats (Goepfrich *et al*, 2013) in 5&20% ethanol three-bottle choice and 6% ethanol two-bottle choice paradigms, respectively. These differences between outbred strains raise the possibility that there is a genetic underlying component on voluntary alcohol intake. Indeed, we have experienced differences in alcohol intake ranging from a mean of 2.5 to 6 g/kg/24hrs depending on the batch we obtained. Long-Evans rats were used in the sucrose intake and operant experiments since they generally perform well on demanding operant tasks and were used before in the same paradigms in an earlier study (Giuliano *et al*, 2012) by the co-author Dr. Giuliano.

3.2 THE DRINKING MODEL (PAPER I-III)

We used the intermittent-access to 20% ethanol in a two-bottle choice paradigm (IA20E, Fig-5) (Simms *et al*, 2008), which was first introduced by Dr. Wise (Wise, 1973). My supervisor Dr. Steensland has reintroduced this drinking model (Simms et al. 2008) demonstrating clear face validity (escalation of voluntary alcohol intake to around 6 g/kg/d, relevant blood alcohol concentration) and predictive validity (Litten *et al*, 2013; Simms *et al*, 2008; Steensland *et al*, 2007). Subsequent studies confirmed face and predictive validity, as well as demonstrated construct validity (Barak *et al*, 2011; Carnicella *et al*, 2014; Hopf *et al*, 2010; Khemiri *et al*, 2015; Steensland *et al*, 2012).

Since rats are nocturnal, alcohol access started during the dark phase, when the biggest drinking bouts occurr (Wise, 1975).

Rats were drinking for at least three months, as long-term access is needed to produce relevant neurobiological changes. This is based on an observation by Dr. Steensland that varenicline decreased alcohol drinking in rats that had been drinking in the IA20E for 12 weeks, but not in rats that had been drinking for only five weeks in this model (unpublished observation).

Rats were single-housed to measure individual alcohol intake. We were weighing the rats daily, in order to get them familiarized with the experimenter. A second aim of the daily handling was to reduce the amount of stress and anxiety-like behavior induced by the single housing (Pritchard *et al*, 2013). In fact, single-housed animals could also experience less stress than group-housed animals, indicated by one study showing lower cortisone levels in single-housed animals compared to group-house animals (Roeckner *et al*, 2017). In our experience the use of filtertops on the animals' cages seemed to reduce the rats stress levels while increasing their alcohol consumption.

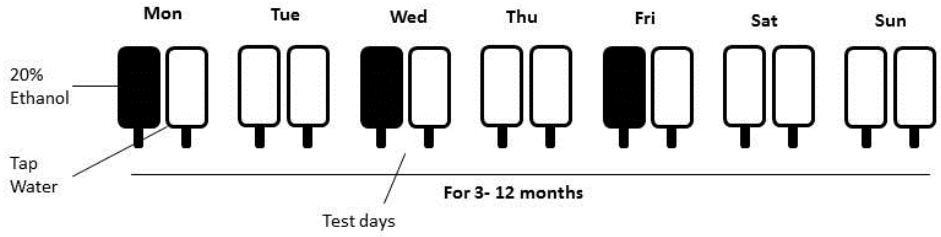

Figure 5. Two-bottle choice intermittent access to 20% ethanol (IA20E) drinking paradigm
Single-housed male rats got access to 20% alcohol and water on three alternating days during the week. Access to alcohol is given shortly after the beginning of the dark phase. Fluid intake is measured after 4 and 24 hours. Alcohol intake is voluntary and escalates during the first weeks. Pharmacological compounds can be administered on Wednesdays to test their acute effect on alcohol intake and a potential rebound effect on alcohol intake on Fridays.

Reflections

We observed that each rat's alcohol intake was individually quite stable after the first few weeks, but varied between individuals. Thus, we usually had batches with individuals voluntarily drinking relatively low (1-2 g/kg/24hrs), moderate (2-3 g/kg/24hrs), or high amounts of alcohol (3.5-6 g/kg/24hrs). Although the overall alcohol intake was higher in previous studies (Simms *et al*, 2008; Wise, 1973) compared to this thesis, I consider this variation in alcohol intake in the present thesis a strength of the model, considering the individual variance of alcohol intake in the human population. Moreover, it is of interest to the development of AUD to examine whether moderate amounts of alcohol intake over a long period of time are able to induce relevant biological changes. Finally, (-)-OSU6162 has been shown to reduce alcohol intake in rats voluntarily drinking high, but not low, amounts of alcohol, proving selectivity for moderate and high drinkers (Steensland *et al*, 2012). Nevertheless, although the IA20E model shows face, constructive and predictive validity, an even better model would be one where (at least some) rats continue to escalate their voluntary alcohol intake to levels of intoxication and physical dependence.

Since both age and experience has an influence on the dopamine system (Ihalainen *et al*, 1999; Jonsson *et al*, 2014), we used an age-matched alcohol-naive control group, i.e. rats that were given water in a two-bottle choice paradigm (paper I-III). These rats were treated in the exact same way (e.g. weighed daily, bottles changed in the same way as in the alcohol group) as the alcohol group with the exception of access to alcohol.

3.3 REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION PCR (PAPER I)

Reverse transcriptase real-time polymerase chain reaction (RTqPCR) is a sensitive technique to quantify mRNA from cells or tissue even if only very small amounts are available.

We used a two-step method, where mRNA is first converted into cDNA with random primers and then the resulting cDNA is amplified with specific primers and quantified with SYBR Green. The SYBR Green dye intercalates with all double-stranded DNA, i.e. with the mRNA-derived cDNA but also genomic DNA, primer dimers and hairpins. Hence, genomic DNA was digested prior to cDNA conversion. Moreover, melt-curves were analyzed and agarose gel of the qPCR-amplified sample and a no-reverse-transcription-control were run to test for unspecific amplification. Furthermore, to avoid amplification from genomic DNA primers were designed to detect exon-exon junctions. No sample controls were always added in each RTqPCR to test for cross-contamination during the preparation of samples. Finally, multiple reference genes were checked to ensure stable expression, however, only

peptidylprolyl isomerase A (PPIA) was used for analysis, since it has been shown to be the most reliable (Engdahl *et al*, 2016).

Reflections

The method used for extraction of mRNA and DNA from tissue (tissue lysis and column-based separation) did not result in very high levels of nucleic acids. Alternative methods such as chloroform extraction exist, which might produce better yields. However, we wanted to be able to detect both RNA and DNA to measure both gene expression and CpG methylation levels. Furthermore, we needed an easy method that could be used for many samples as I had no prior experience with, for example, chloroform-based extraction. Furthermore, based on the 260/280 nm absorbance ratios of above 2.0 and 1.8 for RNA and DNA, respectively, the purity of these nucleic acids seemed satisfactory.

Instead of the intercalating dye method (SYBR-Green), a 5'nuclease assay (TaqMan® or PrimeTime®) could have been used as a more specific method for quantification of the amplified product. 5'nuclease assays use sequence-specific probes that attach to the DNA strand during amplification, releasing a fluorescent probe from its quencher. Moreover, multiplex-qPCR could have been used to quantify multiple genes in one sample simultaneously, in case a greater amount of samples are being analyzed. Although this method needs certain expertise and would have been more expensive, perhaps more brain regions could have been analyzed.

A more unbiased approach than the selection of target genes based on a prior hypothesis would have been to start with a microarray. However, our aim was not to get a full picture of all the genes differentially expressed following long-term drinking. Nevertheless, after a first array, target hits need to be verified by qPCR.

3.4 PROXIMITY LIGATION ASSAY (PAPER I)

PLA is a sensitive method that can detect and visualize receptor-complexes in brain slices. It is based on using specific antibodies for each receptor and secondary antibodies connected to short nucleic acids, which-upon being in close proximity- can form a circle that can be amplified with PCR (Fig.6). The amplified PCR product can be visualized with a fluorescent dye, appearing as specific 'plobs' in the slice. Many studies analyze and quantify only a single selected z-layer. Instead, we scanned 20 layers and stacked them to one single picture. This measure avoids bias and variability based on the selection of certain layers.

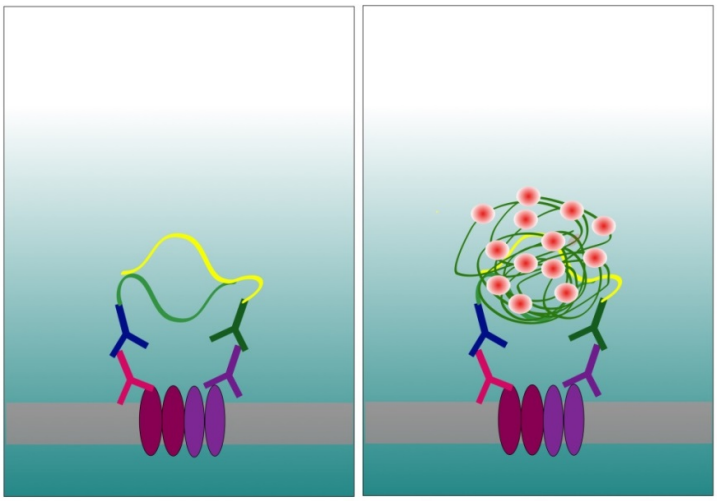

Figure 6. Principle of proximity ligation assay
Left: Two primary antibodies, one for each receptor, derived from different species (e.g. mouse and rabbit) are binding to the receptors. Then, secondary antibodies (e.g. goat-derived anti-mouse & anti-rabbit) attached to oligos (PLA probes) are binding to the primary antibodies. Only when the receptors are in close proximity (10 nm) can these oligos from a ring.
Right: The DNA-ring is ligated and amplified with PCR. The fluorescent probes are binding to the amplified DNA. In the microscopy slice, each receptor complex will appear as one dot.

Reflections

Today, the PLA technique is the only technique that is used to identify receptor-complexes in tissue. However, the existence of receptor complexes such as the D2R-A2AR in cells has been confirmed with bioluminescence resonance energy transfer and co-immunoprecipitation (Borroto-Escuela *et al*, 2011; Kamiya *et al*, 2003). Also, the findings of allosteric receptor-receptor interactions, i.e. the ligand of one receptor affects the affinity for the ligand of the interacting receptor (Fuxe *et al*, 2014a), indicates that a direct interaction might indeed occur as opposed to a secondary interaction on the secondary messenger level. Moreover, these allosteric interactions have been reduced by a point mutation (Borroto-Escuela *et al*, 2010a), further supporting the idea of direct receptor-receptor interactions. However, this technique can only identify changes in receptor density. The effects of these changes on receptor affinity and cell signaling should be investigated.

I have personally not been involved in any optimization since this technique was up and running in the Fuxe laboratory and has been used to identify changes after chronic cocaine self-administration (Borroto-Escuela *et al*, 2017). In fact, I mostly assisted Lucas Pinton and Dasiel Oscar Borroto-Escuela in the execution of the experiments. Then, Lucas analyzed the slices of the pictures and I did the statistical analysis. Therefore, I would have to conduct more PLA experiments to gain true independence and master this technique. Nevertheless, I was involved in creating the hypothesis and interpretation of the data and the concept of receptor-complexes fundamentally changed my understanding of neuropharmacology.

3.5 MICRODIALYSIS (PAPER II)

Microdialysis allows the quantification, as a measurement over time, of several neurotransmitters and their metabolites in the brain of the alive, free moving rodent.

The levels of the desired neurotransmitter or metabolite in the brain region of interest together with the detection limit of the HPLC/electrical cell system, determines the time-resolution. We used an established sampling frequency of 15 min, since we used off-line sampling and were most interested in dopamine changes over several hours. However, the high dopamine levels in the NAc would probably allow for an increased sampling frequency and thus, increased time-resolution. For example, time-resolutions of 1-min have been achieved recently with microdialysis in the striatum (Gu *et al*, 2015).

We constructed the probes ourselves, as this is an inexpensive method to produce a probe at each desired length. Furthermore, we left the probe including the dialysis membrane inside the brain for 48 hours before dialysis started. Since insertion of the probe causes some tissue trauma, this period allows for some tissue recovery before the dialysis and might influence the detection limit. More commonly, purchased probes are inserted into the brain directly before microdialysis by using a previously surgically implanted guide cannula. Since the probe is longer than the guide a fresh trauma is induced and an inflammatory response initiated potentially increasing the noise and the detection threshold.

High performance liquid chromatography (HPLC) was used to separate neurotransmitters and metabolites. Concentrations of methanol and octanesulfonic acid were optimized for each HPLC system to ensure optimal separation. Dopamine, DOPAC, HVA of known concentration (external standards) were used to enable quantification of these molecules in the samples.

Reflections

I had previously done microdialysis in the laboratory of Dr. Svensson (under the supervision of Dr. Schilström), where the HPLC analysis of the samples used in paper II took place. Thus, I had some experience with the surgical techniques, as well as the HPLC analysis. However, for the thesis project, I had to set up the surgery and the microdialysis at another laboratory, where this type of methodology had not been previously used. Mrs. Aronsson and I prepared one room for surgery and another for the dialysis. In the surgery room, to protect me from isoflurane exposure, we used a fan connected to a big tube that could suck isoflurane from the surgical area and transport it to the ventilation system. I also used oxygen from a gas bottle to mix with the isoflurane, preventing hypoxia during longer surgeries. Since the room

was not always only used for surgery, I took great care in cleaning all the surgical areas, the stereotaxic frame and the handles of the microscope with 70% ethanol. I also always used clean scrubs and surgical towels above the animal leaving only the head exposed. As no additional room was available to shave the animal's hair, I cleaned the head using an ethanol-soaked cotton ball and using scissors cut the wet hair at the incision area off. After surgery animals got chow soaked in water to facilitate eating. Onto the box for dialysis, I attached a moveable arm that had a ring attached, through which I threaded the tubings. To protect the tubings from being grabbed and chewed by the rat I threaded them through a rubber tube, whose stiffness also allowed the tubings to go straight towards the ring without bending. The pump was located higher than the boxes to ensure good flow avoiding pumping against gravity. The Hamilton syringes were cleaned with dishwater detergent and scrubbed with a brush after every use to avoid leakages, which would affect the flow.

Moreover, since I used older, bigger animals with a thicker skull compared to the previous studies, I encountered some problems. For example, I had trouble using the surgical anchor screws, which also seemed to have changed between delivery batches. I also used isoflurane for the first time. Using isoflurane enables a better control of the depth of anesthesia compared to injectable anesthetics and allows for a faster awakening of the animal after surgery. Finally, I used off-line sampling for the first time, which brings its own challenges, such as dopamine oxidation, variation in the amount sampled/injected. To avoid dopamine oxidation 0.5 M perchloric acid was added to the collection tubes. However, two added standards to correct for variations in flow and injection into the column would have been useful. Furthermore, due to offline-sampling, I was unable to know, if the dopamine baseline would be stable before the first injection. Nevertheless, the laboratory where the analysis was done had a great experience with microdialysis and up and running HPLC-electrochemical cell systems.

Alcohol was injected during dialysis (Fig. 7 for experimental set-up) to avoid individual differences in time and amount of alcohol intake. Our original plan was to repeat the microdialysis study with rats voluntarily drinking alcohol during dialysis, in order to see if rats would truly restore their reduced dopamine levels back to baseline. This experiment might produce valuable information, however, the possibility that rats would drink less after surgery (Wise and James, 1974) and therefore would need a prolonged recovery period needs to be taken into account. Unfortunately, we had to cancel that follow up experiment because my animal allergy had become severe.

Dialysis was done unilateral, in the right NAc. Based on studies showing lateralized effects of alcohol in humans (Oberlin *et al*, 2015), it might have been informative to study both hemispheres, although rats are probably not as lateralized as humans are.

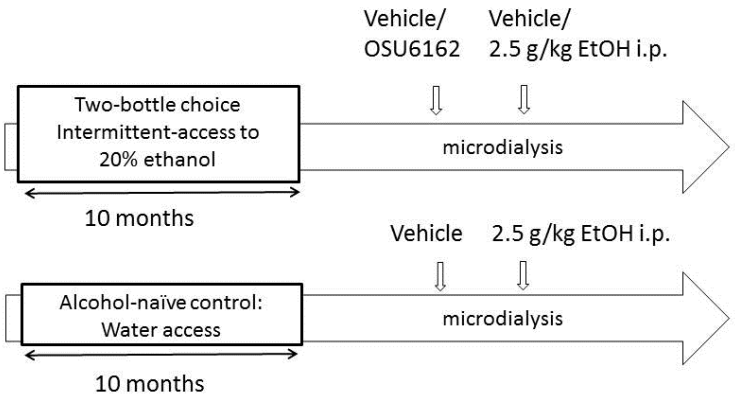

Figure 7. Set-up of microdialysis experiment (paper II)
Vertical arrows indicate injections during dialysis (separated by 60 min). The baseline dopamine values (before injection) and the dopamine output induced by the ethanol injection were compared between the alcohol drinking group and the control group. The effects of (-)-OSU6162 on basal and ethanol-induced dopamine output were evaluated in alcohol-drinking rats.

3.6 BINGE-EATING MODEL (PAPER IV)

The binge-eating model is a relatively simple paradigm to measure binge-eating (eating large amounts of palatable food in a short time) and the anticipation of palatable food (Fig. 8) developed by Cottone and coworkers (Cottone *et al*, 2008).

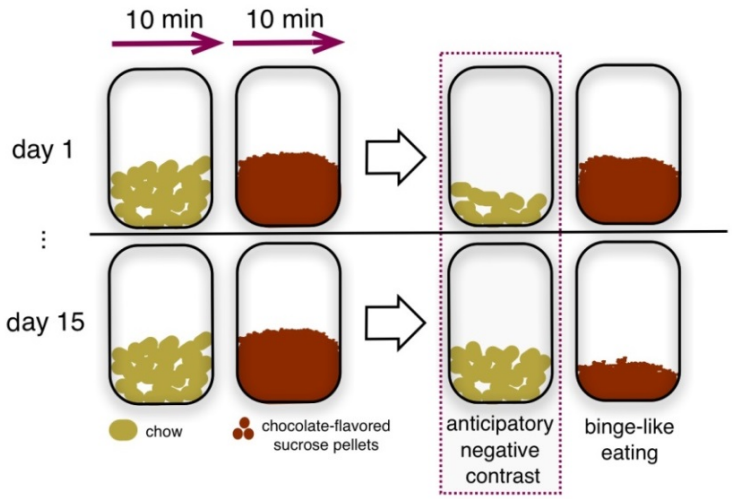

Figure 8. Binge-eating model
During training, rats are daily exposed to a 2 hour-food deprivation, 10-min chow access (first feeder), followed by 10-min chow access (second feeder). After stabilization of food intake rats get access to palatable food in the second feeder. Food intake is measured by weighing the feeders before and after food access (left and right side of the arrow) On day 1, rats continue to eat a substantial amount of chow and an equal amount (in kcal) of sucrose. During 15 days, rats significantly escalate their sucrose intake (binge-like eating), while reducing the prior chow intake in anticipation of the palatable food (anticipatory negative contrast).

In the binge-eating model the anticipatory negative contrast is interpreted as a voluntary self-restriction of food in anticipation of the more palatable food. This effect is compared to the dietary restraint persons suffering from binge-eating disorder (Corwin, 2006). However, persons with this disorder are usually under great distress, trying to control their eating, but

ultimately failing and 'relapsing' into binge-eating. It is therefore, questionable if the present model truly mimics this compulsive behavior and the attempt to control food intake. Nevertheless, the rats display a binge-like eating pattern of high-caloric food, the core feature of BED. Other models have used intermittent access to highly palatable food in the home cage, inducing significant weight gain, an aspect we did not study in the current paper. However, BED is not necessarily associated with weight gain.

Reflections

I learned this method from Dr. Giuliano who had published her results in 2012 (Giuliano *et al*, 2012). I had three groups that were run after each other, which resulted in long days, since each had a roughly 3-hour procedure. In the original version of the method (Giuliano *et al*, 2012), rats got an additional 1-h food access before the 2-h food deprivation, which we excluded. However, we did measure homecage food intake every 24 hours. My group of rats had a substantial greater amount of spillage than Dr. Giuliano was used to. We collected and weighted all spillage to account for it when we measured the food intake. Still, there may be some measurement error based on the spillage as food could get wet or disintegrate into powder. We trained the animals for one month in the hope that spillage would decrease, which however, did not happen.

3.7 SECOND-ORDER SCHEDULE OF REINFORCEMENT (PAPER IV)

This is a complex training schedule with the aim to separate the seeking period before the reward is received from the seeking period after the reward is received (Fig. 9).

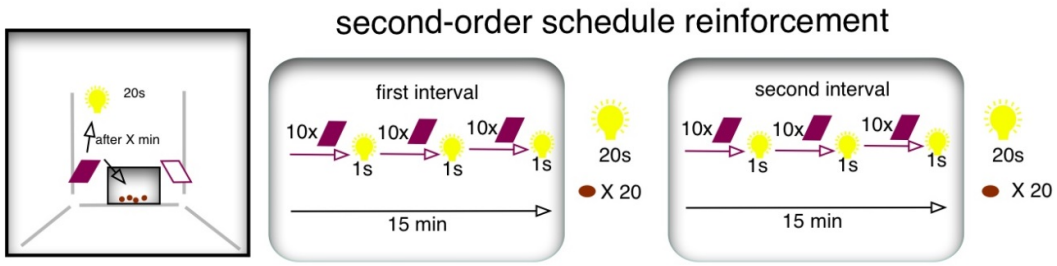

Figure 9. The second-order schedule of reinforcement
The basis of the second-order schedule is a fixed interval schedule, where upon a press of the active lever rats get a certain amount of pellets paired with a 20-s light stimulus (conditioned stimulus, CS) after a fixed time has elapsed (left box). The fixed interval starts at 1 min and is increased gradually over several days to 15 mins. The fixed interval 15-min schedule becomes a second-order schedule of reinforcement when after each 10 active lever presses a short 1-s CS is introduced. Consequently, the 1-s CS increases the amount of lever presses. After 15-min have elapsed (first interval) the rats are rewarded with 20 chocolate-flavored sucrose pellets. Then, an identical 15-min interval (second interval) and a reward delivery follows. Hence, the first (pre-reward) and second (post-reward) interval can be analyzed separately to identify the effects of compounds on sucrose seeking directly (during the first interval) or indirectly by affecting hedonic aspects during food ingestion (during the second interval).

Originally, the second order schedule of reinforcement was developed by Prof. Everitt to study sexual behavior (Everitt *et al*, 1989). Since, drugs of abuse or other centrally active compounds can influence seeking behavior by itself (e.g. cocaine has in itself a motor stimulatory effect), the second-order schedule is a useful tool to study motivation to obtain rewards, especially the cue-controlled seeking behavior (Everitt and Robbins, 2000).

Reflections

I also learned this method by Dr. Giuliano (Giuliano *et al*, 2012). The inherent difficulty with this behavior compared to other behaviors seems to be that the seeking behavior under this schedule is less stable between days for each individual. Hence, there is a bigger error and re-baselining between testing days is harder to assess.

3.8 A WORD ON STATISTICS

In both the alcohol (paper III) and sucrose study (paper IV) we tested pharmacological compounds using a Latin-square design (i.e. every rat received all doses and thus was its own control) when pharmacological compounds were tested, to minimize the effects of inter-individual behavioral differences. When between-group comparisons were required (paper II, microdialysis study), rats were matched for their individual alcohol intake. Similarly, before being designed for different treatments or paradigms (paper IV), rats were matched for baseline responding.

Parametric tests were only used when their assumptions were met, such as Gaussian distributions (e.g. non-skewed), similar standard deviation between groups. If these assumptions were not met, non-parametric tests were used or data was square-rot transformed. For example, in paper II basal levels of dopamine, DOPAC and HVA were compared between alcohol-drinking and alcohol-naïve rats using Mann-Whitney U test and in paper IV FI15 and second-order data was square-rot transformed.

The significance level was set at alpha = 0.05, i.e. the chance of observing the finding by chance is less than 5%. This agreement is based on the aim to keep the risk of a type-I error low, i.e. falsely rejecting the null-hypothesis (falsely claiming a difference). However, care should be taken at interpreting non-significant results as they do not proof absence of differences, i.e. do not proof the null-hypothesis to be true. Moreover, although the significance level could be kept even lower, which can be necessary on large data sets, this could increase the risk of a type II error (failing to reject a true null-hypothesis, i.e. missing a difference).

Whenever possible, I have been blind to the drinking condition (alcohol vs. water), when, for example, scoring the novel object recognition data (paper II) or extracting RNA and DNA

from brain tissue. The PLA experiments have also been analyzed by a person blind to the drinking condition.

Due to ethical concerns the number of animals used in each study is generally kept to a minimum. However, the use of small sample sizes, commonly used (including the present thesis) increases the likelihood of observing extreme, random results. This effect, together with a publication bias for positive results, might have contributed to generally poor reproducibility in preclinical neuroscience studies (Button *et al*, 2013). Hence, it might be crucial to increase sample sizes, attempt to replicate one's own and other people's findings and publish all findings.

3.9 ETHICAL CONSIDERATIONS

All experiments carried out in the study have been approved by the ethical committees in Sweden or England.

As all studies involve animals (mammals) it should be carefully evaluated, if these experiments are relevant and necessary. The aim of this study was to ultimately aid in the development of novel treatments for AUD and BED. Both disorders cause great individual suffering and for both disorders better treatments are needed. Therefore, I believe that the experiments in this thesis were necessary as the study of alcohol's effect on the brain and the investigation of compounds' effect on voluntary alcohol drinking and binge-like eating need complex, higher-order organisms, such as mammals. However, my hope is that the interdisciplinary collaboration, a greater understanding of neurobiology and computer models in the future will ultimately eliminate the need for animal experiments.

Another aspect is that the animals should not suffer in line with EU, British and Swedish ethical guidelines. Only very few animals displayed signs of sickness and had to be sacrificed prior to the end of the experiment.

A number of refinement measures were taken in order to reduce the number of animals required for the experiments. First, rats were handled daily to ensure familiarity with the experimenter, reduce stress and increase stability in behavioral readout. Second, most alcohol-drinking rats were subjected to repeated treatments (separated by wash-out periods) during their life-time. Third, sensitive techniques (PLA, qPCR) and Latin-square designs were used. Finally, long training periods (alcohol drinking, sucrose seeking) allowed the behaviors to become individually stable, which is crucial for the use of Latin-square designs.

4 RESULTS AND DISCUSSION

4.1 EFFECTS OF LONG-TERM ALCOHOL DRINKING ON THE DOPAMINE SYSTEM (PAPER I AND II)

4.1.1 Results

I evaluated the effects of long-term voluntary alcohol drinking on D2R expression (paper I) and dopamine output (paper II) in the NAc using rats that had been drinking in the IA20E model (Fig. 5) and age-matched alcohol-naïve controls. First, alcohol drinking reduced Drd2 gene expression in the NAc (Fig. 11). Second, alcohol drinking significantly decreased densities of D2R-D2R homoreceptor complexes in the striatum (including the NAc shell) and increased densities of A2A-D2R heteroreceptor complexes in the NAc shell (Fig. 12). Together, these results indicate reduced D2R levels and affinity (based on the reciprocal A2AR-D2R antagonism). Third, alcohol drinking reduced basal extracellular dopamine levels in the NAc (Table 2) and blunted the dopamine response to an alcohol challenge (Fig. 13).

Figure 11. Alcohol drinking reduces D2R gene expression in the NAc
A) Rats that had been drinking in the IA20E model for five months (alcohol, n=12) displayed a significantly lower gene expression of the D2R long isoform (D2L) in the NAc compared to the alcohol-naïve age-matched control group (n=7). *p<0.05, two-way ANOVA followed by Bonferroni post-hoc (from analysis including the dorsal striatum not shown here). B) There was a significant correlation between the individual alcohol intake (mean of seven weeks) and the D2L expression levels (Pearson's correlation).
Gene expression was measured using RTqPCR and is presented as 2^ΔCT values relative to PPIA.

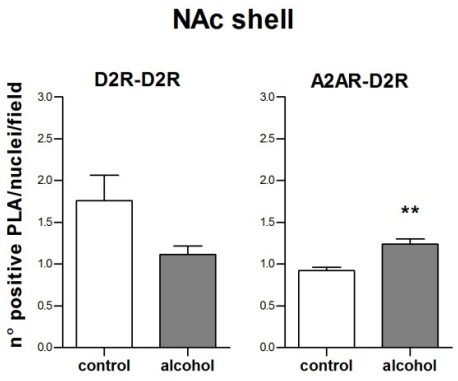

Figure 12. Alcohol drinking changes the densities of D2R homo-and heteroreceptor complexes in the NAc
Rats that had been drinking in the IA20E model for 12 weeks were compared to age-matched alcohol naïve controls (n=4/group) for *in situ* densities of D2R receptor complexes using the proximity ligation assay (PLA, Fig. 6). Alcohol drinking reduced the density of D2R-D2R homoreceptor complexes in the striatum (NAc core, shell and dorsal striatum analyzed together in a two-way ANOVA, significant overall main effect of alcohol drinking) and increased the density of A2AR-D2R heteroreceptor complexes in the NAc shell (right) and dorsal striatum (not shown), ** p<0.01, two-way ANOVA followed by Bonferroni post-hoc.

Table 2. Alcohol drinking reduces basal dopamine levels in the NAc

	Dopamine [fmol/min]
Alcohol-naïve group	2.5 (2.3-3.3)
Alcohol-drinking group	1.8* (1.2-2.7)

Using microdialysis, basal dopamine levels in the NAc of rats that had been drinking in the IA20E model for ten months (n=27) and age-matched alcohol-naïve controls (n=7) were measured. After three hours of dialysis, three baseline samples were collected (over 45 min) and the mean of these samples were used to calculate the group medians (interquartile range) provided here. The experimental set-up can be found in Fig. 7. *p<0.05, Mann-Whitney *U*-Test.

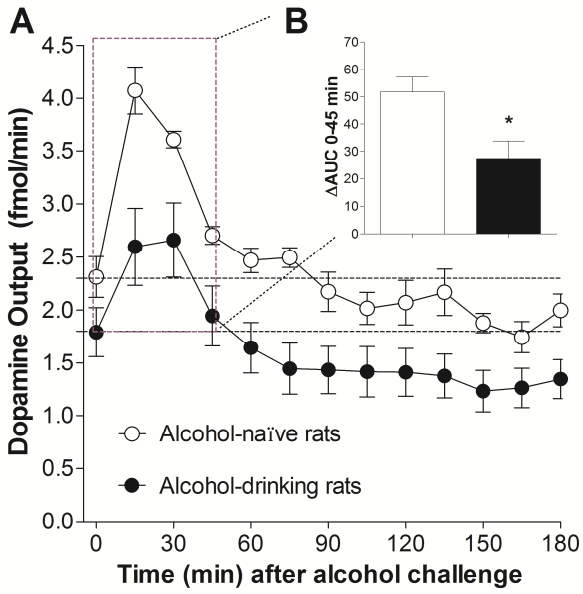

Figure 13. A blunted dopamine response to alcohol in alcohol-drinking rats
A) Microdialysis was used to measure the extracellular dopamine levels in the NAc over time. Rats that had been drinking in the IA20E model for ten months (n=7, filled black circles) and an age-matched alcohol-naïve control group (n=5, filled white circles) were subjected to an alcohol injection (2.5 g/kg, i.p.). The experimental set-up can be found in Fig. 7.
B) The increase in dopamine levels caused by the alcohol injection was measured as the area under the curve increase between 0 to 45 min relative to time-point 0 min. *p<0.05, Student's t-test.

4.1.2 Discussion

The effects of long-term voluntary alcohol drinking on dopamine and D2R expression in the NAc presented above indicate a hypo-functioning dopamine system caused by alcohol drinking. This hypothesis is supported by findings in alcohol-dependent detoxified patients showing a reduced D2R radioligand binding (Hietala *et al*, 1994; Volkow *et al*, 1996), as well as a reduced dopamine response to central stimulants (Martinez *et al*, 2005; Volkow *et al*, 2007) compared to healthy controls. Moreover, reduced D2R binding has also been found in cocaine, methamphetamine and heroin abusers (Volkow *et al*, 2009). Reduced central stimulant-induced dopamine release has also been found in cocaine (Volkow *et al*, 2014; Volkow *et al*, 1997) and in methamphetamine abusers (Wang *et al*, 2012). Hence, a hypo-functioning dopamine system seems to be a common factor of different substance use disorders, including AUD.

Since drugs of abuse consistently release dopamine, repeated counter-adaptive processes of homeostasis might eventually lead to an allostatic state, in which the dopamine system is downregulated outside of the homeostatic range. This theory of allostasis of addiction has been described by Koob and Le Moal (2001), but for a more recent description see (George *et al*, 2012). More specifically, a hypo-functioning dopamine system, together with a hyper-functioning HPA stress axis is thought to underlie a negative emotional state during withdrawal. This theory is in line with the concept that during the development of addiction there is a shift from positive to negative reinforcement (Wise and Koob, 2014).

Although the theory of allostasis suggests a drug-induced downregulation of the dopamine system, reduced D2R levels in the striatum might also be an underlying vulnerability factor, i.e. a neurobiological characteristic that makes the individual more likely to seek and abuse drugs. This suggestion is indicated by studies showing that some rat strains bred for alcohol-preference have reduced D2R levels (McBride *et al*, 1993; Stefanini *et al*, 1992). In contrast, high D2R levels could protect individuals against the development of AUD. This suggestion is supported by one study showing that unaffected members of a family with AUD have higher striatal D2R levels than individuals without a family history of AUD. In these individuals, increased D2R levels correlate with increased frontal lobe metabolism and positive emotionality (Volkow *et al*, 2006).

Similar to the results in the present thesis, several rodent alcohol models had previously shown that chronic alcohol consumption reduces dopamine output in the NAc (Barak *et al*, 2011; Kashem *et al*, 2012; Weiss *et al*, 1996). These studies and the present thesis used outbred rats randomly allocated to alcohol or water access, making underlying vulnerability factors less likely to cause group differences. Furthermore, both the study by Barak and co-workers (2011) and the present thesis used the IA20E paradigm, a model possessing face, construct and predictive validity (see section 3.2). Whereas the former study used ten weeks of alcohol exposure, we used ten months of alcohol exposure, indicating that these changes last and are not masked by an age-related decline in dopamine levels.

The literature has been inconsistent regarding the effects of alcohol consumption on the dopamine output and the D2R levels in the NAc (for details, see section 1.5.2). However, most models used forced alcohol intake, which has no face validity and little translational value. The present thesis shows that long-term alcohol drinking is downregulating D2R-D2R homoreceptor complexes while upregulating A2AR-D2R heteroreceptor complexes. To the best of my knowledge, these receptor complex-specific changes induced by alcohol-drinking have not been reported previously. Furthermore, our collaborator Dr. Borroto-Escuela and

his co-workers (2017) have recently shown that chronic cocaine self-administration also upregulates A2AR-D2R heteroreceptor complexes in the NAc. This result strengthens the previous suggestion that blocking the A2AR-D2R heteroreceptor complex might provide a novel treatment strategy to compensate for the reduced D2R availability in substance use disorders (Kravitz *et al*, 2015). Considering the present results of a hypo-dopaminergic state in alcohol drinking and the upregulation of A2AR-D2R heteroreceptor complexes, this might be a valid treatment strategy for AUD as well.

Finally, the results in the present thesis indicate that the downregulation of D2R-D2R homoreceptor complexes is caused by a reduction in gene expression of the long isoform of the D2R. Jonsson and co-workers (2014) showed recently that rats with continuous access to 6% alcohol in a two-bottle choice paradigm display reduced D2R gene expression in the NAc, compared to age-matched controls, after two and four months, but not at ten months of voluntary alcohol drinking. More specifically, an age-related decline in dopamine levels masked the effects of alcohol. However, the alcohol intake was generally low (less than 1g/kg/24h), due to the continuous access and measurements of fluid intake only twice a weak (Jonsson *et al*, 2014). In the present thesis, rats displayed a higher alcohol intake than in this study, indicating that an alcohol-induced downregulation of D2R expression might still be significant compared to age-matched controls after longer alcohol access than used in the present study (three to five months).

Findings of several studies suggest that a downregulation in dopamine and D2R levels, as found in the present thesis, might contribute to the maintenance of alcohol drinking. First, rats with reduced NAc dopamine levels induced by chronic liquid alcohol diet self-administer just enough alcohol to restore their dopamine baseline (Weiss *et al*, 1996). Second, mice with reduced NAc dopamine levels due to chronic MDMA ('ecstasy') treatment show increased alcohol consumption and preference than saline-treated rats (Izco *et al*, 2007). Third, in humans, the alcohol-induced dopamine release in the ventral striatum correlates with the self-reported levels of intoxication (Oberlin *et al*, 2015; Urban *et al*, 2010). Fourth, reduced D2R levels in the ventral striatum of alcohol-dependent patients are correlated to craving severity and with greater cue-induced activation of the medial prefrontal cortex and anterior cingulate measured by fMRI (Heinz *et al*, 2004). In fact, a downregulated dopamine system in drug abusers is associated with a reduced activity in the prefrontal, cingulate and orbitofrontal cortices, which is thought to underlie impaired executive control, impulsivity and compulsivity, respectively (Volkow *et al*, 2009). Fifth, low levels of dopamine have been associated with earlier relapse in alcohol-dependent patients (Guardia *et al*, 2000). Finally, increasing the dopaminergic tone reduces voluntary alcohol intake rodents (Bass *et al*, 2013;

Cheng *et al*, 2017; Dyr *et al*, 1993; Thanos *et al*, 2004; Thanos *et al*, 2001) as does the dopamine-releasing compound (-)-OSU6162 in the present thesis (see the following section).

The blunted dopamine response to an alcohol challenge found in long-term drinking rats in the present thesis could potentially indicate a tolerance effect to the rewarding effects of alcohol. Hence, the present results might further support the notion that dopamine is involved in the shift from positive to negative reinforcement during the development of AUD. However, in contrast to my finding, one previous study showed that repeated alcohol gavage increases the dopamine response in the NAc to an alcohol challenge (Diana *et al*, 1993). Similarly, one recent study showed that ten weeks of continuous voluntary alcohol intake in female alcohol-preferring rats increases the NAc dopamine response to intra-VTA alcohol infusions (Ding *et al*, 2016). Moreover, a human imaging study revealed that intravenous alcohol administration only releases dopamine in the ventral striatum in alcohol-dependent, but not in social drinkers (Yoder *et al*, 2016). Although these studies are contrasting my findings, one study showed that the alcohol-induced dopamine release is attenuated in MDMA-treated dopamine deficient mice and hypothesized that this result represents an increased reward threshold for alcohol, which in turn, increases alcohol consumption (Izco *et al*, 2007). Furthermore, both methamphetamine and cocaine abusers have a blunted striatal dopamine response to methylphenidate. For example, a small study reported that absence of this dopamine response predicted relapse in methamphetamine abusers (Wang *et al*, 2012). However, although cocaine-dependent patients had a reduced striatal dopamine response, they exhibited an increased thalamic dopamine response that was correlated to craving (Volkow *et al*, 1997). This finding, together with the thalamus' function to filter information from subcortical areas to the cortex ('gate to consciousness') and the discovery of dopaminergic innervation of the thalamus in primates but not rodents (Sanchez-Gonzalez *et al*, 2005), challenges the use of rodent models for addiction. Nevertheless, further studies are needed to test the consistency of a drinking-induced downregulation of the dopamine system and investigate a potential association with dysphoria and maintenance of alcohol intake.

4.2 THE ROLE OF DOPAMINE IN (-)-OSU6162'S EFFECTS TO REDUCE VOLUNTARY ALCOHOL DRINKING (PAPER II AND III)

4.2.1 Results

Potential mechanisms underlying the effects of (-)-OSU6162 to reduce voluntary alcohol intake were investigated. (-)-OSU6162 increased NAc dopamine levels and normalized the alcohol-induced dopamine release in alcohol-drinking rats with a hypodopaminergic tone (Fig. 14, paper II). Pre-treatment with a 5-HT2A antagonist, but not with a D2 antagonist, attenuated the (-)-OSU6162-induced reduction in alcohol intake (Fig. 15) (paper III).

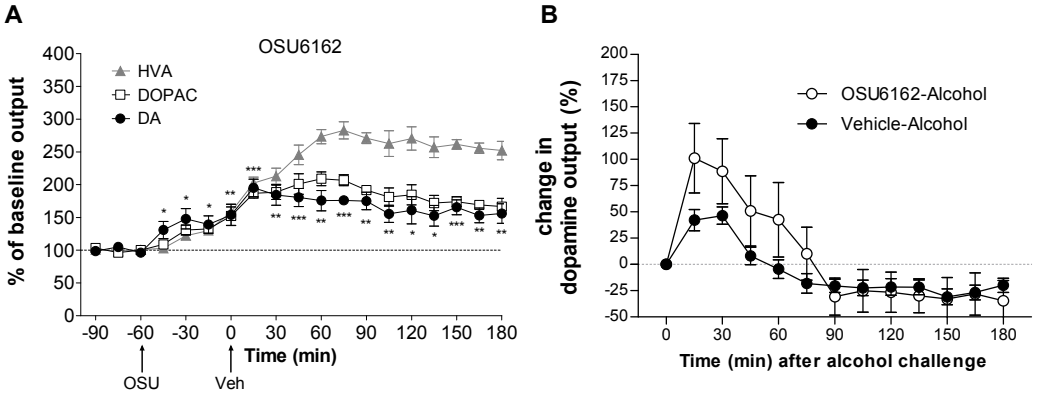

Figure. 14 (-)-OSU6162 restores the dopamine levels in the NAc in long-term drinking rats
During microdialysis, rats that had been drinking in the IA20E model for ten months were treated with (-)-OSU6162 (OSU, 30 mg/kg, s.c.) followed by vehicle (Veh) or alcohol (2.5 g/kg, i.p.) 60 min later (n=7-8 rats, experimental set-up, see Fig. 7). A) (-)-OSU6162 significantly increased dopamine (*p<0.05, **p<0.01, ***p<0.001) compared to baseline. DOPAC and HVA were significantly elevated compared to the baseline from -45 min to 180 min (asterisks omitted); repeated-measures one-way ANOVA followed by Fisher's least significant difference post hoc test. B) Pre-treatment with (-)-OSU6162 had no significant effect on the alcohol-induced increase in dopamine output (no overall effect of treatment or time*treatment interaction effect, two-way ANOVA).

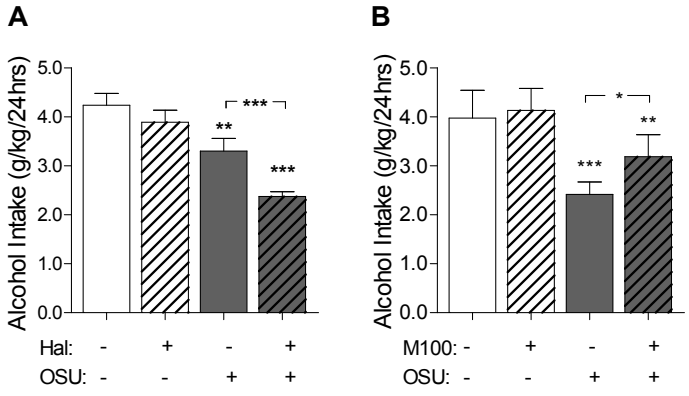

Figure 15. (-)-OSU6162 reduces voluntary alcohol intake via partial 5-HT2A agonism
Rats that had been drinking in the IA20E model for five to sevent months were pre-treated with the D2 antagonist haloperidol (Hal, 0.05 mg/kg) (A) or the 5-HT2A antagonist M100907 (M100, 0.5 mg/kg) (B) followed by (-)-OSU6162 (OSU) 60 min later (n=7-8 rats). Rats got access to alcohol 30 min after the second injection and mean±SEM alcohol intake after 24 hours is presented. Pre-treatment with haloperidol enhanced the (-)-OSU6162-induced reduction in voluntary alcohol intake (A). Pre-treatment with M100907 attenuated the (-)-OSU6162-induced reduction in alcohol intake. *p<0.05, **p<0.01, ***p<0.001 compared to vehicle-vehicle or as indicated, repeated-measures one-way ANOVA followed by Newman-Keuls post-hoc test.

4.2.2 Discussion

The results described in the previous section indicate that the effects of (-)-OSU6162 on the dopamine system, as well as 5-HT2AR partial agonism, are involved in the reduction in voluntary alcohol intake by (-)-OSU6162.

The (-)-OSU6162-induced increase in dopamine levels might underlie its previously found ability to reduce voluntary alcohol intake (Steensland *et al*, 2012). This suggestion is supported by a study showing that optogenetically-induced tonic stimulation of dopamine neurons in the VTA produces stable dopamine elevations in the NAc and dramatically decreases voluntary alcohol intake in rats that had been drinking in the IA20E model for seven weeks (Bass *et al*, 2013). Furthermore, rats self-administer just enough alcohol to restore the dopamine baseline that had been downregulated by chronic alcohol intake (Weiss *et al*, 1996), indicating that an increase in dopamine levels could potentially reduce the need for this compensation. Furthermore, transient over-expression of D2R in the NAc also reduces alcohol consumption (Thanos *et al*, 2004; Thanos *et al*, 2001). Together, these studies indicate that increasing dopamine transmission can reduce voluntary alcohol intake. However, to treat patients pharmacological compounds are needed. Indeed, the dopamine-releasing drug amphetamine reduces voluntary alcohol intake in animals (Feduccia *et al*, 2014; Halladay *et al*, 1999; Pohorecky and Sweeny, 2012), but is obviously a limited treatment option due to its abuse potential. This abuse potential might be caused by a significant peak-like dopamine increase induced by amphetamine. The anti-smoking medication varenicline (agonist/partial agonist at several nicotinic acetylcholine receptor) reduces alcohol drinking in rats (Steensland *et al*, 2007) and humans (Litten *et al*, 2013; McKee *et al*, 2009; Mitchell *et al*, 2012) and was recently shown to increase dopamine output in the NAc in rats (Feduccia *et al*, 2014). Similar to (-)-OSU6162 in the present thesis, varenicline seems to moderately increase dopamine in the NAc of rats and maintains stable levels for at least 2.5 hours. Especially, the long-lasting (at least four hours) increase in dopamine levels by (-)-OSU6162 could explain the reductions of 24-hour alcohol intake (Steensland *et al*, 2012). In conclusion, the increase in dopaminergic tone by (-)-OSU6162 might underlie the effects of (-)-OSU6162 to reduce alcohol drinking in rats and indicate a potential for (-)-OSU6162 to reduce alcohol consumption in AUD.

(-)-OSU6162 is thought to increase dopamine by antagonizing D2R autoreceptors (Carlsson and Carlsson, 2006). In fact, the lack of catalepsy in rats even at 80% of receptor occupancy (Natesan *et al*, 2006), as well as the moderate occupancy in humans (Tolboom *et al*, 2014) might indicate that (-)-OSU6162 does not bind to synaptic D2Rs. Furthermore, in vitro

studies have suggested that (-)-OSU6162 has a low affinity for the D2R and a fast dissociation rate (Dyhring *et al*, 2010), which might contribute to its preference for extrasynaptic D2R. Extrasynaptic receptors have a higher affinity for dopamine than synaptic receptors since they are usually exposed to lower dopamine concentrations (Carlsson and Carlsson, 2006).

Dopamine releasing compounds could be abused and cause addiction. However, the findings of my colleague Dr. Fredriksson that (-)-OSU6162 does not induce conditioned place preference in alcohol-naïve or alcohol-drinking rats (Paper II), indicate that (-)-OSU6162 is not rewarding by itself. Furthermore, a fast increase in dopamine levels and not the absolute dopamine levels seem to be important for drug-induced euphoria, which has been suggested by studies showing that intravenously injected methylphenidate, but not oral methylphenidate causes a feeling of 'high' (Swanson and Volkow, 2003). Swanson and Volkow suggested that the intravenous administration mimics phasic dopamine release, while the oral administration mimics tonic dopamine release. In the present thesis, following treatment with (-)-OSU6162 dopamine levels were slowly increasing and then plateauing for several hours, indicating less risk for abuse liability. Moreover, none of the alcohol-dependent patients in a proof-of-concept human laboratory study (Khemiri *et al*, 2015) reported wanting more of the (-)-OSU6162 tablets and only one patient reported euphoric effects, although even one patient in the placebo group did so (unpublished observation).

Increased dopamine levels can activate both D1R and D2R. One study in alcohol-preferring rats showed that the D2 agonist quinpirole was more effective in reducing voluntary alcohol intake than a D2 antagonist, although D1 agonist and antagonists also showed some effect (Dyr *et al*, 1993). However, in another study quinpirole did not affect alcohol consumption but increased water consumption (Linseman, 1990). Furthermore, the increased alcohol intake in dopamine-deficient mice (induced by MDMA) could be attenuated by a D1 agonist, which in turn, could be abolished by a D1 antagonist (Izco *et al*, 2007). Hence, both D1 and D2 transmission could be involved in (-)-OSU6162's effect on voluntary alcohol intake.

To investigate the role of postsynaptic D2R stimulation in (-)-OSU6162's ability to reduce voluntary alcohol intake, I pre-treated the rats with the D2 antagonist haloperidol and (-)-OSU6162 before access to alcohol was given. This idea is based on a study showing that haloperidol pre-treatment can attenuate (-)-OSU6162's stimulating effect on motor activity in rats with a suspected low dopaminergic tone due to arena-habituation (Carlsson *et al*, 2011). However, haloperidol potentiated the (-)-OSU6162-induced decrease in voluntary alcohol intake in the present thesis. One possibility is that haloperidol reaches the synaptic D2Rs that

(-)-OSU6162 cannot reach and thereby, through additional D2R antagonism reduces alcohol intake. However, we used a low dose of haloperidol that would not affect alcohol or water intake (pilot study, data not shown) and indeed in the present data set, haloperidol alone had only a small effect on alcohol intake. In fact, systemically-administered D2 antagonists reduce water intake at lower doses than those affecting alcohol intake in a two-bottle choice paradigm (Goodwin *et al*, 1996; Linseman, 1990; Silvestre *et al*, 1996). Another possibility is that haloperidol and (-)-OSU6162 in combination antagonize a bigger part of the population of D2R autoreceptors, leading to a prolonged dopamine release than either treatment alone.

Based on the found attenuation of the alcohol-induced dopamine release by (-)-OSU6162 in alcohol-naïve rats (Steensland *et al*, 2012), we originally hypothesized that (-)-OSU6162 is reducing the rewarding effects of alcohol via this mechanism. However, in long-term drinking rats in the present thesis we found the opposite: a trend for a potentiation of the alcohol-induced dopamine release. Although one could argue that this observation indicates that (-)-OSU6162 increases alcohol reinforcement, our behavioral data on alcohol drinking and alcohol seeking argue against this suggestion (Steensland *et al*, 2012). Furthermore, varenicline pre-treatment prolonged the alcohol-induced dopamine release in long-term drinking rats (Feduccia *et al*, 2014), further suggesting that a potentiation in alcohol-induced dopamine release might not be associated with increased alcohol intake. I suggest that (-)-OSU6162 rather normalizes a reduced dopamine response to alcohol caused by long-term drinking. This effect might reduce the amount of alcohol necessary to restore the downregulated dopamine baseline and therefore, reduce alcohol drinking.

The different effects of (-)-OSU6162 on the alcohol-induced dopamine release in alcohol-naïve (Steensland *et al*, 2012) and long-term drinking rats in the present thesis, could further indicate that (-)-OSU6162 affects the dopamine system differently depending on the dopaminergic tone. Nevertheless, this difference in dopaminergic tone might not solely be caused by alcohol intake, since the rats were of different ages and strains (both Wistar rats, but different suppliers) in the two studies. Previous studies showed that (-)-OSU6162 has dopamine-stabilizing properties, such as stabilizing motor activity based on the dopaminergic tone, preventing dyskinesia induced by dopaminergic compounds without causing akinesia (Ekesbo *et al*, 2000; Ekesbo *et al*, 1997) or stabilizing L-[11C]DOPA influx depending on the baseline (Tedroff *et al*, 1998). However, it is not completely understood how (-)-OSU6162 mediates these effects. Potentially, both dopamine-releasing, as well as inhibition of D2R transmission, possibly at extrasynaptic D2R receptors are involved in these effects, but also allosteric mechanisms have been suggested by in vitro studies (Kara *et al*, 2010; Lahti *et al*, 2007).

(-)-OSU6162 is not only a D2 antagonist but also a partial agonist at the 5-HT2AR (Burstein *et al*, 2011). The 5-HT2A antagonist M100907 could prevent the (-)-OSU6162-induced increase in motor activity in reserpinized-mice and arena-habituated rats (Carlsson *et al*, 2011). Similarly, in the present thesis, M100907 attenuated the (-)-OSU6162 induced decrease in voluntary alcohol intake, indicating a role of partial 5-HT2A agonism in (-)-OSU6162's effect to reduce voluntary alcohol intake. The effects of 5-HT2A antagonists on dopamine release (Yamamoto and Meltzer, 1992) and dopamine neuron firing (Grenhoff *et al*, 1990), indicate that modulating the 5-HT2AR activity could influence NAc dopamine levels. Therefore, 5-HT2AR partial agonism of (-)-OSU6162 might contribute to its stabilizing effects on dopamine activity (Ekesbo *et al*, 1997; Natesan *et al*, 2006; Rung *et al*, 2011; Tedroff *et al*, 1998) and in turn, reduce voluntary alcohol intake (Steensland *et al*, 2012).

Although this thesis has focused on the ventral striatum (especially the NAc) the effects of (-)-OSU6162 might also involve the dorsal striatum. A recent chemogenetic study showed that both stimulation of D1-medium spinal neurons (MSNs) and inhibition of D2-MSNs reduces voluntary alcohol intake in mice, implicating a role of both the direct and indirect dopaminergic pathway in alcohol consumption (Cheng *et al*, 2017). (-)-OSU6162 has been shown to release dopamine in the dorsal striatum in rats (Sonesson *et al*, 1994) and displace the D2R radioligand raclopride in the dorsal striatum of humans (Tolboom *et al*, 2014). Thus, the (-)-OSU6162-induced dopamine release or additional extrasynaptic D2R antagonism by (-)-OSU6162 in the striatum could also underlie the effects of (-)-OSU6162 on alcohol intake.

4.3 THE POTENTIAL OF (-)-OSU6162 TO REDUCE BINGE-EATING (PAPER IV)

4.3.1 Results

(-)-OSU6162 significantly decreased binge-like intake of palatable food, but not the prior self-restriction of normal chow in anticipation of this food, i.e. (-)-OSU6162 has no effect on the anticipatory negative contrast (Fig. 16A, for binge-eating model, see Fig. 8). Moreover, (-)-OSU6162 reduced chow intake in the control group that never got access to palatable food and therefore consumed higher amounts of chow (data not shown). (-)-OSU6162 reduced the cue-controlled seeking of palatable food under a second-order schedule of reinforcement during the first interval, i.e. before the first reward delivery (Fig. 16B). These results were replicated when (-)-OSU6162 was infused in the NAc core (Fig. 16C).

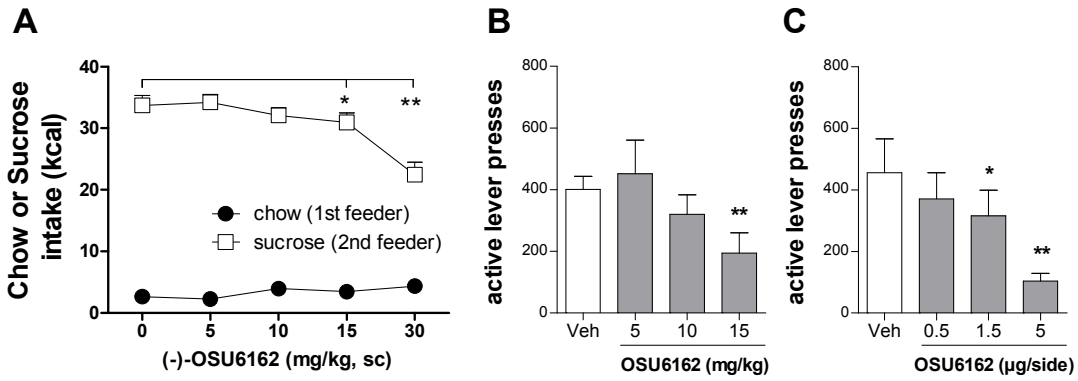

Figure 16. (-)-OSU6162 reduces binge-like eating and cue-controlled seeking of chocolate-flavored sucrose
(A) (-)-OSU6162 reduces binge-like intake of chocolate-flavored sucrose pellets, but does not affect the anticipatory negative contrast in prior chow intake (see model Fig. 8). Values represent mean ±SEM food intake (n=10). *p<0.05. ** p<0.01 repeated-measures one-way ANOVA followed by within-subject contrast to vehicle. Systemic (B, n=8)) and intra-NAc core (C, n=15) administration of (-)-OSU6162 significantly reduced seeking of chocolate-flavored sucrose pellets under a second-order schedule of reinforcement during the first interval, before reward delivery (see model Fig. 9). Values represent mean ±SEM lever presses. *p<0.05, **p<0.01 repeated-measures one-way ANOVA followed by within-subject contrast to vehicle.

4.3.2 Discussion

(-)-OSU6162 reduced both the binge-like consumption of palatable food, as well as motivation to obtain this food as indicated by the effect on cue-controlled seeking. This effect on both consummatory and appetitive behaviors indicate the potential of (-)-OSU6162 as a novel treatment for BED.

The effects of (-)-OSU6162 on binge-like eating of sucrose found in this thesis confirm previous findings of a reduction in voluntary intake of a sucrose solution (Steensland *et al*, 2012). Furthermore, since (-)-OSU6162 reduces intake of sucrose and alcohol, but not of a salt water solution, (-)-OSU6162 might specifically reduce the intake of rewarding substances. However, in the present thesis, (-)-OSU6162 reduced also the intake of regular

chow in control rats of the binge-eating paradigm, arguing against a suggested specificity for palatable food. However, the chow intake in the binge-eating rats was not affected by (-)-OSU6162 and neither did (-)-OSU6162 affect the consumption of the palatable food received after each interval of the second-order schedule of reinforcement. Since both this chow intake and this sucrose intake was low, I suggest that (-)-OSU6162 only affects high levels of food intake. Similarly, (-)-OSU6162 reduced alcohol intake only in rats voluntarily drinking high, but not low, amounts of alcohol (Steensland *et al*, 2012).

The effects of (-)-OSU6162 on sucrose seeking already during the first interval of the second-order schedule of reinforcement, before the food reward is delivered, indicates that (-)-OSU6162 is able to directly affect the motivation to obtain the reward. Instead, compounds that only affect the second interval indicate an indirect effect on motivation through modulation of the hedonic properties of the reward upon ingestion. Furthermore, the finding that (-)-OSU6162 is more specific in reducing cue-controlled seeking under a second-order schedule, but not simpler schedules of reinforcement, in contrast to the D2 antagonist raclopride (see paper IV), indicates that (-)-OSU6162 might be especially beneficial in BED. Obese patients with BED display enhanced cue-induced dopamine activation than obese patients without BED (Wang *et al*, 2011).

These effects of (-)-OSU6162 on sucrose seeking are in line with the effects of (-)-OSU6162 on alcohol seeking under various schedules revealed by my colleague Dr. Fredriksson (Steensland *et al*, 2012). Furthermore, the finding that (-)-OSU6162 does not affect a simpler schedule of reinforcement (paper IV) further strengthens our observations of lack of general motor-inhibiting or sedative effects that could account for an unspecific reduction in lever pressing. For example, (-)-OSU6162 increased the inactive lever press in the cue/priming-induce reinstatement test of alcohol seeking (Steensland *et al*, 2012) and did not affect the latency to respond to a stimulus or collect the reward in a 5-choice serial reaction time task (unpublished observations by Fredriksson and co-workers).

The fact that (-)-OSU6162 infused into the NAc core replicates the findings of systemic administration on seeking under a second-order schedule of reinforcement, confirms a role of dopamine in the NAc on (-)-OSU6162's effects on cue-controlled food seeking. However, the exact mechanism, especially the role of dopamine release, D1R and D2R transmission, as well as 5-HT2A partial agonism, in these found behavioral effects of (-)-OSU6162 should be further investigated.

5 GENERAL CONCLUSIONS

The monoamine stabilizer (-)-OSU6162 has recently been identified as a potential novel treatment for AUD. (-)-OSU6162 reduces voluntary alcohol drinking, seeking and withdrawal symptoms in long-term drinking rats (Steensland *et al*, 2012)

In the present thesis, long-term voluntary alcohol drinking downregulated the dopamine system and (-)-OSU6162 counteracted such dopaminergic deficits (for proposed model of mechanism, see Fig. 17). First, the finding that alcohol drinking reduces dopamine levels in the NAc is in line with previous studies. Second, the alcohol-induced reduction on D2R expression is adding to the relatively scarce literature on the effects of alcohol drinking on D2R levels using validated rodent alcohol models. Furthermore, these results indicate that reduced striatal D2R levels found in alcohol-dependent individuals during abstinence might be directly caused by alcohol drinking. Third, the effects of alcohol drinking on the density of D2R receptor complexes *in situ* have not been investigated previously. Hence, a D2R receptor complex-specific change has been demonstrated with the present thesis for the first time. Moreover, increased A2AR-D2R heteroreceptor complexes might provide a novel treatment target for AUD. Finally, (-)-OSU6162 slowly elevated the dopaminergic tone in the NAc to a new plateau. This effect is likely underlying the ability of (-)-OSU6162 to reduce voluntary alcohol drinking. In fact, several studies show that increasing the dopaminergic tone (e.g. via optogenetics) decreases voluntary alcohol drinking. Finally, pre-treatment with a 5-HT2A antagonist attenuated the (-)-OSU6162-induced reduction in voluntary alcohol intake, indicating an additional role of partial agonism at this receptor in (-)-OSU6162's effects.

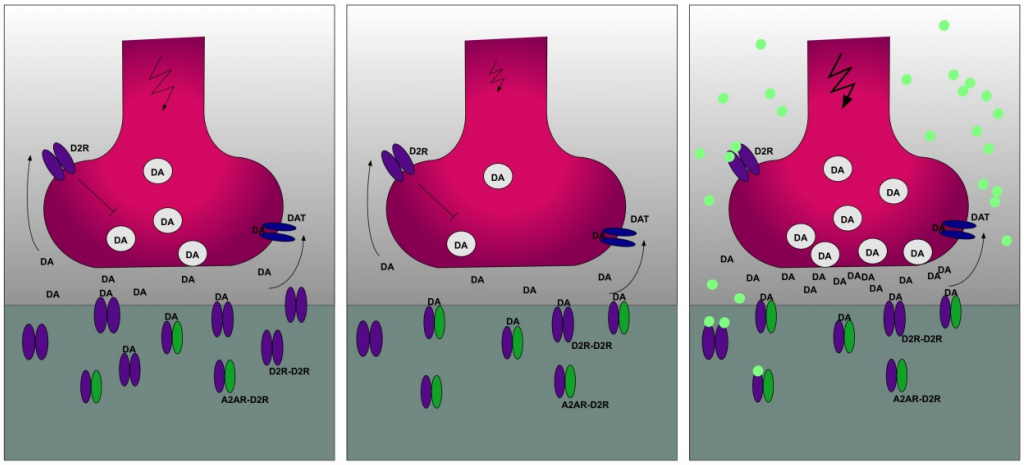

Figure 17. Role of dopamine transmission in the ability of (-)-OSU6162 to reduce alcohol drinking

Left: Acute alcohol intake releases dopamine in the NAc and stimulates synaptic and extrasynaptic receptors. Released dopamine binds to presynaptic D2R autoreceptors, inhibiting further dopamine release ***Middle:*** Long-term alcohol drinking downregulates the dopamine system demonstrated by decreased extracellular dopamine levels, reduced densities of D2R-D2R homoreceptor complexes (likely caused by decreased D2R gene expression) and increased densities of A2A-D2R heteroreceptor complexes (probably decreasing D2R affinity through reciprocal receptor antagonism). ***Right:*** (-)-OSU6162 (light green dot) increases dopamine release via D2R autoreceptor antagonism (at the axon terminal, increasing dopamine release, and/or at the soma, increasing dopamine firing and release). This slow increase in dopamine levels might abolish the need to drink alcohol in order to restore the dopamine system. (-)-OSU6162 might also antagonize extrasynaptic receptors (homo- or heteroreceptor complexes) located on the medium spinal neurons, which could inhibit alcohol-reinforcement.

6 FUTURE STUDIES

Several questions remain regarding the mechanisms of (-)-OSU6162's reduction in alcohol drinking and other alcohol-mediated behaviors, which should be investigated in future studies.

First, the role of the sigma1 receptor in the (-)-OSU6162-induced reduction in voluntary alcohol intake should be investigated. (-)-OSU6162 has shown a high affinity for the sigma1 receptor (Sahlholm *et al*, 2013). This receptor can form a complex with the long isoform of the D2R receptor (Borroto-Escuela *et al*, 2017; Navarro *et al*, 2013) and is often a co-receptor in different D2R heteroreceptor-complexes (Borroto-Escuela *et al*, 2016; Navarro *et al*, 2013). Moreover, in paper I, a reduction in the density of sigma1-D2R in the dorsal striatum by alcohol drinking has been found. A similar reduction has also been found following chronic cocaine intake (Borroto-Escuela *et al*, 2017; Navarro *et al*, 2013) and the sigma1 receptor is thought to play a role in cocaine's inhibition of D2 signaling (Borroto-Escuela *et al*, 2017; Navarro *et al*, 2013). Furthermore, a sigma1 antagonist was shown to reduce voluntary alcohol intake (Blasio *et al*, 2015).

Second, the role of the dorsal striatum in the effects of (-)-OSU6162 to reduce alcohol drinking, but especially alcohol seeking, should be investigated. (-)-OSU6162 releases dopamine in the dorsal striatum (Sonesson *et al*, 1994; Tolboom *et al*, 2014) and reduces dyskinesia in a rat model of Parkinson's disease (Ekesbo *et al*, 1997). Moreover, the dorsal striatum is involved in habitual alcohol (Corbit and Janak, 2016; Corbit *et al*, 2014) and cocaine seeking (Belin and Everitt, 2008; Murray *et al*, 2012). Furthermore, as mentioned above chronic alcohol drinking (paper I) and cocaine intake (Borroto-Escuela *et al*, 2017; Navarro *et al*, 2013) affects the density of D2R heteroreceptor complexes in the dorsal striatum.

Third, the effect of chronic alcohol intake on signaling and interaction between D2R-heteroreceptor complexes, such as the A2AR-D2R (Pintsuk *et al*, 2016) or D2R-5-HT2A receptor complex (Borroto-Escuela *et al*, 2010b), should be further investigated. Moreover, the effect of (-)-OSU6162 treatment on the signaling of these heteroreceptor complexes should be studied.

Fourth, the role of postsynaptic D1R and D2R in (-)-OSU6162-induced reduction of voluntary alcohol intake and other alcohol-mediated behaviors should be investigated, as well as the role of long-term alcohol intake on the D1R receptor.

Fifth, the effect of long-term drinking on the serotonergic system, as well as the effect of 5-HT2A partial agonism on the dopamine system, should be investigated. Finally, the role of different brain areas, such as the prefrontal cortex, and their interaction to the striatum, should be investigated in the effect of (-)-OSU6162 on alcohol-mediated behaviors.

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