LOSS OF CONTROL OVER SUBSTANCE USE

Preclinical studies into the behavioural and neural mechanisms of addiction

Maryse Minnaard

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Preclinical studies into the behavioural and neural mechanisms of addiction

VERLIES VAN CONTROLE OVER MIDDELENGEBRUIK

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(met een samenvatting in het Nederlands)

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Promotoren:
Prof. dr. L.J.M.J. Vanderschuren
Prof. dr. R.A.H. Adan

Copromotoren:
Dr. H.M.B. Lesscher
Dr. G.M.J. Ramakers

Prologue

Amsterdam, 8 April 2008.

It was a cold day in early spring when ten teenagers from a small town were visiting the photography museum FOAM in Amsterdam. The visit was for a school assignment and each of them had to choose a favourite object. At the time, FOAM hosted an exhibition called *The Ninth Floor* created by photojournalist and filmmaker Jessica Dimmock. Her work depicted young heroin addicts living on the ninth floor of an apartment complex in Manhattan, New York City. The images were both intimate and raw, as they captured the chaotic atmosphere of human lives spinning out of control and showed the shocking and gruesome truths about the world of addiction.

In the midst of all the raw and shocking photographs, one girl picked a photo of a young family as her favourite object. It was a photo taken outside of former addicts Dionn, Rachel, and their baby Matilda. Now that they had become parents, Dionn and Rachel attempted to quit drugs and improve their lifestyle. The image was in strong contrast to the photographs taken at the ninth floor; this was an image of hope. She wondered how their story would continue. Would they succeed and live a joyful life in sobriety, or would the addiction pull them back into the life they left behind? Would they reach cloud nine? Or would they be driven back to the ninth floor? Although these questions remained unanswered, the image lingered in her mind long after this trip to Amsterdam.

Little did she know that this city would become the place she would call home.

Little did she know that she would devote her time and effort to research in an attempt to eventually help them.

Perhaps all of this started this very day in early spring.

For Dionn, for Rachel, for all those others.

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Chapter 1

General introduction

Addiction is a major societal problem affecting millions of people. To better understand what drives addictive behaviours, and to improve and develop novel treatment options, it is of great importance to understand the mechanisms underlying loss of control over substance use. Therefore, this thesis aims to expand our current understanding of the changes in brain and behaviour that characterise substance use disorder (SUD). This introductory chapter provides a concise overview of the clinical and epidemiological characteristics of SUD, followed by a discussion of the behavioural and neurobiological mechanisms underlying SUD, and a presentation of rodent models to investigate SUD-related behaviour. Finally, an outline of the chapters included in this thesis is given.

1. Substance use disorder

Substance use disorder (SUD) is a chronic relapsing brain disorder that is manifested by persistent use of alcohol or other drugs, despite harmful consequences (American Psychiatric Association, 2000, 2013; Volkow et al., 2016). These drugs include tobacco, psychostimulants such as cocaine, amphetamine and MDMA, and opiates such as heroin and morphine. The diagnosis for SUD, also commonly referred to as addiction, is often made using the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), published by the American Psychiatric Association (Table 1) (American Psychiatric Association, 2013). SUD has pharmacological features, such as tolerance and withdrawal, and psychological and behavioural features, including an increased motivation to obtain a drug and escalation of drug use. The diagnostic criteria of SUD reflect that it is a clinically heterogenous disorder because clinical manifestations and severity vary considerably between patients. The severity of the disorder can be qualified as mild, moderate, or severe based on how many of the eleven diagnostic criteria are met. A major hallmark of SUD is a loss of control over drug seeking and taking, as is reflected by most (i.e. 9 out of 11) DSM-5 criteria for SUD, for example substance use in hazardous conditions and continued use despite knowledge of substance-related problems.

DSM-5 diagnostic criteria for Substance Use Disorder	Loss of control
Hazardous use	✓
Social/interpersonal problems related to use	✓
Neglected major roles to use	✓
Withdrawal	
Tolerance	
Used larger amounts/longer	✓
Repeated attempts to quit/control use	✓
Much time spent using	✓
Physical/psychological problems related to use	✓
Activities given up to use	✓
Craving	✓

Table 1. DSM-5 diagnostic criteria for Substance Use Disorder. The diagnosis for SUD is based on two or more substance use disorder criteria within a 12-month period. The checkmarks indicate the criteria related to loss of control. Table adapted from Hasin et al., 2013.

Epidemiology

SUD is a major socioeconomic and public health issue and it has been calculated as one of the most financially costly disorders of the central nervous system (Uhl & Grow, 2004; UNODC, 2019). According to recent reports, drug use was responsible for 305,800 and 585,000 deaths in 2016 and 2017, respectively (Tran et al., 2019; UNODC, 2019; World Health Organization, 2018). As such, SUD is among the leading preventable causes of death. Besides mortality, substance use contributes heavily to the burden of disease because substance use is a risk factor for injuries and secondary diseases, and is a cause of substantial harm to users and others in the society (Carvalho et al., 2019; Degenhardt et al., 2018; Nutt et al., 2010; Whiteford et al., 2013). Of all substances of abuse, alcohol is deemed the most harmful substance and alcohol use disorder (AUD) is, together with tobacco addiction, the most common form of substance use disorder (Nutt et al., 2010; Rehm et al., 2015; van Amsterdam et al., 2010; Wittchen et al., 2011). The twelve-month prevalence of AUD is estimated to be 13.9%, versus 3.9% for other drug use disorders (Grant et al., 2015, 2016). SUDs generally affect more men than women and they are associated with a variety of mental health conditions, including major depressive disorder, posttraumatic stress disorder and bipolar disorders (Grant et al., 2015, 2016; Schuckit, 2006).

Treatment options

Currently, a number of treatment options for SUD is available, including cognitive behavioural therapy, pharmacological treatment and social support strategies. The main goal of the available treatments is to achieve and maintain complete

abstinence. Pharmacotherapeutic options for AUD include disulfiram, naltrexone, and acamprosate which have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (Beraha et al., 2016; Lee et al., 2015; van den Brink, 2012). For opioid use disorders, the opioid agonists methadone and buprenorphine are used as opioid replacement therapies (Lee et al., 2015; van den Brink, 2012). However, the efficacy of the treatment strategies for SUD varies across individuals and short-term relapse rates after abstinence treatment are high, ranging from 20% to 60%, depending on the length of follow-up throughout the first few years and the criteria for remission (Miller et al., 2001; Monahan & Finney, 1996; Moos & Moos, 2006). Hence, there is an urgent need for better treatment strategies for individuals suffering from SUD.

Individual vulnerability

Substances of abuse, especially alcohol, are widely used for recreational purposes. For instance, twelve-month alcohol use among American adults was estimated to be 65.4% in 2001-2002 and 72.7% in 2012-2013 (Grant et al., 2017). Although many people consume alcohol and other drugs on a regular basis, only a minority of users become addicted. Susceptibility to SUD differs across individuals as a result of various genetic, environmental, and developmental factors (Volkow et al., 2016). SUD has a significant genetic component as approximately 40% to 60% of the vulnerability to SUD can be attributed to genetic factors (Demers et al., 2014; Goldman et al., 2005; Hiroi & Agatsuma, 2005). The vulnerability for SUD cannot be attributed to a single gene, but is thought to result from complex interactions among multiple genes and genetic interactions with environmental influences (Enoch, 2012). Furthermore, SUD has been associated to several psychological phenotypes including anxiety, sensation seeking, and impulsivity, which are considered risk traits for SUD. For example, impulsivity, i.e. the tendency to engage in inappropriate or maladaptive behaviours with little or inadequate forethought, has been strongly associated with SUD, both as a contributor to use and as a consequence of use (Allen et al., 1998; de Wit, 2009; Perry & Carroll, 2008; Verdejo-García et al., 2008).

2. Behavioural mechanisms of substance use disorder

Several theories have posited a framework to better understand the behaviours associated with SUD. Here, some of these influential theories are discussed, which will be followed by a brief overview of the underlying brain mechanisms involved.

Three-stage addiction cycle

Since SUD is a chronic relapsing brain disorder, it can be viewed as a cycle of recurring stages, i.e. a binge and intoxication stage, a withdrawal and negative affect stage, and a preoccupation and anticipation (or craving) stage (George et al., 2012; Koob & Le Moal, 1997; Koob & Volkow, 2010). It is thought that this cycle intensifies over time, accompanied by neuroplastic changes in the brain reward and executive function systems. This process coincides with a transition from casual use to more compulsive use, giving rise to the pathological state of SUD.

Executive dysfunction

Chronic substance use has been associated with several cognitive deficits, particularly in attention, behavioural flexibility, behavioural inhibition and planning (Bickel et al., 2012). Attention refers to concentrating on one aspect of the environment while ignoring others. Individuals with attentional deficits are easily distracted by other stimuli, making it difficult to maintain focus and to complete required tasks. Attention deficits are found across a range of substance abusing individuals, including alcohol, cocaine, and amphetamine abusers (Di Sclafani et al., 2002; Hester et al., 2006; Iwanami et al., 1995; Johanson et al., 2006; Kalapatapu et al., 2011; Simon et al., 2000; Thoma et al., 2011). Behavioural flexibility is the ability to adjust behaviour appropriately in response to changes in the environment. Individuals with flexible behaviour may seamlessly switch between coping behaviours as they encounter risky situations, whereas individuals with impaired behavioural flexibility may be unable to adapt their responses. Drug users tend to be less flexible in the face of changing contingencies compared to non-using individuals (Giancola et al., 1996; Lane et al., 2007). Behavioural inhibition is an active mechanism that allows for withholding unwanted or prepotent responses. Impairments in behavioural inhibition can be observed in substance users (Colzato et al., 2007; Fillmore & Rush, 2002). Planning may be defined as the ability to organise cognitive behaviour in order to guide behaviour towards the attainment of an immediate or distant goal (Owen, 1997). Indeed, substance-dependent individuals perform poorer on planning tasks compared to healthy individuals (Davydov & Polunina, 2004; Ersche et al., 2006; Fernández-Serrano et al., 2010)

Positive and negative reinforcement

A hallmark of SUD is an enhanced motivation to pursue a substance (American Psychiatric Association, 2013; Kalivas & Volkow, 2005). Regarding motivation, the opponent process theory describes how both positive and negative reinforcement contribute to substance taking (Solomon & Corbit, 1973). Consumption of a substance of abuse will induce a positive, pleasant, hedonic state to which the

body will respond with mechanisms that reduce the intensity of hedonic feelings in order to help restore homeostasis and bring brain states back to normal (Koob & Le Moal, 1997; Solomon & Corbit, 1973). The sum of these processes constitutes the emotional state experienced by the user. Initially, the positive reinforcement process dominates over the negative reinforcement process, causing the individual to experience a positive effect of substance use. With repeated substance use however, the negative process increases in duration and size, eventually leading to the emergence of a negative emotional state that is characterised by withdrawal symptoms, such as dysphoria, anxiety, and irritability (Koob & Le Moal, 2001). At this point, it is thought that the substances of abuse are consumed as an approach to alleviate the experienced negative state, instead of being driven by the pleasurable effects that the substance elicited initially.

Incentive sensitisation

Individuals with SUD have an unusually strong urge to seek substances of abuse, which can persist after long periods of drug abstinence. The incentive sensitisation theory posits that the repeated use of substances of abuse leads to neural adaptations, in particular in the mesolimbic dopamine system (Robinson & Berridge, 1993, 2003). As a consequence of these adaptations, the neuronal system that is involved in motivation is thought to become hypersensitive to substances of abuse and substance -associated stimuli. This process is thought to transform normal 'wanting' into excessive substance craving. According to the incentive sensitisation view, this boosting of 'wanting' the rewarding substance is critical in the enhanced motivational response but it can occur independently of changes in neural systems that facilitate the experienced pleasurable effects of drugs ('liking'). Thus, how much a reward is wanted is dissociable from how much the same reward is liked (Berridge & Robinson, 2016).

Habitual behaviour

Another concept central to SUD is the formation of habits that is thought to contribute to the development of persistent behaviour. Habit refers to an automatic reaction to a specific situation, and it allows for the quick and efficient retrieval of routine actions (Robbins & Costa, 2017). Although habits can be adaptive in everyday life, the inability to quit or shift behaviour directed at substances may underlie impaired decision making as seen in SUD (Everitt & Robbins, 2016). That is, flexible performance relies on outcome expectancy so reward-directed behaviour is usually reduced when the value of the outcome is decreased (Balleine & Dickinson, 1998; Dickinson, 1985). Thus, action control is then considered goal directed. In contrast, habitual or automatic action control signifies responding that is no longer sensitive to changes in the outcome. Instead, actions are evoked by contextual stimuli that have become associated with the outcome, because of their presence at the time of reinforcement, and have become dissociated from the goal. Thus, the behaviour is no longer goal directed. Compulsive substance use, as observed in patients with SUD, has been hypothesised to be driven by an imbalance between goal directed and habitual drug seeking (Everitt & Robbins, 2016).

3. Brain mechanisms of substance use disorder

Over the last decades, research has shown that the neurobiology of SUD is complex and that it involves neuroplastic changes throughout the brain and in numerous subsystems, including the ascending mesocorticolimbic dopamine system and corticostriatal glutamate projections (Koob & Volkow, 2016). A simplified overview of the brain regions and neurotransmitter systems implicated in substance use and SUD is presented in Figure 1. Despite substantial progress that has been made in recent years, it is not yet fully understood how SUD develops and is maintained. This thesis aims to contribute to this knowledge. Although a comprehensive overview is beyond the scope of this general introduction, some of the most important neural systems involved in SUD will be described.

Nucleus accumbens

Substances of abuse are known to activate the brain reward system. The nucleus accumbens, which is part of the ventral striatum, is a crucial node in this reward system. For example, rats have been found to self-administer several substances of abuse, including alcohol, cocaine, and MDMA, directly into the nucleus accumbens (Engleman et al., 2009; Rodd-Henricks et al., 2002; Shin et al., 2008). The two major neurotransmitters that mediate the rewarding effects of substances of abuse are dopamine and opioid peptides. Virtually all known substances of abuse have been shown to directly or indirectly increase dopamine release in the nucleus accumbens (Di Chiara & Imperato, 1988; Volkow et al., 2007). Dopamine is released in the nucleus accumbens by neurons originating in the ventral tegmental area. This dopaminergic projection is referred to as the mesolimbic pathway. In the nucleus accumbens, dopamine exerts its effects via activation of dopamine receptors located on GABAergic medium spiny neurons, the predominant cell type in the nucleus accumbens (Meredith & Totterdell, 1999).

In addition to a strong dopaminergic input from the ventral tegmental area, the nucleus accumbens also receives glutamatergic input from a variety of limbic and cortical regions, including the prefrontal cortex, the ventral hippocampus, and the basolateral amygdala. Glutamate is the most abundant excitatory neurotransmitter in the brain and chronic substance use produces long-lasting neuroadaptations in the glutamatergic corticostriatal circuitry (Kalivas, 2009; Tzschentke & Schmidt, 2003; Wolf & Ferrario, 2010).

Besides an increase in dopamine release, various substances of abuse, including stimulants and alcohol, also have been shown to increase extracellular levels of endogenous opioid peptides in the nucleus accumbens and ventral tegmental area (Mitchell et al., 2012; Murphy, 2015). These ligands can bind to opioid receptors and especially the nucleus accumbens is densely innervated by μ- and δ-opioid receptors (Mansour et al., 1988). Opioids can facilitate the release of dopamine in the nucleus accumbens through the ventral tegmental area, although this may also happen locally in the nucleus accumbens (Fusa et al., 2005; Hirose et al., 2005; Melis et al., 2000; Spanagel et al., 1992; Yoshida et al., 1999). Within the ventral tegmental area, μ-opioid agonists disinhibit dopaminergic neurons indirectly through inhibition of presynaptic GABAergic inputs on dopaminergic neurons (Zhang et al., 2015). The effects of opioids within the nucleus accumbens on dopamine release remains less understood. Nucleus accumbens μ-opioid receptor activation is thought to increase dopamine levels in the nucleus accumbens, perhaps by interactions between μ- and δ-opioid receptors or by inhibition of GABA release onto cholinergic neurons that stimulate dopamine release via excitation of dopamine terminals (Cachope et al., 2012; Hirose et al., 2005; Wenzel & Cheer, 2018). However, activation of μ-opioid receptors has also been implicated in the motivational and hedonic properties of substances of abuse through non-dopaminergic mechanisms. For instance, μ-opioid receptor knockout mice show little to no alcohol consumption (Hall et al., 2001; Méndez & Morales-Mulia, 2008; Roberts et al., 2000).

Dorsal striatum

The transition from controlled to compulsive drug consumption has been associated with a shift from the involvement of ventral striatal subregions to the dorsal striatum (Everitt et al., 2008). More specifically, the dorsolateral striatum seems to be required for habit learning whereas the dorsomedial striatum is thought to be required for goal directed learning (Corbit et al., 2012; O'Hare et al., 2017; Yin et al., 2004). Preclinical evidence supports this view, as animal studies showed that cocaine-seeking behaviour becomes dependent on the dorsal striatum when well-established (Belin & Everitt, 2008; Murray et al., 2015; Vanderschuren et al., 2005; Zapata et al., 2010). Similar results have been found after prolonged exposure in rats trained to respond for heroin or alcohol (Corbit et al., 2012, 2014; Hodebourg et al., 2019). Further, a recent study showed that reliance of alcohol seeking on dorsolateral striatal dopamine signalling predicted the vulnerability to subsequently develop compulsive alcohol seeking (Giuliano et al., 2019). Consistently, human neuroimaging research showed that heavy alcohol drinking subjects presented with alcohol-related cues showed a shift in activation from the ventral to the dorsal striatum when compared to light alcohol drinkers (Vollstädt-Klein et al., 2010). Moreover, an overreliance on habit learning was found behaviourally in alcohol-dependent individuals compared to healthy individuals, which was accompanied by increased recruitment of striatal brain areas implicated in habit learning (Sjoerds et al., 2013).

Prefrontal cortex

Executive control, referring to a range of processes including attention, planning, working memory, cognitive flexibility, and response inhibition, is strongly dependent on prefrontal cortex functioning. The prefrontal cortex normally exerts inhibitory control over behaviours, but increasing evidence suggests that continued exposure to substances of abuse leads to a diminished ability of the prefrontal cortex to maintain this cognitive control over behaviour, resulting in compulsivity (Abernathy et al., 2010). Consistently, prolonged substance use leads to reductions in prefrontal cortex excitability in rats, which is associated with compulsive substance seeking (Chen et al., 2013; Halladay et al., 2020; Hu et al., 2019; Kasanetz et al., 2013; Seif et al., 2013). In particular, the prefrontal cortex-nucleus accumbens projection has been shown to mediate aversion-resistant alcohol intake (Seif et al., 2013). In line with preclinical evidence, clinical studies assessing inhibitory control in SUD patients have repeatedly shown that prefrontal cortex hypoactivation was associated with poor task performance (Goldstein & Volkow, 2011).

The prefrontal cortex is an extensively interconnected region. It receives innervations from various cortical and subcortical regions, including a dopaminergic innervation from the ventral tegmental area. The medial prefrontal cortex can be subdivided into four distinct areas along a dorsal to ventral axis, i.e. the medial precentral area (also known as the second frontal area), the anterior cingulate cortex, the prelimbic prefrontal cortex, and the infralimbic prefrontal cortex (Heidbreder & Groenewegen, 2003). The local medial prefrontal cortex network consists mainly of excitatory pyramidal cells (80% to 90% of the total population) and inhibitory GABAergic interneurons (10% to 20% of the total population) (Riga et al., 2014). From the prefrontal cortex, glutamatergic neurons project to several brain regions, including the nucleus accumbens, the ventral tegmental area, and the dorsal striatum.

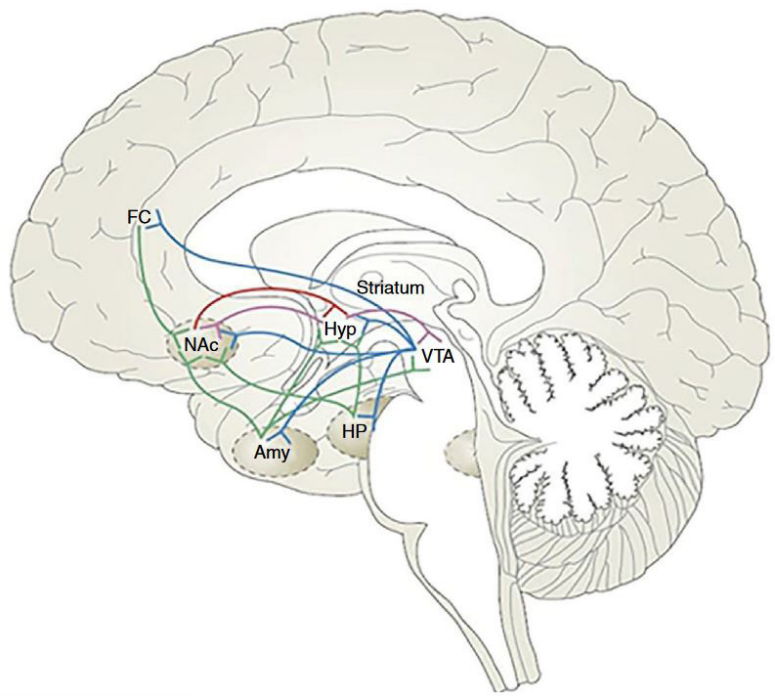

Figure 1. Illustration of brain circuits associated with substance use disorder (SUD). A diagram showing the neurocircuitry involved in SUD, although simplified, with dopaminergic (blue), glutamatergic (green), and GABAergic (red) projections. FC, prefrontal cortex; NAc, nucleus accumbens; Amy, amygdala; Hyp, hypothalamus; HP, hippocampus. Adapted from Miela et al., 2018.

4. Rodent models to study SUD-related behaviour

A wide array of animal models is available to investigate SUD-related behaviour that capture different aspects of substance use, such as reinforcement, motivation, or control over substance use. In the following sections, a number of rodent models and experimental techniques are briefly introduced, which were used in the experimental chapters of this thesis.

Voluntary consumption

A core feature of many rodent models is the voluntary consumption of substances of abuse in operant conditioning chambers. In operant self-administration, an animal needs to perform an action, such as pressing a lever or making a nose poke, in order to obtain a reward. By performing the action and experiencing the outcome of being rewarded, the animal learns to associate the action with the reward which leads to reinforcement, i.e. an increase in the likelihood that a response will occur. The reward can be anything with reinforcing value, including but not limited to sucrose, alcohol and cocaine. Operant conditioning is the basis of many behavioural tasks, which can be used to investigate different phases of SUD, such as acquisition, maintenance, escalation, extinction and reinstatement (Belin-Rauscent et al., 2016).

Alcohol consumption in rodents can be evoked through several experimental manipulations, such as fluid deprivation, schedule-induced polydipsia, sucrose fading, and forced induction of alcohol dependence through injections or vapour chambers (Bell et al., 2012). However, such approaches compromise the voluntary component of drinking alcohol and can provoke other determinants, including stress and taste, which may hamper the interpretation of findings. High levels of voluntary alcohol intake can be achieved by exposing rodents intermittently to alcohol in the home cage. Intermittent access to alcohol has been shown to induce high levels of alcohol intake, higher than those achieved when alcohol is available continuously (Loi et al., 2010; Spoelder et al., 2015). Moreover, several research groups, including our own, have shown that intermittent alcohol access evokes profound individual differences in alcohol intake (Hwa et al., 2011; Lesscher et al., 2015; Momeni & Roman, 2014; Sabino et al., 2013; Simms et al., 2008; Spoelder et al., 2015). Based on these individual differences in alcohol intake, Lister Hooded rats can be distinguished into subgroups of high alcohol drinking (HD), medium alcohol drinking, and low alcohol drinking rats (LD) (Spoelder et al., 2015). Importantly, HD have been associated with compulsive alcohol consumption as they showed more motivation to obtain alcohol and more punishment-resistant alcohol-directed behaviour than LD (Spoelder et al., 2015, 2017).

Motivation and compulsive use

SUD is characterised by an enhanced motivation to pursue a substance of abuse (American Psychiatric Association, 2013; Kalivas & Volkow, 2005). Progressive ratio (PR) schedules of reinforcement provide a robust tool to measure motivation (Hodos, 1961; Richardson & Roberts, 1996). Under PR schedules of reinforcement, rodents respond for a reward and the response requirement increases after each reward delivery until the animal quits responding. The number of rewards obtained, lever presses, and the highest ratio achieved (i.e. the breakpoint) in PR tasks are common measures that are thought to reflect incentive motivation (Richardson & Roberts, 1996; Salamone & Correa, 2012).

Persistent substance use despite negative consequences is a hallmark of SUD considering that many of the criteria for SUD listed in the DSM-5 comprise behaviours that reflect a loss of control over substance intake (American Psychiatric Association, 2013). Recent models have been developed that strive to capture these

compulsive aspects of addictive behaviour. In these models, compulsive use is often operationalised as resistance to punishment. Substance seeking or taking is paired with a punisher, for example a mild electric foot shock. Animals that show control over their substance use, and do not display compulsive substance use, should reduce substance seeking or taking to avoid the risk of being punished, while compulsive rats will continue to pursue their reward despite the possible aversive consequences. In chapter 2 of this thesis, we will elaborate on various types of punishment models of compulsive substance use.

Experimental techniques

Throughout the experiments described in this thesis, several behavioural pharmacological techniques were used which will be briefly introduced here. Behavioural pharmacology is a field that is concerned with the mechanisms through which biologically active compounds exert their effects on behaviour in animals. A common approach is the administration of pharmacological compounds, via systemic injection or local infusion through cannulas that are implanted above a certain brain area. In experiments presented in this thesis we used systemic injections of various pharmacological compounds in chapters 6 and 7, and local infusions through cannulas in chapter 4.

A more recently developed technique is chemogenetics, which enables the manipulation of specific neuronal populations through the use of Designer Receptor Exclusively Activated by Designer Drugs (DREADD) (Rogan & Roth, 2011). DREADDs are muscarinic G-protein coupled receptors that have been modified to become selectively activated by a designer drug, often Clozapine-N-oxide (CNO). Through activation, DREADDs can either facilitate or inhibit neuronal firing, which depends on whether it is coupled to an excitatory (Gq, Gs) or inhibitory (Gi) signalling cascade. Several Gq-DREADDs are available, of which hM3Dq appears to be the most frequently used excitatory DREADD (Roth, 2016). These designer receptors can be introduced into cells by an intracranial infusion of viral vectors, such as adeno-associated viral vectors (AAVs), encoding the DREADD.

A great advantage of DREADD technology is that it allows for manipulation of specific neuronal subpopulations, such as dopaminergic neurons. This selective targeting can be achieved by using a cell type-specific promoter and using recombination technology to drive DREADD expression, thereby making DREADD expression cre-recombinase-dependent. In this case, the coding sequence for the DREADD is inserted into the viral vector using a double-floxed inverted open reading frame (DIO) construct. This DIO comprises an inverted DREADD sequence surrounded by a pair of loxP sites. The cre-recombinase enzyme binds to the DIO-loxP sites, thereby recombining the sequence and allowing expression of the receptor. Thus, the DREADD is only expressed in the presence of cre-recombinase. In chapter 6 of thesis, we used a transgenic rat line that expresses cre-recombinase only in tyrosine hydroxylase (TH) containing cells (Witten et al., 2011). Since TH is a marker for dopamine, injecting a cre-dependent virus containing the hM3Dq DREADD in the midbrain allowed us to specifically target dopaminergic neurons, and activate these neurons with CNO.

5. Scope of this thesis

Aims and approaches

The overarching aim of this thesis is to expand our current understanding of the changes in brain and behaviour that characterise SUD. To this aim, we used existing behavioural models and also developed a novel model, that capture important aspects of addictive behaviour in combination with neuropharmacological interventions. With this approach, novel insight can be gained into the behavioural mechanisms that underlie addictive behaviour, which can contribute to the translation of preclinical findings to the human clinical practice.

Thesis outline

Throughout the last decades, animal models have been extensively used to investigate the behavioural and neural mechanisms of SUD. These models have evolved and contributed to the development of preclinical models that strive to more closely mimic diagnostic criteria of SUD by using punishment tasks. In **chapter 2**, we provide an overview of punishment models of compulsive substance use that use aversive stimuli. These models have been developed to explicitly study loss of control over substance seeking and taking which is operationalised as resistance to punishment. This chapter presents various modes of punishers used, discusses their benefits and disadvantages, and highlights the potential valuable contribution of punishment tasks to increase our knowledge of SUD. In **chapter 3**, we present the novel Seeking under Threat of Adversity (STA) task. Here, we aimed to develop a novel task to measure reward seeking behaviour in the face of adversity in rats, to improve resemblance of addictive behaviour in humans. In **chapter 4**, the STA task that was presented in chapter 3 was utilised to investigate the involvement of the prelimbic prefrontal cortex in reward seeking under threat of adversity. The effects of pharmacological inactivation of the PrL, through local infusions of a GABA agonist mixture (i.e. baclofen and muscimol), were assessed in rats that

were trained to seek alcohol or sucrose rewards. **Chapter 5** was devoted to a behavioural characterisation of alcohol drinking rats. It is generally conceived that SUD is complex in nature and subject to a substantial degree of individual variability. Considering that various behavioural measures are used to determine addiction-like behaviour in rodents, we investigated how different aspects of AUD-like behaviour are related. To that aim, rats were characterised for alcohol consumption, habit formation, motivation for alcohol, and aversion-resistant alcohol consumption, and the interrelationship between these measures was evaluated by computing correlations, cluster tendency analysis, and an addiction severity score. **Chapter 6** focusses on deficits in cost-benefit decision making as this is a core feature of several psychiatric disorders, including SUD, eating disorders, depression, and bipolar disorder. Here, we took a behavioural economics approach combined with the use of DREADDs in investigating the role of dopaminergic neurotransmission in the relationship between price and consumption of sucrose. In **chapter 7**, we explored functional differences in neural systems that may underlie the individual vulnerability to AUD. Therefore, we investigated the involvement of GABAergic, opioid, and glutamatergic neurotransmission in individual differences in alcohol consumption. This chapter reports the effects of treatment with the $GABA_B$ receptor agonist baclofen, the opioid receptor antagonist naltrexone, and the cysteine precursor N-acetylcysteine on alcohol consumption in rats that differed in their levels of alcohol intake. **Chapter 8** is an appendix containing a set of experiments into the neurobiological mechanisms of reward seeking under threat of adversity. Here, we aimed to explore the involvement of the basolateral amygdala, the orbitofrontal cortex, and the nucleus accumbens in sucrose seeking under threat of adversity. For that purpose, we used the STA task in combination with pharmacological inactivation, similar to chapter 4. Finally, the findings presented in this thesis are summarised and their implications discussed in **chapter 9**. We address limitations, future considerations, and evaluate how our findings add to the insights into the changes in brain and behaviour that characterise SUD.

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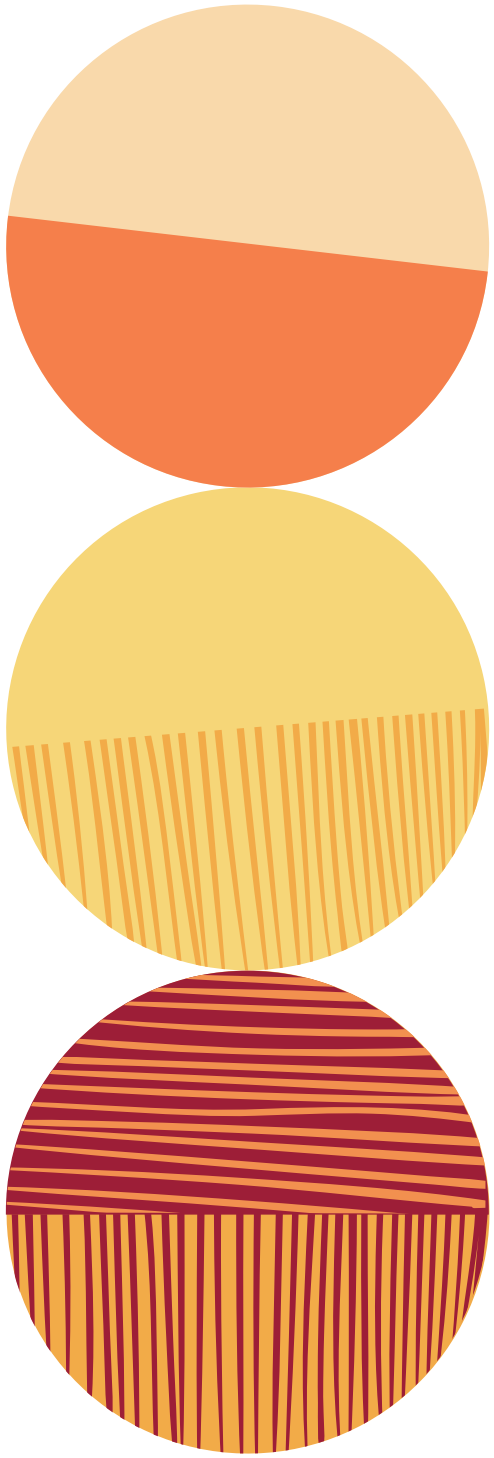

Chapter 2

Punishment models of addictive behaviour

Louk J. M. J. Vanderschuren[1]
A. Maryse Minnaard[1,2]
Johanna A. S. Smeets[1,2]
Heidi M. B. Lesscher[1]

[1] Department of Population Health Sciences, Unit of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2] Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, The Netherlands

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Abstract

Substance addiction is a chronic relapsing brain disorder, characterised by loss of control over substance use. In recent years, there has been a lively interest in animal models of loss of control over substance use, using punishment paradigms. We provide an overview of punishment models of addiction, that use quinine, histamine, lithium chloride and foot shocks as aversive stimuli, and we discuss the merits and drawbacks of these approaches. Importantly, many studies have demonstrated that under certain conditions, animals are willing to endure punishment during the pursuit of substances of abuse, which captures an essential component of addictive behaviour. We conclude that punishment models of addiction represent a valuable contribution to the study of addiction.

Introduction

Addiction to substances of abuse remains an enormous global health problem. It has been estimated that 76 million people worldwide are addicted to alcohol (World Health Organization, 2004), 29 million people are addicted to illicit drugs, such as opiates, psychostimulants and cannabis (United Nations Office on Drugs and Crime, 2016) and 1.1 billion people smoke tobacco (World Health Organization, 2015), a substantial proportion of which can be considered addicted. Alongside the suffering inflicted by the addictive behaviour itself, substance addiction dramatically increases the risk for a wide range of communicable and non-communicable diseases, including lethal conditions such as cardiovascular problems, liver failure and cancer. Indeed, substance addiction is considered to be one of the leading causes of premature death worldwide (United Nations Office on Drugs and Crime, 2016; World Health Organization, 2004, 2015). Remarkably, only 1 in 6 addicts are estimated to be in treatment (United Nations Office on Drugs and Crime, 2016), and the treatment options available are modest in terms of number and efficacy (Pierce et al., 2011; Potenza et al., 2011; van den Brink, 2012). In order to develop improved treatment strategies for addiction, we think that a profound understanding of the neural underpinnings of addictive behaviour is essential.

For more than half a century, animal models have been used to investigate the behavioural and neural mechanisms of addiction. The positive affective, reinforcing properties of substances of abuse have been widely studied using place conditioning (Rossi & Reid, 1976; Tzschentke, 2007) and intracranial self-stimulation methods (Olds & Olds, 1958; Wise, 1996). Arguably the greatest progress in understanding addictive behaviour using animal models has come from oral and intravenous self-administration studies, that derive considerable validity by virtue of the fact that they employ voluntary, active intake of drugs of abuse (O'Connor et al., 2011; Weeks, 1962). Moreover, self-administration setups have shown to be a versatile method to investigate addictive behaviour, in the sense that variants of this paradigm have been developed to study the incentive motivational properties of substances of abuse (Hodos, 1961; Richardson & Roberts, 1996), the role of drug-associated cues in addictive behaviour (Everitt & Robbins, 2000; Goldberg, 1973), and relapse to extinguished drug seeking (Bossert et al., 2013; de Wit & Stewart, 1981).

The most recent development in animal models of addictive behaviour constitutes models that explicitly study loss of control over substance seeking and taking. Inspired by the realization that the majority of the diagnostic criteria for addiction in DSM-IV (American Psychiatric Association, 2000) and DSM5 (American Psychiatric

Association, 2013) comprise behaviours that signify a lack of control over substance use, researchers have started to develop models that capture these compulsive aspects of addictive behaviour. Many of these studies have focused on the DSM criterion of continued substance use despite negative consequences and have operationalised this as resistance to punishment (Lesscher & Vanderschuren, 2012; Vanderschuren & Ahmed, 2013). In the paradigms that have been used, the pursuit of substances was associated with aversive events or circumstances, and the willingness of animals with a certain predisposition or substance taking history to endure this adversity when access to substance is at stake was assessed. In this overview, we will present punishment models of compulsive substance use, highlight their merits and drawbacks, and discuss challenges for future research.

Punishment models of addictive behaviour

Quinine

Perhaps the first use of a punishment setup in the context of addiction research is the work of Wolffgramm and colleagues, who studied alcohol addiction-like behaviour in rats (Wolffgramm, 1991; Wolffgramm & Heyne, 1995). The manipulation they used is to render the taste of orally ingested alcohol aversive using the bitter tastant quinine. They observed that the efficacy of quinine to reduce alcohol intake substantially declined after prolonged periods of alcohol drinking, interspaced with periods of forced abstinence. This reduced sensitivity of alcohol intake to quinine was accompanied by a loss of sensitivity to other factors that influence alcohol drinking, such as social rank and social isolation. Comparable findings were later reported for other substances of abuse, including opiates and psychostimulants (Galli & Wolffgramm, 2004; Heyne & Wolffgramm, 1998; Wolffgramm & Heyne, 1995). The finding of reduced sensitivity of alcohol drinking to quinine after prolonged alcohol intake has subsequently been replicated in rats and mice (Fachin-Scheit et al., 2006; Hopf et al., 2010; Lesscher et al., 2010, 2012; Loi et al., 2010; Seif et al., 2013; Spoelder et al., 2015). In rats, this relative insensitivity to quinine was observed after prolonged exposure to an intermittent (rather than continuous) pattern of alcohol access (Hopf et al., 2010; Loi et al., 2010; Seif et al., 2013), and sometimes in high alcohol consuming rats only (Spoelder et al., 2015). In these experiments in rats, quinine-containing alcohol was the only source of alcohol during the test. Interestingly, experiments in mice have shown comparable findings, for example, willingness to drink bitter, quinine-containing alcohol if water is the only alternative fluid (Fachin-Scheit et al., 2006; Lesscher et al., 2010). Moreover, after two months of voluntary alcohol drinking, mice continued to drink quinine-containing alcohol even if non-adulterated alcohol was simultaneously available (Lesscher et al., 2010). Importantly, in these latter experiments, regardless of experience with alcohol drinking, all mice avoided quinine-containing water, indicating that the persistent intake of quinine-containing alcohol was not the result of altered taste perception (Lesscher et al., 2010).

Lithium chloride and histamine

In order to associate substance intake with interoceptive malaise, post-ingestion treatment with lithium chloride has been used. This approach is widely used to evoke conditioned taste aversion, and to assess the ability of animals to use a representation of the value of a reinforcer to direct operant behaviour (Dickinson, 1985). The first of these studies showed that taste aversion conditioning with lithium chloride profoundly reduced the oral intake of alcohol and cocaine solutions, yet did not alter responding in extinction for alcohol and cocaine (Dickinson et al., 2002; Miles et al., 2003). These findings suggest that acts distal to substance use (i.e. attempts to obtain the substance) are less sensitive to punishment than the actual substance intake, as long as the taste memory trace provides explicit feedback of the degraded value of alcohol and cocaine after its association with interoceptive malaise. Recently, also the sensitivity of intravenous cocaine self-administration in rats to lithium chloride-induced malaise was investigated (Leong et al., 2016). The findings were comparable to those described above (Dickinson et al., 2002; Miles et al., 2003), inasmuch as that cocaine taking was sensitive to devaluation, whereas responding for a cocaine-associated cue was not. Importantly, the sensitivity to lithium chloride was lost in animals with a history of lengthy cocaine self-administration sessions (Leong et al., 2016). Interoceptive aversion has also been employed using intravenous histamine as a punisher in rats and non-human primates (Holtz & Carroll, 2015; Negus, 2005; Woolverton et al., 2012). When histamine was added to the solution for intravenous cocaine self-administration, this reduced responding for cocaine, while at the same time increasing responding for concurrently available food or unadulterated cocaine (Negus, 2005; Woolverton et al., 2012). Importantly, the aversive effects of histamine, by intravenous infusion, are direct (as compared to the delayed aversive effects of lithium chloride treatment after self-administration). Indeed, when infusion of histamine was delayed (i.e. for seconds to minutes after cocaine infusion), its ability to reduce responding for cocaine was found to decline (Woolverton et al., 2012).

Foot shock

The most widely applied punisher in substance self-administration studies is mild electric shock. Originating from Jenkins' obstruction box studies (Jenkins et al.,

1926), initial studies in primates showed that response-contingent shocks reduced cocaine self-administration, whereby shocks of higher intensity were more effective, and delayed shocks less effective (Bergman & Johanson, 1981; Grove & Schuster, 1974). In the last decade, this setup has been widely used in rats (Belin et al., 2008, 2009, 2011; Deroche-Gamonet et al., 2004; Panlilio et al., 2003). In an influential study, Deroche-Gamonet et al. described that response-contingent foot shocks suppressed responding for cocaine in rats (Deroche-Gamonet et al., 2004), but that in a subgroup of rats, the sensitivity to foot shock profoundly declined after a lengthy cocaine taking history. This latter subgroup of animals was also characterised by high levels of cocaine-induced reinstatement of responding after extinction. Moreover, these rats showed other signs of addictive behaviour as well, such as high motivation for cocaine under a progressive ratio of reinforcement and persistence of non-reinforced responding, albeit that these different addiction-like behaviours did not emerge simultaneously (Deroche-Gamonet et al., 2004). Subsequent experiments showed that this addiction-like behaviour could be predicted on the basis of impulsive behaviour (i.e. premature responses in the 5-choice serial reaction time task), irregular patterns of cocaine self-administration and a high preference for a novel environment, but not novelty-induced hyperlocomotion (Belin et al., 2008, 2009, 2011).

In the studies described above, every substance taking episode was punished, and in the studies by Deroche-Gamonet, Belin and colleagues (Belin et al., 2008, 2009, 2011; Deroche-Gamonet et al., 2004), the response preceding the one that lead to cocaine infusion was punished as well (i.e., the fourth and fifth response under a fixed-ratio 5 schedule of reinforcement). Since in humans, not every instance of substance taking has inevitable and direct negative consequences, other studies have used somewhat different punishment procedures. For example, foot shock punishment was made probabilistic, whereby one in eight responses was punished with a foot shock, and one in three responses was reinforced with alcohol (Seif et al., 2013). Thus, even though alcohol taking was punished, delivery of alcohol was more frequent than punishment. With this approach, a subgroup of rats was shown to become insensitive to foot shock punishment. Other studies have moved punishment of responding forward in time, for example, to the acts directed at obtaining cocaine. To achieve this, Pelloux, Everitt and colleagues (Jonkman et al., 2012b; Pelloux et al., 2007, 2015) have used a seeking-taking chain schedule of reinforcement, in which rats were trained to respond on one lever ('seeking lever') in order to gain access to a second, 'taking' lever, responding on which produced an intravenous infusion of cocaine. After training, half of the seeking episodes did not lead to presentation of the taking lever but was punished with a mild electric foot shock.

Whereas the majority of animals showed profoundly reduced cocaine seeking when the punishment contingency was introduced, a subgroup of animals did not, albeit after a prolonged cocaine taking history (Pelloux et al., 2007). Comparable findings were reported by others, in setups in which seeking, when punished, did (Xue et al., 2012), or did not allow for subsequent cocaine taking (Chen et al., 2013). Further analysis of this behaviour showed that insensitivity to punishment was the result of excessive drug exposure rather than experiencing a large number of cocaine-cue associations (Jonkman et al., 2012b). In a subsequent study, punishment of seeking (i.e. foot shock after fulfilling the response requirement on the seeking lever) or taking (i.e. foot shock after responding on the taking lever) was compared. The data showed that rats were more willing to endure punished taking than seeking (Pelloux et al., 2015), suggesting that punishment of distal substance seeking acts is more effective in reducing addictive behaviour than punishment of the actual use of the substance. Importantly, the availability of response-contingent sucrose increased the effectiveness of punishment to reduce cocaine seeking. Threat of adversity has also been used in the context of addictive behaviour, as an alternative to immediate and inevitable punishment. To this aim, auditory cues previously associated with mild electric foot shocks were used to influence cocaine seeking (Kearns et al., 2002; Limpens et al., 2014; Vanderschuren & Everitt, 2004). These experiments revealed that presentation of a foot shock-associated cue suppressed cocaine seeking, but after limited drug taking experience only. Thus, after an extended cocaine self-administration history, the effectiveness of the foot shock-associated cue to alter cocaine seeking profoundly declined (Limpens et al., 2014; Vanderschuren & Everitt, 2004). A different threat model has been used in studies on eating disorders, in which rats or mice have to enter an aversive, brightly lit environment in order to get access to a preferred food (Cottone et al., 2012; Teegarden & Bale, 2007). This approach has as yet not been used in the context of self-administration of substances of abuse.

Punishment of cocaine and heroin self-administration has recently also been performed in studies in which Jenkins' obstruction box (Jenkins et al., 1926) was revisited. Thus, in these experiments, rats had to cross an electrified grid to reach the lever, pressing which produced an infusion of the drug (Barnea-Ygael et al., 2012; Cooper et al., 2007; Peck et al., 2013; Saunders et al., 2013). For each individual animal, the shock intensity that completely suppressed responding for the drug was determined, after which reinstatement of responding for drug-associated cues was assessed. Interestingly, reinstatement of responding for cocaine was only observed in about half of the rats, whereas in the case of heroin, all rats showed cue-induced reinstatement of responding (Cooper et al., 2007; Peck et al., 2013). Last, foot shock-induced punishment of alcohol and methamphetamine self-administration has also

been used as a method to make rats cease responding for the respective substance, in order to assess context- (Marchant et al., 2013) or cue-induced reinstatement of responding (Krasnova et al., 2014).

Punishment models of addictive behaviour: merits and drawbacks

The studies discussed above describe approaches aimed at emulating persistent substance use despite negative consequences. Clearly, these have substantially moved the preclinical addiction field forward by demonstrating that aversive stimuli of different modalities, including gustatory (quinine), interoceptive (lithium chloride, histamine) and tactile (foot shock) ones, can inhibit behaviour directed at substances of abuse. More importantly, a substantial proportion of these studies also reports that animals with a certain predisposition and/or self-administration history display reduced sensitivity to aversive interference (Belin et al., 2008, 2009, 2011; Chen et al., 2013; Deroche-Gamonet et al., 2004; Dickinson et al., 2002; Hopf et al., 2010; Jonkman et al., 2012b; Leong et al., 2016; Lesscher et al., 2010; Limpens et al., 2014; Miles et al., 2003; Pelloux et al., 2007, 2015; Seif et al., 2013; Spoelder et al., 2015; Vanderschuren & Everitt, 2004; Wolffgramm, 1991; Wolffgramm & Heyne, 1995; Xue et al., 2012), which resembles the aberrant, unflagging pursuit of substances of abuse in human addicts (American Psychiatric Association, 2000, 2013). These contemporary setups of addiction-like behaviour hold great promise to increase our understanding of the neural and behavioural structure of substance use disorders. Indeed, recent years have seen explicit progress in the study of the neural underpinnings of addiction using punishment models (Chen et al., 2013; Jonkman et al., 2012a; Kasanetz et al., 2010, 2013; Lesscher et al., 2012; Limpens et al., 2015; Pelloux et al., 2012, 2013; Seif et al., 2013, 2015; Xue et al., 2012).

Quinine and histamine

An issue that needs to be considered with care is which aspect of substance use is being punished in these models. Indeed, gustatory, interoceptive and tactile punishers have all been scrutinised for their validity to study human addictive behaviour. The bitter taste of quinine is a gustatory punisher, that is immediately apparent following ingestion of alcohol (as well as other substances of abuse in oral consumption experiments (Galli & Wolffgramm, 2004; Heyne & Wolffgramm, 1998; Wolffgramm & Heyne, 1995)). As such, it is an immediate punisher of alcohol drinking, and the sensation of its bad taste actually precedes the perception of the subjective effects of alcohol. It is useful to realise that taste is an important aspect of alcohol ingestion, and that one of the behavioural characteristics of alcohol addiction is the ingestion of unpalatable (cheap, but with high alcohol content) liquors, in order to maximise alcohol intake at minimal financial cost. In extreme cases, alcohol addicts even ingest unsavoury alcohol-containing products not intended for human consumption, such as mouthwash and aftershave (Leon et al., 2007; Soo Hoo et al., 2003), whereby taste has obviously become less important than alcohol content. The willingness of animals to endure the bitter taste of quinine, if this is the only way of obtaining alcohol (Lesscher et al., 2010; Seif et al., 2013; Spoelder et al., 2015; Wolffgramm, 1991; Wolffgramm & Heyne, 1995), reflects the reduced importance of taste in alcohol addiction, which is perhaps even better exemplified by the continued ingestion of quinine-containing alcohol when non-adulterated alcohol is simultaneously available (Lesscher et al., 2010). Comparable to quinine in terms of its immediacy is the interoceptive discomfort induced by intravenous histamine, which has been shown to be an efficient punisher of cocaine self-administration (Holtz & Carroll, 2015; Negus, 2005; Woolverton et al., 2012). Resistance to histamine punishment has so far not been demonstrated in an animal study, although this may be a matter of histamine dose and/or cocaine self-administration experience rather than histamine being a stronger punisher than quinine, lithium chloride or foot shock.

Lithium chloride

Somewhat different to quinine and histamine, the aversive effects of lithium chloride-induced malaise emerge with a delay after substance taking. This delay stems both from the slower onset (and probably longer duration) of the lithium chloride-induced interoceptive effects compared to the rapid subjective substance effects, but also from the practical point that lithium is passively administered to the animal after drug exposure (Dickinson et al., 2002; Leong et al., 2016; Miles et al., 2003). In this regard, lithium chloride may more closely emulate the visceral discomfort that follows substance taking episodes, such as the gastrointestinal pain that alcohol addicts may suffer from, as well as the physical malaise that characterises an alcohol hangover or cocaine crash. Remarkably, the studies that have employed lithium chloride to punish addictive behaviour have found that it only reduces proximal substance taking acts (i.e. drinking alcohol and cocaine solutions, intravenous cocaine self-administration) but not behaviours distal to substance use, such as responding for cocaine or alcohol in extinction (i.e. without immediate gustatory feedback about the degraded reinforcer) and responding for cocaine cues (Dickinson et al., 2002; Leong et al., 2016; Miles et al., 2003). This indicates that the effectiveness of punishment declines with increasing temporal distance, consistent with the classic observation that the strength of a learning process declines with the delay between action and outcome (Dickinson et al., 1992).

Foot shock

As is clear from the studies discussed here, mild electric shocks are the most widely employed punisher in preclinical addiction research (Barnea-Ygael et al., 2012; Belin et al., 2008, 2009, 2011; Bergman & Johanson, 1981; Chen et al., 2013; Cooper et al., 2007; Cottone et al., 2012; Deroche-Gamonet et al., 2004; Grove & Schuster, 1974; Jenkins et al., 1926; Jonkman et al., 2012b; Kearns et al., 2002; Krasnova et al., 2014; Limpens et al., 2014; Marchant et al., 2013; Panlilio et al., 2003; Peck et al., 2013; Pelloux et al., 2007, 2015; Saunders et al., 2013; Seif et al., 2013; Teegarden & Bale, 2007; Vanderschuren & Everitt, 2004; Xue et al., 2012). This has probably both scientific reasons, as the large number of fear conditioning studies in the literature yields an enormous database of methodological and neural background information, as well as practical reasons. Thus, the intensity, quantity and probability of foot shocks can easily be varied, which renders this a very versatile way of interfering with behaviour. Comparable to quinine and histamine, foot shocks are often used as an immediate punisher of substance use, but the manner in which addictive behaviour is punished is likely to be different. That is, the sensation of foot shock is immediate, noxious, and brief, and the expectation of foot shocks generates a state of conflict and fear. This may emulate the emerging adverse consequences of persistent substance use in humans, in which the user has to weigh the immediate positive experience of substance use against the possible adverse consequences, such as job loss, relationship crisis or disease. Comparable to quinine and lithium chloride, it has also been shown that under certain conditions, animals are willing to endure mild electric foot shocks in order to obtain cocaine or alcohol (Belin et al., 2008, 2009, 2011; Chen et al., 2013; Deroche-Gamonet et al., 2004; Jonkman et al., 2012b; Pelloux et al., 2007, 2015; Seif et al., 2013; Xue et al., 2012). From a naturalistic point of view, the validity of foot shocks for human addictive behaviour may be less than the other punishers discussed here. Thus, the pursuit or use of substances in humans is typically not followed by noxious, physical punishment, whereas, as discussed above, addicts are confronted with bad taste or interoceptive malaise as a result of their substance use. That said, a recent study in humans has shown that cocaine addicts are less proficient in the avoidance of electric shocks, suggesting that reduced sensitivity to physical punishment does play a role in addictive behaviour (Ersche et al., 2016).

The validity of immediate punishment

A limitation that is often noted for experimental approaches as discussed here is the immediacy of punishment. Although the timing of its consequences remains largely unclear, substance use in humans is usually not punished immediately and inevitably. Rather, the negative consequences of addictive behaviour are often delayed, probabilistic and difficult to trace back to single substance use episodes. In fact, the observation that delayed punishment (compared to immediate punishment) is substantially less effective in interfering with cocaine self-administration (Grove & Schuster, 1974; Woolverton et al., 2012) perhaps illustrates the very nature of addiction, in that substance abuse persists despite negative, but often delayed consequences. In order to use foot shock punishment in a way that more closely emulates the human situation where the adverse sequelae of substance use can be rather unpredictable, researchers have therefore also used probabilistic shocks (Jonkman et al., 2012b; Pelloux et al., 2007, 2015; Seif et al., 2013; Xue et al., 2012). An alternative approach has used threat of foot shock punishment, rather than the shocks themselves (Kearns et al., 2002; Limpens et al., 2014; Vanderschuren & Everitt, 2004), to model seeking substances in a situation where this entails danger (for example, trying to buy drugs while there is police surveillance on the street). Likewise, these approaches have revealed conditions in which animals endure shock or threat when seeking or taking substances of abuse (Jonkman et al., 2012b; Limpens et al., 2014; Pelloux et al., 2007, 2015; Seif et al., 2013; Vanderschuren & Everitt, 2004). One could therefore argue that models using threat of adversity or unpredictable adversity more closely capture the anticipation of adverse consequences at the time of substance use, that probably better reflects the internal conflict that human addicts experience. In any event, understanding the relative timing between substance use and adverse consequences, and how this impacts on use, is one of the main challenges in the management of addiction, and this knowledge should be incorporated into the design of animal models of addictive behaviour.

Understanding interventions

The overarching aim of the studies discussed here has been to develop and use animal models to elucidate the neural underpinnings of addictive behaviour. Subsequently, these approaches can be used to test the effects neural manipulations on addiction (Chen et al., 2013; Jonkman et al., 2012a; Pelloux et al., 2012; Seif et al., 2013). In addition, they can also help understand the effectiveness of behavioural strategies to influence addictive behaviour. For example, the findings that behaviours proximal to substance use are more sensitive to punishment than distal ones if substance intake is punished (Dickinson et al., 2002; Leong et al., 2016; Miles et al., 2003) is very informative about the structure of addictive behaviour. Thus, even if substance taking has negative consequences, this may not alter their procurement, since the temporal distance between seeking substances and the sensation of punishment after substance intake may be too long (Dickinson et al., 1992). An important study in this regard has been performed by Pelloux and colleagues (Pelloux et al., 2015), who reported that punishing distal behaviours (i.e., cocaine seeking) is more effective than punishing cocaine taking, suggesting that interfering with substance

use in an early stage of the chain of substance-directed behaviours may yield better results. Also encouraging is the finding in this study (Pelloux et al., 2015) that the availability of an alternative source of reinforcement (i.e., response-contingent sucrose) further reduces cocaine seeking and taking, suggesting that positive (i.e. an alternative source of reinforcement) and negative incentives (i.e. punishment) can have additive beneficial effects on addictive behaviour.

Other aspects of addictive behaviour

A limitation of punishment studies discussed here is that they only model part of the addictive behaviour in humans. Thus, whereas one can argue that 9 out of 11 diagnostic criteria in DSM5 comprise behaviours representing loss of control over substance use (American Psychiatric Association, 2013), punishment setups emulate only two of those (i.e., recurrent use in situations in which it is physically hazardous; continued use despite knowledge of substance-related problems). Therefore, if one aspires to generate an animal model that captures multiple aspects of addiction, other signs of addictive behaviour should be incorporated as well (Vanderschuren & Ahmed, 2013). These include high motivation to work for substances (as a model of devoting a great deal of time to procuring, consuming and recovering from use), responding in extinction (to model persistent desire or unsuccessful attempts to restrict use), reinstatement of substance seeking (as a model of craving), choosing substances over natural reinforcers (to model the neglect of alternative, social and professional, sources of reward), and the effects of social isolation and social rank (as a model of continued use despite persistent social problems caused by use and giving up important social activities in favour of use) (Baarendse et al., 2014; Belin et al., 2009; Bossert et al., 2013; Deroche-Gamonet et al., 2004; Heilig et al., 2016; Lenoir et al., 2007, 2013; Lesscher et al., 2015; Morgan et al., 2002; Shaham et al., 2003; Trezza et al., 2014). The validity of these models is beyond the scope of this paper, but we do acknowledge the value of these approaches for the study of addictive behaviour. On the other hand, we think that employing single-aspect models allows for the investigation of the neurobiological underpinnings of distinct aspects of addiction in isolation. In this regard, it is important to keep in mind that addiction is a multi-faceted disorder, in which different aspects, criteria or behavioural aberrations may play a role, depending on, for example, the substance abused, the history of the individual, or the environmental circumstances. Importantly, neurobiological studies in which different aspects of addictive behaviour have been combined have provided evidence that exaggerated motivation, responding in extinction, reinstatement of extinguished responding and resistance to punishment rely on distinct neural mechanisms (de Jong et al., 2015; Radke et al., 2015, 2017).

Conclusion

The last two decades have seen a remarkable interest in the use of punishment paradigms to model the persistent aspects of substance use disorders. These models have used punishments from different sensory modalities, and methodological variations in these setups allow for the assessment of distinct aspects of loss of control over substance use. Although these punishment setups may arguably still be in development, we expect that their optimisation and integration with other models, capturing yet other aspects of addictive behaviour such as exaggerated motivation for substances and relapse, will make a valuable contribution to our knowledge about the neural and behavioural structure of addiction. This may ultimately contribute to the development of more effective treatments for this devastating disorder.

Conflicts of interest

The authors declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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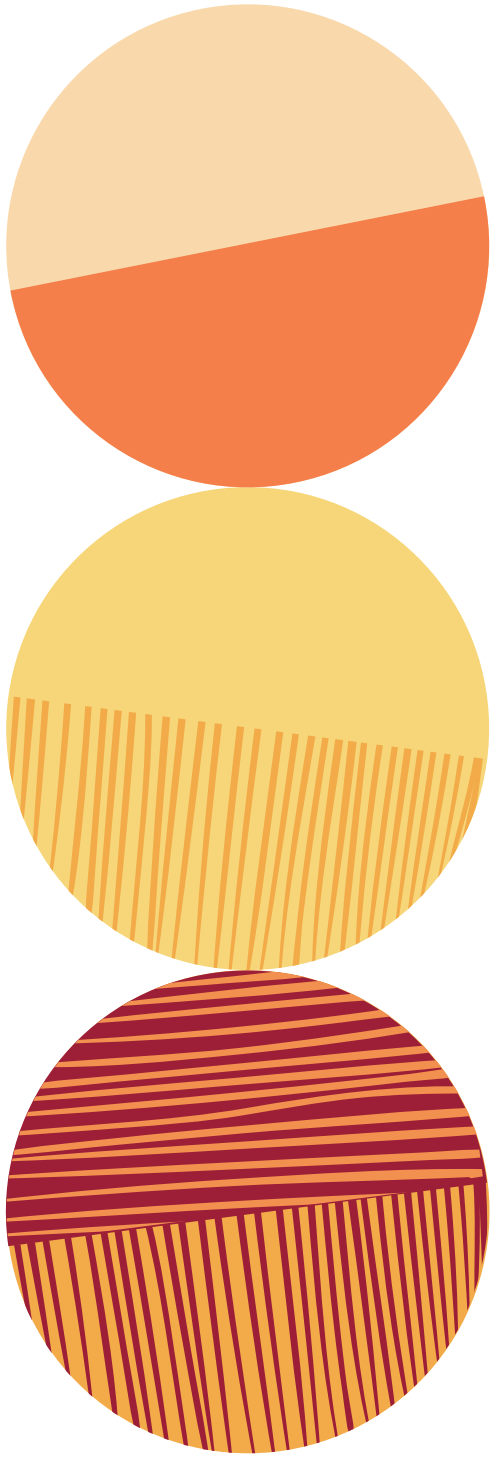


Chapter 3

Seeking under threat of adversity: Assessing control over reward pursuit in rats

A. Maryse Minnaard[1,2]*
Johanna A. S. Smeets[1,2]*
Geert M. J. Ramakers[2]
Roger A. H. Adan[2]
Heidi M. B. Lesscher[1]
Louk J. M. J. Vanderschuren[1]

* shared first authorship

[1] Department of Population Health Sciences, Unit of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2] Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, The Netherlands

Abstract

Rationale
Substance use disorder (SUD) is a chronic relapsing disorder that is characterised by loss of control over substance use. A variety of rodent models employing punishment setups have been developed to assess persistent substance use despite negative consequences, in order to facilitate the translation of findings from animal studies to the human situation.

Objectives
Since the negative consequences of addictive behaviour are typically unpredictable, we here developed and validated the Seeking under Threat of Adversity (STA) task in rats, that incorporates probabilistic, response-contingent punishment of reward seeking.

Methods
Rats were trained to lever press for alcohol or sucrose and were subsequently tested in the STA task. In this task, a tone cue is presented during reward seeking. This cue functions as a warning signal, since responding during tone presentation results in a probabilistic foot shock punishment. We first determined the optimal shock intensity to induce a moderate suppression of seeking. Next, we assessed the stability of punished reward seeking over repeated tests.

Results
Responding for both alcohol and sucrose was suppressed in the STA task. Suppression of responding was relatively stable with repeated testing.

Conclusions
The STA task is a novel behavioural task that includes two important aspects of human substance use despite negative consequences, i.e. response contingency and unpredictability of punishment. This task can be used to assess the neural and behavioural underpinnings of control over substance seeking.

Keywords
Alcohol
Adversity
Punishment
Rats
Substance use disorder
Sucrose

Introduction

Substance use disorder (SUD) is a major socioeconomic and public health issue, which has been calculated as one of the most financially costly disorders of the central nervous system (Uhl & Grow, 2004; UNODC, 2019). SUD is a chronic relapsing disorder that is characterised by loss of control over substance use. Indeed, many of the criteria for SUD listed in the DSM-5 (American Psychiatric Association, 2013) comprise behaviours that reflect persistent substance intake despite negative consequences. This loss of control is thought to develop gradually with prolonged and excessive substance use, but the underlying neural mechanisms are not fully known. At present, treatment options for SUD are limited in number and efficacy, and they are not directed at restoring control over substance use (O'Brien, 2008; van den Brink, 2012). Therefore, a better understanding of the neural underpinnings of loss of control over substance intake might contribute to the development of more effective treatments for SUD.

Over the last decades, rodent models have been developed to investigate behavioural and neural mechanisms of SUD, generally by trying to capture key features of the SUD symptomatology, including loss of control over substance use. These models are often variations of punishment tasks, in which animals are confronted with a conflict situation wherein they can refrain from pursuing a substance of abuse, in order to avoid adverse consequences or rather continue seeking despite negative consequences. Usually, this conflict situation is operationalised by a negative stimulus or outcome, such as a foot shock or a bitter taste, that is paired to the seeking or taking of a substance or to the substance-taking context (for recent reviews see Goltseker et al., 2019; Hopf & Lesscher, 2014; Vanderschuren et al., 2017; Vanderschuren & Ahmed, 2020).

For the development of animal models for loss of control over substance use, i.e. continued substance use despite adverse consequences, the modality and pattern of exposure to negative stimuli should mimic human addictive behaviours in the best possible way. Importantly, in rodent models, foot shock punishment of responding for a substance usually coincides with reward delivery, but negative consequences of substance use in humans typically do not occur immediately or solely at the moment of substance taking. In an attempt to circumvent the direct punishment, other studies have implemented response-independent adversity, for example through the presentation of a tone that was previously associated with foot shocks in another context (Limpens et al., 2014, 2015; Spoelder et al., 2017; Vanderschuren & Everitt, 2004). The latter approach, however, may not be optimal since the presentation

of the punishment stimulus is not contingent upon responding for the substance. Moreover, negative consequences of substance use are often unpredictable in the human situation, while the user has typically been confronted with warning signals on multiple occasions. In fact, the warning signals pose a certain threat of adversity, but the exact timing of this adversity remains unforeseeable. Therefore, the incorporation of unpredictable, yet cued and response-contingent adversity in models for SUD might be more reminiscent of the human situation.

Taking these considerations into account, we aimed to develop a task to measure reward seeking behaviour under the threat of adversity in rats, to improve resemblance of addictive behaviour in humans. In this setup, which we named the Seeking under Threat of Adversity (STA) task, we first trained rats to lever press for alcohol or sucrose under a random interval schedule of reinforcement. Subsequently, a tone cue was presented during reward seeking, that functioned as a warning signal, since lever pressing during tone presentation resulted in a probabilistic foot shock. The conditioning cue and the probabilistic nature of the punishment combined provided a context in which seeking behaviour was associated with a risk for punishment, which was avoidable if animals refrained from seeking responses. In this study, we determined the impact of punishment intensity and reinforcer type on reward seeking under the threat of adversity in the STA task. For this purpose, four cohorts of rats that responded for either alcohol or sucrose were tested in the STA task, with variations in punishment intensity. We predicted that controlled substance seeking would be suppressed upon presentation of the warning cue, thereby avoiding punishment. Alternatively, compulsive seeking would be expressed as an inability to refrain from lever pressing to obtain a reward, despite awareness of the warning cue and the threat of a negative outcome.

Materials and methods

Subjects

A total of 49 adult male Lister Hooded rats (Charles River, Germany), weighing 150-300 grams (~5-10 weeks old) at the start of the experiment, were used in this study. The rats were individually housed in Macrolon type III sawdust bedded cages (42.5 x 26.6 x 18.5 cm) with ad libitum access to tap water and standard chow (Rat and Mouse Breeder and Grower Expanded-CRM(E), Special Diet Service, UK), except during operant training and testing. A polycarbonate rat tunnel (9 x 9 x 15 cm) and a tissue were provided for cage enrichment. The rats were kept under controlled temperature and humidity conditions (21 ± 2°C and 50 - 70% humidity) under a reversed 12 h/12 h light/dark cycle (lights off at 7.00 AM - lights on at 7.00 PM) to allow for behavioural testing in the dark phase. Background noise was provided by a constantly playing radio. The rats were acclimatised to the housing conditions for at least eight days prior to behavioural testing, and they were weighed and handled at least once per week throughout the course of the experiment. Behavioural experiments were conducted in four independent cohorts of animals:

Cohort A: n = 16 animals responding for alcohol.
Cohort B: n = 8 animals responding for sucrose.
Cohort C: n = 16 animals responding for alcohol.
Cohort D: n = 9 animals responding for sucrose.

Experimental procedures were approved by the Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee, and conducted in agreement with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU).

Behavioural procedures

Intermittent alcohol access

In order to facilitate subsequent responding for alcohol, the animals of cohorts A and C were given intermittent access to alcohol (IAA) in a two-month two-bottle choice setup in the home cage for three days a week (i.e., Monday, Wednesday, Friday) as previously described (Spoelder et al., 2015) (Figure 1A). In the first four weeks of IAA, alcohol exposure sessions lasted for 7 hours, approximately between 9.30 AM and 16.30 PM (i.e. during the dark phase of the day-night cycle); sessions were extended to 24 hours during the second month of IAA. The bottles were weighed before and after each drinking session and the placement of the alcohol bottle was alternated between sessions to avoid the development of a side bias. Alcohol (99.5%, Klinipath, The Netherlands) was freshly diluted with tap water once per week to a final concentration of 20% (v/v).

Apparatus

The animals were trained and tested in operant conditioning chambers (29.5 x 24 x 25 cm, Med Associates Inc., USA) equipped with two retractable levers (4.8 x 1.9 cm; ENV-112CM) and a white cue light (28 V, 100mA; ENV-221M) present above each lever. A recessed liquid dipper and food receptacle were situated in between the levers. The wall on the opposite side of the box contained a white house light (28 V, 100mA; ENV-215M) and a tone generator (85 dB, 2900 Hz; ENV-223AM). The floor of the chamber was covered with a metal grid with bars that were separated by 1.57 cm and were connected to a shock generator (ENV-414SA standalone aversive stimulator). All chambers were situated in light- and sound-attenuating cubicles

equipped with a ventilation fan, and were controlled by MED-PC IV software (version 4.2) for Windows.

Fixed ratio and random interval schedules of reinforcement

The rats were trained to respond for alcohol (cohorts A and C) or sucrose (cohorts B and D) in 30 minute operant sessions, during which the house light was illuminated, once daily, 4-6 days per week. The position of the active and inactive levers was counterbalanced between rats. The presentation of the reward was paired with the retraction of both levers and the illumination of the cue light above the active lever. Animals were first trained under a fixed ratio (FR) 1 schedule of reinforcement. In the case of alcohol reinforcement, pressing the active lever raised the dipper cup containing an alcohol reward (0.1 ml, 20% v/v). The dipper cup remained in the raised position until 10 seconds after the animal entered the recessed receptacle, which was detected by interruption of an infrared light beam. The cue light was turned off and the levers were reintroduced, signalling the start of a new trial. The alcohol solution was refreshed between sessions to prevent a decline in alcohol concentration by evaporation of the alcohol between sessions. In the case of sucrose reinforcement, pressing the active lever activated a pellet dispenser that delivered a 45 mg sucrose pellet (TestDiet, USA) into the food receptacle. Collection of the sucrose reward, which was detected by interruption of an infrared light beam, was followed by a 0.5 second inter-trial interval. After this period, the cue light was turned off and the levers were reintroduced, signalling the start of a new trial. For both reinforcers, inactive lever presses were recorded but were without programmed consequences.

After acquisition of self-administration under an FR 1 schedule (i.e. when animals obtained an inactive to active lever press ratio of approximately 1:7), the animals were trained under a random interval (RI) 5 schedule of reinforcement (Figure 1A). Under this schedule, each trial commenced with the presentation of the active and inactive lever. Pressing the active lever initiated an interval with an average duration of 5 seconds (interval duration picked in pseudo-random order), during which both levers remained extended. All lever presses during this interval were recorded but were without programmed consequences. The first active lever press (ALP) after completion of the interval resulted in the delivery of the reward (alcohol or sucrose), as described for the FR 1 schedule of reinforcement. The rats were subsequently trained with increasing interval lengths (RI 15, RI 30, RI 60), before reaching the final RI 120 schedule (Figure 1A). The animals were trained under the RI 120 schedule until stable responding was achieved for three consecutive days, i.e. until the average ALPs at group level on subsequent days fell within an 80-120% variability range of the total average of ALPs across those three consecutive days.

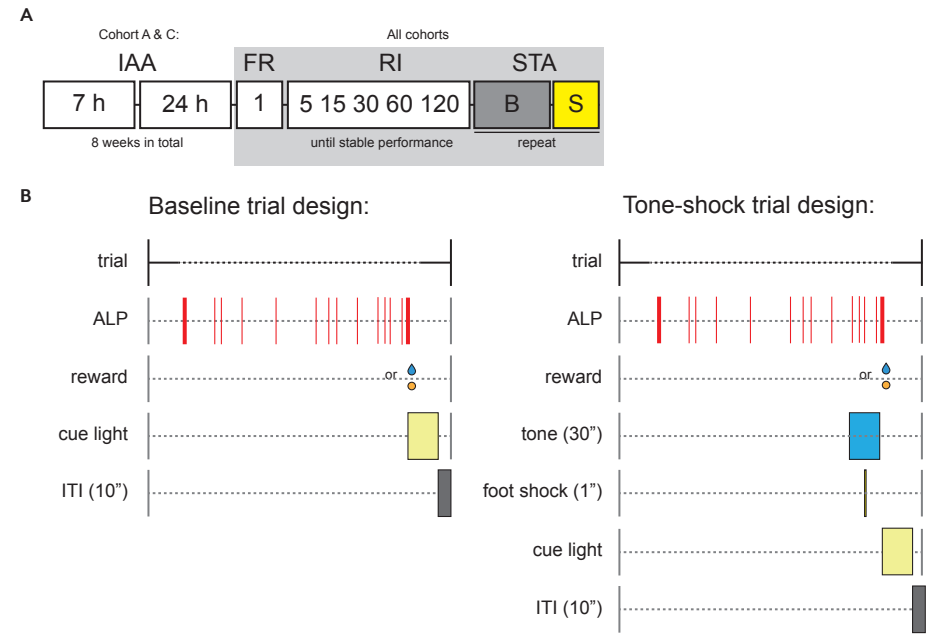


Figure 1. Procedural outline. A. Schematic flowchart of the experimental outline. Cohorts A and C were first exposed to intermittent alcohol access (IAA) in the home cage for eight weeks. All cohorts were subjected to a minimum of ten fixed ratio (FR) 1 operant training and random interval (RI) operant training sessions. Subsequently, the animals were tested in the seeking under threat of adversity (STA) task, which consisted of a minimum of three baseline (B) sessions and two tone-shock (S) sessions. After two S sessions, rats were retrained for a minimum of three B sessions until stable responding before being tested in another two S sessions. **B.** Schematic overview of the trial design for B session trials (left panel) and S session trials (right panel). In B trials, pressing the active lever (signified by the red lines) initiated an interval with an average duration of 120 seconds. All lever presses during this interval were recorded but were without programmed consequences. The first active lever press (ALP) after completion of the interval resulted in the delivery of the reward (alcohol solution or sucrose pellet) and the illumination of the cue light, followed by a 10 second inter-trial interval (ITI). Trials in S sessions had a similar set-up as trials in a B session, with the exception that a tone was presented during the last 30 seconds of each interval. During the tone presentation, ALPs were punished in a probabilistic manner (25% chance) with a 1 second foot shock (intensity ranging between 0.05 - 0.35 mA). If a rat made at least one ALP during the tone presentation, the first ALP following completion of the interval resulted in reward delivery. However, if animals refrained from lever pressing during tone presentation, both levers were retracted immediately upon completion of the random interval and the 10 second ITI was initiated.

Table 1. Possible outcomes during trials of a tone-shock session.

	Tone trials	
active lever press during 30 s tone	**shock**	**reward**
Yes	+	+
Yes	-	+
No	-	-

Seeking under threat of adversity (STA) task

After acquisition of stable responding under the RI 120 schedule of reinforcement, the rats progressed to the STA task. The schedule of reinforcement in the STA task is a modified version of the RI 120 schedule, divided into two types of sessions: baseline and tone-shock sessions. Baseline sessions were similar to RI 120 sessions, with the exception that intervals of all trials lasted at least 30 seconds and an inter-trial interval of 10 seconds was introduced (Figure 1B, left panel). The animals were trained until stable responding was achieved (i.e. 80-120% variability range of average ALPs) on three consecutive days, before testing in two consecutive tone-shock sessions (tone-shock block) commenced. Trials in tone-shock sessions had a similar set-up as trials in a baseline session, with the exception that a tone was presented during the last 30 seconds of each interval. During the tone presentation, ALPs were punished in a probabilistic manner (25% chance) with a 1 second foot shock (ranging between 0.05 - 0.35 mA, depending on the cohort and phase of the study) (Figure 1B, right panel).

If a rat made an ALP during the tone presentation, the first ALP following completion of the random interval resulted in reward delivery, regardless of whether it had received shock punishment during the preceding 30 seconds or not (see Table 1, Figure 1B). However, if animals refrained from lever pressing during tone presentation, both levers were retracted immediately upon completion of the random interval and the 10 second inter-trial interval was initiated. In this way, only trials in which rats took the risk of shock punishment by pressing the lever during the tone were rewarded. After each tone-shock block, the animals were retrained in baseline sessions until stable responding was achieved (80-120% variability range of average ALPs) on three consecutive days.

Initially, we set out to determine the optimal foot shock intensity for the STA task, i.e. the intensity at which a moderate suppression of seeking was observed. Therefore, for cohort A and cohort B, the intensity of foot shock punishment was increased across tone-shock blocks, from 0.05 mA up to 0.35 mA. Based on the data from cohorts A and B (as later described in detail in the results section), 0.25 mA was determined to be the optimal shock intensity, which was subsequently used for cohorts C and D.

Data analysis and statistics

For cohorts A and C, home cage alcohol intake and preference were calculated as follows. Fluid intake was calculated by subtracting the bottle weights at the end of every drinking session by the starting weights. Alcohol intake (ml) was calculated by the following equation: (Δ alcohol bottle weight in grams) / (0.8+(0.2*0.789)) in which the density of ethanol (i.e., 0.789 g/ml), is included. Alcohol intake (g/kg) was calculated by the following equation: ((alcohol fluid intake in ml) * (0.2*0.789)) / (bodyweight in kg). Preference for alcohol (%) was calculated according to the following equation: (alcohol intake in ml) / ((alcohol intake in ml) + (water intake in ml)) * 100. To assess gradual escalation of alcohol consumption, as animals in cohort A and C progressed to the 24 hour sessions, differences in mean alcohol intake (g/kg) and total volume intake (ml/kg) between the 7 hour (group mean week 1-4) and 24 hour (group mean week 5-8) sessions were compared with a paired samples t-test. Changes in preference between the 7 hour (group mean week 1-4) and 24 hour sessions (group mean week 5-8) were assessed with a paired samples t-test for cohort A, and Wilcoxon matched-pairs signed rank test for cohort C, as the difference between alcohol preference between the 7 hour and 24 hour sessions were not normally distributed in cohort C.

Cohorts A and B were subjected to shock intensities in the STA task that gradually increased over sessions, to determine the optimal shock intensity at which a moderate suppression of seeking was observed. To measure the effect of gradually increasing (in cohorts A and B) or consistent (in cohorts C and D) punishment intensities on seeking behaviour, we assessed the total number of responses (i.e. ALPs) in consecutive baseline and tone-shock sessions, the total amount of rewards obtained in consecutive baseline and tone-shock sessions, and the amount of shocks received in tone-shock sessions. The number of seeking responses was assessed within-subjects, by comparing the mean ALPs during baseline blocks (i.e. average of three consecutive baseline sessions) with that during tone-shock blocks (i.e. average of two tone-shock sessions) using two-way repeated measures analyses of variance (ANOVA) with *shock intensity* as the within-subjects factor for cohorts A and B or *time* as the within-subjects factor for cohorts C and D and with *session type* (i.e. baseline or tone-shock) as the within-subjects factor. Similarly, the number of rewards was assessed within-subjects, by comparing the mean number of obtained rewards during baseline blocks (i.e. average of three consecutive baseline sessions) with that during tone-shock blocks (i.e. average of two tone-shock sessions) using two-way repeated measures ANOVAs. The number of shocks was assessed by comparing the mean number of shocks per block using one-way repeated measures ANOVAs and Friedman tests whenever the data was not normally distributed. Significant main effects were followed up by Tukey's post hoc analyses with correction for multiple comparisons. Significant interaction effects were followed up by pairwise comparisons with a Bonferroni correction. ALP data were normally distributed as assessed by Shapiro-Wilk's test ($p > 0.05$ at each shock intensity or time point) in cohort A, B, C, and D. Mauchly's test of sphericity was used to determine if variances

of the differences between timepoints were equal. Whenever the assumption of sphericity was violated, a Greenhouse-Geisser correction was used. Corrected degrees of freedom are presented, rounded to the nearest integer.

Furthermore, we expressed suppression of seeking using two measures. First, we calculated the number of ALP during shock sessions as a fraction of the number of ALP during baseline, i.e. (average ALP of a tone-shock block) / (average ALP of three consecutive baseline sessions). A value of 1.0 would indicate no difference between baseline and shock sessions, and a value 0.8 or lower (i.e. below the lower cut-off of the 80-120% variability range for stable responding during baseline training) was considered suppression of seeking behaviour. Second, we calculated a suppression ratio, as (average ALP of three consecutive baseline sessions - average ALP during tone-shock block) / (average ALP of three consecutive baseline sessions + average ALP during tone-shock block). Here, a suppression ratio of 0.0 means no suppression, while a suppression ratio of 1.0 indicates complete suppression of seeking during a tone-shock block. Suppression ratios were compared using one-way repeated measures ANOVAs with *shock intensity* as the within-subjects factor for cohorts A and B and *time* as the within-subjects factor for cohorts C and D. Significant effects were followed up by Tukey's post hoc analyses with correction for multiple comparisons. Suppression ratios were normally distributed as assessed by Shapiro-Wilk's test ($p > 0.05$ at each shock intensity or time point) in cohort A, B, C, and D. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated in suppression ratio data of cohort D ($p = 0.043$), and therefore, a Greenhouse-Geisser correction was used.

Data are expressed as mean ± SEM, unless otherwise stated. Data were analysed and visualised using Microsoft Excel, Graphpad Prism (version 8.3.0, Graphpad Software Inc., USA) and SPSS for Windows (version 25.0.0.1, IBM Corp., USA). No outliers were detected in the data, as assessed by studentised residuals values greater than ± 3. A significance criterion of $p < 0.05$, two-tailed, was used for all the statistical analyses.

Results

Voluntary alcohol consumption in the home cage

In order to facilitate operant responding for alcohol, the rats of cohorts A and C were exposed to IAA in the home cage for eight weeks. Weekly averages of alcohol intake were calculated (Figure 2A-B) and alcohol intake and preference during the 7 hour and 24 hour sessions were compared (Table 2). In both cohorts, animals drank significantly more during the 24 hour as compared to the 7 hour sessions (alcohol intake: cohort A: $t(15) = 4.682$, $p < 0.001$; cohort C: $t(15) = 3.427$, $p = 0.004$; total volume intake cohort A: $t(15) = 17.42$, $p < 0.001$; cohort C: $t(15) = 11.19$, $p < 0.0001$). These results show that rats readily consumed alcohol in the home cage and that alcohol intake increased when sessions were prolonged from 7 hours to 24 hours.

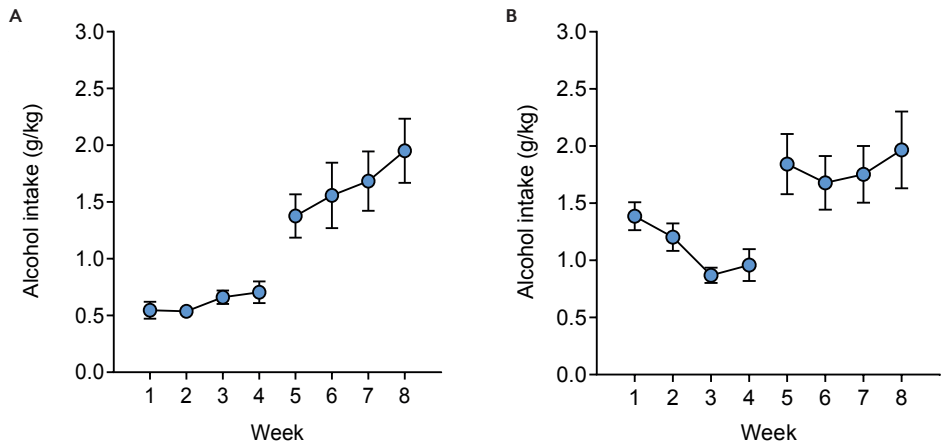
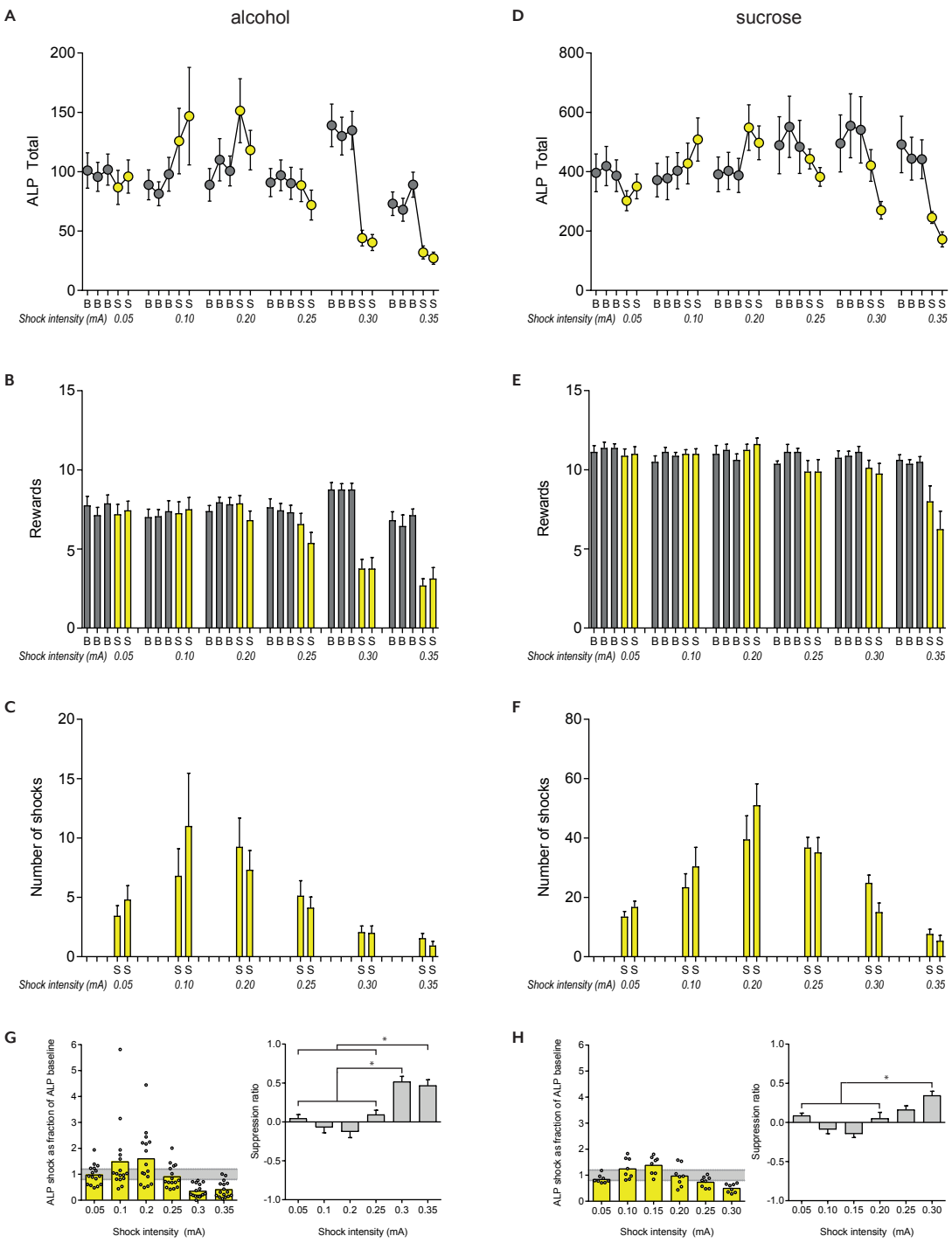


Figure 2. Voluntary alcohol intake in the home cage. Weekly averages of intake of alcohol (g/kg) for cohort A (n = 16) (**A**) and cohort C (n = 16) (**B**) during eight weeks of intermittent alcohol access (IAA) in the home cage prior to operant training for responding for alcohol. Data expressed as mean ± SEM.

Table 2. Mean ± SEM daily alcohol intake, alcohol preference, and total fluid intake for the 7 hour and 24 hour sessions of intermittent alcohol access (IAA) in the home cage (Figure 2). Significant differences ($p < 0.05$) are indicated with *.

	Cohort A		Cohort C	
Alcohol intake (g/kg)				
7 h sessions	0.61 ± 0.04	*	1.11 ± 0.08	*
24 h sessions	1.64 ± 0.24		1.77 ± 0.20	
Alcohol preference (%)				
7 h sessions	14.96 ± 1.53		17.19 ± 1.89	
24 h sessions	19.83 ± 2.97		18.48 ± 2.81	
Total volume (ml/kg)				
7 h sessions	28.38 ± 1.28	*	44.18 ± 2.34	*
24 h sessions	54.34 ± 0.91		63.15 ± 2.12	



< **Figure 3. Gradual suppression of seeking behaviour induced by threat of foot shock punishment.** **A.** Average of total active lever presses (ALP) for alcohol during baseline (B; grey) and tone-shock (S; yellow) sessions. **B.** Average number of alcohol rewards obtained during baseline (B; grey) and tone-shock (S; yellow) sessions. **C.** Average number of shocks received during tone-shock sessions for rats responding for alcohol. Foot shock intensity (mA) increased over tone-shock blocks: 0.05, 0.10, 0.20, 0.25, 0.30, and 0.35 mA. **D.** Average of total ALPs for sucrose during baseline (B; grey) and tone-shock (S; yellow) sessions. **E.** Average number of sucrose rewards obtained during baseline (B; grey) and tone-shock (S; yellow) sessions. **F.** Average number of shocks received during tone-shock sessions for rats responding for sucrose. Foot shock intensity (mA) increased over tone-shock blocks: 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mA. **G-H.** ALPs during shock sessions as a fraction of ALPs during baseline sessions at different shock intensities (bar: mean; dots: individual animals) for alcohol (**G**, left panel) and for sucrose (**H**, left panel). Grey insert indicates the 80-120% variability range in lever pressing (i.e., the stability criterium). Suppression ratios for alcohol (**G**, right panel) and for sucrose (**H**, right panel) at different shock intensities. Data expressed as mean ± SEM unless otherwise stated. Asterisk (*) denotes significance at a $p < 0.05$ level.

Gradual suppression of seeking behaviour induced by the threat of punishment

Two independent cohorts of animals were trained to lever press either for alcohol (cohort A, n = 16) or sucrose (cohort B, n = 8) in the STA task (Figure 3). To assess which shock intensity would be required to suppress seeking behaviour in the face of a possible adverse outcome, we gradually increased the intensity of foot shocks across tone-shock blocks from 0.05 mA to 0.30 mA for animals that were responding for sucrose, and to 0.35 mA for animals that were responding for alcohol.

In both cohorts, ALPs during baseline vs. tone-shock blocks were compared (Table 3). For the tone-shock sessions, a gradual reduction in ALPs was observed compared to baseline, starting from shock intensities of 0.25 mA and higher for both alcohol and sucrose (Figure 3A-F). For both rewards, responding for the reward during tone-shock sessions changed as a function of shock intensity (cohort A: $F_{sessiontype}$ (1,15) = 3.703, p = 0.074; $F_{shockintensity}$ (2,33) = 5.807, p = 0.006; $F_{sessiontype \times shockintensity}$ (2,35) = 8.048, p < 0.001; cohort B: $F_{sessiontype}$ (1,7) = 2.634, p = 0.149; $F_{shockintensity}$ (5,35) = 3.162, p = 0.019; $F_{sessiontype \times shockintensity}$ (5,35) = 8.957, p < 0.001).

For alcohol, post hoc analyses showed that responding during tone-shock sessions was significantly suppressed as compared to baseline, when shock intensities of 0.30 mA (p < 0.001) and 0.35 mA (p < 0.001) were used. For sucrose, post hoc analyses showed that responding during tone-shock sessions was significantly suppressed as compared to baseline, when shock intensities of 0.05 mA (p = 0.028), 0.25 mA (p = 0.031) and 0.30 mA (p = 0.005) were used.

Remarkably, at the lower shock intensities, i.e. up to 0.20 mA, an unexpected increase was seen in the total number of ALPs during tone-shock blocks when compared to the respective baseline sessions. Post hoc analyses showed that responding for sucrose during tone-shock sessions was significantly increased as compared to baseline when a shock intensity of 0.15 mA ($p = 0.031$) was used. This pattern of gradual increase in ALPs at lower shock intensities, followed by a reduction at higher shock intensities was also reflected in the number of shocks the animals received (Figure 3C and F; cohort A: $\chi^2(5) = 29.400$, $p < 0.001$; cohort B: $F_{shockintensity}(2,11) = 10.960$, $p = 0.004$). Similarly, the number of rewards the animals earned was occasionally slightly higher at a lower shock intensity and gradually decreased across the tone-shock blocks with increasing shock intensity (Figure 3B and E; cohort A: $F_{sessiontype}(1,15) = 36.248$, $p < 0.001$; $F_{shockintensity}(3,42) = 10.275$, $p < 0.001$; $F_{sessiontype \times shockintensity}(5,75) = 18.346$, $p < 0.001$; cohort B: $F_{sessiontype}(1,7) = 11.336$, $p = 0.012$; $F_{shockintensity}(2,17) = 6.698$, $p = 0.005$; $F_{sessiontype \times shockintensity}(5,35) = 5.789$, $p = 0.001$).

For both reinforcers, seeking behaviour was suppressed below 80% as compared to baseline lever pressing, set as the lower cut-off for stable responding, when a shock intensity of 0.30 mA or higher was used in the tone-shock sessions (Figure 3G-H). Shock intensity had a significant effect on suppression ratios for animals lever pressing for alcohol ($F_{shockintensity}(5,75) = 18.600$, $p < 0.001$) and on suppression ratios for animals that were lever pressing for sucrose ($F_{shockintensity}(5,35) = 14.890$, $p < 0.001$). Post hoc analyses showed that suppression ratios at 0.30 mA and 0.35 mA were significantly increased compared to all of the lower intensities for animals lever pressing for alcohol ($p \leq 0.005$). Likewise, post hoc analyses showed that suppression ratios at 0.30 mA were significantly increased compared to all of the lower intensities for animals lever pressing for sucrose ($p \leq 0.004$), except for the comparison with 0.25 mA ($p = 0.080$). Together, these results show a gradual suppression of seeking behaviour in tone-shock sessions compared to baseline sessions.

Based on these data, a shock intensity of 0.30 mA appeared to be most suitable to suppress reward seeking. However, seeking behaviour was increased beyond the 120% cut-off (and suppression ratios were lower than 0) in approximately half of the animals at the lower shock intensities. This increase in seeking during tone-shock sessions suggests that the tone was interpreted as a positive, reward-predictive, instead of a negative cue. An initial positive appraisal of the tone might therefore have raised the shock intensity threshold for suppression of seeking in these animals. Therefore, a slightly lower shock intensity of 0.25 mA was used for the following cohorts.

Consistent suppression of reward seeking during repeated shock sessions

Next, we set out to test whether suppression of reward seeking was consistent when a fixed shock intensity was used, in two separate groups of animals. These cohorts of animals were trained to lever press for either alcohol (cohort C, n = 16) or sucrose (cohort D, n = 9) in the STA task. We assessed whether lever pressing for either reinforcer would be consistently suppressed when animals were repeatedly tested in tone-shock blocks with a shock intensity of 0.25 mA.

ALPs during baseline vs. tone-shock blocks were compared for both cohorts (Table 4). For alcohol, animals showed significantly reduced seeking behaviour in tone-shock blocks (94.08 ± 6.53) as compared to baseline blocks (171.2 ± 13.65) (Figure 4A-C; $F_{sessiontype}(1,15) = 25.090$, $p < 0.001$). There was no significant difference in ALPs between

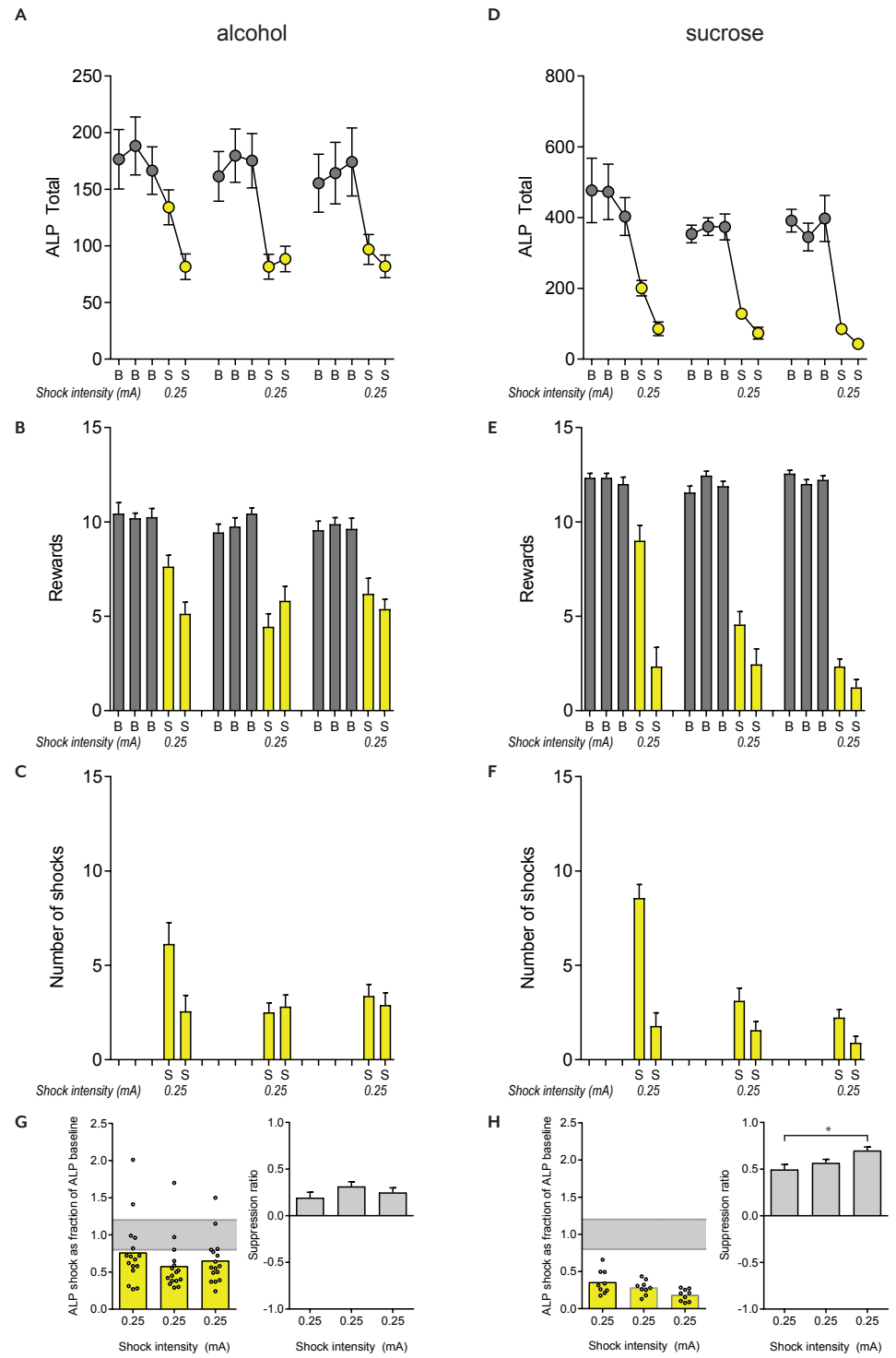
Table 3. Mean ± SEM active lever presses (ALP), rewards, and shocks of animals trained for different reinforcers (alcohol or sucrose) across different blocks of repeated baseline and tone-shock sessions (Figure 3). Significant differences ($p < 0.05$) are indicated with *. A trend ($p = 0.056$) is indicated with #.

		Alcohol					Sucrose				
mA		ALP		Rewards		Shocks	mA		ALP		Rewards		Shocks
	Baseline	99 ± 13		7.58 ± 0.43				Baseline	401 ± 56	*	11.29 ± 0.30		
0.05	Tone-shock	91 ± 13		7.31 ± 0.47		4.13 ± 0.92	0.05	Tone-shock	326 ± 35		10.94 ± 0.43		15.13 ± 1.72
	Baseline	89 ± 12		7.15 ± 0.45				Baseline	385 ± 61		10.83 ± 0.18		
0.10	Tone-shock	136 ± 31		7.38 ± 0.71		8.91 ± 3.01	0.10	Tone-shock	468 ± 70		11.00 ± 0.19		26.88 ± 5.32
	Baseline	100 ± 14		7.71 ± 0.29				Baseline	394 ± 57	*	10.96 ± 0.26	*	
0.20	Tone-shock	135 ± 20		7.34 ± 0.43		8.28 ± 1.90	0.15	Tone-shock	523 ± 65		11.44 ± 0.22		45.25 ± 7.28
	Baseline	93 ± 12		7.46 ± 0.38	*			Baseline	508 ± 94		10.88 ± 0.23		
0.25	Tone-shock	80 ± 12		5.97 ± 0.52		4.63 ± 0.97	0.20	Tone-shock	413 ± 23		9.88 ± 0.73		35.94 ± 3.86
	Baseline	117 ± 15	*	8.75 ± 0.34	*			Baseline	530 ± 103	*	10.92 ± 0.34	#	
0.30	Tone-shock	42 ± 6		3.75 ± 0.60		2.03 ± 0.54	0.25	Tone-shock	346 ± 41		9.94 ± 0.47		19.94 ± 2.76
	Baseline	85 ± 10	*	6.79 ± 0.42	*			Baseline	459 ± 74	*	10.50 ± 0.17	*	
0.35	Tone-shock	30 ± 5		2.91 ± 0.48		1.25 ± 0.35	0.30	Tone-shock	209 ± 22		7.13 ± 1.00		6.50 ± 1.70

the consecutive sequences ($F_{time}(2,30) = 0.877$, $p = 0.427$; $F_{sessiontype \times time}(2,30) = 0.384$, $p = 0.684$). The number of rewards obtained was significantly lower in tone-shock blocks as compared to baseline blocks ($F_{sessiontype}(1,15) = 75.710$, $p < 0.001$; $F_{time}(2,30) = 4.489$, $p = 0.020$; $F_{sessiontype \times time}(2,30) = 1.498$, $p = 0.240$). The number of shocks varied over time, with the number of shocks in the first block significantly higher than in the second block ($F_{time}(1,22) = 4.831$, $p = 0.027$). There was some variation in the suppression animals displayed in that not all animals suppressed seeking behaviour for alcohol consistently throughout the three consecutive tone-shock blocks (Figure 4G, left panel).

For sucrose, the animals showed significantly reduced seeking behaviour in tone-shock blocks (102.78 ± 9.40) as compared to baseline blocks (398.75 ± 29.53) (Figure 4D-F; $F_{sessiontype}(1,8) = 55.560$, $p < 0.001$). There was no significant difference in ALPs between the consecutive sequences ($F_{time}(1,9) = 3.769$, $p = 0.081$; $F_{sessiontype \times time}(1,10) = 0.548$, $p = 0.512$). The number of rewards obtained was significantly lower in tone-shock blocks as compared to baseline blocks and this difference between session types increased over time ($F_{sessiontype}(1,8) = 405.937$, $p < 0.001$; $F_{time}(2,16) = 13.425$, $p < 0.001$; $F_{sessiontype \times time}(2,16) = 22.289$, $p < 0.001$). The number of shocks decreased over time, with the number of shocks in the first block significantly higher than in the second and third block ($\chi^2(2) = 15.760$, $p < 0.001$). Furthermore, all animals suppressed their seeking behaviour - i.e. responded below the 80% of baseline lever pressing set as the lower cut-off criterium for stable responding - when a shock intensity of 0.25 mA in tone-shock blocks was applied (Figure 4H, left panel).

> **Figure 4. Consistent suppression of reward seeking behaviour induced by threat of foot shock punishment.**
A. Average of total active lever presses (ALP) for alcohol during baseline (B; grey) and tone-shock (S; yellow) sessions. **B.** Average number of alcohol rewards obtained during baseline (B; grey) and tone-shock (S; yellow) sessions. **C.** Average number of shocks received during tone-shock sessions when responding for alcohol. Foot shock intensity (mA) was maintained at 0.25 mA over tone-shock blocks. **D.** Average of total ALPs for sucrose during baseline (B; grey) and tone-shock (S; yellow) sessions. **E.** Average number of sucrose rewards obtained during baseline (B; grey) and tone-shock (S; yellow) sessions. **F.** Average number of shocks received during tone-shock sessions when responding for sucrose. Foot shock intensity (mA) was maintained at 0.25 mA over tone-shock blocks. **G-H.** ALPs during shock sessions as a fraction of ALPs during baseline sessions (bar: mean; dots: individual animals) for alcohol (**G**, left panel) and for sucrose (**H**, left panel). Grey insert indicates the 80-120% variability range in lever pressing (i.e., stability criterium). Suppression ratios for alcohol (**G**, right panel) and for sucrose (**H**, right panel) at 0.25 mA shock intensities. Data expressed as mean ± SEM unless otherwise stated. Asterisk (*) denotes significance at a $p < 0.05$ level.



Suppression ratios were calculated for both cohorts (Figure 4G-H, right panels). For alcohol, suppression ratios did not significantly change with repeated testing (F_{time} (2,30) = 1.754, p = 0.190). For sucrose, suppression ratios significantly increased over time (F_{time} (1,10) = 7.172, p = 0.019). Post hoc analyses showed that the suppression ratio measured at the third sequence of baseline and tone-shock block was significantly higher from the first (p = 0.041) and second sequence (p = 0.004), whereas the first and second sequence did not significantly differ (p = 0.481).

Table 4. Mean ± SEM active lever presses (ALP), rewards, and shocks of animals trained for different reinforcers (alcohol or sucrose) across repeated cycles of baseline and tone-shock sessions (Figure 4). Significant differences (p < 0.05) are indicated with *.

		Alcohol			Sucrose		
mA		ALP	Rewards	Shocks	ALP	Rewards	Shocks
	Baseline	177 ± 23	10.29 ± 0.40		451 ± 73	12.22 ± 0.22	
0.25	Tone-shock	108 ± 12	6.38 ± 0.55	4.34 ± 0.87	143 ± 17	5.67 ± 0.78	5.17 ± 0.65
	Baseline	172 ± 22	9.86 ± 0.35		367 ± 26	11.96 ± 0.14	
0.25	Tone-shock	85 ± 10	5.13 ± 0.64	2.66 ± 0.49	101 ± 11	3.50 ± 0.57	2.33 ± 0.55
	Baseline	165 ± 27	9.69 ± 0.39		378 ± 44	12.26 ± 0.14	
0.25	Tone-shock	89 ± 11	5.78 ± 0.64	3.13 ± 0.60	64 ± 7	1.78 ± 0.22	1.56 ± 0.36

Discussion

The aim of this study was to develop a task in which control over reward seeking behaviour was measured under threat of adversity, to closely resemble aspects of human addictive behaviour. Using this operant task, we validated whether responding for different reinforcers would be suppressed in the context of risk of a negative outcome, which was operationalised using a conditioning cue that predicted a probabilistic foot shock punishment. Our findings show that threat of adversity reduced reward seeking. A moderate shock intensity was sufficient to consistently suppress seeking behaviour for both alcohol and sucrose when tested repeatedly. Altogether, we show that the STA task can be used as a tool to assess control over substance intake.

To measure (loss of) control over reward seeking behaviour in the face of risk of a negative outcome, our task comprised several elements: i) a random interval schedule of reinforcement, ii) response dependent punishment that is paired to the reward seeking action but not to reward taking, and iii) a cued but unpredictable punishment. Random interval schedules induce a high and constant number of active seeking responses; they are therefore suited to mimic the complex substrate of drug seeking behaviour in addicted individuals (Belin-Rauscent et al., 2016; Robbins & Costa, 2017). That is, temporally dissociating the seeking responses from obtaining the reward allows for the investigation of seeking behaviour without interference of direct effects of the substance itself. Moreover, in interval reinforcement schedules the amount of responding and the number of earned rewards are weakly correlated, thereby minimising confounds by variability in intoxication between animals. Additionally, we chose a foot shock punishment that is paired to the reward seeking action rather than to the reward taking as it has been suggested that the effectiveness of a punishment may be reduced when paired to the reward (Dickinson & Pearce, 1976; Pelloux et al., 2007). In other words, by unpairing the foot shock punishment and the reward taking, any attenuation of the intrinsic aversiveness of the shock through counterconditioning was avoided. A foot shock punishment also allows for finetuning of punishment severity as the intensity is adjustable and delivery can be precisely timed in contrast to some other forms of punishment, such as histamine or lithium chloride (Vanderschuren et al., 2017). Response-dependent punishment is by definition avoidable and therefore an animal has the option to prevent punishment by refraining from reward seeking, which may resemble the conflicts human substance addicted individuals face. Finally, a tone cue was included to function as a warning signal with the purpose of creating a threat of negative consequences. Nevertheless, the exact timing of the punishment remained unpredictable, since not all responses during the cue were punished. This probabilistic component was included in the task to mimic the human situation, in which heavy substance users are often warned for negative consequences of their use (e.g. loss of a job or relationship, getting involved in an accident), while whether and when these harmful consequences will actually happen remains unknown.

Rats trained in the STA task showed sensitivity to foot shock punishment as suppression of seeking was observed at group level when tested repeatedly with a mild foot shock intensity. Importantly, other studies on compulsive alcohol or cocaine

seeking have reported resistance to foot shock punishment in (subpopulations of) animals after an extensive history of drug taking, even at higher shock intensities (Augier et al., 2018; Chen et al., 2013; Deroche-Gamonet et al., 2004; Seif et al., 2013). It is likely that the expression of loss of control only emerges when prior exposure, intoxication and duration of daily use negatively affects the ability to process warning cues that signal adversity and change decision-making behaviour accordingly, as it is suggested that insight and awareness are compromised in drug addicted individuals (Goldstein et al., 2009). Indeed, ample substance exposure, which can be implemented through for instance the extended length of the training period or the duration of daily training sessions, has been associated with an insensitivity to punishment (Hopf et al., 2010; Pelloux et al., 2007; Seif et al., 2013; Spoelder et al., 2017; Vanderschuren & Everitt, 2004). In the current study, the history of alcohol exposure may not have been sufficient to induce compulsive seeking under threat of adversity, however. That is, the levels of alcohol consumed by the rats in this study did not resemble intake levels of rats previously termed 'high drinkers', i.e. a subset within a larger population that was shown to be prone to show compulsive alcohol seeking behaviour when facing quinine adulteration or threat of foot shock punishment (Lesscher et al., 2010; Spoelder et al., 2015, 2017). For example, in contrast to high alcohol drinking rats, alcohol preference did not change in the current cohorts when sessions were extended from 7 hours to 24 hours. Moreover, our voluntary drinking paradigm was followed by a relatively short period of daily training sessions in which animals generally earned no more than 2.0 ml of 20% alcohol. For future endeavours, prolonging the home cage exposure and daily training duration prior to testing in the STA task, will allow us to investigate which conditions will make animals more prone to resist negative outcome and thus induce loss of control over reward seeking.

Besides prior substance exposure, whether seeking behaviour will be suppressed in the context of adversity depends on the type of reinforcer that is used. Thus, it has repeatedly been shown that unlike seeking for cocaine, animals suppressed seeking for sucrose regardless of training experience (Kearns et al., 2002; Limpens et al., 2014; Miles et al., 2003; Pelloux et al., 2007; Vanderschuren & Everitt, 2004). This is in line with the data from our sucrose-reinforced animals. Seeking alcohol and cocaine is likely more prone to resistance to punishment compared to seeking sucrose, since the addictive potential of substances of abuse is thought to be higher than that of natural food reinforcers (see e.g. de Jong et al., 2013). Together, the consistent suppression of sucrose seeking behaviour and sensitivity to mild foot shock punishment is not surprising.

The STA task can be used as a tool to assess control over substance use in future investigations. First, by manipulation of the underlying neural circuitry of behavioural control, with the aim of inducing loss of control and therefore compulsive seeking. This can be achieved by means of reversible pharmacological inactivation of brain regions thought to mediate executive control over behaviour, or reversibly manipulating activity in neural projections that might play a role in behavioural control. The observation that seeking behaviour was consistently suppressed using this task will enable a within-subject comparison of the effect of neural activity manipulations, since animals can reliably be tested repeatedly with the STA task. Second, it would be of interest to implement the STA task within an array of behavioural tests that assess other aspects of addictive behaviour. Thus, characterisation of substance seeking behaviour using the current task in combination with behavioural measurements of motivation for reward, continued seeking despite unavailability, and vulnerability to relapse may be essential to fully grasp the behavioural and biological mechanisms that underlie addictive behaviour. The fact that animals can serve as their own control in the STA task, is of added practical value when combining different behavioural tasks. Finally, although there was a clear suppression of seeking for alcohol in the face of adversity at group level in the present study, the extent to which individual animals expressed this suppression of seeking varied between sessions, even with our relatively small sample sizes. Therefore, the STA task may be applied as a tool to investigate individual differences in control over seeking behaviour, which is of utmost importance considering that only a subgroup of users of substances of abuse develops addictive behaviour (Anthony et al., 1994). Future studies are needed in which interindividual variability in sensitivity or resistance to punishment is evaluated in larger cohorts of animals, with prolonged pre-exposure to substances of abuse and with extended daily training sessions.

To conclude, we present a novel behavioural task to repeatedly measure reward seeking behaviour under threat of adversity in rats. This task is intended to mimic an important aspect of addictive behaviour in humans and to study the neural underpinnings of SUD. Combined with other behavioural tasks and neural manipulations, the STA task may contribute to a more exact translation of findings from animal studies to the human psychopathology of SUD.

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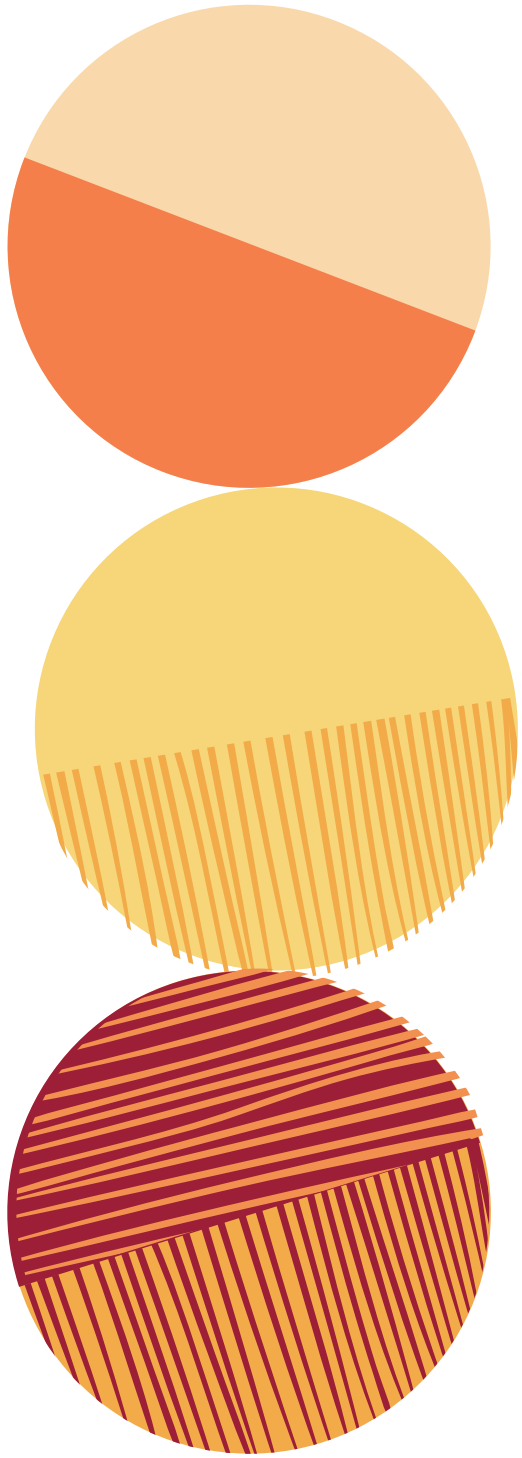

Chapter 4

Involvement of the prelimbic prefrontal cortex in reward seeking under threat of adversity in rats

A. Maryse Minnaard[1,2]
Johanna A. S. Smeets[1,2]
Annemarie M. Baars[1]
Geert M. J. Ramakers[2]
Roger A. H. Adan[2]
Heidi M. B. Lesscher[1]
Louk J. M. J. Vanderschuren[1]

[1] Department of Population Health Sciences, Unit of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2] Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, The Netherlands

Abstract

Rationale

Substance use disorder (SUD) is characterised by loss of control over use but the underlying mechanisms are incompletely understood. Identifying the neural substrates of loss of control over reward seeking is important to better understand the mechanisms that are involved in the development of SUD, which may eventually contribute to the development of improved treatment options. Considering its potential involvement in response inhibition and compulsive reward seeking, the prelimbic prefrontal cortex (PrL) may be particularly important in situations involving behavioural control over reward pursuit.

Objectives

The present study evaluated the role of the PrL in reward seeking behaviour under threat of adversity, as a model for loss of control over substance seeking.

Methods

Rats were trained to lever press for alcohol or sucrose. They were subsequently tested in the Seeking under Threat of Adversity (STA) task in which presentation of a tone cue signalled a response-contingent and probabilistic foot shock. Next, the impact of pharmacological PrL inactivation on STA task performance was assessed.

Results

Reward seeking was suppressed under threat of adversity. Pharmacological inactivation of the PrL reduced this suppression of responding for alcohol, but not for sucrose.

Conclusions

These findings suggest that reduced neural activity in the PrL promotes persistent alcohol seeking behaviour, which may underlie compulsive drug use in SUD. Conversely, inactivation of the PrL did not affect suppression of responding for sucrose, suggesting that the functional involvement of the PrL in control over reward seeking is specific for substances of abuse.

Keywords

Alcohol
Baclofen
Muscimol
Prefrontal cortex
Rats
Reward seeking
Substance use disorder
Sucrose

Introduction

Substance use disorder (SUD) is a major global health problem. It contributes heavily to the burden of disease, is a cause of substantial harm to society, and remains one of the leading preventable causes of death (Carvalho et al., 2019; Degenhardt et al., 2018; Nutt et al., 2010; Tran et al., 2019; UNODC, 2019; Whiteford et al., 2013; World Health Organization, 2018)(Carvalho et al., 2019; Degenhardt et al., 2018; Nutt et al., 2010; Whiteford et al., 2013). SUD can be defined as a chronic relapsing disorder characterised by criteria described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013). Many of the DSM-5 criteria for SUD reflect loss of control over drug seeking and taking, e.g. substance use in hazardous conditions and continued use despite knowledge of substance-related problems. However, therapeutic options for SUD are limited in number and efficacy, and generally not aimed at restoring control over substance use (Potenza et al., 2011; van den Brink, 2012). A better understanding of the neural underpinnings of loss of control over substance use may therefore contribute to the development of more effective treatment options for SUD, that are directed at restoring control over substance use.

In preclinical studies, loss of control can be operationalised in behavioural tests as resistance to punishment (Vanderschuren et al., 2017). In SUD, one typically has knowledge of negative consequences of substance abuse, but the exact timing of those consequences is often unpredictable in humans. In an attempt to model this aspect of human SUD, we recently developed the Seeking under Threat of Adversity (STA) task, which incorporates two important aspects of human substance use despite negative consequences, i.e. response contingency and unpredictability of punishment (Chapter 3). With this task, we showed that rats display suppression of responding for alcohol and sucrose when facing threat. Therefore, the STA task is a promising tool to investigate the neural underpinnings of (loss of) control over substance use, for instance when combined with neuropharmacological interventions.

The prefrontal cortex has been extensively associated with control over reward seeking and taking. Clinical and preclinical studies have indicated that the prefrontal cortex is involved in mediating cognitive control over substance intake (Dalley et al., 2004; Goldstein & Volkow, 2011; Koob & Volkow, 2016; Volkow & Fowler, 2000). Within the prefrontal cortex, the prelimbic prefrontal cortex (PrL) has been implicated in response inhibition and compulsive drug seeking. That is, PrL inactivation was shown to disrupt avoidance behaviour in tasks where rats had to either initiate or inhibit

a response to avoid foot shock punishment (Capuzzo & Floresco, 2020; Verharen et al., 2019). Consistently, flexible inhibitory control over cocaine seeking selectively depended on neuronal activity within the PrL and lesioning this region increased perseverative responding (Allen & Leri, 2014; Mihindou et al., 2013). Moreover, compulsive cocaine and sucrose seeking was observed upon PrL inactivation in rats that initially showed controlled reward seeking (Chen et al., 2013; Limpens et al., 2015). Taken together, these findings suggest that the PrL may be particularly important in situations involving behavioural control over reward pursuit.

The aim of this study was to investigate the involvement of the PrL in behavioural control over reward seeking under threat of adversity. To that aim, Lister Hooded rats were trained to respond for alcohol or sucrose and were tested in the STA task. Subsequently, the PrL was pharmacologically inactivated by local infusions of a GABA agonist mixture (i.e. baclofen and muscimol), to assess its involvement in reward seeking under threat of adversity. Considering the findings that implicate the PrL in response inhibition and compulsive reward seeking, we hypothesised that pharmacological inactivation of the PrL would reduce suppression of responding for alcohol and sucrose when facing threat.

Materials and methods

Subjects

A total of 30 adult male Lister Hooded rats (Charles River, Germany), weighing 250-300 grams at the start of the experiments, were used in this study. The rats were individually housed in Macrolon type III sawdust bedded cages (42.5 x 26.6 x 18.5 cm) with ad libitum access to tap water and standard chow (Rat and Mouse Breeder and Grower Expanded-CRM(E), Special Diet Service, UK), except during operant training and testing. A polycarbonate rat tunnel (9 x 9 x 15 cm) and a tissue were provided for cage enrichment. The rats were kept under controlled temperature and humidity conditions (21 ± 2°C and 50 - 70% humidity) under a reversed 12 h/12 h light/dark cycle (lights off at 7.00 AM - lights on at 7.00 PM) to allow for behavioural testing in the dark phase. Background noise was provided by a constantly playing radio. The rats were acclimatised to the housing conditions for at least eight days prior to behavioural testing, and they were weighed and handled at least once per week throughout the course of the experiment. All animals used for this study were experimentally naive. Experimental procedures were approved by the Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee, and conducted in agreement with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU).

Behavioural procedures

Intermittent alcohol access

In order to facilitate responding for alcohol, the rats that would later be trained to respond for alcohol (n = 18) were given intermittent access to alcohol (IAA) in a two-month two-bottle choice setup in the home cage for three days a week (i.e., Monday, Wednesday, Friday) as previously described (Spoelder et al., 2015) (Figure 1A). In the first four weeks of IAA, alcohol exposure sessions lasted for 7 hours, approximately between 9.30 AM and 16.30 PM (i.e. during the dark phase of the day-night cycle), and sessions were subsequently extended to 24 hours during the second month of IAA. The bottles were weighed before and after each drinking session and the placement of the alcohol bottle was alternated between sessions to avoid the development of a side bias. Alcohol (99.5%; Klinipath, The Netherlands) was freshly diluted with tap water once per week to a final concentration of 20% (v/v).

Apparatus

The animals were trained and tested in operant conditioning chambers (29.5 x 24 x 25 cm, Med Associates Inc., USA) equipped with two retractable levers (4.8 x 1.9 cm; ENV-112CM) and a white cue light (28 V, 100mA; ENV-221M) present above each lever. A recessed liquid dipper and a food receptacle were situated between the levers. The wall on the opposite side of the box contained a white house light (28 V, 100mA; ENV-215M) and a tone generator (85 dB, 2900 Hz; ENV-223AM). The floor of the chamber was covered with a metal grid with bars that were separated by 1.57 cm and were connected to a shock generator (ENV-414SA standalone aversive stimulator). All chambers were situated in light- and sound-attenuating cubicles equipped with a ventilation fan, and were controlled by MED-PC IV software (version 4.2) for Windows.

Fixed ratio and random interval schedules of reinforcement

The rats were trained to respond for alcohol (n = 18) or sucrose (n = 12) in 30 minute operant sessions, during which the house light was illuminated, once daily, 4-6 days per week. The position of the active and inactive levers was counterbalanced between rats. The presentation of the reward was paired with the retraction of both levers and the illumination of the cue light above the active lever. Animals were first trained under a fixed ratio (FR) 1 schedule of reinforcement. In the case of alcohol reinforcement, pressing the active lever raised the dipper cup containing an alcohol reward (0.1 ml, 20% v/v). The dipper cup remained in the raised position until 10

seconds after the animal entered the recessed receptacle, which was detected by interruption of an infrared light beam. The cue light was turned off and the levers were reintroduced, signalling the start of a new trial. The alcohol solution was refreshed between sessions to prevent a decline in alcohol concentration by evaporation of the alcohol between sessions. In the case of sucrose reinforcement, pressing the active lever activated a pellet dispenser that delivered a 45 mg sucrose

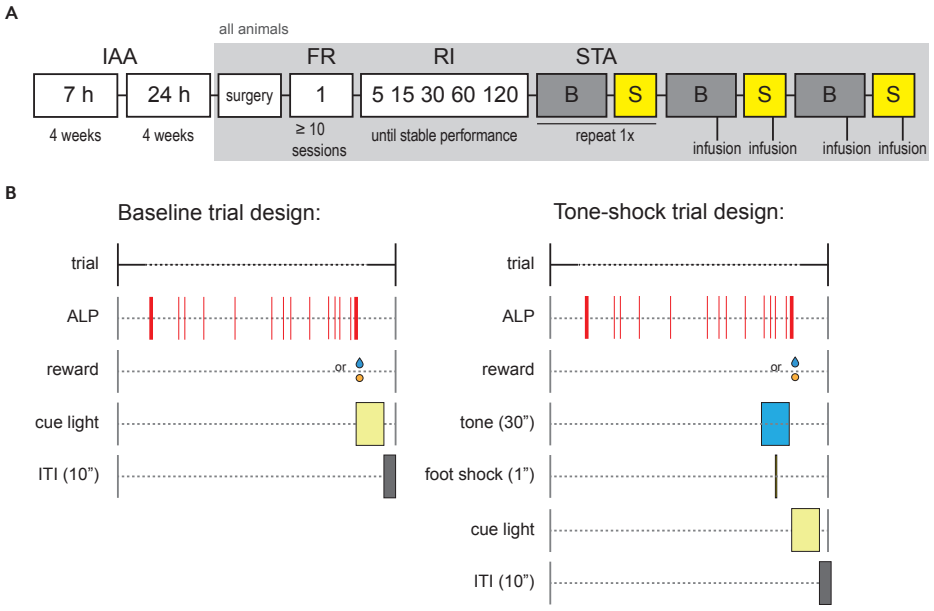

Figure 1. Procedural outline. A. Schematic flowchart of the experimental outline. Only animals that later would be trained to respond for alcohol, were first exposed to intermittent alcohol access (IAA) in the home cage for eight weeks. All animals underwent surgery in which intracranial cannulas were placed and all animals were subjected to fixed ratio (FR) 1 operant training and random interval (RI) operant training sessions. Subsequently, the animals were tested in the seeking under threat of adversity (STA) task, which consisted of a minimum of three baseline (B) sessions and two tone-shock (S) sessions. After two S sessions, the rats were retrained for a minimum of three B sessions until stable responding before being tested in another two S sessions. Next, animals were retrained in B sessions and intracranial infusions were administered before a baseline session and before a tone-shock session. The latter was repeated so that all animals received baclofen/muscimol and vehicle treatment in a counterbalanced design. **B.** Schematic overview of the trial design for B session trials (left panel) and S session trials (right panel). In B trials, pressing the active lever (signified by the red lines) initiated an interval with an average duration of 120 seconds. All lever presses during this interval were recorded but were without programmed consequences. The first active lever press (ALP) after completion of the interval resulted in the delivery of the reward (alcohol solution or sucrose pellet) and the illumination of the cue light, followed by a 10 second inter-trial interval (ITI). Trials in S sessions had a similar set-up as trials in a B session, with the exception that a tone was presented during the last 30 seconds of each interval. During the tone presentation, ALPs were punished in a probabilistic manner (25% chance) with a 1 second foot shock of 0.25 mA. If a rat made at least one ALP during the tone presentation, the first ALP following completion of the random interval resulted in reward delivery. However, if animals refrained from lever pressing during tone presentation, both levers were retracted immediately upon completion of the random interval and the 10 second ITI was initiated.

pellet (TestDiet, USA) into the food receptacle. Collection of the sucrose reward, which was detected by interruption of an infrared light beam, was followed by a 0.5 second inter-trial interval. After this period, the cue light was turned off and the levers were reintroduced, signalling the start of a new trial. For both reinforcers, inactive lever presses were recorded but were without programmed consequences.

After acquisition of self-administration under an FR 1 schedule, the animals were trained under a random interval (RI) 5 schedule of reinforcement (Figure 1A), where each trial commenced with the presentation of the active and inactive lever. Pressing the active lever initiated an interval with an average duration of 5 seconds, during which both levers remained extended. All lever presses during this interval were recorded but were without programmed consequences. The first active lever press (ALP) after completion of the interval resulted in the delivery of the reward (alcohol or sucrose), as described for the FR 1 schedule of reinforcement. Rats were subsequently trained with increasing interval lengths (RI 15, RI 30, RI 60), before reaching the final RI 120 schedule (Figure 1A). The animals were trained under the RI 120 schedule until stable responding was achieved for three consecutive days, i.e. until the average ALPs at group level on subsequent days fell within an 80-120% variability range of the total average of ALPs across those three consecutive days.

Seeking under threat of adversity (STA) task

After acquisition of stable responding under the RI 120 schedule of reinforcement, the rats progressed to the STA task. The schedule of reinforcement in the STA task was a modified version of the RI 120 schedule, divided into two types of sessions: baseline and tone-shock sessions. Baseline sessions were similar to RI 120 sessions, with the exception that intervals of all trials lasted at least 30 seconds and an inter-trial interval of 10 seconds was introduced (Figure 1B). The animals were trained until stable responding was achieved (i.e. 80-120% variability range of average ALPs) on three consecutive days, before testing in two consecutive tone-shock sessions (tone-shock block) commenced. Trials in tone-shock sessions had a similar set-up as trials in a baseline session, with the exception that a tone was presented during the last 30 seconds of each interval. During the tone presentation, ALPs were punished in a probabilistic manner (25% chance) with a 1 second foot shock of 0.25 mA (Figure 1B).

If a rat made an ALP during the tone presentation, the first ALP following completion of the random interval resulted in a reward delivery, regardless of whether the rat had received shock punishment during the preceding 30 seconds or not (Figure 1B). However, if animals refrained from lever pressing during tone presentation, both levers were retracted immediately upon completion of the random interval and the

10 second inter-trial interval was initiated. In this way, only trials in which rats took the risk of shock punishment by pressing the lever during the tone were rewarded. After each tone-shock block, animals were retrained in baseline sessions until stable responding was achieved (80-120% variability range of average ALPs) on three consecutive days.

Infusion procedure

Before testing commenced, all animals received an infusion of saline (0.9% NaCl) to habituate them to the infusion procedure. Test infusions took place after two tone-shock blocks (i.e. four tone-shock sessions in total), so that the animals were familiarised with the tone cue, and whenever responding in the following baseline sessions was stable (Figure 1A). On test days, the animals received an infusion with saline or a mixture of baclofen (1 nmol; Sigma-Aldrich, The Netherlands) and muscimol (0.1 nmol; Sigma-Aldrich, The Netherlands) dissolved in saline. Infusions were performed before a baseline session and before a tone-shock session, separated by one tone-shock session without infusions, in a within-subject Latin square design. After responding in baseline sessions re-stabilised, the subsequent infusions were performed.

For the infusion, the dummy injectors were removed and replaced by 33-gauge injectors (Plastics One, USA) that extended 1.0 mm below the guide cannulas and were connected to a 10-µl syringe. Using a syringe pump (Harvard apparatus, USA), 0.3 µl/side baclofen/muscimol mixture or saline was infused bilaterally over 1 minute. After the infusion, the injectors were kept in place for one additional minute to allow for diffusion of the injection volume. Injectors protruded 1.0 mm below the guide cannulas. After the infusion, the dummy injectors were placed back into the guide cannulas and the animals were returned to their home cage for 10 minutes, after which operant experimental testing commenced.

Surgery

All rats were equipped with 26-gauge bilateral guide cannulas (Plastics One, USA) that were aimed at the PrL (AP +2.8, ML ±1.8, DV -2.7 (20° angle), n = 18 (responding for alcohol) and n = 12 (responding for sucrose)), with coordinates in mm relative to bregma (Paxinos & Watson, 2004). Prior to stereotactic surgery, rats were anesthetised with a ketamine/dexmedetomidine mixture (0.2 ml mixture/100 g body weight intraperitoneal: 75 mg/kg ketaminehydrochloride, Narketan, 0.25 mg/kg Dexdomitor®, Pfizer Animal Health BV, The Netherlands). Rats were given eye cream (CAF, CEVA Sante Animale BV, The Netherlands) and were subsequently placed in a stereotaxic apparatus (David Kopf Instruments, USA). Next, rats received a subcutaneous injection of carprofen (5 mg/kg, subcutaneously, Carporal, AST Farma BV, The Netherlands) as analgesic and a subcutaneous injection of 8 ml saline (0.9% NaCl) for rehydration. An incision was made along the midline of the skull and additional local analgesia was provided by xylocaine, sprayed on the skull (Lidocaine 100 mg/ml, AstraZeneca BV, The Netherlands). Two small craniotomies were made bilaterally above the brain region of interest and the guide cannulas were lowered to the desired coordinates. Next, the guide cannulas were secured to the skull using stainless steel screws and antibiotic cement (Simplex P bone cement with tobramycin, Stryker Nederland BV, The Netherlands). Upon completion of the surgery, dummy injectors (Plastics One, USA) were placed inside the guide cannulas and anaesthesia was terminated through the application of atipamezole (1.0 mg/kg, subcutaneously, Antisedan®, Pfizer Animal Health BV, The Netherlands). For postoperative analgesia, the rats were treated with carprofen (5 mg/kg, subcutaneously, Carporal, AST Farma BV, The Netherlands) at 24 hour intervals for 2 days after surgery. Animals were monitored and weighed daily for one week after surgery, and were allowed to recover for at least 8 days prior to operant training.

Histological verification

After the behavioural experiments, animals were killed using carbon dioxide inhalation and microinjected with 0.3 µl of black ink (Parker) over 1 minute through the guide cannulas to aid visual localisation of the infusion sites. Next, the rats were immediately decapitated, and the brains were removed. The brains were flash-frozen in methyl-butane isopentane (-80°C) and subsequently stored at -80°C. Coronal sections (40 µm) were sliced using a cryostat and microinjection sites were verified by light microscopy using a rat brain atlas (Paxinos & Watson, 2004).

Exclusion criteria

Data from rats that lost one or both of their cannulas before the experiment was finished were excluded. Data from rats with one or both cannulas placed outside the target area were also discarded from all analyses. Based on these criteria, a total of 4 rats from the PrL-alcohol group and 4 rats from the PrL-sucrose group were excluded.

Data analysis and statistics

To assess alcohol consumption, home cage alcohol intake, water intake and alcohol preference were calculated as follows. Fluid intake was calculated by subtracting the bottle weights at the end of every drinking session by the starting weights. Alcohol intake (ml) was calculated by the following equation: (Δ alcohol bottle weight in grams) / (0.8+(0.2*0.789)) in which the density of ethanol (i.e., 0.789 g/ml), is included.

Alcohol intake (g/kg) was calculated by the following equation: ((alcohol fluid intake in ml) * (0.2*0.789)) / (bodyweight in kg). Preference for alcohol (%) was calculated according to the following equation: (alcohol intake in ml) / ((alcohol intake in ml) + (water intake in ml)) * 100. To assess gradual escalation of alcohol consumption, as animals progressed to the 24 hour sessions, differences in mean alcohol intake (g/kg) and total volume intake (ml/kg) between the 7 hour (group mean week 1-4) and 24 hour (group mean week 5-8) sessions were compared with a paired samples t-test. Changes in preference between the 7 hour (group mean week 1-4) and 24 hour sessions (group mean week 5-8) were assessed with a paired samples t-test.

For each PrL inactivation experiment, the number of seeking responses and rewards obtained were assessed using two-way repeated measure analyses of variance (ANOVA) with *treatment* (i.e. baclofen/musicimol or saline) and *session type* (i.e. baseline or tone-shock) as the within-subject factors. Prior to analysis, normality of the data was assessed. Each active lever press variable was log transformed prior to statistical analyses to obtain a normal distribution of the data.

Suppression of substance seeking was assessed by calculating a suppression ratio as [(active lever presses during baseline session - active lever presses during tone-shock session) / (active lever presses during baseline session + active lever presses during tone-shock session)]. A suppression ratio of 0.0 or lower means no suppression, while a suppression ratio of 1.0 indicates complete suppression of responding during a tone-shock session. Suppression ratios were analysed using paired t-tests. In case the difference scores of paired data were non-normally distributed, the data were analysed using a Wilcoxon matched-pairs signed rank test.

Data are expressed as mean ± SEM, unless otherwise stated. Data were analysed and visualised using Microsoft Excel, Graphpad Prism (version 8.3.0, Graphpad Software Inc., USA) and SPSS for Windows (version 25.0.0.1, IBM Corp., USA). A significance criterion of $p < 0.05$, two-tailed, was used for all the statistical analyses.

Results

Voluntary alcohol consumption in the home cage

In order to facilitate operant responding for alcohol, rats were exposed to IAA in the home cage for eight weeks. Weekly averages of alcohol intake and preference were calculated (Figure 2A-B) and alcohol intake and preference during the 7 hour and 24 hour sessions were compared (Table 1). The rats consumed significantly more alcohol during the 24 hour as compared to the 7 hour sessions ($t(13) = 4.954$, $p < 0.001$). Preference for alcohol and total fluid intake were also significantly higher for the 24 hour sessions compared to the 7 hour sessions ($t(13) = 4.078$, $p = 0.001$; $t(13) = 16.559$, $p < 0.001$). Together, these results show that rats readily consumed alcohol in the home cage and that alcohol intake increased when sessions were prolonged from 7 hours to 24 hours.

Table 1. Mean ± SEM daily alcohol intake, alcohol preference, and total fluid intake for the 7 hour and 24 hour sessions of intermittent alcohol access (IAA) in the home cage. (Figure 2). Significant differences ($p < 0.05$) are indicated with *.

	alcohol group (n = 14)	
Alcohol intake (g/kg)		
7 h sessions	0.72 ± 0.06	*
24 h sessions	2.57 ± 0.41	
Alcohol preference (%)		
7 h sessions	15.92 ± 1.59	*
24 h sessions	33.54 ± 5.52	
Total volume (ml/kg)		
7 h sessions	32.79 ± 1.52	*
24 h sessions	50.82 ± 1.05	

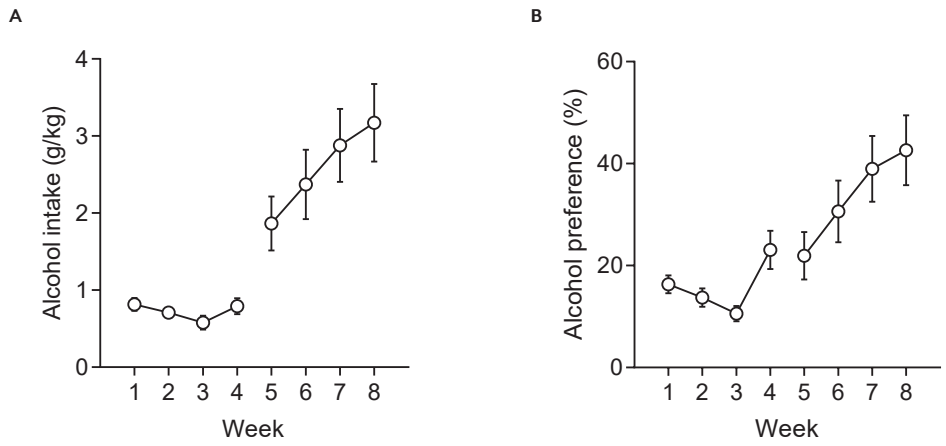

Figure 2. Voluntary alcohol intake in the home cage. Weekly averages of intake of alcohol (g/kg) and alcohol preference (%) during eight weeks of intermittent alcohol access (IAA) in the home cage prior to operant training for responding for alcohol. Data expressed as mean ± SEM.

Effects of PrL inactivation on control over alcohol and sucrose seeking

Alcohol

To determine the role of the PrL in behavioural control over alcohol seeking, the PrL was inactivated immediately prior to STA tests (Figure 3A). The number of ALPs was affected by baclofen/muscimol infusions, and this effect was dependent on session type (Figure 3B; $F_{sessiontype}$ (1,13) = 2.660, p = 0.127; $F_{treatment}$ (1,13) = 3.939, p = 0.069; $F_{sessiontype\ x\ treatment}$ (1,13) = 5.551, p = 0.035). Post hoc analyses showed that baclofen/muscimol infusion increased the number of ALPs specifically in the tone-shock session compared to saline (p = 0.027), whilst the number of ALPs during baseline sessions were not affected by inactivation of the PrL (p = 0.621). The number of rewards the rats obtained was significantly lower in tone-shock sessions compared to baseline sessions, but was not affected by infusion of baclofen/muscimol (Figure 3C; $F_{sessiontype}$ (1,13) = 22.977, p < 0.001; $F_{treatment}$ (1,13) = 0.214, p = 0.651; $F_{sessiontype\ x\ treatment}$ (1,13) = 1.260, p = 0.282). Analysis of the suppression ratios revealed significantly lower suppression ratios when baclofen/muscimol was infused into the PrL, compared to saline infusions (Figure 3D-E; t(13) = 2.785, p = 0.015). These findings show that suppression of alcohol seeking was reduced by infusion of baclofen/muscimol into the PrL.

Sucrose

To determine the role of the PrL in behavioural control over sucrose seeking, the PrL was inactivated immediately prior to STA tests (Figure 4A). The number of ALPs was significantly lower in tone-shock sessions compared to baseline sessions, but this was not affected by baclofen/muscimol infusion (Figure 4B; $F_{sessiontype}$ (1,7) = 13.560, p = 0.008; $F_{treatment}$ (1,7) = 2.061, p = 0.194; $F_{sessiontype\ x\ treatment}$ (1,7) = 0.172, p = 0.691). The number of rewards obtained tended to be reduced in the tone-shock sessions, but this was not affected by infusion with baclofen/muscimol (Figure 4C; $F_{sessiontype}$ (1,7) = 5.512, p = 0.051; $F_{treatment}$ (1,7) = 0.208, p = 0.662; $F_{sessiontype\ x\ treatment}$ (1,7) = 0.406, p = 0.544). Subsequent analysis of the suppression ratios revealed no significant difference between baclofen/muscimol and saline treatments (Figure 4D-E; t(7) = 0.429, p = 0.681). Taken together, baclofen/muscimol infusions into the PrL did not affect suppression of sucrose seeking.

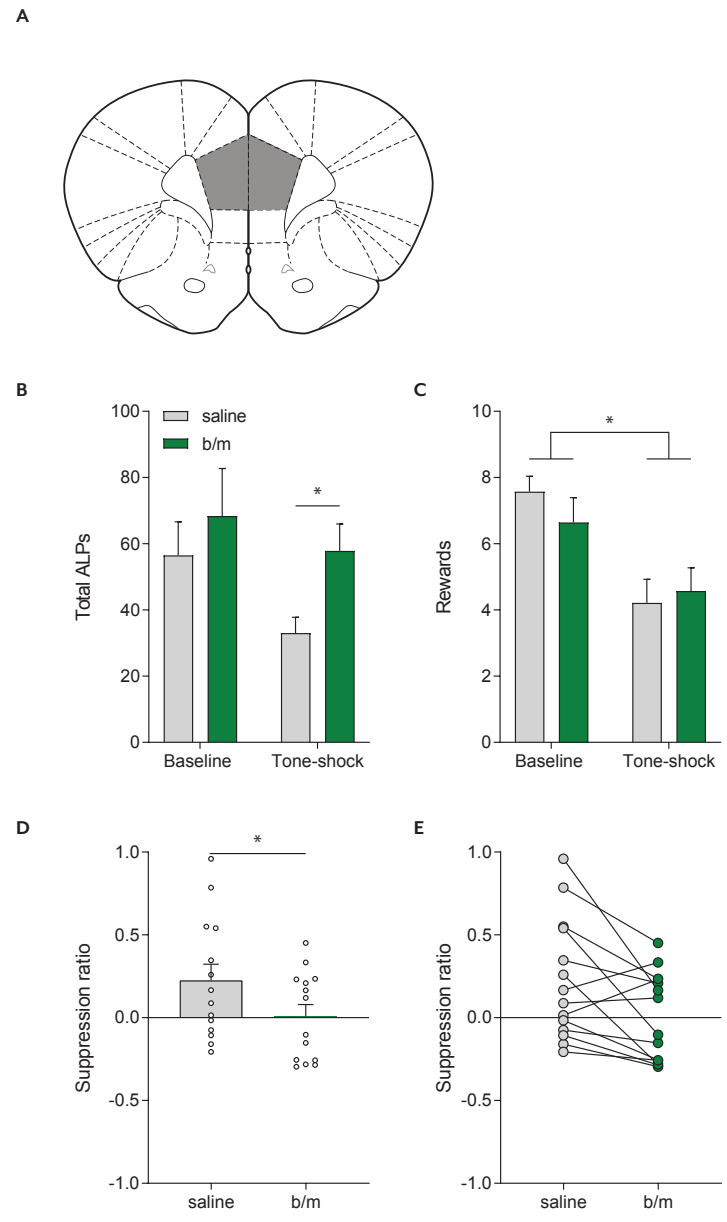

Figure 3. Effects of pharmacological inactivation of the prelimbic prefrontal cortex (PrL) on alcohol seeking behaviour in the STA task. A. Infusions were administered into the PrL, which is indicated in grey in the coronal brain section. **B.** Total active lever presses (ALPs) made in baseline and tone-shock sessions. **C.** Number of rewards obtained in baseline and tone-shock sessions. **D.** Suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. **E.** Individual suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. Data expressed as mean + SEM. Significant differences (p < 0.05) are indicated with *.

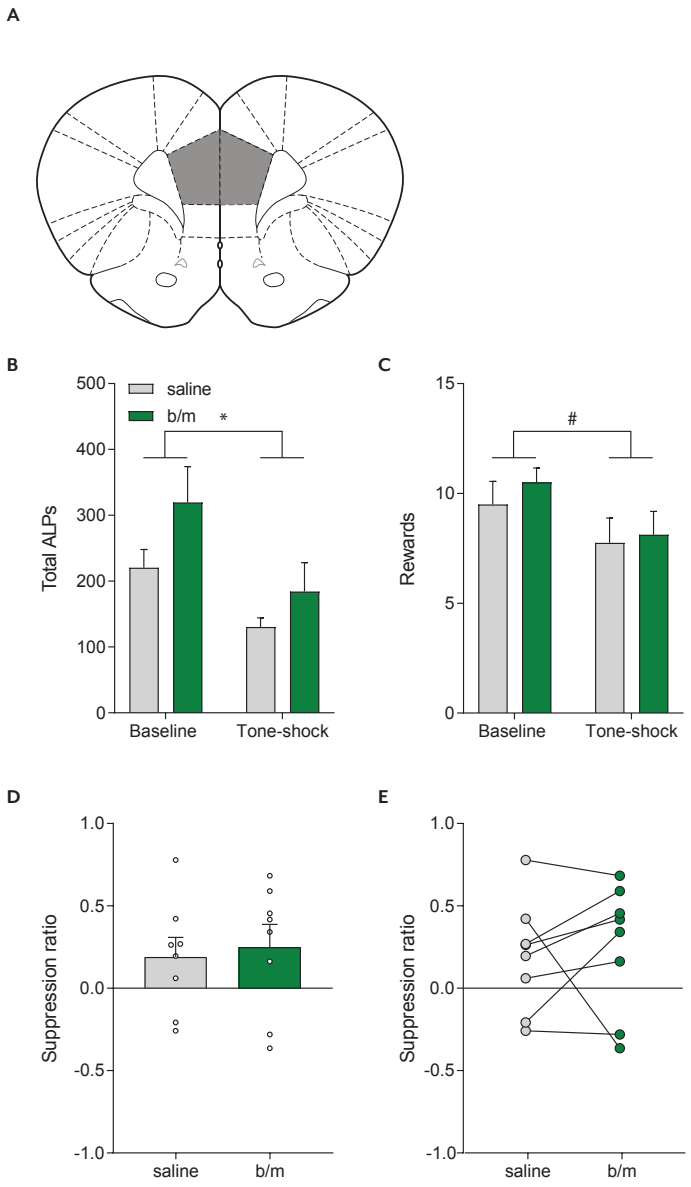

Figure 4. Effects of pharmacological inactivation of the prelimbic prefrontal cortex (PrL) on sucrose seeking behaviour in the STA task. A. Infusions were administered into the PrL, which is indicated in grey in the coronal brain section. **B.** Total active lever presses (ALPs) made in baseline and tone-shock sessions. **C.** Number of rewards obtained in baseline and tone-shock sessions. **D.** Suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. **E.** Individual suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. Data expressed as mean + SEM. Significant differences ($p < 0.05$) are indicated with *. A trend towards a main effect of session type ($p = 0.051$) is indicated with #.

Discussion

The present study set out to investigate the involvement of the PrL in behavioural control over reward seeking. Rats were trained in a task where responding for a reward was measured in the context of a possible negative outcome and the effect of pharmacological inactivation of the PrL on task performance was assessed. We utilised the STA task, in which the negative outcome is operationalised as a response-contingent, probabilistic foot shock. Our findings show that pharmacological inactivation of the PrL reduced suppression of responding for alcohol, but not for sucrose in the STA task. These findings suggest that reduced neural activity in the PrL promotes persistent alcohol seeking behaviour which may underlie compulsive alcohol use and alcohol use disorder.

Prelimbic prefrontal cortex involvement in reward seeking

The observation that PrL inactivation led to a phenotype that is indicative of loss of control over alcohol seeking is in line with previous studies investigating reward seeking in the face of adversity (Chen et al., 2013; Limpens et al., 2015; Verharen et al., 2019). However, others report no significant effects of silencing the PrL on punished lever pressing for alcohol or cocaine (Halladay et al., 2020; Jean-Richard-dit-Bressel & McNally, 2016; Pelloux et al., 2013). These conflicting findings within the field emphasise the complexity of the role of the PrL in control over reward seeking.

It has been suggested that the role of the PrL might depend on the valence of the outcome. For instance, PrL inactivation impaired active but not inhibitory avoidance of a foot shock punishment in a discriminative task, and did not alter avoidance behaviour when a single cue was used (Capuzzo & Floresco, 2020). On the contrary, PrL inactivation disrupted inhibitory but not active sucrose reward seeking (Capuzzo & Floresco, 2020). Thus, how the PrL may contribute to behavioural control over reward seeking might depend on whether the outcome is appetitive or aversive and on which action (i.e. initiating or withholding a response) is required.

In the STA task, both the valence of the outcome and the required action are twofold and conflicting. The initiation of a lever press is needed in order to obtain an appetitive reward, whereas withholding of lever pressing is needed in order to avoid an aversive foot shock. Thus, rats are confronted with a motivational conflict as reward seeking is associated with a threat of a foot shock punishment that is signalled by a tone cue. A contingent but probabilistic foot shock was incorporated in the task to integrate multiple aspects of SUD in order to improve the resemblance to the conflict situations SUD patients face. The contingent but probabilistic foot shock

puts the animals in a motivational conflict, which is a core trait of SUD (Heather, 1998). Previous studies implicated the PrL to be involved in punished responding for rewards using different behavioural tasks (Chen et al., 2013; Seif et al., 2013; Verharen et al., 2019). The current findings confirm the involvement of the PrL in control over substance use, extending the evidence for its involvement in control over alcohol in a behavioural task where responding is both conditional and probabilistic.

The role of the PrL might also depend on whether the punishment is associated with a cue. In the STA task, a tone cue is included which functions as a warning signal that might lead to activation of the PrL. In a study in which aversive instrumental associations were studied, the PrL did not appear to be involved, perhaps because the foot shock was not signalled in this case (Jean-Richard-dit-Bressel & McNally, 2016). Indeed, another study reported no effects of PrL silencing on foot shock punished alcohol self-administration, in which the punishment was not cued either (Halladay et al., 2020). When a punishment is cued, the cue might elicit a fear response through activation in the PrL. Consistently, the PrL is thought to contribute to the processing of threat signals as projections to and from the PrL have been implicated in fear responses and punishment learning (Burgos-Robles et al., 2017; Li et al., 2019).

Alcohol versus sucrose seeking

The differential effects of PrL inactivation on alcohol versus sucrose seeking indicate that the involvement of the PrL in suppression of substance seeking is specific for alcohol. This may speak for reinforcer specificity of the STA task, since the studies discussed above have shown that PrL inactivation may lead to a more general loss of control over reward seeking. In addition, seeking behaviour for sucrose in the STA task was relatively unaffected by inactivation of the basolateral amygdala (BLA), the orbitofrontal cortex (OFC), and the nucleus accumbens (NAc) (see Appendix of this thesis for more details). Follow-up studies in which these brain regions are inactivated prior to testing in the STA task for alcohol may yield further insight into the neurobiological mechanisms involved in control over alcohol use.

These differential effects might be explained by the nature of the reinforcer. Signalling in the PrL has been implicated in incentive salience and alcohol could be a more salient reinforcer compared to sucrose (Batten et al., 2018). That said, a recent study showed that when offered a choice, only a minority of rats choose alcohol over an alternative sweet reward (i.e. the noncaloric sweetener saccharin) (Augier et al., 2018). Therefore, whether reinforcer salience or reinforcer preference underlies the differential effects of PrL inactivation on suppression of responding for alcohol versus sucrose awaits further investigation.

Repeated alcohol exposure causes neuroplastic changes in the prefrontal cortex that lead to differential involvement of the PrL in the regulation of control over seeking compared to the natural reward sucrose. The alcohol group was pre-exposed to alcohol for a time period that we have previously shown to be sufficient to induce loss of control over alcohol seeking (Spoelder et al., 2015, 2017). Although under baseline conditions, loss of control was not detected in the STA task at group level after alcohol pre-exposure, it is likely that the consumption of alcohol evoked neuroadaptations that may affect responding in the STA task. For example, repeated alcohol exposure is known to induce upregulation of NMDA receptor subunits, increases in NMDA receptor functionality, neuronal dendritic expansions in the dorsolateral striatum, and impaired synaptic plasticity in the nucleus accumbens (DePoy et al., 2013; Gass & Olive, 2008; Kasanetz et al., 2010; Stuber et al., 2010). Of these, especially changes in corticostriatal NMDA receptor signaling have been implicated in punished alcohol intake (Seif et al., 2013, 2015). The PrL is part of a larger interconnected network with other brain areas implicated in reward-directed behaviour, including the BLA. Future studies could focus on determining the contribution of pathways between the PrL and the BLA as this connection has been implicated in driving fear responses (Burgos-Robles et al., 2017).

Limitations and future directions

A limitation of the present study is that individual differences could not be statistically examined because of the small sample sizes. We observed individual differences in sensitivity to threat of adversity in the control condition, reflected by a substantial variability in suppression ratios. For example, we and others have previously shown that after two months of IAA, profound individual differences in alcohol intake and motivation can be observed, whereby high alcohol drinking rodents displayed more motivation to obtain alcohol and more punishment-resistant alcohol-directed behaviour than low alcohol drinking rodents (Hopf et al., 2010; Lesscher et al., 2010; Seif et al., 2013; Spoelder et al., 2015, 2017). Moreover, punishment-resistant reward seeking after prolonged cocaine exposure has been associated with decreased excitability of neurons in the PrL. Together, this suggests that rats that consumed high levels of alcohol during IAA are the ones more likely to show little suppression, and excitability in the PrL in those animals might be reduced (Chen et al., 2013).

Moreover, the present study was focused on the involvement of the PrL in behavioural control over alcohol and sucrose seeking, but other subregions of the prefrontal cortex might also play a role in the loss of control as seen in SUD. Various processes associated with several subregions of the prefrontal cortex are disrupted in SUD (Goldstein & Volkow, 2011). Subregions such as the infralimbic cortex (IL) might also

contribute to behavioural control over substance seeking, since the IL has been implicated in avoidance behaviour and thereby may possibly aid in suppressing responses (Capuzzo & Floresco, 2020; Martinez et al., 2013; Moscarello & LeDoux, 2013). Additionally, the OFC may be involved in behavioural control, as it plays a role in processing reward information about opposing options and through that influences choice and decision making (Guillem & Ahmed, 2018; Izquierdo, 2017; Rich et al., 2018). Thus, other subregions of the prefrontal cortex are important targets to investigate in relation to behavioural control over reward seeking.

Conclusion

To conclude, the present study showed that PrL inactivation selectively reduced suppression of alcohol seeking in the face of a threat of punishment, while no effect on sucrose seeking was detected. This implies that neuroplastic changes in the PrL might contribute to the progression from controlled, moderate alcohol consumption to uncontrolled, compulsive use, which is a hallmark of SUD. Together, our data demonstrate that PrL functioning is necessary for maintaining control over the pursuit of alcohol.

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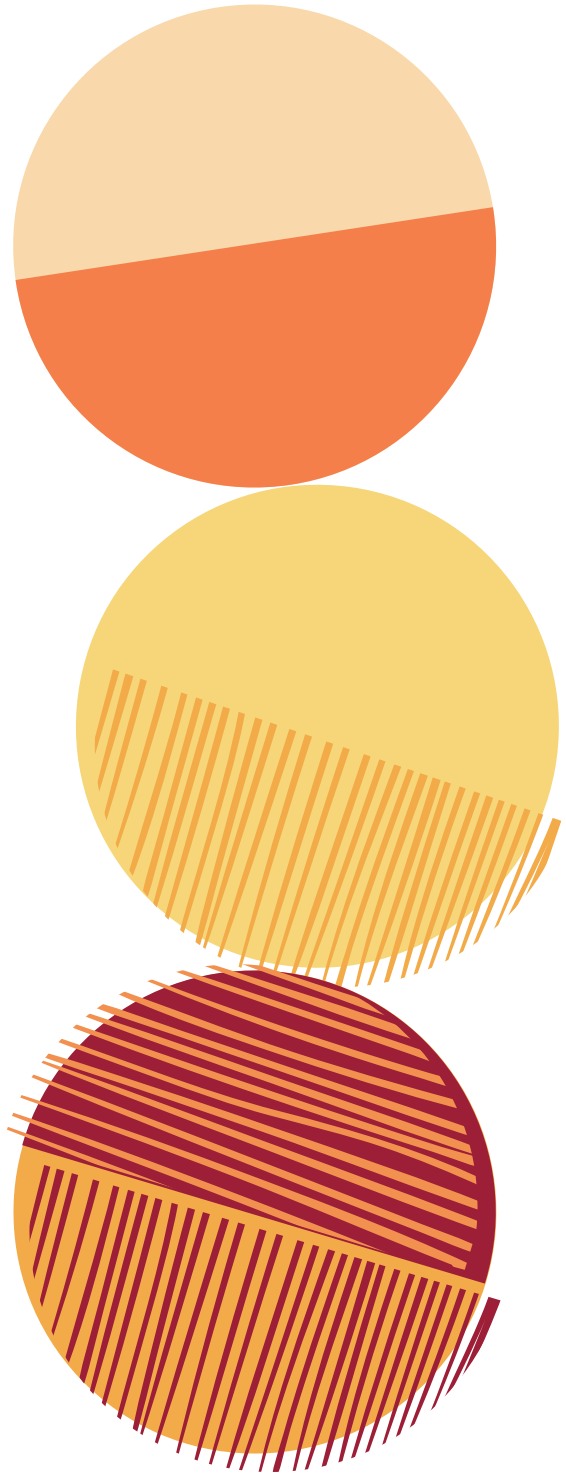


Chapter 5

On the interrelation between alcohol addiction-like behaviours in rats

Johanna A. S. Smeets*[1,2]
A. Maryse Minnaard*[1,2]
Geert M. J. Ramakers[2]
Roger A. H. Adan[2]
Louk J. M. J. Vanderschuren[1]
Heidi M. B. Lesscher[1]

*shared first authorship

[1] Department of Population Health Sciences, Unit of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2] Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, The Netherlands

Abstract

Rationale

Alcohol use disorder (AUD) is a complex and heterogeneous disorder which is subject to a substantial degree of individual vulnerability. A variety of behavioural mechanisms is thought to be involved in AUD, but the relationship between those mechanisms is unclear. In addition, it may be that the different behavioural subcomponents contribute differently to the AUD pathology in different individuals.

Objectives

The aim of the present study was to explore the relation between four behavioural mechanisms that have been associated with AUD, i.e. alcohol intake, habit formation, motivation, and aversion resistance in rats. We hypothesised that a subpopulation of rats could be identified that, based on these measures, display characteristics of AUD-like behaviour.

Methods

A group of Lister Hooded rats (n = 47) was characterised for alcohol consumption, habit formation, motivation for alcohol, and aversion-resistant alcohol consumption. The interrelationship between these measures was evaluated by determining correlations and by evaluating the tendency of the data to cluster, by means of Hopkins statistics. Moreover, an addiction severity score was computed using normalised scores of the different combinations of the four measures, and the consistency of the identified AUD-like subpopulation was assessed.

Results

Aversion-resistant alcohol consumption correlated with alcohol consumption. Overall, the data was uniformly distributed as there was no significant tendency of the behavioural measures to cluster in the dataset. We assessed whether animals were consistently categorised as AUD-like, on the basis of multiple ranked addiction severity scores. Five animals were consistently classified as displaying AUD-like behaviours. The composition of the remaining subpopulation of animals with the highest addiction severity score varied substantially, depending on the combination of measures used for the calculation.

Conclusions

Out of a population of alcohol drinking rats, only a small minority showed consistent AUD-like behaviour. The variable composition of the remaining AUD-like subpopulation suggests that the four behavioural components contribute differently to the AUD phenotype across individual animals, which is consistent with the heterogeneity in the human AUD pathology. Together, the findings emphasise the importance of considering the heterogeneity in underlying behavioural constructs driving AUD-like behaviour in preclinical studies.

Keywords

Alcohol consumption
Alcohol use disorder
Aversion
Habit
Motivation
Rats

Introduction

Alcohol use disorder (AUD) is a chronic relapsing disorder characterised by a lack of control over alcohol use. It is associated with major socioeconomic problems, thereby contributing substantially to the global burden of disease (Connor et al., 2016; Rehm et al., 2009, 2013). The pathology of AUD is heterogeneous, and its diagnosis relies on a variety of behavioural criteria such as a strong desire to use alcohol and continued alcohol use despite having persistent problems caused or exacerbated by alcohol consumption (American Psychiatric Association, 2013). In Europe, approximately 5.5% of the population meet criteria for AUD while more than 75% of the population has been estimated to consume alcohol (Eurostat, 2018; Rehm et al., 2009, 2013). These numbers illustrate that only a minority of all individuals who drink alcohol will eventually develop AUD (Anthony et al., 1994). To better understand the risk for AUD, it is therefore of great importance to investigate factors that underlie AUD.

Different neurobehavioural processes are thought to be involved in AUD, and animal studies have been used to gain insight into the underlying mechanisms. To that aim, behavioural procedures, building upon self-administration concepts, have been developed to model one or more of the diagnostic criteria of AUD. First, excessive alcohol drinking is a hallmark of AUD which can be studied using limited access choice procedures. This intermittent exposure to alcohol entails repeated cycles of voluntary alcohol intake which can result in a gradual escalation of alcohol consumption (Carnicella et al., 2014; Lesscher et al., 2010; Loi et al., 2010; Spoelder et al., 2015). Second, AUD may be driven by automated, habitual behaviour, whereby alcohol intake devolves from a voluntary, goal-directed to an automated, cue-

driven structure, in which alcohol use becomes disconnected from its consequences (Corbit et al., 2012; Dickinson et al., 2002; Everitt & Robbins, 2016; Lopez et al., 2014). Outcome devaluation procedures are typically used to dissociate habitual from goal-directed behavioural control (Barker & Taylor, 2014; Dickinson, 1985; McKim et al., 2016; Robbins & Costa, 2017). Third, the progression from casual to compulsive alcohol use is thought to be accompanied by increased motivation to obtain alcohol (American Psychiatric Association, 2013). In animals, this increased exertion of effort can be measured using progressive ratio (PR) schedules of reinforcement (Hodos, 1961; Richardson & Roberts, 1996; Spoelder et al., 2015). Fourth, insensitivity to negative consequences is a major characteristic of AUD. Behaviourally, this can be conceptualised as resistance to punishment or aversion, for example by rendering alcohol unsavoury using the bitter tastant quinine (Vanderschuren et al., 2017). Resistance to quinine adulteration after prolonged alcohol experience has been reported, which is indicative of continued alcohol use despite negative consequences (Hopf et al., 2010; Lesscher et al., 2010; Wolffgramm, 1991). Altogether, this wide range of behavioural tests provides tools to study the complexity of human AUD pathology in rodents. As such, animal studies allow for elucidation of neural mechanisms that underlie AUD, knowledge that may ultimately contribute to the development of better treatment options for AUD.

In order to advance knowledge on the neurobehavioural underpinnings of AUD, a great deal of effort has been made to develop preclinical models that mimic the complexity of AUD in humans (Belin-Rauscent et al., 2016). Various procedures have been proposed as rodent analogues of substance use disorder (SUD) in which multiple behavioural measures are combined (Ahmed, 2012; Belin et al., 2009; Deroche-Gamonet et al., 2004; Domi et al., 2019; Jadhav et al., 2017; Kasanetz et al., 2010; O'Neal et al., 2020; Radke et al., 2017). In these models, the individual variation in behaviour is captured and employed by defining subpopulations that portray a SUD-like phenotype. These subpopulations are often further investigated for differences in e.g. genetic and behavioural predispositions or in neurobiological mechanisms (Augier et al., 2018; Domi et al., 2019; Giuliano et al., 2015; O'Neal et al., 2020; Radwanska & Kaczmarek, 2012). However, the interrelations between frequently used behavioural measures, for example between individual consumption levels, habitual behaviour and loss of control, remain elusive.

Therefore, the aim of this study was to determine how different aspects of AUD-like behaviour are related. To that end, we characterised various aspects of AUD-like behaviour within a sample of rats of the Lister Hooded strain. Each rat was characterised for four AUD-like behavioural measures and the variability in each measure was assessed. First, voluntary home cage alcohol drinking was determined using an intermittent every-other-day voluntary alcohol drinking paradigm. Second, sensitivity to outcome devaluation was determined after extended operant alcohol self-administration training, in order to examine whether alcohol seeking would progress from a goal-directed to a habitual structure. Third, motivation for alcohol was assessed using a PR schedule of reinforcement. Fourth, the consumption of quinine adulterated alcohol was measured as an indicator of aversion-resistant alcohol consumption. Distributions of all individual measurements were compared to explore how these measures are related to one another. Moreover, we investigated the consistency of the classification of a subpopulation of rats as AUD-like, using addiction severity scores. We hypothesised that a subgroup of rats could be identified that, based on these behavioural measures, display characteristics of AUD-like behaviour.

Materials and methods

Subjects

A total of 47 adult male Lister Hooded rats (Charles River, Sulzfeld, Germany), weighing 200-250 grams (~8-10 weeks old) at the start of the experiment, were used in this study. The rats were individually housed in Macrolon type III sawdust bedded cages (42.5 x 26.6 x 18.5 cm) with ad libitum access to tap water and chow (Rat and Mouse Breeder and Grower Expanded-CRM(E), Special Diet Service, UK). A polycarbonate rat tunnel (9 x 9 x 15 cm) and a tissue were provided for cage enrichment. The rats were kept under controlled temperature and humidity conditions (21 ± 2°C and 50 – 70% humidity) and on a reversed 12 h/12 h light/dark cycle (lights off at 7.00 AM - lights on at 7.00 PM) to allow for behavioural testing in the dark phase. The rats were acclimatised to the housing conditions for eleven days prior to behavioural testing and they were weighed and handled at least once per week throughout the course of the experiment. All animals were experimentally naïve. All experimental procedures were approved by the Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee and were conducted in accordance with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU).

Behavioural procedures

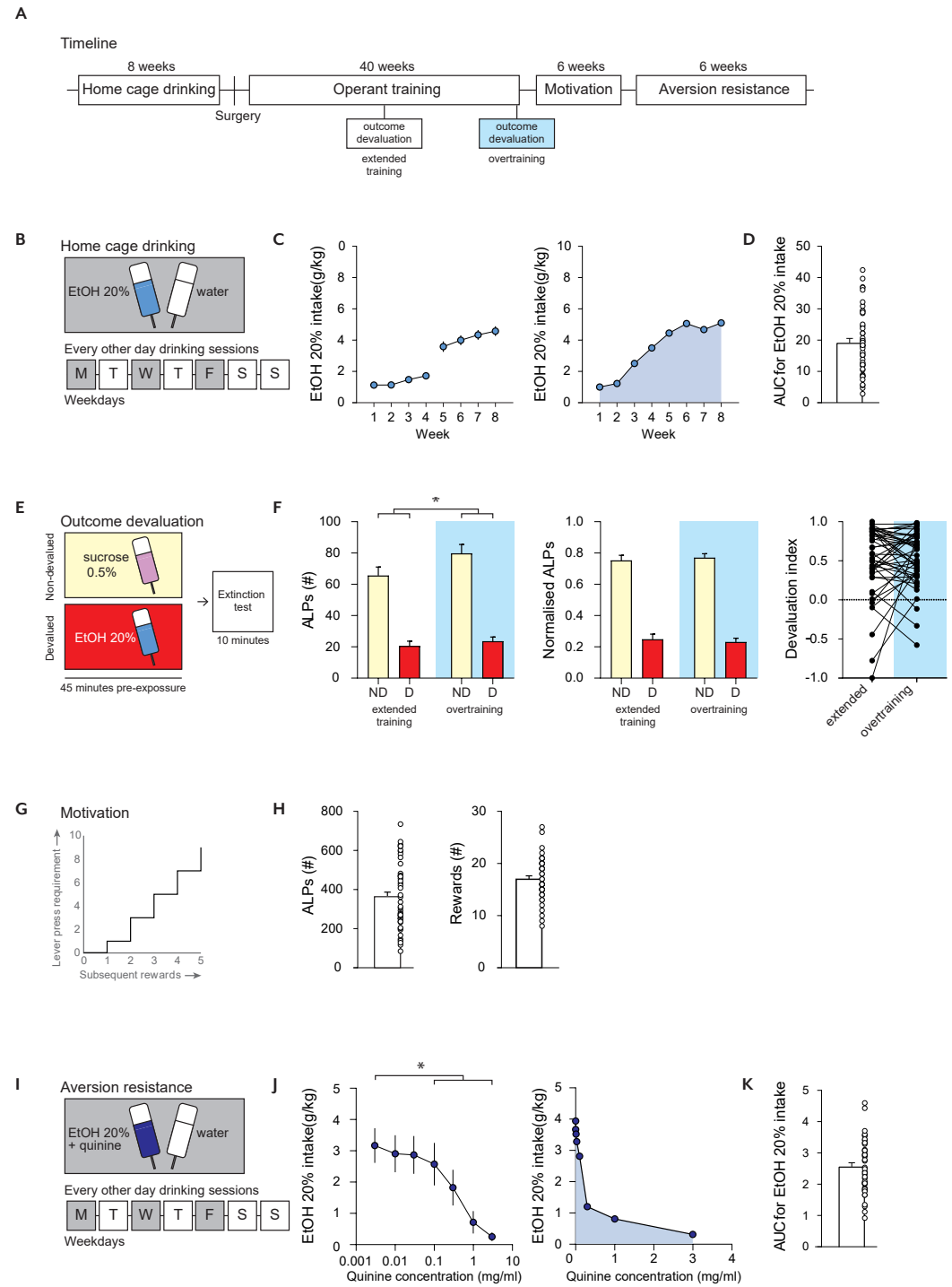
An overview of the behavioural procedures is provided in Figure 1A. The specific experiments are described below.

Intermittent alcohol access

Intermittent alcohol access (IAA) was performed as previously described (Spoelder et al., 2015, 2016). The rats were exposed to 20% (v/v) alcohol and tap water in a two-bottle choice setup in the home cage for three days a week (Monday, Wednesday, Friday) according to an IAA schedule (Figure 1A-B). In the first four weeks, alcohol exposure sessions lasted for 7 hours between 9.30 AM and 16.30 PM (i.e. during the dark phase) and sessions were extended to 24 hours in the second month. After eight weeks of IAA, animals were exposed to alcohol in the home cage one weekend day (i.e. when no behavioural training or testing took place) per week for the remainder of the experiment to maintain alcohol consumption. Alcohol (99.5%, Klinipath, The Netherlands) was freshly diluted with tap water once per week to a final concentration of 20% (v/v). The position of the bottles was alternated between drinking sessions to avoid the development of side bias.

Apparatus

The animals were trained and tested in operant conditioning chambers (29.5 x 24 x 25 cm, Med Associates Inc., USA) equipped with two retractable levers (4.8 x 1.9 cm; ENV-112CM) and a white cue light (28 V, 100mA; ENV-221M) present above each



> **Figure 1. Individual variation in alcohol intake, habit formation, motivation, and aversion resistance.** **A.** Schematic of the experimental design. **B.** Schematic of the two-bottle choice intermittent access home cage drinking procedure. The animals initially received 7 hours (week 1-4) and subsequently 24 hours (week 5-8) drinking sessions every other day on Mondays, Wednesdays and Fridays. **C.** Group average of alcohol intake (g/kg) across 8 weeks of home cage drinking (left panel). For each animal, area under the curve (AUC) values were calculated (blue shaded area) based on their alcohol intake (g/kg) across 8 weeks of home cage drinking (right panel shows a representative animal). **D.** Group average (bar) and distribution of individual AUC values for alcohol intake across 8 weeks of home cage drinking. **E.** Schematic of the outcome devaluation procedure to test habit formation. All animals were pre-exposed (45 minutes) to a control solution (sucrose 0.5%, non-devalued) or an alcohol solution (EtOH 20%, devalued). Following pre-exposure, active lever presses (ALPs) were measured for 10 minutes in the absence of reinforcer delivery (extinction test). **F.** Group averages of the number of ALPs during the extinction test for the non-devalued (ND) and devalued (D) condition after extended training and overtraining are shown (blue shaded) (left panel). In addition, normalised ALPs are shown that reflect the distribution of lever pressing for the non-devalued and devalued test after extended training and overtraining (blue shaded) (middle panel). Individual values of the devaluation index after extended training and overtraining (blue shaded) (right panel). **G.** Schematic of the progressive ratio schedule of reinforcement to assess motivation. **H.** Group average (bar) and distribution of individual values for ALPs averaged across three PR sessions (left panel). Group average (bar) and distribution of individual values for alcohol rewards obtained averaged across three PR sessions (right panel). **I.** Schematic of quinine modulation to test aversion resistance. **J.** Group average of alcohol intake (g/kg) across drinking sessions with increasing quinine concentrations (left panel). Area under the curve (AUC) values for all animals were calculated (shaded area) based on their alcohol intake (g/kg) across drinking sessions with increasing quinine concentrations (representative animal, right panel). **K.** Group average (bar) and distribution of individual AUC values for alcohol intake (g/kg) across drinking sessions with increasing quinine concentrations.
Group data are presented as the mean ± SEM. Asterisk (*) denotes significance at a $p < 0.05$ level.

lever. One lever was designated as 'active', responding on which was reinforced with alcohol access, the other lever was designated as 'inactive', responding on which had no programmed consequences. The position of active and inactive levers was counterbalanced between rats. A recessed liquid dipper and food receptacle were situated in between the levers, equipped with an infrared beam for nosepoke detection. The wall on the opposite side of the box contained a white house light (28 V, 100 mA; ENV-215M). The floor of the chamber was covered with a metal grid with bars separated by 1.57 cm. All chambers were equipped with Med Associates Inc. equipment and were situated in light- and sound-attenuating cubicles equipped with a ventilation fan. All chambers were controlled by MED-PC IV software (version 4.2) for Windows. The alcohol solution was refreshed in between test sessions to prevent that a decline in alcohol concentration by evaporation of the alcohol between sessions would influence the animals' behaviour.

Operant alcohol self-administration

The rats were trained to respond for alcohol during 30-minute operant sessions, once daily, 2-5 days per week. The house light was illuminated throughout the session. Animals were first trained under a fixed ratio (FR) 1 schedule of reinforcement for alcohol. Pressing the active lever (active lever press; ALP) raised the dipper cup containing an alcohol reward (0.1 ml, 20% v/v). The dipper cup remained in raised position until 10 seconds after the animal entered the recessed receptacle, which was detected by interruption of the infrared light beam in the recessed receptacle. The presentation of the dipper cup was paired with the retraction of both levers and the illumination of the cue light above the active lever. Ten seconds after the animal entered the recessed receptacle, the cue light was turned off and the levers were reintroduced, signalling the start of a new trial. All inactive lever presses were recorded but were without programmed consequences.

After acquisition of alcohol self-administration under an FR1 schedule, animals were next trained twice on a random ratio (RR) 2 schedule of reinforcement during 30-minute sessions. The response requirement varied pseudo-randomly per trial between one, two, three or four ALPs, and was on average two ALPs across trials. Finally, all animals were trained on a RR3 schedule of reinforcement during 30-minute sessions once daily, 2-4 days per week. Here, the response requirement pseudo-randomly varied per trial between one, two, three, four, five, or six ALPs, and was on average three ALPs across trials. Performance in this operant task was measured by the number of ALPs.

Outcome devaluation testing

Habit formation was measured by outcome devaluation tests in which there were two conditions (Figure 1E). In the devalued condition, rats had access to alcohol (EtOH 20%) in the home cage for 45 minutes. In the non-devalued condition, rats received a 0.5% sucrose solution in the home cage for 45 minutes, to control for general satiation. Animals had ad libitum access to chow during pre-exposure. Next, the rats were tested in a 10-minute extinction test to measure lever pressing in the absence of reinforcer delivery, followed by a regular 30-minute RR3 self-administration session. Outcome devaluation tests were performed according to a within-subjects Latin square design, whereby each test day was followed by at least one day with a regular 30-minute RR3 session. Sensitivity to outcome devaluation was examined after 50 training sessions (extended training) and after minimally 100 training sessions (overtraining) (Figure 1A). After each pair of outcome devaluation tests, rats were trained on RR3 sessions once daily, 2-4 days per week.

Furthermore, two additional outcome devaluation tests were performed as controls. (1) To control for potential satiation effects of sucrose, an outcome devaluation test was performed after extended training in which water pre-exposure was used as the non-devalued condition instead of a sucrose solution. (2) To evaluate whether the sedative effects of alcohol influenced responding during the subsequent test, an outcome devaluation test was performed with a lower alcohol concentration (i.e. EtOH 10%) as the devalued condition.

Progressive ratio

Motivation for alcohol was measured using a progressive ratio (PR) schedule of reinforcement (Figure 1A). The response requirement was set according to a linear PR, in which 2 additional ALPs were required for each subsequent alcohol reward (i.e. 1, 3, 5, 7, 9, etc., Figure 1G). Sessions ended after 100 minutes and PR performance was considered stable at group level when the average number of ALPs on individual days fell within a 75-125% variability range of the total average of ALPs across those three consecutive days.

Quinine modulation of alcohol intake

Aversion resistance was determined by quinine modulation of alcohol intake (Figure 1A). Alcohol and water consumption were examined using a two-bottle choice setup in the home cage for 24 hours every other day. Rats were presented with one bottle containing tap water and one bottle with alcohol solution that was adulterated with increasing concentrations of quinine (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 mg/ml; Sigma-Aldrich, Germany) in tap water (Figure 1I). Each quinine concentration was offered for

two consecutive sessions. Bottle positions were switched between sessions to avoid side bias.

Data analysis and statistics

Data were analysed and visualised using Microsoft Excel, GraphPad Prism (version 8.3.0, GraphPad Software Inc., USA) and RStudio (version 1.2.1335, RStudio Inc., USA). Three rats were excluded from all analyses because they did not complete all behavioural procedures. Results are presented as mean ± SEM unless otherwise stated. A significance criterion of $p < 0.05$, two-tailed, was adopted in the statistical analyses of correlations.

Home cage drinking

Bottles were weighed before and after each drinking session. Fluid intake was calculated by subtracting the bottle weights at the end of every drinking session by the starting weights. Alcohol intake (ml) was calculated according to the following equation: (Δ alcohol bottle weight in grams) / (0.8+(0.2*0.789)) in which the density of ethanol (i.e., 0.789 g/ml), is included. Alcohol intake (g/kg) was calculated as follows: ((alcohol fluid intake in ml) * (0.2*0.789)) / (bodyweight in kg). Water intake (ml/kg) was calculated as: (water fluid intake in ml) / (bodyweight in kg). Preference for alcohol (%) was calculated according to the following equation: (alcohol fluid intake in ml) / ((alcohol fluid intake in ml) + (water fluid intake in ml)) * 100. Weekly averages of alcohol intake throughout intermittent alcohol access were calculated for all animals and the area under the curve (AUC) value was calculated for each animal using GraphPad Prism (version 8.3.0, GraphPad Software Inc., USA). To assess gradual escalation of alcohol consumption as animals progressed to the 24 hour sessions, differences in mean alcohol intake (g/kg) and alcohol preference (%) between the 7 hour (group mean week 1-4) and 24 hour (group mean week 5-8) sessions were compared with a paired samples t-test.

Outcome devaluation testing

Bottles were weighed before and after pre-exposure. Pre-exposure intake was calculated by subtracting the bottle weights at the end of pre-exposure from the starting weights. Alcohol fluid intake (ml) and alcohol intake (g/kg) were calculated as described above. Sucrose intake was calculated using the following equation: (Δ sucrose bottle weight in grams) / (0.995+(0.05*1.69)) in which the density of sucrose (i.e., 1.69 g/ml), is included. For the outcome devaluation tests, output data were the number of ALPs during the 10-minute extinction test. Mean ALPs during outcome devaluation testing were assessed with two-way repeated measures ANOVA with *condition* (i.e. non-devalued or devalued) and *timepoint* (i.e. extended training or overtraining) as the within-subjects factors. Mean ALPs of each control outcome devaluation test were compared across the two conditions (i.e. non-devalued or devalued) with a paired t-test. Moreover, ALPs in the non-devalued and devalued states were normalised to the total number of lever presses (non-devalued + devalued) in each condition. Devaluation indexes were calculated by the following equation: ((#ALP in non-devalued condition)-(#ALP in devalued condition)) / ((#ALP in non-devalued condition)+(#ALP in devalued condition)). Habit formation was calculated as devaluation index after extended training – devaluation index after overtraining, such that a positive score indicated greater habit formation.

Progressive ratio

Output data were the number of ALPs and rewards obtained within the session, averaged per animal across three consecutive stable days.

Quinine modulation of alcohol intake

Bottles were weighed before and after each drinking session. Fluid intake, alcohol fluid intake (ml), alcohol intake (g/kg), and preference for alcohol (%) were calculated as described above. Alcohol intake (g/kg) was averaged across both sessions for all quinine concentrations and mean alcohol intake was assessed with a one-way repeated measures ANOVA with *quinine concentration* as the within-subjects factor. Post hoc pairwise Bonferroni comparisons were used to compare intake at each quinine concentration with intake at a quinine concentration of 0. The AUC value was calculated for each animal using GraphPad Prism (version 8.0.1, GraphPad Software Inc., USA).

Correlations

To investigate relations between the different behavioural measurements associated with AUD, the following measurements were selected for correlation analyses:

- Alcohol intake: AUC of weekly home cage alcohol intake (g/kg) during week 1-8 of IAA. A high value indicates high alcohol consumption during IAA.
- Habit formation: difference score between the devaluation index after extended training and after overtraining. Values range between -2 and 2. A positive value (i.e. devaluation index after overtraining is lower than after extended training) indicates a decreased sensitivity to outcome devaluation over time, i.e. habit formation.
- Motivation: average of ALPs across three PR sessions. A higher value is indicative of higher motivation to seek alcohol.
- Aversion resistance: AUC of alcohol intake (g/kg) across increasing concentrations of quinine adulteration. A higher value is indicative of an increased resistance to aversion, i.e. persistent alcohol drinking despite an aversive taste.

One rat was excluded from the correlation analyses as it was detected as an outlier by a Z-score > 3.29.

Cluster tendency

The cluster tendency in the data was assessed using the Hopkins statistics (Adolfsson et al., 2019; Banerjee & Dave, 2004; Hopkins & Skellam, 1954; Lawson & Jurs, 1990). The Hopkins statistics, or H-value, can be considered as a hypothesis test of spatial randomness with the null hypothesis that the dataset is uniformly distributed (i.e. no meaningful clusters) and the alternative hypothesis that the data is not uniformly distributed (i.e. contains meaningful clusters). Highly clusterable datasets will have an H-value that is close to 1 and completely random data will have an H-value that is close to 0.5. Thus, if the H-value < 0.5, then it is unlikely that the dataset contains statistically significant clusters. The analysis was done using RStudio (version 1.2.1335) and to obtain the Hopkins statistics, we used the *get_clust_tendency()* function from *factoextrapackage*.

Addiction severity scores

Based on alcohol intake, habit formation, motivation, and aversion resistance (as described earlier under *Correlations)*, an addiction severity score was computed according to Belin and colleagues (Belin et al., 2009). Normalisation of each measure was done by subtracting the mean of all animals from the measure for every individual animal that was subsequently divided by the standard deviation of the whole group. This resulted in a score with an average of 0 and a standard deviation of 1 for each measure.

The total addiction severity score was then repeatedly calculated as a sum of the normalised scores using various compositions of three out of the four behavioural measures, i.e. each time excluding one of the four measures (Figure 3A). Animals were ranked on their addiction severity score and the highest quartile (n = 11) was selected and categorised as the subgroup showing typical AUD behaviour. Next, we counted the amount of times each animal fell into the highest quartile. As such, we assessed whether animals were consistently categorised as AUD-like, i.e. were selected in the highest quartile regardless of the varying composition of the measures used for the addiction severity score. The subgroups of animals that were consistently (i.e. belonging to the highest quartile in 4/4 addiction severity score computations) and animals that were never (i.e. belonging to the highest quartile in 0/4 addiction severity score computations) assigned as AUD-like were compared on each of the four behavioural measures using unpaired t-tests.

Results

Individual variation in alcohol intake, habit formation, motivation, and aversion resistance

Home cage drinking

The animals gradually increased their alcohol intake throughout the first 8 weeks of home cage drinking (7 hour sessions: mean 1.4 g/kg, SEM 0.1; 24 hour sessions: mean 4.3 g/kg, SEM 0.3; $t(43) = 13.510$, $p < 0.001$) (Figure 1B-D). Similarly, alcohol preference over water also gradually increased over the course of the experiment (7 hour sessions: mean 25.7%, SEM 2.3; 24 hour sessions: mean 44.7%, SEM 3.1; $t(43) = 10.577$, $p < 0.001$) (Supplementary Figure 1). Individual animals showed a widespread variation in alcohol intake, which was most pronounced during the 24 hour sessions (Supplementary Figure 1).

Outcome devaluation testing

All animals were trained daily on a RR3 schedule of reinforcement over the course of several months. Depicted in Supplementary Figures 2A-D is the performance in this operant task, measured by the number of ALPs, and the number of rewards obtained in the five sessions prior to testing outcome devaluation in the extended as well as the overtraining phase. At the moment of outcome devaluation testing, the animals were pre-exposed to either alcohol or sucrose in the home cage, followed by an extinction test in the operant cage (Figure 1E; Supplementary Figure 2E, right panel). Alcohol pre-exposure reduced responding on the lever associated with alcohol compared with sucrose pre-exposure, both after extended training and overtraining, demonstrating a significant outcome devaluation effect ($F_{condition}(1,43) = 132.337$, $p < 0.001$; $F_{timepoint}(1,43) = 2.434$, $p = 0.126$; $F_{timepoint\ x\ condition}(1,43) = 2.036$, $p = 0.161$) (Figure 1F, left and middle panel). Individual animals showed variation in the development of habit formation over time: for some animals the devaluation index increased whereas for others the devaluation index decreased or remained comparable (Figure 1F, right panel).

To control for potential palatability-induced stimulation of responding in the non-devalued condition, an additional outcome devaluation test was performed after extended training, where water pre-exposure was used as the non-devalued condition instead of sucrose pre-exposure. This yielded very similar results in that there was a significant outcome devaluation effect ($t(43) = 11.124$, $p < 0.001$) (Supplementary Figure 2F). Thus, the higher number of ALPs that was observed in the non-devalued condition as compared to the devalued condition was not dependent on sucrose consumption. To rule out whether the reduced number of

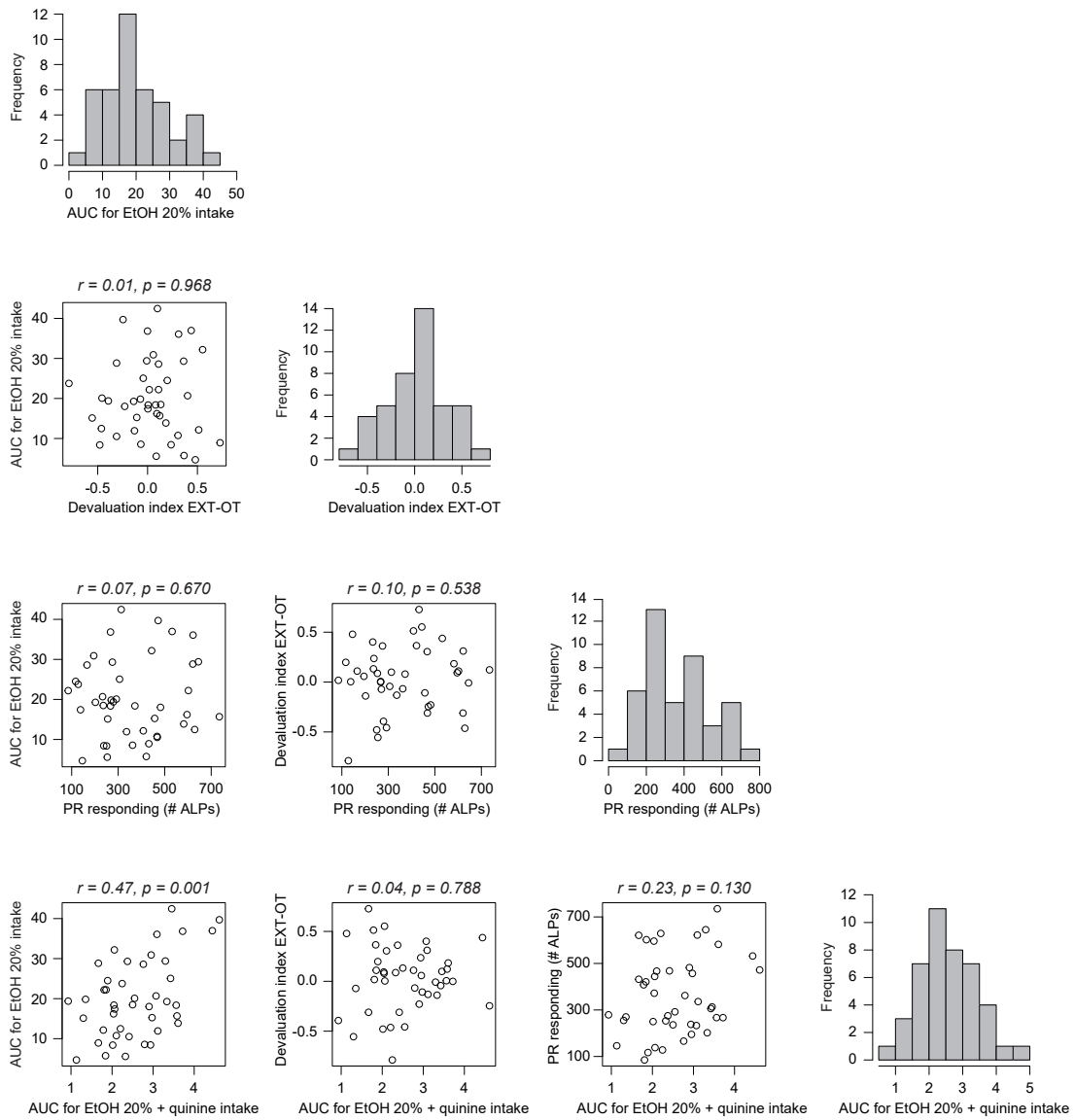


Figure 2. Correlation matrix and frequency plots of alcohol intake, habit formation, motivation, and aversion resistance. Alcohol intake is defined as area under the curve (AUC) of weekly home cage alcohol intake (g/kg) during week 1-8 of intermittent alcohol access (IAA) (first column). A high AUC value indicates high alcohol consumption.
Habit formation is defined as a difference score between the devaluation index after overtraining and after extended training (second row, second column). A habit formation score > 0 indicates a decreased sensitivity to outcome devaluation, i.e. a higher degree of habitual behaviour. Motivation for alcohol is defined as the average number of active lever presses made during three progressive ratio (PR) sessions (third row, third column). A higher value is indicative of higher motivation to seek alcohol. Aversion resistance is defined as AUC of alcohol intake (g/kg) across increasing concentrations of quinine adulteration. A higher value is indicative of resistance to aversion, i.e. persistent alcohol drinking despite the aversive taste (fourth row, fourth column).
Above every correlation plot, Pearson correlation coefficient (*r*) and the associated p-value are presented.

ALPs in the devalued condition was driven by the sedative effects of alcohol intake during pre-exposure, outcome devaluation was determined using a solution with a lower alcohol concentration. The animals consumed less alcohol during the pre-exposure, yet a similar significant outcome devaluation effect was observed under these conditions ($t(43) = 11.865$, $p < 0.001$) (Supplementary Figure 2G).

Progressive ratio

Motivation for alcohol was assessed using a PR schedule of reinforcement (Figure 1G). On average, the rats made 366 ALPs (SEM 25.7) and earned 17 rewards (SEM 0.6), and individual animals showed considerable variation in their levels of responding (Figure 1H).

Quinine modulation of alcohol intake

Aversion resistance was assessed through quinine modulation of alcohol intake (Figure 1I). Alcohol intake at baseline (i.e. without quinine) was on average 3.03 g/kg (SEM 0.08). As the quinine concentration increased, the animals reduced their alcohol intake from more than 3 g/kg (mean 3.17 g/kg, SEM 0.08) at a quinine concentration of 0.003 mg/ml to less than 1 g/kg (mean 0.25 g/kg, SEM 0.02) at a quinine concentration of 3 mg/ml ($F_{Quinineconcentration}(5,200) = 422.781$, $p < 0.001$) (Figure 1J). Post hoc analyses showed that alcohol intake at quinine concentrations of 0.1 mg/ml and higher was significantly lower than alcohol intake without quinine ($p < 0.001$). Individual animals showed considerable variation in their levels of quinine modulated of alcohol intake (Figure 1K).

Interrelation between the behavioural measures for AUD

To assess the interrelation between the different behavioural measures (i.e. alcohol intake, habit formation, motivation, and aversion resistance), correlation analyses, cluster tendency evaluation, and classifications based on addiction severity scores were performed.

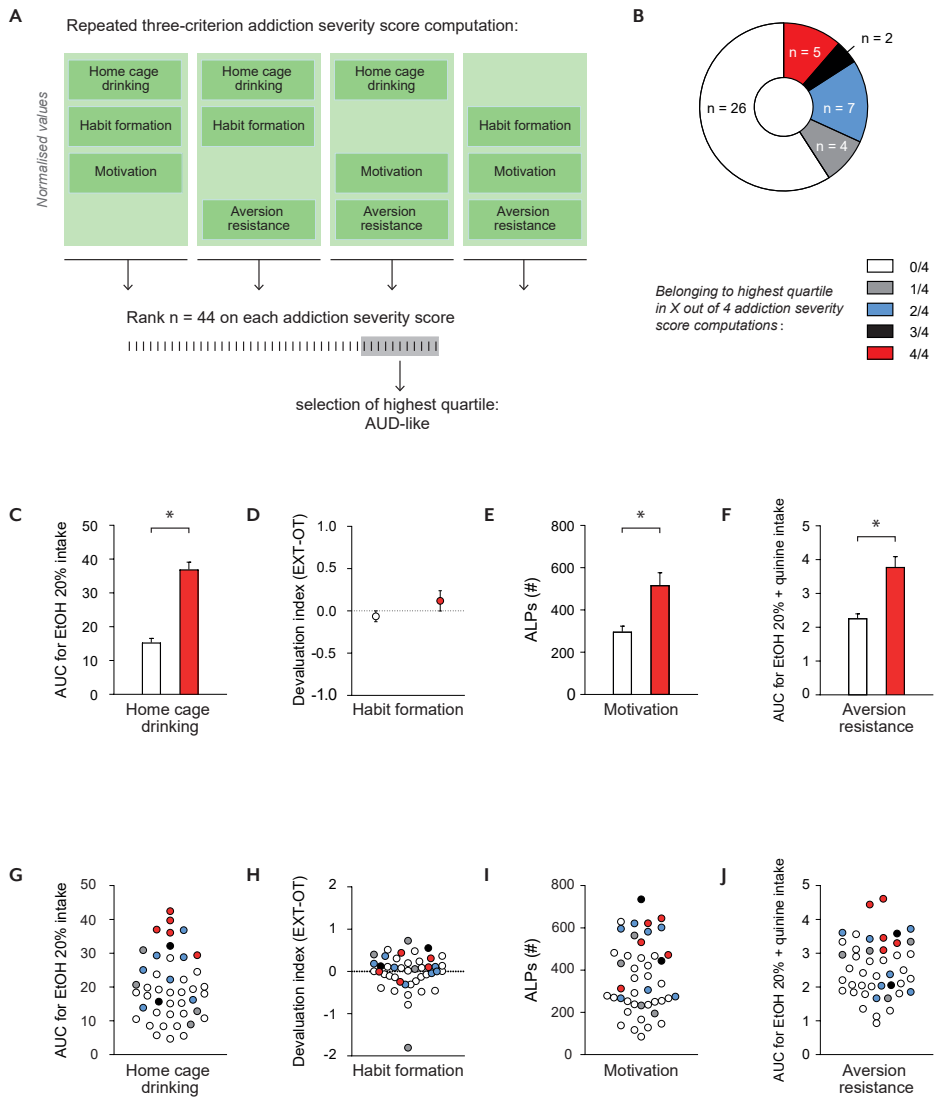
Correlations and cluster tendency

The relation between alcohol intake, habit formation, motivation, and aversion resistance was assessed by correlation analyses. The correlation plots are shown in Figure 2. There was a statistically significant, positive correlation ($r = 0.47$, $p = 0.001$) between alcohol intake during IAA and quinine-adulterated alcohol intake, indicating that rats that consumed more alcohol during IAA were also likely to consume more alcohol during quinine adulteration. Other correlations between any of the measures were weak (i.e. $-0.25 < r < 0.25$) and not significant ($p \geq 0.130$). The tendency to cluster in the complete dataset was evaluated using the Hopkins

statistic. The H-value was 0.475, which indicates that overall the data set was uniformly distributed rather than clustered into a meaningful grouping structure.

Classification of AUD-like animals based on three-criterion addiction severity scores

Similar to Belin and colleagues (2009), we computed an addiction severity score which was based on alcohol intake, habit formation, motivation, and aversion resistance. Specifically, multiple three-criteria addiction severity scores were calculated by consecutively excluding one of the measures (i.e. resulting in four calculations of the three-criteria addiction severity scores). Next, we identified animals with the highest addiction severity scores for each computation whereby the highest quartile (n = 11) was labelled as showing AUD-like behaviour (Figure 3A). We assessed the consistency of the classification of animals as AUD-like, among the various compositions of the three-criterion addiction severity scores (Figure 3B).



> **Figure 3. Addiction severity score based on combinations of alcohol intake, habit formation, motivation, and aversion resistance.**
The addiction severity score was computed (based on alcohol intake, habit formation, motivation, and aversion resistance) and the highest quartile (n = 11) was identified as rats showing typical AUD-like behaviour. When any of the four measures was excluded from the addiction severity score computation, the selection of rats in the highest quartile changed in composition. **A.** Schematic of the repeated three-criterion addiction severity score computation procedure. **B.** Division of animals based on individual rats belonging to the highest quartile in the addiction severity score computations. Individual animals were labelled according to the frequency in which they were assigned to the highest quartile, i.e. never (0/4; white), once (1/4; grey), twice (2/4; blue), three times (3/4; black), consistently (4/4; red). **C.** Average area under the curve (AUC) values for alcohol intake (g/kg) across 8 weeks of home cage drinking for animals that never fell into the highest quartile (n = 26) and for animals that always fell into the highest quartile (n = 5). **D.** Average of the difference score between the devaluation indexes after extended training versus overtraining for animals that never fell into the highest quartile (n = 26) and for animals that always fell into the highest quartile (n = 5). **E.** Average active lever presses (ALPs) averaged across three progressive ratio (PR) sessions for animals that never fell into the highest quartile (n = 26) and for animals that always fell into the highest quartile (n = 5). **F.** Average AUC values for alcohol intake (g/kg) across drinking sessions with increasing quinine concentrations for animals that never fell into the highest quartile (n = 26) and for animals that always fell into the highest quartile (n = 5). **G.** Distributions of individual AUC values for alcohol intake (g/kg) across 8 weeks of home cage drinking. Individual animals were labelled on the basis of selection in the highest quartile, i.e. least (0/4; white), once (1/4; grey), twice (2/4; blue), three times (3/4; black), consistently (4/4; red) AUD-like. **H.** Individual values of the difference score between the devaluation indexes after extended training versus overtraining. Individual animals were labelled on the basis of selection in the highest quartile, i.e. never (0/4; white), once (1/4; grey), twice (2/4; blue), three times (3/4; black), consistently (4/4; red) AUD-like. **I.** Distributions of individual values for ALPs averaged across three PR sessions. Individual animals were labelled on the basis of selection in the highest quartile, i.e. never (0/4; white), once (1/4; grey), twice (2/4; blue), three times (3/4; black), consistently (4/4; red) AUD-like. **J.** Distributions of individual AUC values for alcohol intake (g/kg) across drinking sessions with increasing quinine concentrations. Individual animals were labelled on the basis of selection in the highest quartile, i.e. never (0/4; white), once (1/4; grey), twice (2/4; blue), three times (3/4; black), consistently (4/4; red) AUD-like.
Group data are presented as the mean ± SEM. Asterisk (*) denotes significance at a $p < 0.05$ level.

A subgroup of five animals was consistently classified as AUD-like (4/4; n = 5). The majority of rats was never classified as AUD-like (n = 26), independent of the composition of the three-criterion addiction severity score. The remaining animals were classified as AUD-like once (1/4; n = 4), twice (2/4; n = 5), or three out of four times (3/4; n = 2). Thus, repeatedly calculating a three-criteria addiction severity score based on different combinations of behavioural measures resulted in the attribution of different animals to the AUD-like subpopulation, while a small group of animals displayed consistent AUD-like behaviour.

The subgroup of animals that was consistently classified as AUD-like was compared to the subgroup of animals that were never classified as AUD-like on each of the behavioural measures. The consistent AUD-like animals showed significantly higher levels of alcohol intake, PR responding, and higher levels of quinine-adulterated alcohol intake compared to the animals that were never classified as AUD-like (alcohol intake: $t(29) = 7.173$, $p < 0.001$; PR responding: $t(29) = 3.316$, $p = 0.0025$; quinine-adulterated alcohol intake: $t(29) = 4.581$, $p < 0.001$). However, habit formation levels were comparable between the two subgroups ($t(29) = 1.166$, $p = 0.253$). These findings confirm that the rats that were consistently classified as AUD-like consumed more alcohol, showed higher motivation, and were more aversion resistant than the rats that were never classified as AUD-like.

Although subgroups could be identified based on the addiction severity scores, there was considerable individual variation on each of the four behavioural parameters (Figure 3G-J). Whether or not an animal was consistently (i.e. belonging to the highest quartile in 4/4 addiction severity score computations) or never (i.e. belonging to the highest quartile in 0/4 addiction severity score computations) assigned as AUD-like matched most clearly with a high or low level of alcohol intake, respectively, since all five animals of the consistent AUD-like subgroup fell into the highest quartile for alcohol intake (Supplementary Table 1). Similarly, the animals of the consistent AUD-like subgroup generally showed high levels of quinine-adulterated alcohol intake as most of them were in the highest quartile when ranked based on quinine-adulterated alcohol intake. However, scores of consistently assigned AUD-like animals and never assigned as AUD-like animals overlapped, and this overlap was most prominent for the habit formation and motivation parameters. When also considering the intermediate groups, this overlap in scores on each of the behavioural measures was even more pronounced. Thus, while a subgroup could be identified that consistently showed AUD-like behaviour, there was considerable overlap with the other animals due to the widespread individual variation in each of the four behaviours.

Discussion

The present study aimed to explore the relation between four behavioural measures in rats that have been associated with AUD: alcohol intake, habit formation, motivation, and aversion resistance. Within the population of rats used for this study, we report considerable individual variation on all of the four AUD-like behaviours. We found a correlation between alcohol consumption and aversion-resistant alcohol consumption across the population. Overall, the selection of animals that had a high addiction severity score varied substantially, depending on which of the four measures were included. This means that being designated as AUD-like based on one AUD behaviour is not necessarily indicative of a high score on the other AUD behaviours. However, a group of five animals were consistently classified as displaying AUD-like behaviour.

Using a similar home cage drinking paradigm, we replicated the previously reported individual variability in voluntary alcohol consumption and aversion resistance in our population of Lister Hooded rats (Spoelder et al., 2015, 2017). Interestingly, overtraining in our operant self-administration task did not result in habit development for alcohol seeking at group level. A similar degree of sensitivity to outcome devaluation was shown both after an extended period of training and after overtraining, suggesting that responding for alcohol was and remained goal-directed at both timepoints. The absence of habit detection is in contrast to studies that reported reduced sensitivity to changes in outcome value after a prolonged training period for alcohol, cocaine, and nicotine (Clemens et al., 2014; Corbit et al., 2012; LeBlanc et al., 2013; Zapata et al., 2010). However, conflicting results have also been reported (Halbout et al., 2016; Samson et al., 2004). Moreover, studies that demonstrate a decrease in sensitivity to devaluation as a function of behavioural repetition in humans are scarce. Some studies found overreliance on habit learning in AUD or a reduced sensitivity to devaluation after extensive training (Sjoerds et al., 2013; Tricomi et al., 2009), but recent studies report no evidence for habit formation in human subjects after prolonged training (de Wit et al., 2018; Hogarth et al., 2019; Luijten et al., 2020). It is also notable that when the different measures were compared between animals that showed most and least AUD-like behaviour, habit formation score was the only parameter that did not significantly differ between these subgroups of animals (Figure 3D). This might suggest that habit formation played only a minor role in the AUD-like behaviour detected in the present study. Taken together, the current findings underscore the complexity of the role of habits in AUD.

To assess how the different AUD-like behavioural measures are related to one another, a combination of analyses was used. Correlation analyses revealed that the strongest association was observed between alcohol consumption and aversion-resistant alcohol consumption, as animals drinking large quantities of alcohol exhibited more resistance to quinine adulteration. This is in line with previous studies that reported greater aversion resistance in quinine-adulterated alcohol intake in rats with a high alcohol drinking phenotype (Hopf et al., 2010; Spoelder et al., 2015). None of the other correlations reached significance, however. It should be noted that, as a result of the longitudinal set-up of the current experiments, some of

the behavioural assessments were separated in time by months, e.g. alcohol intake during IAA and PR responding, which may explain the absence of a significant correlation. Indeed, it has been suggested that AUD-associated behaviours are not static, but rather can change over time during alcohol exposure (Jadhav et al., 2017; Spoelder et al., 2017). Therefore, we cannot exclude the possibility that some measures would have shown a different association when they were performed closer to one another in time. On the other hand, it is noteworthy that the IAA period and the quinine-adulterated alcohol exposure were most distant in time, but showed the strongest correlation. Interestingly, it has been shown that resistance to punishment initially did not correlate with responding for alcohol in rats, but did so after >75 alcohol training sessions (Jadhav et al., 2017). In this same study, the correlation between excessive motivation for alcohol and responding for alcohol increased over time (Jadhav et al., 2017). Additionally, we cannot exclude that the order in which the experimental procedures were set out may have influenced the outcome of our correlational analyses. That said, apart from the potential effects of prolonged alcohol exposure, we have no reason to assume that the measurements affect one another, except for quinine adulteration of the alcohol solution perhaps. Therefore, aversion resistance was assessed as a final measurement to prevent long-term reduced valuation of alcohol due to the association of alcohol with the bitter taste caused by the quinine-adulterated alcohol exposure. Together, the weak (and non-significant) correlations between most of our parameters suggest that the measures capture distinct features of AUD-like behaviour. Similar results were reported for heroin addiction in a recent study that reported highly variable relationships between metrics (O'Neal et al., 2020).

To gain more insight into the interrelation between alcohol intake, habit formation, motivation, and aversion resistance, the tendency to cluster within the data was assessed. For this purpose, a test of spatial randomness was used, which can reveal whether a feature is distributed randomly across the dataset or not (Adolfsson et al., 2019; Lawson & Jurs, 1990). No significant tendency to cluster was detected suggesting that on the basis of the four alcohol drinking components used in this study, no distinct AUD-like subpopulation could be identified whose scores are clearly dissociable from the others. However, the four behavioural measures were continuous variables and assumed to be normally distributed. Consequently, the fact that there is no tendency to cluster in the data does not exclude the possibility that there might be individual animals that score consistently high or low on the different measures. Therefore, further analysis of the data was performed using addiction severity scores.

The addiction severity score was computed repeatedly, each time excluding one of the four variables, to assess variability in the composition of the group exhibiting AUD-like behaviour. Five animals were consistently classified as displaying AUD-like behaviours across all addiction severity score computations. However, the remaining selection of animals that were attributed to the highest quartile of the addiction severity score varied substantially, depending on which of the four measures were included. This suggests that a significant proportion of animals score higher in one of more categories, but lower in others, and therefore may or may not be assigned to the AUD-like group depending on the combination of behavioural measures. Thus, although the majority of animals did not (consistently) display AUD-like behaviour, a small proportion of all animals consistently scored high on the different AUD-like behaviours. Importantly, this is comparable to the situation in humans, where only a minority of alcohol drinkers develops AUD (Anthony et al., 1994).

Individual variation in alcohol-directed behaviour was investigated by evaluating the distributions of all parameters in which each animal was labelled, ranging from least AUD-like (selected in the highest quartile of the addiction severity score computation 0/4 times) increasing up to consistently AUD-like. Five rats (i.e. 11% of the sample) were consistently labelled as having a high addiction severity score and thus consistently displaying AUD-like behaviour. Compared to the rats selected as least AUD-like, the consistent AUD-like animals portrayed higher alcohol intake levels, higher motivation for alcohol, and more aversion resistance. Nevertheless, the distribution of the whole population revealed that there was substantial overlap in scores between the groups on each of the behaviours. Thus, designating individual animals as increasingly consistent AUD-like, was not necessarily reflected by a higher score on all of the behavioural measures for every individual animal. This implies that the relative contribution of separate behavioural subcomponents to the AUD phenotype varies for different individual animals. Our findings emphasise the importance of considering this heterogeneity in relative contribution of behavioural constructs ultimately driving AUD-like behaviour in animals. First, this heterogeneity may have implications for pinpointing underlying neural substrates and predispositions of AUD using preclinical studies. Second, taking this heterogeneity into account might facilitate the translation to the human psychopathology, as AUD in human individuals is also considered as a very heterogeneous pathology. Large clinical heterogeneity can be seen in terms of symptom dimensions, AUD severity, treatment response, and comorbidities (Schuckit, 2006).

Conclusion

Aversion-resistant alcohol consumption was associated with alcohol consumption and the data on alcohol intake, motivation, habit formation, and aversion resistance were uniformly distributed. Although the composition of the subpopulation of animals with the highest addiction severity score varied substantially, depending on the combination of the behaviours that were included, five animals were consistently classified as displaying AUD-like behaviours. The variable composition of the AUD-like subpopulation suggests that the behavioural subcomponents contribute in different degrees to the AUD-like phenotype across individual animals. This is consistent with the behavioural heterogeneity seen in human AUD pathology. Together, the findings emphasise the importance of considering the individual variation and heterogeneity in behavioural components that together constitute the essence of AUD-like behaviour in preclinical studies.

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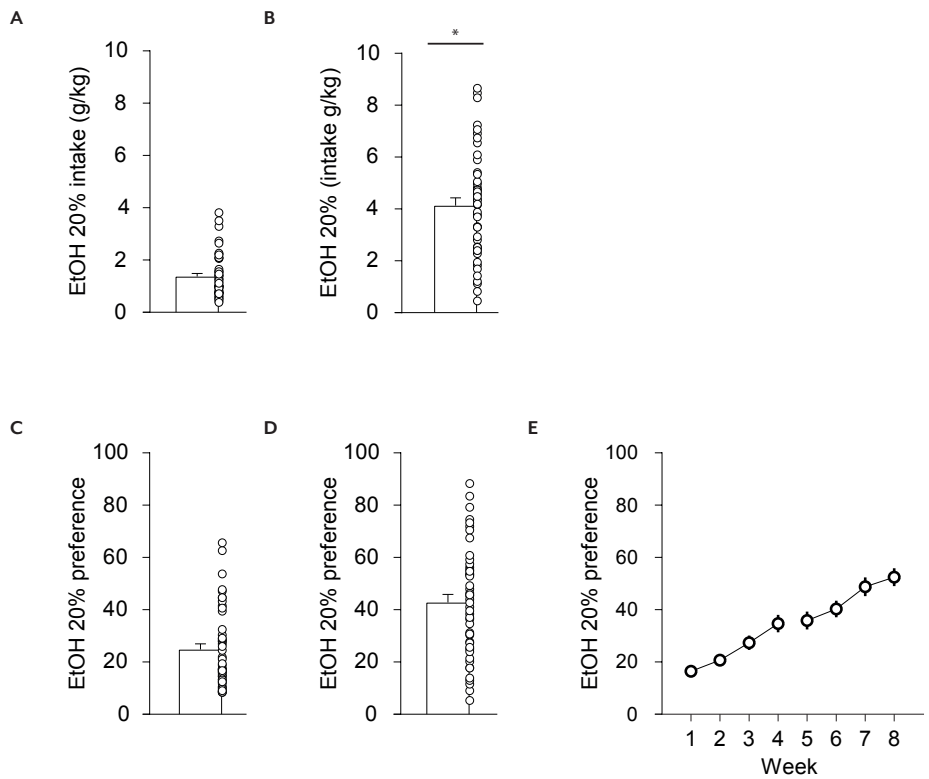
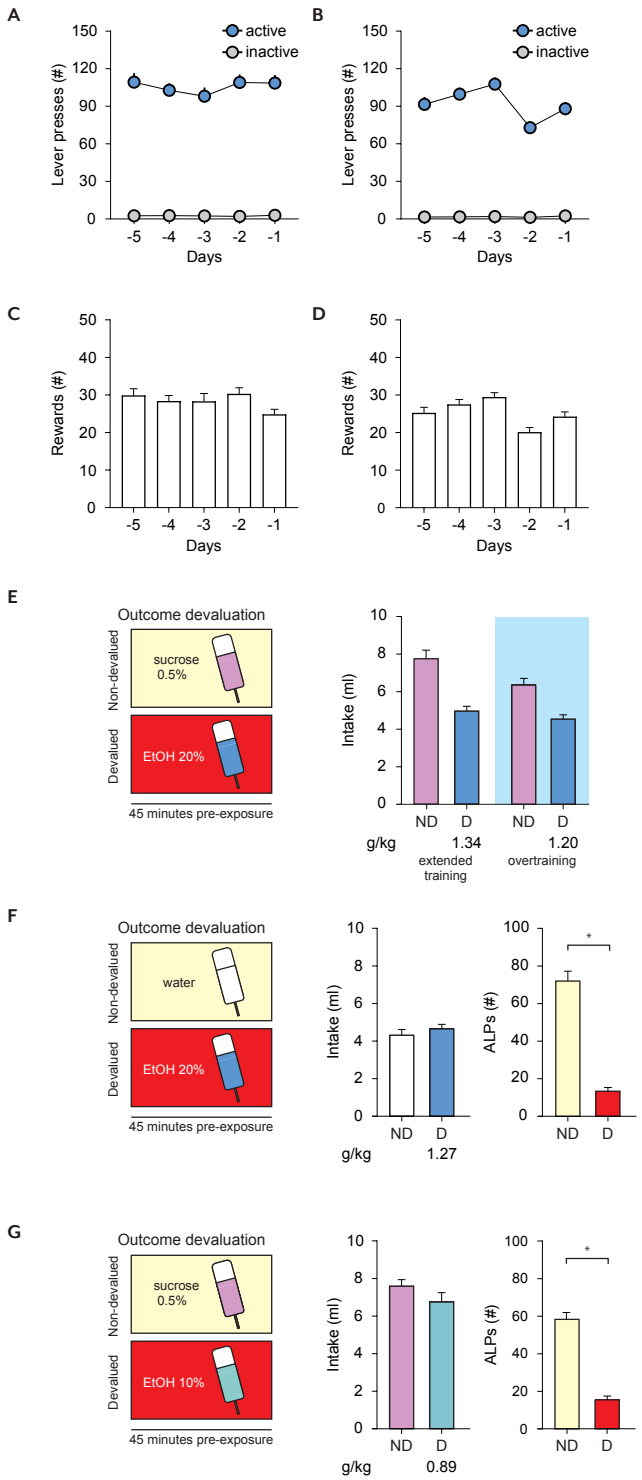


Supplementary Figure 1. Home cage alcohol intake.
A. Group average (bar) and distribution of individual values for alcohol intake averaged across all 7 hour sessions. **B.** Group average (bar) and distribution of individual values for alcohol intake averaged across all 24 hour sessions. Asterisk (*) denotes significance at a $p < 0.05$ level as compared to Supplementary Figure 1A. **C.** Group average (bar) and distribution of individual values for alcohol preference over water averaged across all 7 hour sessions. **D.** Group average (bar) and distribution of individual values for alcohol preference over water averaged across all 24 hour sessions. **E.** Group average of alcohol preference over water across 8 weeks of home cage drinking.
Group data are presented as the mean ± SEM.

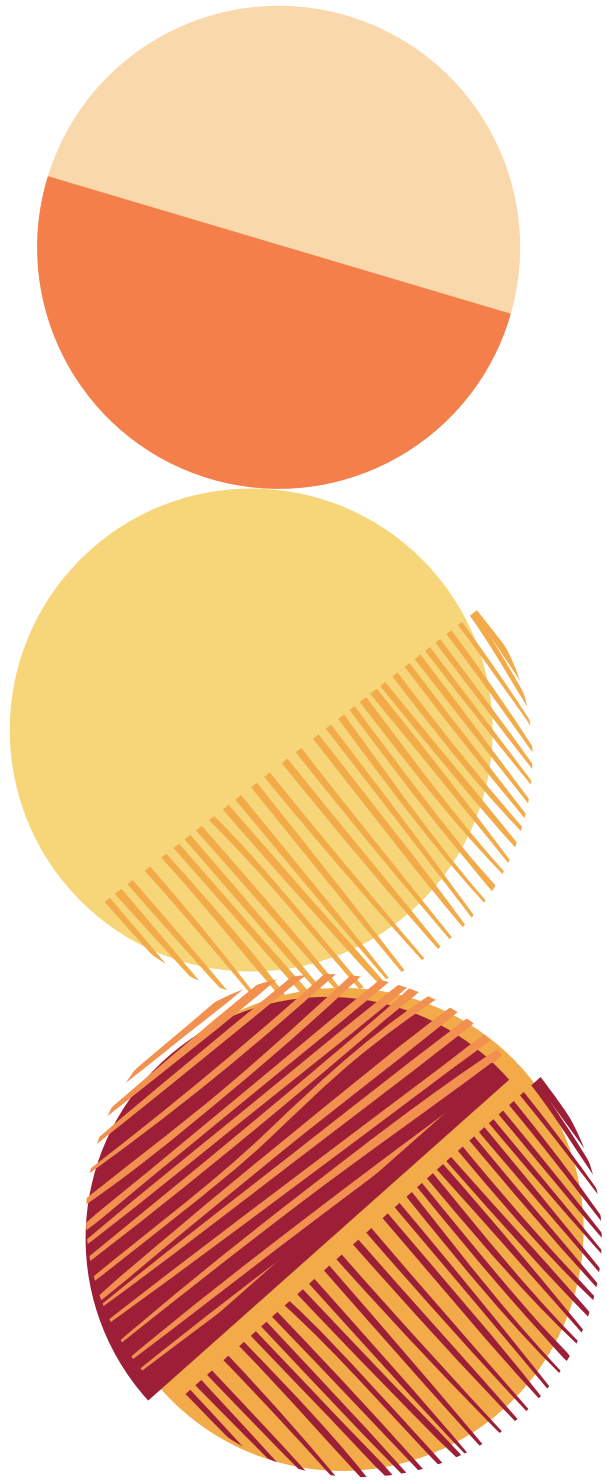


< Supplementary Figure 2. Habit formation.

A. Group average of the RR3 training response rate (active lever presses (blue), inactive lever presses (grey)) five days prior to outcome devaluation in the extended training phase. **B.** Group average of the RR3 training response rate (active lever presses (blue), inactive lever presses (grey)) five days prior to outcome devaluation in the overtraining phase. **C.** Group average of the rewards obtained during RR3 training five days prior to outcome devaluation in the extended training phase. **D.** Group average of the rewards obtained during RR3 training five days prior to outcome devaluation in the overtraining phase. **E.** Schematic of the pre-exposure procedure (left panel). Group average of solution intake during pre-exposure for the non-devalued (sucrose 0.5%, non-devalued, pink) or devalued (EtOH 20%, devalued, blue) condition for the extended training and overtraining (blue shaded) (right panel). Average intake (g/kg) of alcohol is indicated below the respective devalued conditions. **F.** Schematic of the pre-exposure procedure. All animals were pre-exposed (45 minutes) to a control solution (water, non-devalued, white) or an alcohol solution (EtOH 20%, devalued, blue) (left panel). Group average of solution intake during pre-exposure for the non-devalued (water, non-devalued, white) or devalued (EtOH 20%, devalued, blue) (middle panel). Average intake (g/kg) of alcohol is indicated below the devalued condition. Group averages of active lever presses (ALPs) made during the extinction test for the non-devalued (ND) and devalued (D) condition (right panel). **G.** Schematic of the pre-exposure procedure. All animals were pre-exposed (45 minutes) to a control solution (sucrose 0.5%, non-devalued, pink) or an alcohol solution with a lower concentration (EtOH 10%, devalued, blue) (left panel). Group average of solution intake during pre-exposure for the non-devalued (water, non-devalued, white) or devalued (EtOH 10%, devalued, blue) (middle panel). Average intake (g/kg) of alcohol is indicated below the devalued condition. Group averages of ALPs made during the extinction test for the non-devalued (ND) and devalued (D) condition (right panel).
Group data are presented as the mean ± SEM. Asterisk (*) denotes significance at a $p < 0.05$ level.

Supplementary Table 1. Ranking of the population on each behavioural measure.
All animals were ranked on their scores on alcohol intake, habit formation, motivation, and aversion resistance. 1 refers to the highest score within the population, 44 refers to the lowest score within the population.

	Ranking place	*Ranking place*	*Ranking place*	*Ranking place*
animal ID	**Alcohol intake**	**Habit formation**	**Motivation**	**Aversion resistance**
3	2	35	12	1
14	3	5	10	2
35	8	27	2	11
40	1	18	23	7
49	5	9	4	13
6	28	15	1	5
42	6	2	16	30
15	12	28	24	8
16	27	19	7	32
18	31	13	8	4
26	15	17	6	35
34	9	8	27	24
38	4	26	30	3
44	10	37	5	40
10	38	1	17	39
21	17	6	36	14
32	32	44	9	9
37	7	22	38	16
1	44	4	40	43
2	25	34	11	18
4	43	20	32	25
5	18	39	25	21
7	13	12	43	34
8	24	24	29	6
9	11	16	39	20
11	39	29	21	19
12	30	42	31	42
13	37	36	13	23
17	42	7	18	36
20	41	41	33	33
22	40	11	34	17
24	16	23	44	37
25	22	14	35	22
27	35	32	22	12
29	29	31	15	15
30	20	38	26	44
31	33	40	3	27
33	26	25	41	29
36	34	3	19	38
41	19	30	28	41
43	36	10	14	28
46	21	33	37	10
47	23	21	20	31
50	14	43	42	26



Chapter 6

Increased elasticity of sucrose demand during hyperdopaminergic states in rats

A. Maryse Minnaard[1,2]
Mieneke C. M. Luijendijk[2]
Annemarie M. Baars[1]
Lisa Drost[1]
Geert M. J. Ramakers[2]
Roger A. H. Adan[2]
Heidi M. B. Lesscher[1]
Louk J. M. J. Vanderschuren[1]

[1] Department of Population Health Sciences, Unit of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2] Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, The Netherlands

Abstract

Rationale
Deficits in cost-benefit decision making are a core feature of several psychiatric disorders, including substance addiction, eating disorders, depression, and bipolar disorder. Mesocorticolimbic dopamine signalling has been implicated in various reward- and cognition-related processes but its precise role in reward valuation and cost-benefit trade-off decisions remains incompletely understood.

Objectives
We aimed to determine the role of mesocorticolimbic dopamine signalling in the relationship between price and consumption of sucrose, to better understand its role in cost-benefit decisions.

Methods
Dopamine neurons in the ventral tegmental area (VTA) were chemogenetically activated in rats, and a behavioural economics approach was used to quantify the relationship between price and consumption of sucrose. Motivation for sucrose was also assessed under a progressive ratio (PR) schedule of reinforcement. To further gauge the role of dopamine in cost-benefit trade-offs for sucrose, the effects of treatment with D-amphetamine and the dopamine receptor antagonist alpha-flupentixol were assessed.

Results
Chemogenetic activation of VTA dopamine neurons increased demand elasticity, while responding for sucrose under a PR schedule of reinforcement was augmented upon stimulation of VTA dopamine neurons. Treatment with amphetamine partially replicated the effects of chemogenetic dopamine neuron activation, whereas treatment with alpha-flupentixol reduced free consumption of sucrose and had mixed effects on demand elasticity.

Conclusions
Stimulation of mesocorticolimbic dopaminergic neurotransmission altered cost-benefit trade-offs in a complex manner. It reduced the essential value of palatable food, increased incentive motivation and left free consumption unaltered. Together, these findings imply that mesocorticolimbic dopamine signalling differentially influences distinct components of cost-benefit decision making processes.

Keywords
Behavioural economics
Demand
Dopamine
DREADD
Motivation
Rats
Ventral tegmental area

Introduction

Every day we are confronted with situations requiring judgements and decisions. As such, the ability to make decisions on the basis of costs and benefits can be considered a cornerstone of adaptive behaviour. Value-based decision making entails a process in which humans and animals choose between competing courses of action by assessing the expected costs and the relative outcome values of each choice (Rangel et al., 2008; Tang et al., 2016). Deficits in cost-benefit trade-offs are a core feature of several psychiatric disorders, including substance addiction, eating disorders, depression, and bipolar disorder (American Psychiatric Association, 2013; Cáceda et al., 2014; Goschke, 2014). Therefore, a better understanding of the neurobiological mechanism underlying cost-benefit trade-off decisions will provide more insight into these pathologies.

Derived from the field of behavioural economics, operant-based methods have been developed to study the relationship between price and consumption (i.e. cost and benefit). Behavioural economics concentrates on the consumption of a commodity as a fundamental index of demand (Hursh et al., 2005), based on the consumer demand theory, which considers how demand for a certain commodity varies as a function of price (Hursh, 1980; Hursh et al., 1988). In rodent studies, the number of operant responses required per unit of reinforcer can be considered the price. The relation between the consumption of a commodity and its cost can then be described by a demand curve, which generally follows the law of demand: as its price increases the demand for a good decreases (Hursh et al., 1988; Watson & Holman, 1977).

A behavioural economic analysis of demand offers insightful measures derived from operant self-administration data: (1) demand elasticity (signified by α), which is the degree at which consumption decreases as price increases and (2) demand intensity (signified by Q_0), which is the consumption at a minimally constrained price (Hursh & Silberberg, 2008). When applying these concepts to operant rodent studies, demand

elasticity reflects the degree at which the number of earned rewards decreases as the required effort per reward increases, while demand intensity reflects the hypothetical number of rewards an animal would consume if the price was zero. Demand for a commodity is considered elastic when it decreases in response to proportionately small increases in price, whereas an inelastic demand reflects low sensitivity to price changes. Note that the distinction between elastic and inelastic demand is defined as a continuum since consumption of all goods eventually declines if the price is elevated sufficiently (Hursh et al., 2005). Moreover, the value of α also reflects the strength of a reinforcer as it is inversely related to the essential value (Hursh & Silberberg, 2008). That is, reinforcers with a steep declining demand curves have a higher α value and thus a lower essential value than reinforcers with an inelastic demand curve that declines slowly when price increases. The measure of demand elasticity is thought to go beyond a response rate-based measure by more fully characterising the relationship between the price of a reinforcer and its consumption. As such, behavioural economics offers a valuable tool to study the relationship between price and consumption and the underlying neurobiological mechanisms.

Another operant-based method to study the exertion of effort for appetitive rewards is the progressive ratio (PR) schedule of reinforcement (Hodos, 1961). PR schedules offer a robust tool to test the degree to which an animal maintains operant responding for reward as the response requirement increases after each reward delivery. The number of obtained rewards, lever presses, and the highest ratio achieved (i.e. breakpoint) in PR tasks are common measures that are thought to reflect incentive motivation (Richardson & Roberts, 1996; Salamone & Correa, 2012).

The mesocorticolimbic dopamine system is one of the most prominent neurobiological systems involved in reward-directed behaviour, specifically in processes such as behavioural activation, salience, reward prediction error signalling and incentive motivation (Berridge & Robinson, 1998; Bromberg-Martin et al., 2010; Hamid et al., 2016; Keiflin & Janak, 2015; Robbins & Everitt, 2007; Salamone & Correa, 2012; Schultz, 2016). The mesocorticolimbic dopamine system refers to the dopamine system originating in the ventral tegmental area (VTA), projecting to the nucleus accumbens and the prefrontal cortex. Especially dopamine signalling in the nucleus accumbens is thought to contribute to effort-based choice behaviour (Floresco, 2015; Floresco et al., 2008; Mai et al., 2012; Salamone et al., 2016b, 2016a). This has been shown, for instance, in studies where administration of dopamine D1 and D2 receptor antagonists reduced responding and breakpoints under a PR schedule of reinforcement in rats (Randall et al., 2012, 2014). Nucleus accumbens dopamine depletions have also been shown to make rats more sensitive to the effort requirements under ratio schedules (Aberman & Salamone, 1999; Ishiwari et al., 2004). Conversely, stimulation of forebrain dopamine signalling, through increased expression of nucleus accumbens dopamine D2 receptors, reduced expression of midbrain dopamine D2 autoreceptors or chemogenetic mesocorticolimbic dopamine neuron activation, increased motivational behaviour measured as increased responding under a PR schedule (Boekhoudt et al., 2018; Boender et al., 2014; de Jong et al., 2015; Trifilieff et al., 2013). Recently, behavioural economic studies have yielded comparable results for the role of dopamine in demand elasticity. Blockade of dopaminergic neurotransmission, using the dopamine receptor antagonist haloperidol, the dopamine depleting agent tetrabenazine or dopamine D2 receptor knockout mice, led to increased elasticity of demand (Salamone et al., 2017; Soto et al., 2016). Conversely, demand elasticity for cocaine was found to decrease upon chemogenetic midbrain dopamine neuron activation (Mahler et al., 2019). However, the way in which mesocorticolimbic dopamine signalling is involved in cost-benefit trade-offs remains incompletely understood. The role of dopamine in cost-benefit trade-offs might vary depending on the type of costs (i.e. physical or cognitive effort), for example (Hosking et al., 2015). A full characterisation of the relationship between price and consumption may provide insight into how dopamine modulates the willingness to work for a reward as the effort-based costs change.

Therefore, the purpose of this study was to determine the role of dopaminergic neurotransmission in the relationship between price and consumption of sucrose, using a chemogenetics approach in rats. We selectively activated dopamine neurons in the VTA and determined the effects on demand elasticity and demand intensity for sucrose within single sessions. Motivation for sucrose was also assessed in the same animals under a progressive ratio (PR) schedule of reinforcement. To further gauge the role of dopamine in cost-benefit trade-offs for sucrose, the effects of treatment with D-amphetamine and the dopamine receptor antagonist alpha-flupentixol were assessed. We hypothesised that, in accordance with previous rodent studies, increased VTA dopamine activation would increase responding to self-administer sucrose at higher costs, resulting in a decreased demand elasticity.

Materials and methods

Animals

A total of 49 male rats, comprising three experimental groups, were used in this study. Tyrosine hydroxylase (TH)::Cre transgenic rats (Witten et al., 2011) were bred in-house, by crossing heterozygous TH::Cre+/- (cre+) rats with wild type Long Evans mates. Experimental group I consisted of 17 TH::cre+ rats. Experimental group II was

a control group that consisted of 16 homozygous TH::Cre-/- (cre-) littermates of the TH::cre+ rats. Experiments with group I and II were performed in two batches (batch 1: n = 13 of which 7 TH::cre+; batch 2: n = 20 of which 10 TH::cre+) for practical reasons. Rats from experimental group I and II were approximately 10 weeks old and weighed 220 – 300 grams at the time of surgery (bodyweight (g), mean ± SEM: batch 1, 280 ± 7; batch 2, 265 ± 4). Experimental group III consisted of 16 adult Lister Hooded rats (Charles River, Sulzfeld, Germany), weighing 200 – 250 grams (approximately 8 – 10 weeks old) at the start of the experiment. Upon arrival, rats from experimental group III had eight days for acclimatisation, after which operant training commenced. All animals were experimentally naive.

The rats were individually housed in Macrolon type III sawdust bedded cages (42.5 x 26.6 x 18.5 cm) with ad libitum access to tap water. Initially, all rats had ad libitum access to standard chow (Rat and Mouse Breeder and Grower Expanded-CRM(E), Special Diet Service, UK). After the completion of training under the fixed ratio (FR) 1 schedule of reinforcement (see below), rats were food restricted (4 g of normal chow per 100 g body weight on training and test days, 6 g per 100 g body weight on remaining days) to approximately 85% of their free-feeding body weight in order to enhance the reinforcing value of sucrose (Yang et al., 2020). A polycarbonate rat tunnel (9 x 9 x 15 cm), a wood block and a tissue were provided for cage enrichment. The rats were kept under controlled temperature and humidity conditions (21 ± 2°C and 50 – 70% humidity) on a reversed 12 h/12 h light/dark cycle (lights off at 7.00 AM – lights on at 7.00 PM) to allow for behavioural testing in the dark phase. Background noise was provided by a constantly playing radio. The rats were weighed and handled at least once per week throughout the course of the experiment. All experimental procedures were approved by the Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee, and were conducted in accordance with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU).

Surgery

Prior to behavioural training, the rats from experimental groups I and II underwent intracranial surgery. The rats were anaesthetised with a ketamine/dexmedetomidine mixture (0.2 ml mixture/100 g body weight, intraperitoneal: 75 mg/kg ketaminehydrochloride, Narketan, 0.25 mg/kg Dexdomitor®, Pfizer Animal Health B.V., The Netherlands). Local anaesthesia was provided by xylocaine, sprayed on the skull (Lidocaine 100 mg/ml, AstraZeneca BV), once the animals were placed in a stereotactic apparatus (Kopf Instruments). The rats were injected bilaterally into the VTA with 0.8 µl of AAV-hSyn-DIO-hM3Dq-mCherry (1.3 x 10^{12} genomic copies/ml; UNC Vector Core), using the following coordinates: AP -5.5, ML +1.3, DV -8.1 (5° angle), in mm relative to Bregma. The virus was infused at a rate of 0.2 µl/min for 4 minutes, and the needle was left in place for 10 more minutes to allow for diffusion. Upon completion of the surgery, anaesthesia was terminated through the application of atipamezole (1.0 mg/kg, subcutaneously, Antisedan®, Pfizer Animal Health B.V., The Netherlands), and carprofen (5.0 mg/kg, subcutaneously, Carporal, AST Farma BV) was administered to the rats for pain relief on the day of surgery and the two following days. The rats were housed individually after surgery for the remainder of the experiment. Rats were housed one week under DM-II conditions after surgery to recover, followed by transportation to the animal facility where behavioural training and testing took place. After transportation, the rats had a minimum of eight days for acclimatisation to the reversed day light/dark cycle, after which operant training commenced (see Figure 1 for the experimental outline).

Drugs

Experimental groups I and II were treated with the selective Designer Receptor Exclusively Activated by Designer Drugs (DREADD) ligand clozapine-N-oxide (CNO) or vehicle. CNO (Enzo Life Sciences BVBA, Belgium) was dissolved in milliQ and dissolved CNO was kept at 4˚C in between injections, for a maximum of one week. The doses of 0.3 mg/kg and 1.0 mg/kg CNO were chosen based on previous work (Boekhoudt et al., 2016, 2017b, 2018; Boender et al., 2014).

Experimental group III was treated with D-amphetamine and flupentixol. D-Amphetamine (d-amphetamine sulphate; Spruyt Hillen bv, The Netherlands) and flupentixol (cis-[Z]-a-flupentixol dihydrochloride; Sigma-Aldrich, The Netherlands) were dissolved in sterile saline (0.9% NaCl). Doses of 0.5 mg/kg and 1.0 mg/kg D-amphetamine and of 0.25 mg/kg and 0.5 mg/kg flupentixol were chosen based on previous work (Boekhoudt et al., 2017a; Cardinal et al., 2000; Mayorga et al., 2000; Veeneman et al., 2011, 2012).

Prior to test injections, the rats were injected once with saline to habituate them to the injection procedure. All injections were given intraperitoneally at a volume of 1 ml per kg bodyweight. After injection, the rats were returned to their home cage, after which testing under the relevant training schedule started 30 minutes after each injection. For all experiments, each rat received drug and vehicle injections according to a within-subjects Latin-square design.

Apparatus

The rats were trained and tested in operant conditioning chambers (29.5 x 24 x 25 cm; Med Associates Inc., USA) equipped with two retractable levers (4.8 x 1.9 cm; ENV-112CM) and a white cue light (28 V, 100mA; ENV-221M) present above each lever. A recessed liquid dipper and food receptacle were situated in between the levers, equipped with an infrared beam for nose-poke detection. The wall on the opposite side of the box contained a white house light (28 V, 100mA; ENV-215M). The floor of the chamber was covered with a metal grid with bars separated by 1.57 cm. All chambers were situated in light- and sound-attenuating cubicles equipped with a ventilation fan, and were controlled by MED-PC IV software (version 4.2) for Windows.

Fixed ratio schedule of reinforcement

All rats were trained to respond for sucrose in 30 minutes operant sessions, once daily, 4 – 5 days per week. The house light was illuminated throughout the session. The position of the active and inactive levers was counterbalanced between rats. The animals were first trained to respond for sucrose under a FR 1 schedule of reinforcement. Pressing the active lever activated a pellet dispenser that delivered a 45 mg sucrose pellet (TestDiet, USA) into the food receptacle. Simultaneous with reward delivery, both levers were retracted and the cue light above the active lever was illuminated until 1 second after the animal entered the food receptacle. Next, the cue light was turned off and the levers were reintroduced, signalling the start of a new trial. All inactive lever presses were recorded but were without programmed consequences. After 8 – 12 FR 1 sessions, the animals were trained under a FR 5 schedule of reinforcement for sucrose for 8 – 10 sessions (Figure 1).

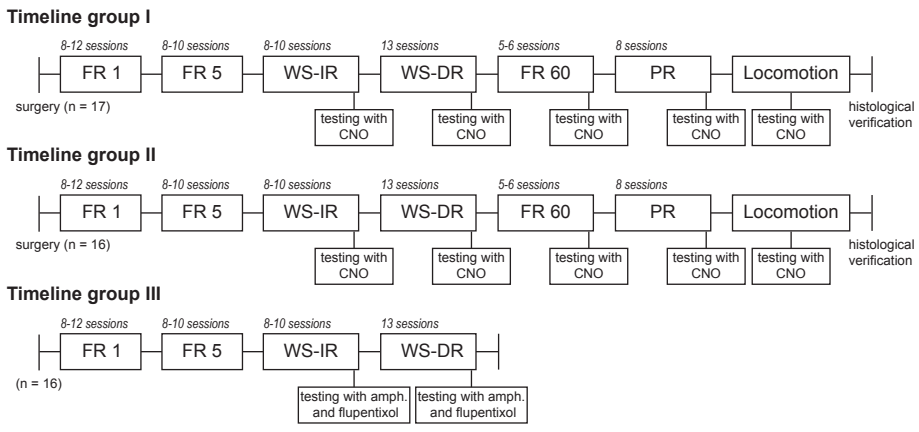


Figure 1. Schematic of experimental design. Timelines for experimental groups I, II, and III. The number of training sessions before testing is indicated in italics above each block. WS-IR: within session increasing ratio, WS-DR: within session decreasing ratio, amph.: D-amphetamine.

Within-session increasing/decreasing ratio schedule of reinforcement

Next, in order to determine demand curves, the rats were subjected to tests in which the ratio requirement increased within sessions (WS-IR) from 5 to 15, 30, 60, and 80. Each ratio requirement was offered for a block of 8 minutes separated by a 2-minute inter-block interval, that was signalled by retraction of the levers. Subsequently, the animals were trained in sessions in which the ratio requirement was decreased within sessions (WS-DR), from 80 to 60, 30, 15, and 5. Similar to WS-IR sessions, each session consisted of five blocks of 8 minutes, separated by 2-minute inter-block intervals. Experimental groups I, II and III were tested in WS-IR sessions, after 8 – 10 training sessions, and in WS-DR, after 13 training sessions (Figure 1).

Fixed ratio 60 schedule of reinforcement

After completion of the WS-IR and WS-DR tests, experimental groups I and II were trained (5 – 6 sessions) and tested under a FR 60 schedule of reinforcement (Figure 1). This schedule had a similar block design as the WS-IR and WS-DR ratio sessions. In an FR 60 session, 60 active lever presses were required to obtain a sucrose pellet in all five blocks of 8 minutes each with 2-minute inter-block intervals in between.

Progressive ratio schedule of reinforcement

Experimental groups I and II were subsequently trained (for 8 sessions) and tested under a progressive ratio (PR) schedule of reinforcement (Figure 1), in which the response requirement for a sucrose pellet progressively increased after each obtained reward (response requirement: 1, 2, 4, 6, 9, 12, 15, 20, 25, etc.) (Richardson and Roberts, 1996). A PR session ended when no reward was earned for 30 consecutive minutes.

Locomotor activity

Locomotor activity was assessed in experimental groups I and II (Figure 1). Subjects were placed individually in smooth, grey-painted plastic arenas (50 x 30 x 40 cm) 30 minutes after injection. Horizontal locomotor activity was registered using a camera positioned approximately 2 meters above the setup. Distance travelled (cm) and velocity (cm/s) were recorded and analysed using video tracking software (EthoVision XT 13, Noldus, The Netherlands) which determined the position of the animals five times per second. Locomotor activity was measured for 1 hour.

Tissue preparation and immunohistochemical analysis

The animals from experimental groups I and II were euthanised by an intraperitoneal injection of sodium pentobarbital (0.2 ml/100 g; Euthanimal 1709296-08, Alfasan,

the Netherlands), followed by a transcardial perfusion with 1x phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) (P6148, Sigma-Aldrich, the Netherlands) in PBS. Brains were dissected and post-fixed in 4% PFA in PBS at 4 ˚C for at least 24 hours, after which they were transferred to 30% sucrose in PBS at 4°C for at least three days.

Using a cryostat, 40 µm coronal slices were cut and stored in PBS with 0.05% sodium azide. The slices were washed 3x 15 minutes in PBS and then blocked for 1 hour in PBS containing 10% v/v normal goat serum (Ab156046, Abcam plc, UK) and 0.25% v/v Triton-X100 at room temperature. Next, the slices were placed in PBS containing the primary antibodies (Rabbit anti-dsRed 1:750, #632496, Clontech, Takara Bio USA Inc, USA; Mouse anti-Th 1:750, MAB318, Sigma Aldrich) and 10% normal goat serum overnight at 4°C. At room temperature, the slices were subsequently washed 3x 15 minutes in PBS and placed in PBS containing the secondary antibodies (Goat anti-Rabbit 568, 1:750, A11011, Thermo Fisher; Goat anti-Mouse 488, 1:750, ab150113, Abcam plc, UK) and 2% normal goat serum for 2h in the dark. Finally, the slices were washed 3x 15 minutes in PBS and mounted onto microscope slides (Thermo Superfrost), dried and covered using Fluorsave (EMD Millipore Corporation, USA) and a coverslip.

To check for co-localisation of TH and hM3Dq-mCherry expression, images were captured at 2x magnification using Olympus BX60 upright microscope and Leica Application Suite software (Leica Microscopy B.V., the Netherlands). Slides were illuminated with bright-field, fluorescein isothiocyanate (FITC) (515 nm; green) and tetramethylrhodamine (TRITC) (640 nm; red).

Exclusion criteria

Two animals, both TH::cre+, from batch 1 were excluded from the experiment before operant training started because they did not recover sufficiently from surgery. Histological verification of infusion sites and viral expression was performed as an inclusion criterion. For experimental group I (i.e. TH::cre+ rats), only animals that showed bilateral expression of hM3Dq-mCherry in the VTA were included in analyses. Two TH::cre+ rats were excluded because no viral expression was detected. For experimental group II (i.e. TH::cre- rats), all animals were included in analyses as none of them showed expression of hM3DGq-mCherry.

Data analysis

The number of rewards obtained during the WS-IR, WS-DR, FR 60, and PR sessions was measured per subject. Sessions in which an animal obtained < 5 rewards were excluded from further analyses since such low levels of responding hamper reliable analyses of the data. Based on this criterion, we had to exclude six animals from the flupentixol analyses as their response rates were strongly diminished when tested with the 0.5 mg/kg flupentixol dose. To avoid bias, all data from the 0.5 mg/kg flupentixol dose was excluded from further analyses. Moreover, one animal from experimental group II was excluded from analysis of the WS-DR data and one animal from experimental group III was excluded from analysis of the flupentixol treatment during WS-DR data due to obtaining < 5 rewards.

For experimental groups I and II, the effects of CNO treatment on the number of rewards obtained under the WS-IR, WS-DR and FR60 schedules were analysed using repeated measures analysis of variance (ANOVA) tests with *dose* and *block* as within-subject variables and *group* (TH::cre+ or TH::cre-) as the between-subject variable. Number of rewards and breakpoint data from PR sessions were analysed using repeated measures ANOVAs with *dose* as the within-subject variable and *group* (TH::cre+ or TH::cre-) as the between-subject variable. Since behavioural effects of CNO treatment compared to vehicle did not differ between experimental batches, these data were pooled for analysis. For experimental group III, the effects of D-amphetamine and flupentixol on the number of rewards were analysed using repeated measures ANOVAs with *dose* and *block* as the within-subject variables.

Subsequently, a behavioural demand analysis was executed on the data (i.e. number of rewards obtained) derived from the WS-IR and WS-DR sessions. Demand can be modelled using the exponential demand function (Hursh & Silberberg, 2008). The exponential demand function is defined as: $\log Q = \log(Q_0) + k \times (e^{-\alpha \times Q_0 \times C} - 1)$. In this function, Q is units of consumption (i.e. 3 rewards equals $Q = 3$), Q_0 is consumption at a minimally constrained price, and C is the cost requirement (i.e. FR 15 would have $C = 15$). Parameter k is the number of logarithmic units spanned by the demand curve e indicates the base of the natural logarithm and α represents the rate constant of the exponential. Both α and Q_0 are estimated from the best fit function. Additionally, the gauge of the substantive significance of the model is given as R^2. The R^2 reflects how well the model fits the data by denoting the proportion of data variance that the equation accommodates.

Consumption during the WS-IR and WS-DR sessions was measured per subject and per ratio with units defined as 'number of rewards earned'. To prevent zero values and to permit logarithmic transformations, 0.001 was added to each consumption value. Next, exponential demand functions, as detailed above, were fit to the data using the GraphPad Prism template that was kindly provided by the Institute for Behavioral Resources, Inc. website (http://ibrinc.org/software/). The overall mean

performance was first analysed to determine the best-fitting *k* parameter, which was used across all demand curve fits. For experimental groups I and II the value of the *k* parameter used was 2.116, and for experimental group III, the best-fitting *k* parameter used was 1.667. Separate demand curves were fit to consumption values for individual subjects to determine individually fit α and Q_0 values and R^2, which indicates the goodness of fit. Curves, and derived α and Q_0 values, with poor model fit ($R^2 \leq 0.30$) were discarded from analyses (Bentzley et al., 2013; Cohn et al., 2020; Fragale et al., 2017; Leonard et al., 2017; Murphy et al., 2009). Due to poor model fit, a total of five individual curves was excluded: one curve from the WS-DR in experimental group I, three curves from the WS-IR in experimental group II, and three curves from the WS-DR in experimental group III.

Behavioural economics provides a framework for studying demand from the level of the individual through that of large numbers of consumers (Hursh & Roma, 2016; Hursh & Silberberg, 2008). To fully exploit this asset, demand curves were analysed both at an individual level as well as at a population level. Individually fit α and Q_0 values were used for the primary analysis to determine group and dose differences. For experimental groups I and II, ANOVAs were performed with *dose* as a within-subject variable and *group* (TH::cre+ or TH::cre-) as the between-subject variable. For experimental group III, ANOVAs were performed with *dose* as a within-subject variable to assess the effects of D-amphetamine, and with paired t-tests and Wilcoxon matched-pairs signed rank tests to assess the effects of flupentixol. A secondary analysis on the demand functions per population was conducted using an extra sum-of-squares F-test to determine whether the best-fit values for demand curve parameters significantly differed over dosages. The null hypothesis was that parameters did not differ and therefore a single demand curve fit the data from different doses. A significant F-statistic indicated that a single demand curve could not accommodate data from different doses. In that case, separate demand curves per dose offer a better accommodation of the data.

Horizontal locomotor activity was expressed as travelled distance (cm) in 5 min time bins. The effects of CNO administration on locomotor activity were analysed in experimental groups I and II using a repeated measures ANOVA tests with *dose* and *time bin* as the within-subject variables and *group* (TH::cre+ or TH::cre-) as the between-subject variable.

For every ANOVA and t-test, normal distribution of the data was assessed. Locomotor activity, demand elasticity variables derived from experimental group I and II, and demand elasticity variables from D-amphetamine tests derived from experimental group III were transformed prior to statistical analyses by natural log to allow for parametric testing. Whenever the difference scores of the flupentixol measurements compared to vehicle were not normally distributed, a Wilcoxon matched-pairs signed rank test was used. This was the case for the demand elasticity WS-IR and demand intensity WS-DR variables. A total of twelve statistical outliers were removed from the analyses: three datapoints from the WS-IR in experimental group I, three datapoints from the WS-IR in experimental group II, and six datapoints from experimental group III. Mauchly's test of sphericity was used to test whether variances of the differences between levels were equal. If the assumption of sphericity was violated, degrees of freedom were corrected using Greenhouse-Geisser (GG) estimates of sphericity or Huynh-Feldt estimates of sphericity when the GG estimate was > 0.75. Corrected degrees of freedom are presented rounded to the nearest integer. When significant main effects or interactions were detected, post hoc analyses were conducted using pairwise comparisons with Bonferroni corrections.

Data were analysed and visualised using Microsoft Excel, GraphPad Prism (version 8.3.0, GraphPad Software Inc., USA) and SPSS for Windows (version 25.0.0.1, IBM Corp., USA). Results are presented as mean ± SEM unless otherwise stated. A significance criterion of $p < 0.05$, two-tailed, was used for all statistical analyses.

Results

Experimental group I and II: effects of chemogenetic activation of VTA dopamine neurons

Virus expression

Immunohistochemical analysis confirmed DREADD expression (hM3Dq-mCherry) in dopamine neurons in the VTA in TH::cre+ animals (Figure 2). In absence of Cre (in TH::cre- animals), no DREADD expression was observed (data not shown).

Within-session increasing ratio

Responding for sucrose was assessed following treatment with CNO under a within-session increasing ratio (WS-IR) schedule of reinforcement. CNO treatment significantly reduced the number of rewards obtained in TH::cre+, but not in TH::cre- rats, and this effect was dependent on block (Figure 3A-B; $F(2,40)_{dose} = 21.861$, $p < 0.001$; $F(2,40)_{dose \times group} = 19.724$, $p < 0.001$; $F(4,116)_{dose \times block} = 5.409$, $p < 0.001$; $F(4,116)_{dose \times block \times group} = 6.473$, $p < 0.001$). Post-hoc analyses showed that both the 0.3 mg/kg and 1.0 mg/kg CNO dose reduced the number of rewards obtained compared to vehicle in every block in TH::cre+ animals ($p < 0.01$). In contrast,

CNO treatment had no significant effect on the number of rewards in any of the blocks in TH::cre- animals. Thus, chemogenetic activation of VTA dopamine neurons decreased the number of rewards obtained across the ratio requirements.

Individual demand curves were plotted and the derived parameters R^2, α, and Q_0 were compared. For all treatments, individual demand data fitted well to the model as the average R^2 was above 0.80 (i.e. [mean ± standard deviation] $R^2_{vehicle}$: 0.85 ± 0.21; $R^2_{CNO\ 0.3}$: 0.89 ± 0.12; $R^2_{CNO\ 1.0}$: 0.86 ± 0.18). CNO treatment significantly increased demand elasticity in TH::cre+, but not in TH::cre- rats (Figure 3C; $F(2,42)_{dose} = 9.101$, $p = 0.001$; $F(2,42)_{dose\ x\ group} = 19.854$, $p < 0.001$). Post-hoc analyses showed that both the 0.3 mg/kg and the 1.0 mg/kg CNO dose increased demand elasticity compared to vehicle in TH::cre+ animals ($p < 0.001$). In contrast, CNO treatment had no significant effect on demand elasticity in TH::cre- animals. Moreover, demand intensity (Q_0) was not affected by CNO treatment in either group (Figure 3D; $F(2,42)_{dose} = 0.553$,

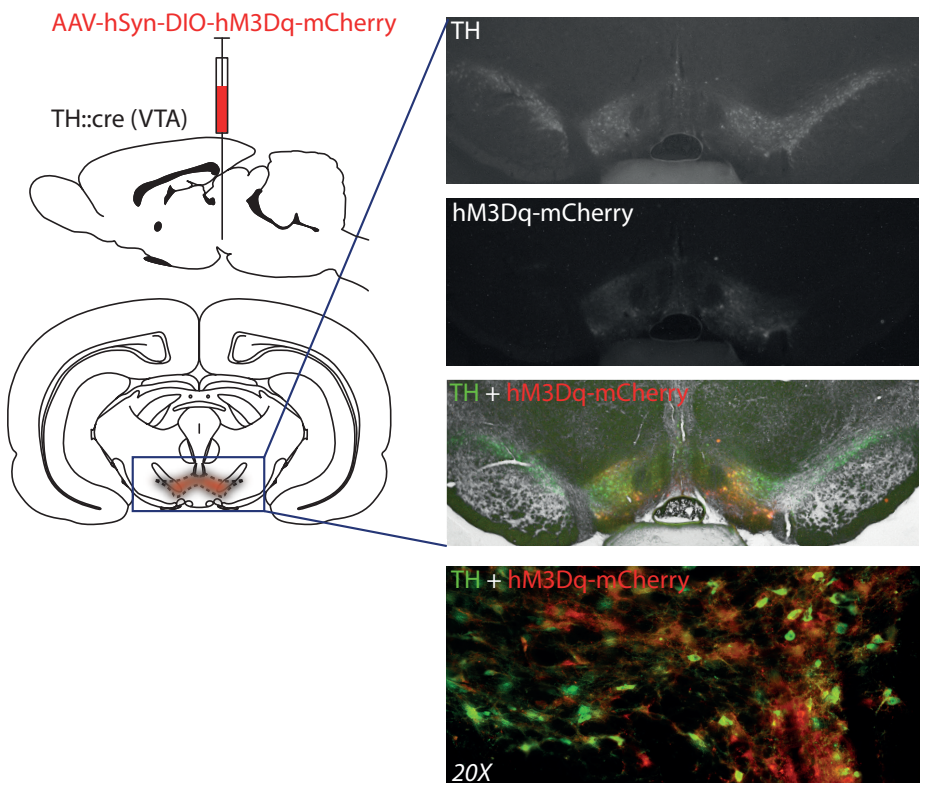


Figure 2. Expression of AAV-hSyn-DIO-hM3Dq-mCherry in experimental group I (TH::cre+) animals. Representative example of a TH::cre+ rat injected with AAV-hSyn-DIO-hM3Dq-mCherry of the ventral tegmental area (VTA). Expression is shown in coronal slices -5.6 mm posterior to Bregma. Atlas image adapted from Paxinos and Watson, 2004.

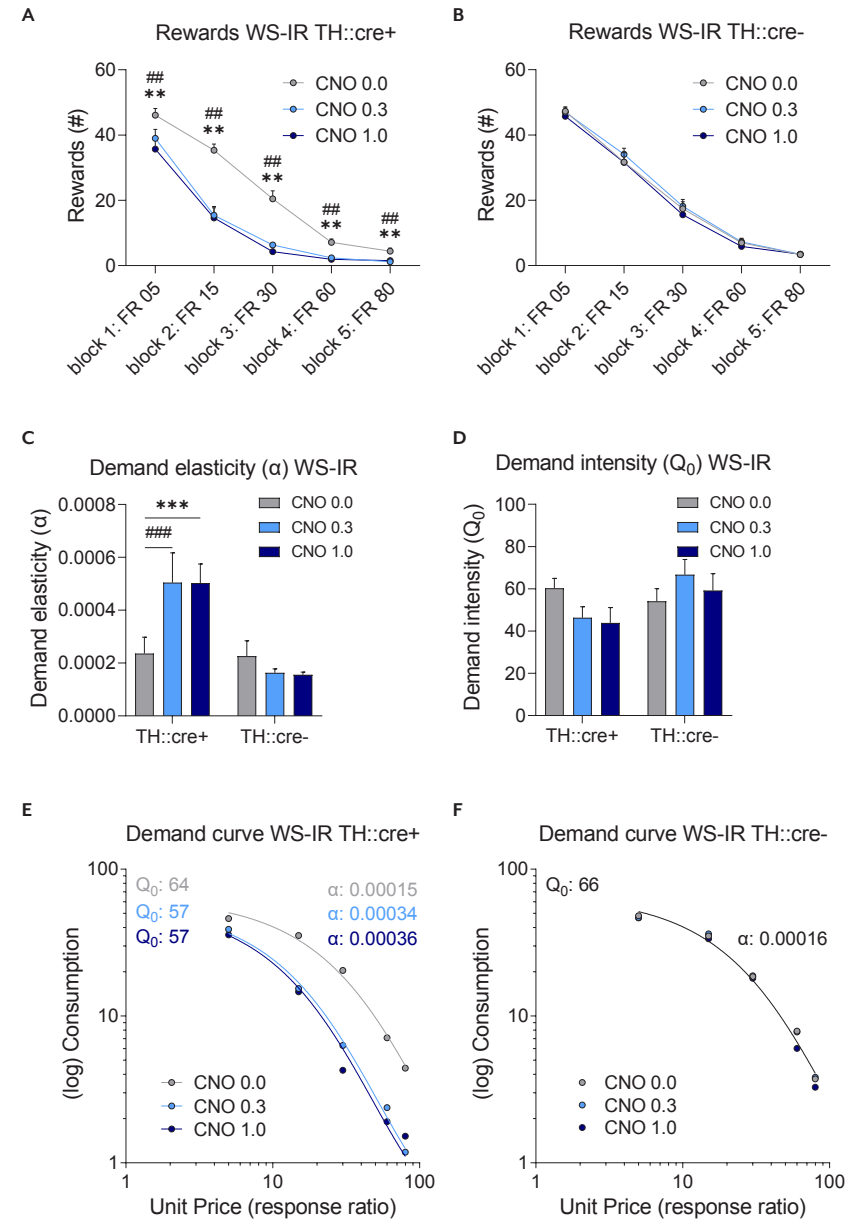


Figure 3. The effects of CNO on the number of rewards, demand elasticity (α) and intensity (Q_0), and demand curve when measured under a within session increasing ratio (WS-IR) schedule in experimental groups I (TH::cre+) and II (TH::cre-). Effects of CNO on number of rewards obtained in TH::cre+ rats (**A**) and TH::cre- rats (**B**) when assessed in a WS-IR task. Effects of CNO on demand elasticity (**C**) and demand intensity (**D**) based on individual demand curve analysis. Effects of CNO on population demand curve in TH::cre+ rats (**E**) and TH::cre- rats (**F**). Data in panels A-D are presented as the mean + SEM. ** CNO 1.0 different from vehicle, $p < 0.01$; ## CNO 0.3 different from vehicle, $p < 0.01$; *** CNO 1.0 different from vehicle, $p < 0.001$; ### CNO 0.3 different from vehicle, $p < 0.001$.

p = 0.579; $F(1,21)_{group}$ = 2.096, p = 0.162; $F(2,42)_{dose \times group}$ = 2.925, p = 0.065). Taken together, chemogenetic activation of VTA dopamine neurons increased demand elasticity without significantly affecting demand intensity.

Next, demand curves based on group means were plotted and analysed separately per TH::cre group. The extra sum-of-squares F-test showed that a global fit could not accommodate all data in the TH::cre+ group (F(4,9) = 28.000, p < 0.001). Therefore, demand curves and the derived best-fit values of α and Q_0 were different for each dose in the TH::cre+ group, with an increased α and decreased Q_0 when under the influence of CNO (Figure 3E). In the TH::cre- group, the extra sum-of-squares F-test indicated that parameters did not differ across the different doses and that a single demand curve with an R^2 of 0.99 fit the data from different doses (Figure 3F; F(4,9) = 0.930, p = 0.489). This confirms that CNO treatment had no significant effect on demand in the TH::cre- animals. Thus, analysis of group demand curves suggests that chemogenetic activation of VTA dopamine neurons shifted the demand curve to one with an increased demand elasticity and decreased demand intensity (Q_0).

Within-session decreasing ratio

To rule out potential satiety effects that might arise as the session and number of obtained rewards progressed, the animals were also tested under a reversed schedule of reinforcement (i.e. within-session decreasing ratio, WS-DR). Similar ratio requirements as for the WS-IR schedule of reinforcement were used, but the ratio requirements were presented in a reversed order, such that the required effort per reward decreased over blocks.

Analysis of the data for responding under the WS-DR schedule of reinforcement revealed that CNO treatment significantly decreased the number of rewards obtained in TH::cre+, but not in TH::cre- rats, and the effect was not dependent on block (Figure 4A-B; $F(2,52)_{dose}$ = 7.366, p = 0.002; $F(2,52)_{dose \times group}$ = 8.606, p = 0.001; $F(4,116)_{dose \times block}$ = 1.043, p = 0.392). Post-hoc analyses showed that both the 0.3 mg/kg and 1.0 mg/kg CNO dose decreased the number of earned rewards compared to vehicle in TH::cre+ animals (p < 0.005). This effect of CNO was consistent over blocks ($F(4,116)_{dose \times block \times group}$ = 1.813, p = 0.124). In contrast, CNO treatment had no significant effect on the number of rewards in TH::cre- animals. Thus, chemogenetic activation of VTA dopamine neurons decreased the number of rewards earned across the ratio requirements.

Individual demand curves were plotted and the derived parameters R^2, α, and Q_0 were compared. For all treatments, individual demand data fitted well to the model as the average R^2 was above 0.90 (i.e. [mean ± standard deviation] $R^2_{vehicle}$:

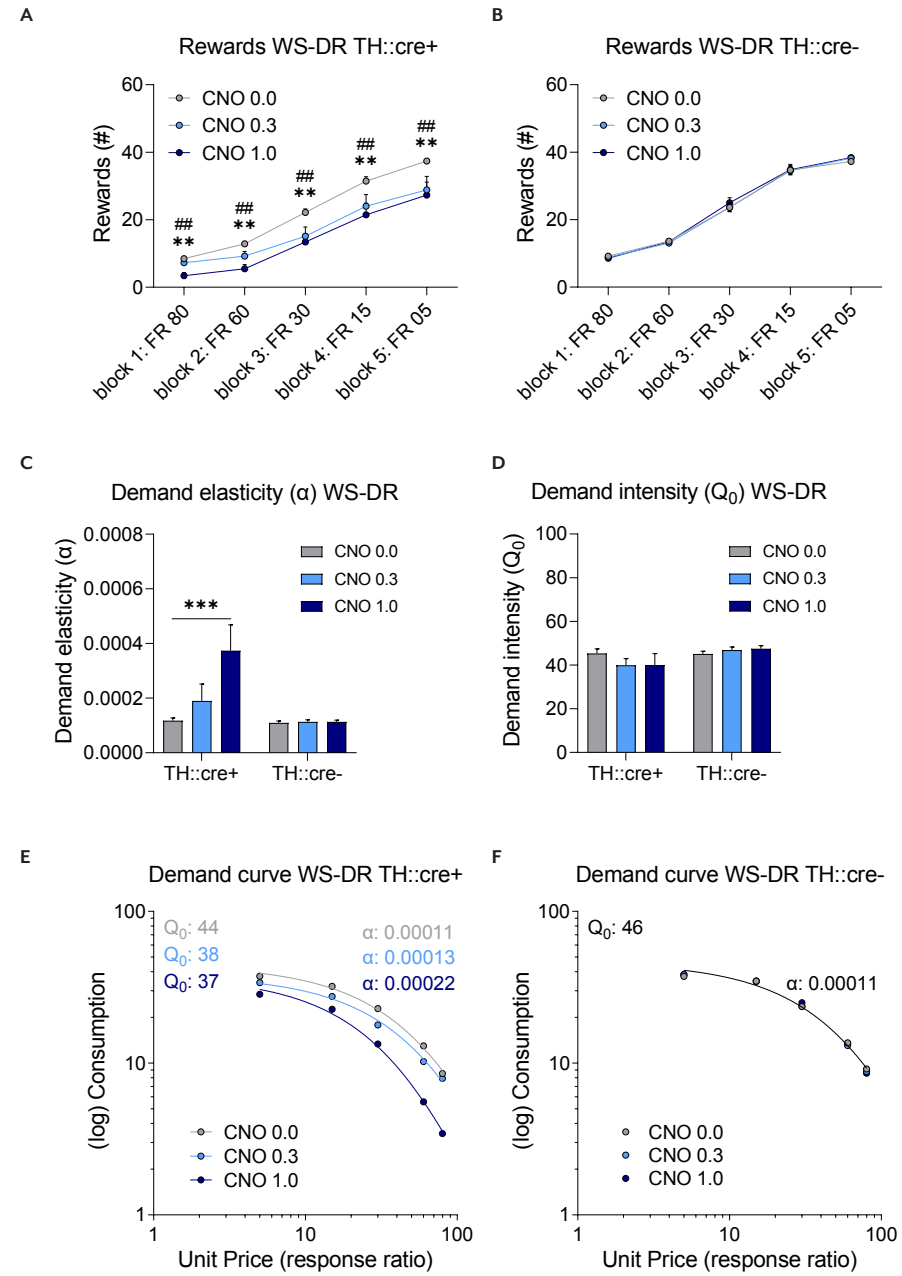


Figure 4. The effects of CNO on the number of rewards, demand elasticity (α) and intensity (Q_0), and demand curve when measured under a within session decreasing ratio (WS-DR) schedule in experimental groups I (TH::cre+) and II (TH::cre-). Effects of CNO on number of rewards obtained in TH::cre+ rats (**A**) and TH::cre- rats (**B**) when assessed in a WS-DR task. Effects of CNO on demand elasticity (**C**) and demand intensity (**D**) based on individual demand curve analysis. Effects of CNO on population demand curve in TH::cre+ rats (**E**) and TH::cre- rats (**F**). Data in panels A-D are presented as the mean + SEM. ** CNO 1.0 different from vehicle, p < 0.01; ## CNO 0.3 different from vehicle, p < 0.01; *** CNO 1.0 different from vehicle, p < 0.001.

0.96 ± 0.04; $R^2_{CNO\ 0.3}$: 0.95 ± 0.10; $R^2_{CNO\ 1.0}$: 0.93 ± 0.08). CNO treatment significantly increased demand elasticity in TH::cre+, but not in TH::cre- rats (Figure 4C; $F(2,48)_{dose} = 11.623$, $p < 0.001$; $F(2,48)_{dose\ x\ group} = 10.552$, $p < 0.001$). Post-hoc analyses showed that the 1.0 mg/kg CNO dose increased demand elasticity compared to vehicle in TH::cre+ animals ($p < 0.001$). In contrast, CNO treatment had no effect on demand elasticity in TH::cre- animals. Moreover, demand intensity (Q_0) was not affected by CNO treatment in either group (Figure 4D; $F(1,24)_{group} = 3.211$, $p = 0.086$; $F(1,33)_{dose} = 0.483$, $p = 0.551$; $F(1,33)_{dose\ x\ group} = 2.286$, $p = 0.132$). These findings indicate that chemogenetic activation of VTA dopamine neurons increased demand elasticity without significantly affecting demand intensity.

Next, demand curves based on group means were plotted and analysed separately per TH::cre group. The extra sum-of-squares F-test showed that a global fit could not accommodate all data in the TH::cre+ group ($F(4,9) = 86.000$, $p < 0.001$). Demand curves and the derived best-fit values of α and Q_0 were different for each dose in the TH::cre+ group, with an increased α and decreased Q_0 when under the influence of CNO (Figure 4E). In the TH::cre- group, the extra sum-of-squares F-test indicated that parameters did not differ across the different doses and that a single demand curve with an R^2 of 0.99 fit the data from different doses (Figure 4F; $F(4,9) = 0.160$, $p = 0.952$). Together, these group demand curve analyses revealed that chemogenetic activation of VTA dopamine neurons shifted the demand curve to one with an increased demand elasticity and decreased demand intensity (Q_0).

Fixed ratio 60

As these results were obtained in sessions in which the required effort per reward changed over time blocks, a certain level of behavioural flexibility might be required, which may be compromised as a result of hyperactivity of VTA dopamine cells (Floresco, 2013; Izquierdo et al., 2017; Verharen et al., 2019). Therefore, to control for potential CNO treatment effects on flexibility in reward seeking behaviour, responding of the animals, following treatment with CNO or vehicle, was also assessed under a FR 60 schedule of reinforcement. In this schedule, the required effort per reward was high but did not change over time blocks.

CNO treatment significantly decreased the number of rewards obtained in TH::cre+, but not in TH::cre- rats, and the effect was not dependent on block (Figure 5A-B; $F(1,39)_{dose} = 13.266$, $p < 0.001$; $F(1,39)_{dose\ x\ group} = 27.009$, $p < 0.001$; $F(5,137)_{dose\ x\ block} = 1.623$, $p = 0.157$). Post-hoc analyses showed that both the 0.3 mg/kg and the 1.0 mg/kg CNO dose decreased the number of rewards earned compared to vehicle in TH::cre+ animals ($p < 0.001$). This effect of CNO in the TH::cre+ group was consistent

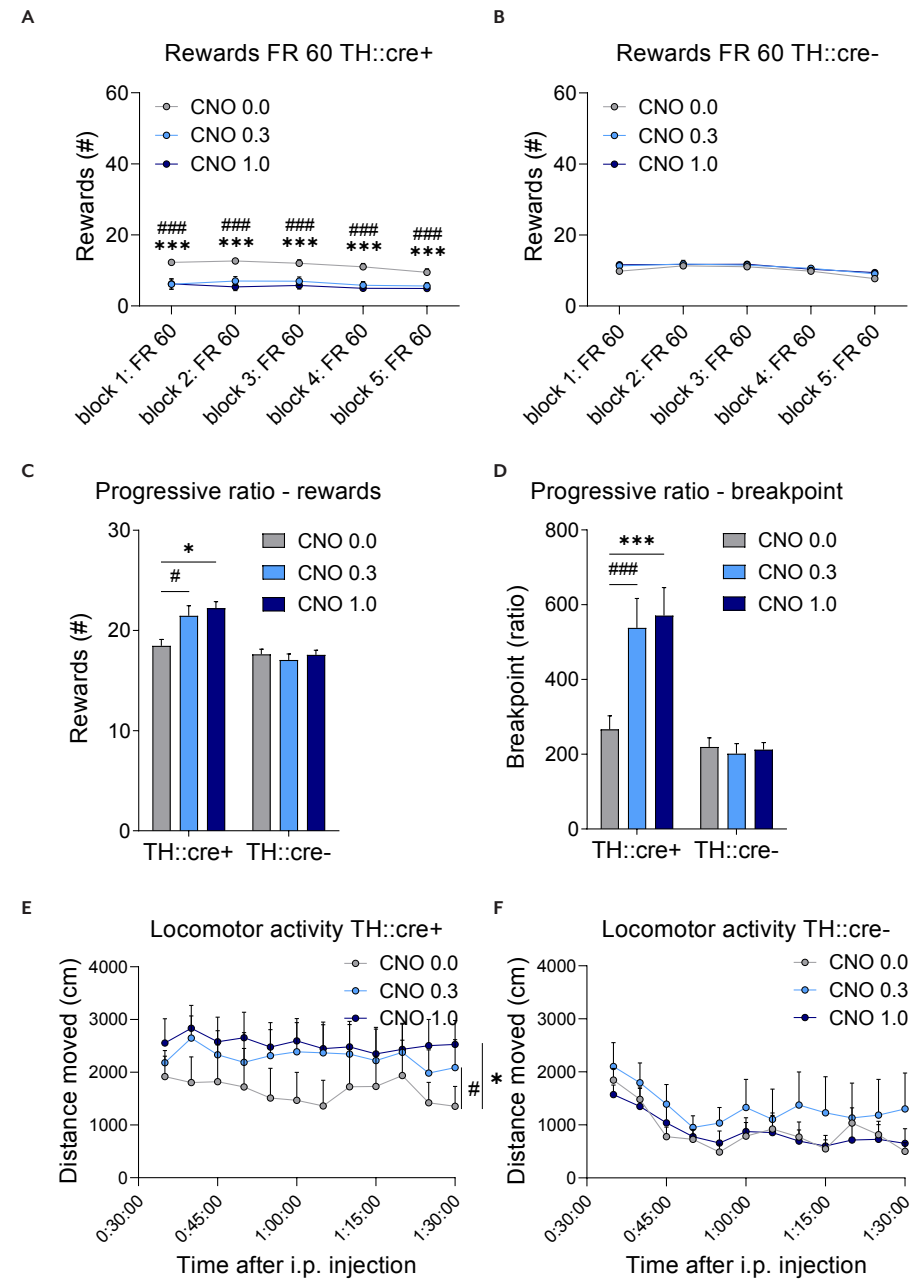


Figure 5. The effects of CNO on performance in fixed ratio (FR) 60 sessions, progressive ratio (PR) sessions, and locomotor activity in experimental groups I (TH::cre+) and II (TH::cre-). Effects of CNO on number of rewards obtained in TH::cre+ rats (**A**) and TH::cre- rats (**B**) when assessed in a FR 60 task. Effects of CNO on number of rewards obtained (**C**) and breakpoint (**D**) in TH::cre+ and TH::cre- rats when assessed in a PR task. Effects of CNO on distance moved in TH::cre+ rats (**E**) and TH::cre- rats (**F**) when locomotor activity was assessed. Data are presented as the mean + SEM. *** CNO 1.0 different from vehicle, $p < 0.001$; ### CNO 0.3 different from vehicle, $p < 0.001$; * CNO 1.0 different from vehicle, $p < 0.05$; # CNO 0.3 different from vehicle, $p < 0.05$.

over blocks ($F(5,137)_{dose\ x\ block\ x\ group} = 0.650$, $p = 0.664$). In contrast, CNO treatment had no effect on the number of rewards in the TH::cre- animals. The number of rewards slightly decreased during the session as an effect of block was observed, but this was not different between the groups ($F(2,63)_{block} = 12.136$, $p < 0.001$; $F(2,63)_{block\ x\ group} = 0.760$, $p = 0.491$). Post-hoc analyses showed that the number of rewards obtained was significantly lower in the final block compared to all other blocks ($p < 0.05$), which might reflect a satiation effect. Thus, chemogenetic activation of VTA dopamine neurons decreased the number of rewards even when flexibility was not required in the task.

Progressive ratio

Besides demand curve analyses, the effects of CNO treatment on motivation were assessed using a PR schedule of reinforcement in the same animals. Dopamine has been strongly associated with motivation and incentive motivational aspects can influence the cost-benefit trade-offs. Therefore, assessment of the effects of CNO treatment on PR task performance in the same animals would provide a deeper understanding of the role of dopaminergic neurotransmission in incentive motivational aspects and the relationship between price and consumption of sucrose.

CNO treatment significantly increased the number of rewards obtained and the breakpoint in TH::cre+, but not in TH::cre- rats (Figure 5C-D; rewards: $F(2,46)_{dose} = 7.153$, $p = 0.003$; $F(2,46)_{dose\ x\ group} = 9.343$, $p = 0.001$; breakpoint: $F(2,54)_{dose} = 10.847$, $p < 0.001$; $F(2,54)_{dose\ x\ group} = 12.715$, $p < 0.001$). Post-hoc analyses showed that both the 0.3 mg/kg and 1.0 mg/kg CNO dose increased the number of rewards and the breakpoint compared to vehicle in TH::cre+ animals (rewards: $p < 0.05$; breakpoint: $p < 0.001$). In contrast, CNO treatment had no effect on the number of rewards and the breakpoint in TH::cre- animals. These results show that chemogenetic activation of VTA dopamine neurons increased the number of rewards obtained and the breakpoint under a PR schedule of reinforcement.

Locomotor activity

As a non-motivation-related functional control for activation of VTA dopamine neurons, the effect of CNO on locomotor activity was assessed. Chemogenetic activation of these neurons was previously shown to induce a hyperactive phenotype (Boekhoudt et al., 2016; Wang et al., 2013). CNO treatment significantly increased the distance travelled in TH::cre+, but not in TH::cre- rats (Figure 5E-F; $F(2,46)_{dose} = 4.741$, $p = 0.018$; $F(2,46)_{dose\ x\ group} = 3.370$, $p = 0.049$). Post-hoc analyses showed that both the 0.3 mg/kg and 1.0 mg/kg CNO dose increased distance travelled compared to vehicle in TH::cre+ animals ($p < 0.05$). By contrast, CNO treatment had no effect on locomotor activity in TH::cre- animals. The effect of CNO was constant throughout the test as no significant interaction effect of CNO with time bin was observed ($F(7,200)_{dose\ x\ time\ bin} = 0.997$, $p = 0.437$; $F(7,200)_{dose\ x\ time\ bin\ x\ group} = 0.735$, $p = 0.651$). Thus, chemogenetic activation of VTA dopamine neurons increased locomotor activity in TH::cre+ rats.

Experimental group III: effects of D-amphetamine and flupentixol

Within-session increasing ratio

Responding for sucrose under a WS-IR schedule of reinforcement was determined following systemic treatment with D-amphetamine and flupentixol. D-amphetamine treatment significantly decreased the number of rewards obtained (Figure 6A; $F(1,21)_{dose} = 9.958$, $p = 0.002$). Post-hoc analysis showed that the number of rewards at the 1.0 mg/kg D-amphetamine dose was significantly lower when compared to vehicle treatment (mean difference: 5.7, $p = 0.003$). As the required ratio increased over blocks, the number of rewards obtained significantly decreased ($F(3,38)_{block} = 319.872$, $p < 0.001$). The dampening effect of D-amphetamine on the number of rewards obtained was independent of the ratio requirement ($F(3,47)_{dose\ x\ block} = 1.224$, $p = 0.312$). Flupentixol also significantly decreased the number of rewards obtained compared to vehicle (Figure 6B; $F(1,15)_{dose} = 34.013$, $p < 0.001$). As the required ratio increased over blocks, the number of rewards obtained significantly decreased ($F(2,27)_{block} = 242.688$, $p < 0.001$). The dampening effect of flupentixol on number of rewards was not related to the ratio requirements ($F(4,60)_{dose\ x\ block} = 2.038$, $p = 0.100$). Taken together, these data show that both D-amphetamine and flupentixol reduced the number of rewards obtained under a WS-IR schedule of reinforcement.

Similar to the TH::cre animals, individual demand curves were plotted and the derived parameters R^2, α, and Q_0 were analysed for both D-amphetamine and flupentixol treatments. For D-amphetamine, individual demand data fitted well to the model as the average R^2 was above 0.80 (i.e. [mean ± standard deviation] $R^2_{D\text{-amphetamine}}$: 0.93 ± 0.09; $R^2_{D\text{-amphetamine 0.5}}$: 0.88 ± 0.13; $R^2_{D\text{-amphetamine 1.0}}$: 0.80 ± 0.19). D-amphetamine significantly increased demand elasticity (Figure 6C; $F(2,28)_{dose} = 8.493$, $p = 0.001$). Post-hoc analysis showed that demand elasticity at the 1.0 mg/kg D-amphetamine dose was significantly higher compared to vehicle (mean difference: 0.216, $p = 0.001$). D-amphetamine did not significantly affect demand intensity (Figure 6D; $F(1,18)_{dose} = 1.232$, $p = 0.291$). After flupentixol treatment, individual demand data fitted only marginally to the model as the average R^2 values were relatively low (i.e. [mean ± standard deviation] $R^2_{flupentixol\ 0.00}$: 0.86 ± 0.19; $R^2_{flupentixol\ 0.25}$: 0.54 ± 0.23). Flupentixol significantly increased demand elasticity compared to vehicle (Figure 6E; $Z = -2.897$, $p = 0.004$). Flupentixol also significantly reduced demand intensity (Figure 6F; t(15)

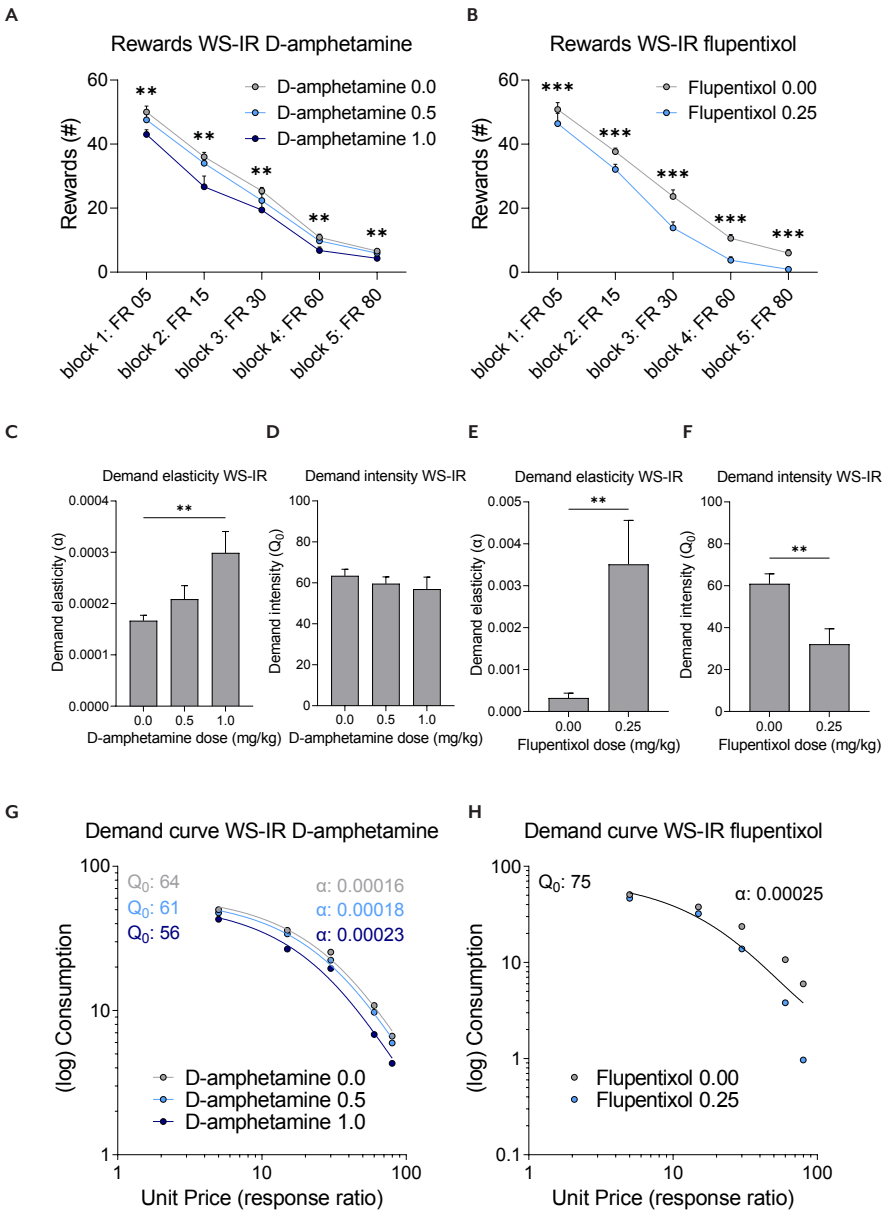


Figure 6. The effects of D-amphetamine and flupentixol on the number of rewards, demand elasticity (α) and intensity (Q_0), and demand curve when measured under a within session increasing ratio (WS-IR) schedule in experimental group III. Effects of D-amphetamine (**A**) and flupentixol (**B**) on number of rewards obtained when assessed in a WS-IR task. Effects of D-amphetamine on demand elasticity (**C**) and demand intensity (**D**) based on individual demand curve analysis. Effects of flupentixol on demand elasticity (**E**) and demand intensity (**F**) based on individual demand curve analysis. Effects of D-amphetamine (**G**) and flupentixol (**H**) on population demand curves. Data in panels A-D are presented as the mean + SEM. ** D-amphetamine 1.0 / Flupentixol 0.25 different from vehicle, $p < 0.01$; *** flupentixol different from vehicle, $p < 0.001$.

= 3.471, p = 0.003). Together, these results show that D-amphetamine increased demand elasticity without significantly affecting demand intensity, while flupentixol increased demand elasticity and decreased demand intensity.

Analysis of the demand curves based on dose means for D-amphetamine treatment using the extra sum-of-squares F-test indicated that a global fit could not accommodate all data (F(4,9) = 8.200, p = 0.005). Therefore, demand curves and the derived best-fit values of α and Q_0 were different for each D-amphetamine dose (Figure 6G). These analyses suggest that D-amphetamine shifted the demand curve to one with an increased demand elasticity and decreased demand intensity. For flupentixol treatment, the extra sum-of-squares F-test indicated that parameters did not differ across the different doses and that a single demand curve with an R^2 of 0.80 fit the data from different doses (Figure 6H; F(2,6) = 3.900, p = 0.082). This analysis suggests that flupentixol did not alter the demand curve.

Within-session decreasing ratio

The effects of D-amphetamine and flupentixol on responding for sucrose under a WS-DR schedule of reinforcement were also determined. D-amphetamine treatment significantly decreased the number of rewards obtained (Figure 7A; $F(1,20)_{dose}$ = 6.421, p = 0.014). Post-hoc analysis showed that the number of rewards at the 1.0 mg/kg D-amphetamine dose was significantly lower than vehicle (mean difference: 3.4, p = 0.038). As the required ratio decreased over blocks, the number of rewards obtained significantly increased ($F(2,29)_{block}$ = 155.212, p < 0.001). The dampening effect of D-amphetamine on the number of rewards was not affected by the ratio requirement ($F(2,35)_{dose\ x\ block}$ = 2.006, p = 0.144). Treatment with flupentixol did not significantly affect the number of rewards obtained, although a trend towards a decrease in the number of rewards was observed (Figure 7B; $F(1,14)_{dose}$ = 4.328, p = 0.056). As the required ratio decreased over blocks, the number of rewards obtained significantly increased ($F(1,21)_{block}$ = 81.278, p < 0.001). The effect of flupentixol on the number of rewards was dependent on the ratio requirement ($F(2,26)_{dose\ x\ block}$ = 3.531, p = 0.048). Post-hoc analysis indicated no significant differences between flupentixol and vehicle treatment in any of the blocks, although a trend towards a suppression in responding by flupentixol was observed in block 4, i.e. FR 15 (p = 0.053), and in block 5, i.e. FR 5 (p = 0.073). Together, these results show that D-amphetamine significantly decreased the number of earned rewards and revealed a trend towards a reduction in the number of rewards upon treatment with flupentixol, especially at the lowest ratio requirements.

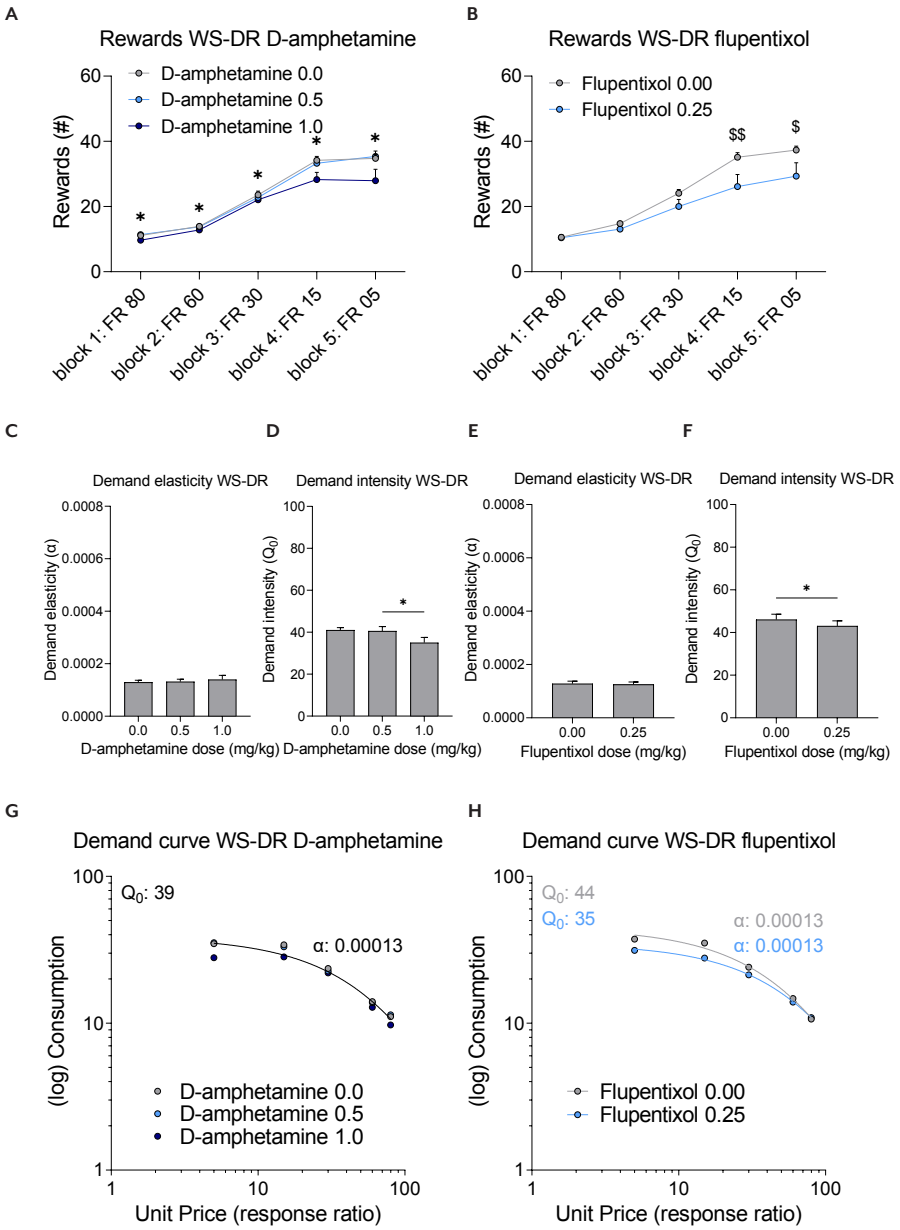


Figure 7. The effects of D-amphetamine and flupentixol on the number of rewards, demand elasticity (α) and intensity (Q_0), and demand curve when measured under a within session decreasing ratio (WS-DR) schedule in experimental group III. Effects of D-amphetamine (**A**) and flupentixol (**B**) on number of rewards obtained when assessed in a WS-DR task. Effects of D-amphetamine on demand elasticity (**C**) and demand intensity (**D**) based on individual demand curve analysis. Effects of flupentixol on demand elasticity (**E**) and demand intensity (**F**) based on individual demand curve analysis. Effects of D-amphetamine (**G**) and flupentixol (**H**) on population demand curves. Data in panels A-D are presented as the mean + SEM. * different from highest dose, $p < 0.05$; $$ $p = 0.053$; $ $p = 0.073$.

Individual demand curves were plotted and the derived parameters R^2, α, and Q_0 were analysed. For D-amphetamine treatment, individual demand data fitted well to the model as the average R^2 was above 0.80 (i.e. [mean ± standard deviation] $R^2_{\text{D-amphetamine 0.0}}$: 0.94 ± 0.06; $R^2_{\text{D-amphetamine 0.5}}$: 0.92 ± 0.08; $R^2_{\text{D-amphetamine 1.0}}$: 0.83 ± 0.14). D-amphetamine did not significantly affect demand elasticity (Figure 7C; $F(1,18)_{\text{dose}} = 0.049$, $p = 0.891$). There was a significant effect of dose on demand intensity (Figure 7D; $F(2,28)_{\text{dose}} = 4.792$, $p = 0.016$), but post-hoc analyses showed that demand intensity at both D-amphetamine doses was not significantly different from vehicle ($p > 0.07$). For flupentixol treatment, individual demand data fitted well to the model as the average R^2 was above 0.90 (i.e. [mean ± standard deviation] $R^2_{\text{flupentixol 0.00}}$: 0.95 ± 0.03; $R^2_{\text{flupentixol 0.25}}$: 0.94 ± 0.02). Flupentixol did not significantly affect demand elasticity (Figure 7E; $t(11) = 0.433$, $p = 0.674$). However, flupentixol significantly decreased demand intensity compared to vehicle (Figure 7F; $Z = -2.515$, $p = 0.012$). Thus, treatment with D-amphetamine did not affect demand elasticity and demand intensity whereas flupentixol decreased demand intensity without significantly affecting demand elasticity.

		VTA DA activation			D-amphetamine			flupentixol		
		# rewards	α	Q_0	# rewards	α	Q_0	# rewards	α	Q_0
Incr. ratio	individual	↓	↑	=	↓	↑	=	↓	↑	↓
	population		↑	↓		↑	↓		=	=
Decr. ratio	individual	↓	↑	=	↓	=	=	↓	=	↓
	population		↑	↓		=	=		=	↓
FR 60		↓			ND			ND		
PR		↑			ND			ND		

Table 1. Summary of the results. Arrows represent a significant increase (↑) or decrease (↓) upon chemogenetic activation of mesocorticolimbic dopamine neurons or upon pharmacological dopamine manipulation. = represents no significant difference between drug and vehicle. A dot was placed when results were not tested. 'individual' refers to the analysis of individual demand curves, 'population' refers to the analysis of population demand curves. Incr.: increasing, Decr.: decreasing, α: demand elasticity, Q_0: demand intensity, ND: not determined.

Analysis of the demand curves based on dose means for D-amphetamine treatment using the extra sum-of-squares F-test indicated that parameters did not differ across the different doses ($F(4,9) = 2.600$, $p = 0.104$). Therefore, a single demand curve with an R^2 of 0.96 fit the data from different doses (Figure 7G). These results suggest that D-amphetamine did not shift the demand curve. For flupentixol treatment, the extra sum-of-squares F-test showed that a global fit could not accommodate all data ($F(2,6) = 12.000$, $p = 0.008$). Therefore, demand curves and the derived best-fit

value Q_0 were different for vehicle and flupentixol, with a similar α (Figure 7H). Thus, analysis of mean demand curves suggested that flupentixol shifted the demand curve to one with a similar demand elasticity and decreased demand intensity.

Discussion

In this study, we aimed to determine the role of dopaminergic neurotransmission in the relationship between price and consumption of sucrose. In contrast to our predictions, chemogenetic activation of VTA dopamine neurons increased demand elasticity, reflecting an increased sensitivity to price elevations and a reduced essential value. When assessing demand at a population level, we also observed a decrease in demand intensity upon VTA dopamine neuron activation. Moreover, VTA dopamine neuron activation increased responding for sucrose under a PR schedule of reinforcement, which is indicative of an increased incentive motivation as was previously reported (Boekhoudt et al., 2018; Boender et al., 2014). Furthermore, treatment with D-amphetamine partially replicated the effects of chemogenetic mesocorticolimbic dopamine neuron activation, whereas treatment with alpha-flupentixol reduced free consumption of sucrose and had mixed effects on demand elasticity. Together, these findings imply that mesocorticolimbic dopamine signalling differentially influences distinct aspects of value-based decision making processes.

Paradoxical effects of chemogenetic activation of VTA dopamine neurons on the value of sucrose

The observed effects of chemogenetic activation of VTA dopamine neurons on responding for sucrose under the WS-IR/WS-DR and the PR schedules of reinforcement seem paradoxical. Demand analyses revealed that stimulation of VTA dopamine neurons reduced essential value, while incentive motivation was increased in the PR task. Although using other types of reinforcers, the finding of different outcomes for the two measures is not unprecendented in preclinical studies. For example, THC exposure increased the essential value of nicotine without affecting the PR breakpoint in rats, and mice lacking the serotonin transporter gene showed lower PR breakpoints but similar essential value of alcohol compared to wild type mice (Lamb & Daws, 2013; Panlilio et al., 2013). The observation in the present study that increased demand elasticity and increased incentive motivation occur concurrently suggests that these measures reflect different aspects of reward seeking behaviour.

The differential effects of VTA dopamine neuronal activation may be related to the inherent differences for the schedules of reinforcement. Demand curve procedures allow for varying levels of reward obtainment across multiple ratios on a continuous scale. Conversely, reward obtainment under a PR schedule of reinforcement is binary: either a reward is obtained or not at each ratio. As such, essential value reflects sensitivity to price changes while the breakpoint under a PR schedule may also reflect the degree of resistance to extinction (Kearns et al., 2016) or the sensitivity to a declining rate of reinforcement (Verharen et al., 2018). Thus, although responding under both schedules is related to reward seeking, the essential value and breakpoint likely involve distinct behavioural processes. Moreover, the behavioural components underlying essential value and breakpoint seem dissociable in terms of their dependence on mesocorticolimbic dopamine signalling. While in agreement with earlier studies on incentive motivation, the present findings imply that dopamine signalling differentially affects essential value as a hyperdopaminergic state led to a lower attributed essential value.

The current study adds to a growing body of literature that investigates the role of mesocorticolimbic dopamine in reinforcement and motivation. We assessed essential value and motivation, whereby expectancy is a component process that may influence both. An influential theory of reinforcement learning posits that VTA dopamine encodes the reward prediction error (RPE), i.e. the discrepancy between anticipated and experienced reward (Bayer & Glimcher, 2005; Schultz, 2016). VTA dopamine neuronal activity increases when an experienced reward is better than expected, whereas VTA dopamine neuronal activity decreases when an experienced reward is less than anticipated. Consistent with this notion, inducing a positive dopamine RPE signal by optogenetic excitation at the moment of reward delivery made rats less sensitive to increases in response requirement (Schelp et al., 2017). The increase in breakpoint in the PR task suggests that rats may have experienced the obtained rewards as better than expected in a hyperdopaminergic state, while the decreased essential value implicates that the expectations were not met when a price (i.e. ratio requirement) was provided for more than one reward. However, rats were extensively trained in each of the behavioural tasks, the reward size was stable, and the delivery of a reward was never preceded by a cue. Therefore, potential effects on reward expectation are not likely to explain the present pattern of effects. Related to its role in RPE processing, dopamine has also been implicated in behavioural flexibility. Recently, we have shown, for example, that hyperactivity of the mesoaccumbens pathway leads to impaired flexible decision making through interference with negative RPE processing (Verharen et al., 2018). However, we think that impaired flexibility does not play a major role in the findings in the present

study, as VTA dopamine neuron activation decreased the number of rewards also throughout the FR 60 sessions, in which the requirement was relatively high but behavioural flexibility was not required.

An important methodological difference between the schedules of reinforcement used in the current study is the session duration. In a PR session, the rats have more time (30 minutes) to obtain a subsequent reward than in the behavioural economics schedule that was applied, which required rats to respond within a restricted 8-minute time period per ratio requirement, regardless of the number of rewards obtained. Dopaminergic neurotransmission has been implicated in time perception and attention (Boekhoudt et al., 2017b; Lewis & Miall, 2006; Marinho et al., 2018), rendering it conceivable that awareness of time and attention may be affected in a hyperdopaminergic state. Due to the inherent time restriction in behavioural economic tests, the influence of dopamine modulation of time perception and attention might be more pronounced on demand curves than on PR responding. However, the effects on attention were relatively mild for dopamine neuron stimulation specifically in the VTA when compared to substantia nigra dopamine neuron stimulation (Boekhoudt et al., 2017b) and the five-choice serial reaction time task, measuring attention, can be considered cognitively more demanding than lever pressing for a reward. Taken together, we therefore think that it is unlikely that impaired attention contributed substantially to the contrasting findings of CNO on demand and PR analyses.

D-amphetamine and flupentixol treatment

Besides chemogenetics, pharmacological modulation of dopamine neurotransmission was used to gauge the role of dopamine in cost-benefit trade-offs for sucrose. Similar to the effects of chemogenetic stimulation, D-amphetamine decreased responding for sucrose under the WS-IR and WS-DR schedules of reinforcement and increased demand elasticity under WS-IR conditions, reflecting an increased sensitivity to price elevations and a reduced essential value. This confirmed our finding that enhanced dopamine signalling could decrease the essential value based on analyses of demand curves.

Regarding the ratio schedules, chemogenetic VTA dopamine neuron activation yielded similar results in the WS-IR and WS-DR sessions. Remarkably, the effects of D-amphetamine and flupentixol on demand varied depending on the ratio scheme and on whether demand analysis was based on individual or population curves. Stimulation of dopaminergic activity through D-amphetamine reduced responding for sucrose and increased demand elasticity under the WS-IR schedule, which is similar to the effects of chemogenetic activation. However, D-amphetamine did not affect demand elasticity under the WS-DR schedule, while chemogenetic activation reduced the number of rewards and increased demand elasticity under this schedule of reinforcement. Moreover, although there was a suppressing effect of D-amphetamine on the number of rewards under the WS-DR schedule, this effect was constant across the various ratio requirements. Therefore, price sensitivity was not affected by D-amphetamine despite the decrease in the number of rewards obtained.

The dopamine receptor antagonist alpha-flupentixol also reduced the number of rewards obtained in the WS-IR and WS-DR tasks. A reduction in demand elasticity was detected once, whereas almost all demand analyses revealed lower demand intensity. These results are in line with previous studies that reported reductions in the motivation to exert effort for food rewards upon suppression of dopaminergic neurotransmission (Aberman et al., 1998; Aberman & Salamone, 1999; Caul & Brindle, 2001; Reilly, 1999). Remarkably, these effects of flupentixol were similar to those of D-amphetamine. This suggests that dopamine activity is normally at an optimum and that deviations from this optimum, by either increasing or decreasing dopamine signalling, might lead to behavioural impairments.

Although the results from pharmacological stimulation, through D-amphetamine, are largely in line with the results from chemogenetic stimulation, the minor differences in findings between both techniques might be partly explained by the manner and extent of dopaminergic activation. That is, the effects of CNO treatment in TH::cre+ rats and D-amphetamine treatment are mechanistically different: D-amphetamine elevates extracellular dopamine concentrations in a largely impulse-independent manner by acting as a false substrate on the dopamine transporter (Calipari & Ferris, 2013; Carboni et al., 1989; Jones et al., 1998), whereas the designer receptor hM3Dq is a G-protein-coupled receptor (GPCR) and hM3Dq activation upon CNO administration induces intracellular calcium release, thereby enhancing neuronal firing. As a result, the net effects of D-amphetamine on extracellular dopamine levels are much larger (Calipari & Ferris, 2013; Carboni et al., 1989; Jones et al., 1998; Verharen et al., 2018). Moreover, in addition to dopaminergic signalling, other neurotransmitter systems are affected by D-amphetamine and flupentixol. D-amphetamine affects norepinephrine and serotonin signalling and flupentixol is thought to have antagonistic properties at serotonin receptors as well (Leysen et al., 1993; Meltzer et al., 1989; Pum et al., 2007; Rothman et al., 2001; Sloviter et al., 1978; Soyka & de Vry, 2000). Furthermore, D-amphetamine and flupentixol were administered systemically, thereby exerting effects on other brain structures than only the VTA and its projection areas. These divergent neurochemical effects

might explain the observed differential effects of D-amphetamine and flupentixol on responding under the ratio schedules which were not observed in the chemogenetic manipulations.

Strengths and limitations

A strength of the current study is the within-session approach, which enabled us to derive demand curves from single sessions. Conventionally, in preclinical behavioural studies demand curves are predominantly derived from between-sessions approaches through series of daily sessions that each determine the demand at one specific price. However, this is time consuming and may hamper reliable testing as neural manipulations have to be repeated several times (Bentzley et al., 2013; Oleson & Roberts, 2019). We took a within-session approach to construct demand curves based on single sessions.

A limitation of our approach is the potential influence of satiety on task performance. Both increasing and decreasing ratio schedules were used and animals were mildly food restricted to minimise effects of satiety and to enhance the reinforcing value of food (Yang et al., 2020). Food restriction has been shown to alter dopamine neurotransmission (Avena et al., 2008; Sevak et al., 2008). However, a pilot study with chemogenetic activation of VTA dopamine neurons and similar WS-IR and WS-DR schedules of reinforcement for non-food deprived rats (data not shown) revealed overall lower responding, but similar effects of CNO treatment in TH::cre+ animals. Therefore, it is not likely that food restriction contributed to the effects of CNO on demand and PR analyses.

Another limitation is that dopamine activity was chemogenetically increased throughout the forebrain, including the striatum, prefrontal cortex and amygdala. Therefore, there is a lack of specificity to discern the effects of dopamine on distinct brain regions regarding cost-benefit trade-offs. Especially dopamine signalling in the nucleus accumbens has been widely implicated in effort-based choice behaviour, but likely not exclusively because dopamine terminal release in the nucleus accumbens has been shown to lead to similar results as VTA dopamine stimulation on demand, but the accumbal stimulation effects were weaker (Schelp et al., 2017). Moreover, optically enhanced dopamine in the nucleus accumbens was sufficient to shift preference towards a choice with higher costs (i.e. a longer delay), but did not alter preference to a lower benefit (i.e. a smaller reward magnitude) (Saddoris et al., 2015). This suggests that dopamine signalling in other brain regions than the nucleus accumbens contributes to effects on demand, and that dopamine signalling in different brain regions might differentially affect separate aspects of cost-benefit balances.

Furthermore, VTA dopaminergic neuronal activity was enhanced throughout the entire task in the present study, leading to increased demand elasticity, while the temporal pattern of dopamine signalling might determine the effect on demand elasticity and motivational processes. Recently, the effect of VTA dopaminergic neuron stimulation on demand elasticity of sucrose was found to be dependent on timing of the stimulation. Stimulation of these neurons by optogenetics prior to reward delivery at cue presentation made rats more sensitive to price, whereas stimulation at reward delivery made rats less sensitive to price (Schelp et al., 2017). Although dopamine stimulation through DREADD has a lower temporal resolution than optogenetics, we further extend the notion that increasing dopamine release causally modifies sensitivity to price.

Conclusion

To conclude, these findings show that stimulation of dopaminergic neurotransmission altered cost-benefit decision making in a complex manner. It reduced the essential value of palatable food, increased sensitivity to price elevations and increased incentive motivation, while leaving free consumption unaltered. Together, these data extend the notion that aberrant dopamine signalling might underlie deficits in cost-benefit trade-offs in a complex manner as seen in several psychiatric disorders. Future research into the process of cost-benefit assessment and the relative contribution of distinct aspects of reward valuation will be needed to comprehend how this eventually converts to value-based decision making.

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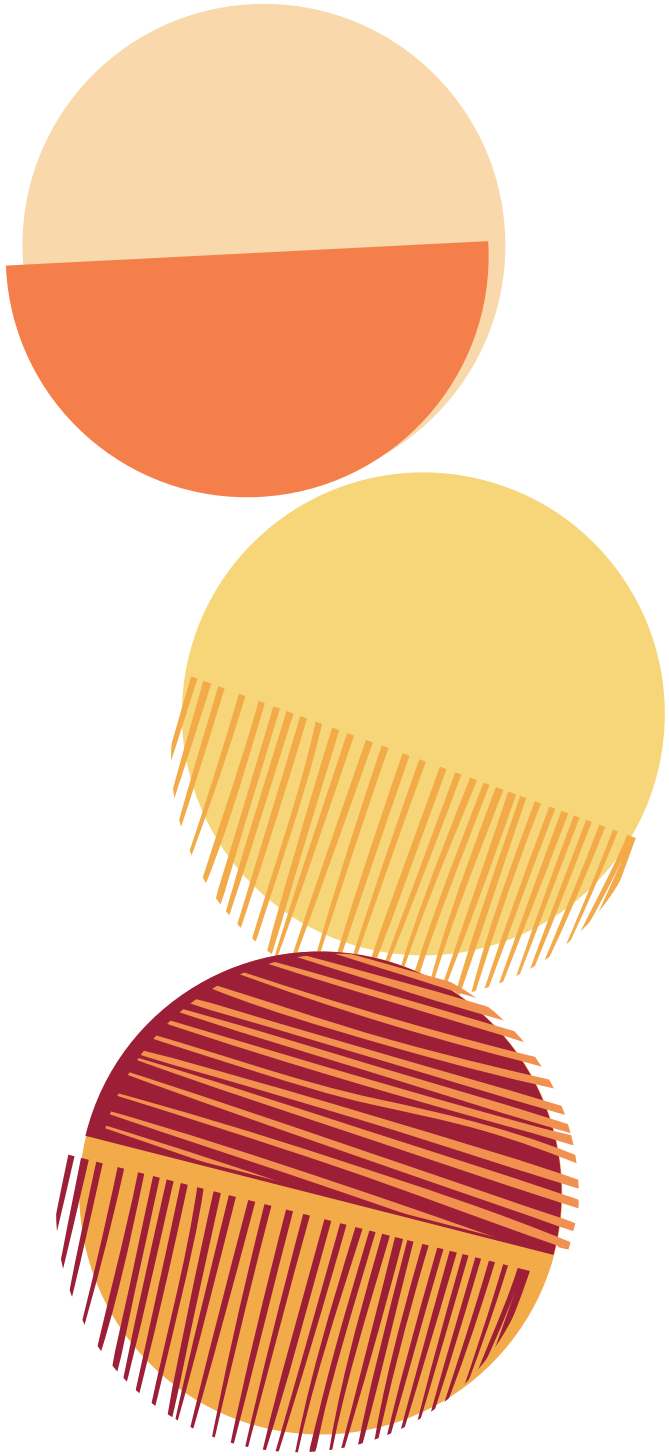

Chapter 7

Baclofen and naltrexone, but not N-acetylcysteine, affect voluntary alcohol drinking in rats regardless of individual levels of alcohol intake

A. Maryse Minnaard[1,2]
Geert M. J. Ramakers[2]
Roger A. H. Adan[2]
Louk J. M. J. Vanderschuren[1]
Heidi M. B. Lesscher[1]

[1] Department of Population Health Sciences, Unit of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2] Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, The Netherlands

Abstract

Rationale

In humans, there is a profound individual variation in the risk of alcohol use disorder (AUD). Since GABA, opioid, and glutamate neurotransmission have been implicated in AUD, functional differences in these neural systems may underlie the individual vulnerability to AUD.

Objectives

We aimed to determine the involvement of GABAergic, opioid, and glutamatergic neurotransmission in individual differences in alcohol consumption. To that aim, the effects of treatment with the $GABA_B$ receptor agonist baclofen, the opioid receptor antagonist naltrexone, and the cysteine precursor N-acetylcysteine on alcohol consumption were assessed in rats that differed in their levels of alcohol intake.

Methods

Subgroups of low, medium, and high alcohol drinking rats (LD, MD, and HD) were selected based on alcohol consumption using an intermittent-every-other-day two-bottle choice procedure. The subgroups were treated with baclofen, naltrexone, and N-acetylcysteine, and the effects on alcohol intake and preference were measured at different timepoints after administration of these compounds.

Results

Baclofen significantly decreased alcohol intake and tended to reduce alcohol preference. Naltrexone caused a modest reduction in alcohol intake without altering alcohol preference. The effects of baclofen and naltrexone on alcohol intake and preference were comparable in LD, MD, and HD. By contrast, N-acetylcysteine did not affect alcohol consumption.

Conclusions

These data confirm that $GABA_B$ and μ-opioid receptor signalling contribute to alcohol consumption in rats. However, GABAergic and opioid pathways do not play a major role in individual differences in alcohol consumption.

Keywords

Alcohol consumption
Baclofen
GABA
Glutamate
Individual differences
N-Acetylcysteine
Naltrexone
Opioids
Rats

Introduction

Alcohol use disorder (AUD) is a chronic relapsing disorder that is characterised by continued alcohol use despite adverse consequences. Alcohol consumption is a major medical and socioeconomic problem. It is causally linked to more than fifty diseases and contributes to approximately 4% of the global burden of disease (Connor et al., 2016; Rehm et al., 2003, 2009). Currently, a wide array of treatment options for AUD is available, including cognitive behavioural therapy, pharmacological treatment and social support strategies. However, treatment efficacy varies and short-term relapse after treatment is common with estimates ranging from 20% to 50%, depending on the length of follow-up and the criteria for remission (Miller et al., 2001; Monahan & Finney, 1996; Moos & Moos, 2006). A better understanding of AUD and the underlying psychopathology is needed for the development of more effective treatment strategies.

Importantly, the quantity and pattern of alcohol intake varies substantially between consumers and only a minority develops an AUD. This individual vulnerability to AUD is likely driven by neural factors. To study these underlying neural processes, models that capture the individual variability in alcohol consumption can be employed. In preclinical studies, individual variation in alcohol consumption can be modelled by exposing rodents intermittently to alcohol (Hwa et al., 2011; Momeni & Roman, 2014; Sabino et al., 2013; Simms et al., 2008). We have previously shown that after two months of intermittent alcohol access (IAA), profound individual differences in alcohol intake and motivation can be observed in outbred rats, whereby subgroups of high alcohol drinking (HD), medium alcohol drinking (MD), and low alcohol drinking rats (LD) could be distinguished (Spoelder et al., 2015). HD display a typical AUD phenotype as they showed more motivation to obtain alcohol and more punishment-resistant alcohol-directed behaviour than LD (Spoelder et al., 2015, 2017). Moreover, IAA can induce a transition from moderate to escalated alcohol consumption, which is another hallmark of AUD (Hopf et al., 2010; Lesscher

et al., 2010; Spoelder et al., 2015). Therefore, distinguishing LD, MD, and HD using the IAA procedure provides a valuable tool to study the underlying neurobiological mechanisms that contribute to variation in alcohol consumption and individual differences in the development of AUD.

A variety of mechanisms may underlie the individual vulnerability to AUD. Rather than having a single primary target, alcohol affects multiple neurotransmitter systems that are involved in emotion, cognition, and motivation (Spanagel, 2009). Alcohol is thought to directly increase GABA activity in the brain by acting on the presynaptic GABA-releasing neuron and on the postsynaptic neuron where it may facilitate GABA receptor function (Abrahao et al., 2017; Förstera et al., 2016; Kelm et al., 2011; Roberto et al., 2003; Roberto & Varodayan, 2017). Alcohol also acts on the opioid system, inducing the release of endogenous opioids, such as β-endorphin in the orbitofrontal cortex, the ventral tegmental area (VTA), the hypothalamus, and nucleus accumbens (Font et al., 2013; Hermann et al., 2017; Mitchell et al., 2012; Olive et al., 2001). Besides affecting β-endorphins, alcohol also increases enkephalin levels in the hypothalamus, and nucleus accumbens (Font et al., 2013). These endogenous opioids activate μ-opioid receptors, that contribute to the reinforcing effects of alcohol, which is for example evident from studies with μ-opioid receptor knockout mice that show little to no alcohol consumption (Hall et al., 2001; Méndez & Morales-Mulia, 2008; Roberts et al., 2000). Furthermore, alcohol has been suggested to have an inhibitory effect on glutamatergic neurotransmission through N-methyl-D-aspartate (NMDA) and mGlu5 metabotropic glutamate receptors (Bäckström et al., 2004; Gonzales & Jaworski, 1997; Hodge et al., 2006; Lominac et al., 2006; Schroeder et al., 2005; Tsai et al., 1995).

Compounds acting on GABAergic, opioid or glutamate systems, such as baclofen, naltrexone, and N-acetylcysteine, have received great attention as possible treatment options for AUD. Baclofen is a potent $GABA_B$ receptor agonist used as a skeletal muscle relaxant in the treatment of spasticity (Davidoff, 1985). Naltrexone is a competitive opioid receptor antagonist with a high affinity for μ-opioid receptors, and to a lesser extent for δ- and κ-opioid receptors (Davis & Nelson, 1995). Both baclofen and naltrexone have been used for pharmacological treatment of AUD (van den Brink, 2012). N-Acetylcysteine is a cysteine precursor commonly used in, amongst others, the treatment of respiratory diseases (Berk et al., 2013; Elbini Dhouib et al., 2016; Mokhtari et al., 2017; Sansone & Sansone, 2011). N-acetylcysteine modulates glutamatergic neurotransmission, because cysteine is a constituent of the antioxidant glutathione and its oxidised dimer form, cystine, serves as a substrate for the cystine-glutamate antiporter (Baker et al., 2003). Preclinical studies have shown that baclofen, naltrexone, and N-acetylcysteine reduce alcohol consumption and motivation for alcohol, suggesting their potential for pharmacological treatment in AUD (Colombo et al., 2000; Coonfield et al., 2002, 2004; Daoura & Nylander, 2011; Daoust et al., 1987; Ferraro et al., 2002; Higley & Kiefer, 2006; Hill & Kiefer, 1997; Lebourgeois et al., 2018, 2019; Momeni et al., 2015; Parkes & Sinclair, 2000; Perfumi et al., 2002; Quintanilla et al., 2016; Simms et al., 2008; Steensland et al., 2012; Stromberg, 2004; Walker & Koob, 2007). However, clinical studies show large individual variations in the treatment response to these compounds in AUD patients (Addolorato et al., 2002, 2007; Beraha et al., 2016; Garbutt et al., 2010; Kiefer et al., 2008; Müller et al., 2015; Spanagel & Kiefer, 2008).

Considering that alcohol affects GABA, opioid, and glutamate signalling, individual variation in alcohol intake may be related to differences in these systems. Thus, the aim of this study was to determine the involvement of GABAergic, opioid, and glutamatergic pathways in individual differences in alcohol consumption under IAA conditions. Subpopulations of HD, MD, and LD rats were identified and the effects of baclofen, naltrexone, and N-acetylcysteine on voluntary alcohol intake and preference were assessed. We hypothesised that, if variations in GABAergic, opioid or glutamatergic signalling underlie individual variation in alcohol consumption, treatment with baclofen, naltrexone or N-acetylcysteine, respectively, should have differential effects on alcohol consumption in HD, MD, and LD rats.

Materials and methods

Subjects

A total of fifty adult male Lister Hooded rats (Charles River, Germany), weighing 200-250 grams (~8-10 weeks old) at the start of the experiment, were used in this study. The rats were individually housed in Macrolon type III sawdust bedded cages (42.5 x 26.6 x 18.5 cm) with ad libitum access to tap water and standard chow (Rat and Mouse Breeder and Grower Expanded-CRM(E), Special Diet Service, UK). A polycarbonate rat tunnel (9 x 9 x 15 cm) and a tissue were provided for cage enrichment. The rats were kept under controlled temperature and humidity conditions (21 ± 2°C and 50 – 70% humidity) and on a reversed 12 h/12 h light/dark cycle (lights off at 7.00 AM - lights on at 7.00 PM) to allow for behavioural testing in the dark phase. Background noise was provided by a constantly playing radio. The rats were acclimatised to the housing conditions for eleven days prior to behavioural testing, and they were weighed and handled at least once per week throughout the course of the experiment. All animals used were experimentally naive. All experimental procedures were approved by the

Animal Ethics Committee of Utrecht University and the Dutch Central Animal Testing Committee, and were conducted in accordance with Dutch (Wet op de Dierproeven, 1996; Herziene Wet op de Dierproeven, 2014) and European legislation (Guideline 86/609/EEC; Directive 2010/63/EU).

Alcohol consumption

IAA procedures were used as previously described (Spoelder et al., 2015, 2016). The rats were exposed to 20% (v/v) alcohol and tap water in a two-bottle choice setup in the home cage for three days a week (Monday, Wednesday, Friday), i.e. intermittent every other day. In the first four weeks of IAA, alcohol exposure sessions lasted for 7 hours, approximately between 9.30 AM and 16.30 PM (i.e. during the dark phase of the day-night cycle), and sessions were subsequently extended to 24 hours in the following months. Alcohol intake during the first two months of alcohol exposure was used to assign rats to the drinking subgroups, after which testing of the pharmacological compounds began. We have previously shown that two months of alcohol consumption is sufficient to discern subgroups of rats displaying substantial differences in alcohol intake, motivation, and resistance to punishment (Spoelder et al., 2015, 2017). The bottles were weighed before and after each session and the placement of the alcohol bottle was alternated between sessions to avoid the development of a side bias. Alcohol intake (g/kg) and alcohol preference (%) were calculated per rat per session and averaged per week. After two months, rats were ranked based on the animals' average alcohol intake (g/kg) per week and were assigned ranking scores. These weekly ranking scores of the first eight weeks of the experiment were summed to compute a total ranking score which was used to assign rats to the drinking subgroups. Rats within the lower and upper 24% of the total ranking score were selected as low and high alcohol drinking rats (LD, n = 12; HD, n = 12), respectively. From the remaining group, rats that listed within the median 24% of the total population at least three times out of the eight weekly ranking scores were selected as medium alcohol drinking rats (MD, n = 16).

Drugs

Alcohol (99.5%; Klinipath, The Netherlands) was freshly diluted with tap water once per week to a final concentration of 20% (v/v). Baclofen ((RS)-4-Amino-3-(4-chlorophenyl)butanoic acid; Tocris Bioscience, United Kingdom) was administered intraperitoneally (i.p.) at the doses of 0, 0.3, 1, and 3 mg/kg (based on Colombo et al., 2003a, 2003b; Janak & Gill, 2003; Maccioni et al., 2005; Quintanilla et al., 2008; Walker & Koob, 2007), 30 minutes before the start of alcohol drinking sessions. Naltrexone hydrochloride ((5a)-17-(Cyclopropylmethyl)-4,5-epoxy-3,14-dihydromorphinan-6-one hydrochloride, Abcam, UK) was administered subcutaneously (s.c.) at the doses of 0, 0.3, and 1 mg/kg (based on Daoura & Nylander, 2011; Momeni et al., 2015; Simms et al., 2008; Steensland et al., 2012), 30 minutes before the start of alcohol drinking sessions. N-Acetylcysteine (N-Acetyl-L-cysteine ((2R)-2-(acetylamino)-3-sulfanylpropanoic acid); Sigma-Aldrich, The Netherlands) was administered i.p. after pH adjustment with NaOH to pH 7.4 at the doses of 0, 25, 50, and 100 mg/kg (based on Kau et al., 2008; Lebourgeois et al., 2018; Reichel et al., 2011), 60 minutes before the start of alcohol drinking sessions because N-acetylcysteine is a prodrug. All drugs were dissolved in sterile physiological saline (0.9% NaCl) and injected at 1 ml per kg bodyweight.

Experimental procedure

All rats received a saline injection (1.0 ml/kg, i.p.) one week before drug testing started to habituate them to the injection procedure. All LD, MD, and HD rats received all doses according to a within-subject Latin square design per drug. The order in which the drugs were administered was similar for each animal: the rats were first treated with baclofen, followed by naltrexone, and N-acetylcysteine. Alcohol and water bottles were weighed before each session and 2 hours, 7 hours, and 24 hours after the start of the session. Each treatment session was always followed by at least one day without alcohol access, according to the IAA schedule. Additionally, there was at least one drug-free 24 hour alcohol exposure session between treatment sessions for the same drug and there were at least three drug-free 24 hour alcohol exposure sessions between different drugs. In order to circumvent carry-over effects, naltrexone was administered with a 1-week wash-out period between each injection (Daoura & Nylander, 2011).

Data analysis and statistics

Alcohol intake, water intake, and alcohol preference were calculated as follows. Fluid intake was calculated by subtracting the bottle weights at the end of every drinking session from the starting weights. Alcohol intake (ml) was calculated according to the following equation: (Δ alcohol bottle weight in grams) / (0.8+(0.2*0.789)) in which the density of ethanol (i.e., 0.789 g/ml), is included. Alcohol intake (g/kg) was calculated as follows: ((alcohol fluid intake in ml) * (0.2*0.789)) / (bodyweight in kg). Water intake (ml/kg) was calculated as: (water fluid intake in ml) / (bodyweight in kg). Preference for alcohol (%) was calculated according to the following equation: (alcohol fluid intake in ml) / ((alcohol fluid intake in ml) + (water fluid intake in ml)) * 100. Two rats in the LD group were excluded from the baclofen analyses because of errors in the bottle weight documentation for these animals.

Alcohol intake, water intake and alcohol preference were analysed using two-way repeated-measures ANOVAs with *week* as the within-subject variable and *group* (LD, MD, HD) as the between-subject variable. In case of significant effects, post hoc analyses were performed using pairwise comparisons with a Bonferroni correction. The effects of the pharmacological treatments on alcohol intake, water intake, and alcohol preference were analysed using three-way repeated measures ANOVA tests with *dose* and *time* (2, 7, and 24 hours) as within-subject variables and *group* (LD, MD, HD) as the between-subject variable. On cumulative alcohol intake and water intake data, main effects of time are not reported, because intake will by definition increase when analysing cumulative data. In case of significant effects involving the drug dose, post hoc analyses were performed using pairwise comparisons of each drug dose with vehicle with a Bonferroni correction. For each ANOVA, Mauchly's test of sphericity was used to test whether variances of the differences between levels were equal. If the assumption of sphericity was violated, degrees of freedom were corrected using Greenhouse-Geisser (GG) estimates of sphericity or Huynh-Feldt estimates of sphericity when the GG estimate was > 0.75. Corrected degrees of freedom are presented rounded to the nearest integer.

Data were analysed and visualised using Microsoft Excel, GraphPad Prism (version 8.3.0, GraphPad Software Inc., USA), and SPSS for Windows (version 25.0.0.1, IBM Corp., USA). Results are presented as mean ± SEM unless otherwise stated. A significance criterion of $p < 0.05$, two-tailed, was used in all statistical analyses.

Results

Intermittent alcohol access

Alcohol intake and preference during the first two months of IAA are summarised in Figure 1. Statistical analyses confirmed that the selected groups (LD, MD, and HD) differed in their levels of alcohol intake and alcohol preference (intake: F_{group} (2,37) = 110.053, $p < 0.001$; preference: F_{group} (2,37) = 78.305, $p < 0.001$). During the first two months of alcohol exposure, alcohol intake significantly increased over weeks (F_{week} (3,123) = 149.237, $p < 0.001$) and this increase was more pronounced in HD compared to MD and LD, and more pronounced in MD compared to LD ($F_{week \times group}$ (7,123) = 30.404, $p < 0.001$). Similarly, alcohol preference significantly increased over weeks (F_{week} (4,139) = 61.569, $p < 0.001$) and this increase was more pronounced in HD compared to MD and LD, and more pronounced in MD compared to LD ($F_{week \times group}$ (8,139) = 15.950, $p < 0.001$). Post hoc analyses showed that alcohol intake and alcohol preference were significantly higher in HD compared to LD in each of the eight weeks ($p < 0.05$), and were also significantly higher in HD compared to MD starting from week 2 ($p < 0.01$). Moreover, alcohol intake and preference were significantly higher in MD compared to LD starting from week 5 ($p < 0.01$). Taken together, these data show that alcohol intake and alcohol preference increased over the course of the initial two months of IAA and subpopulations of LD, MD, and HD could be distinguished.

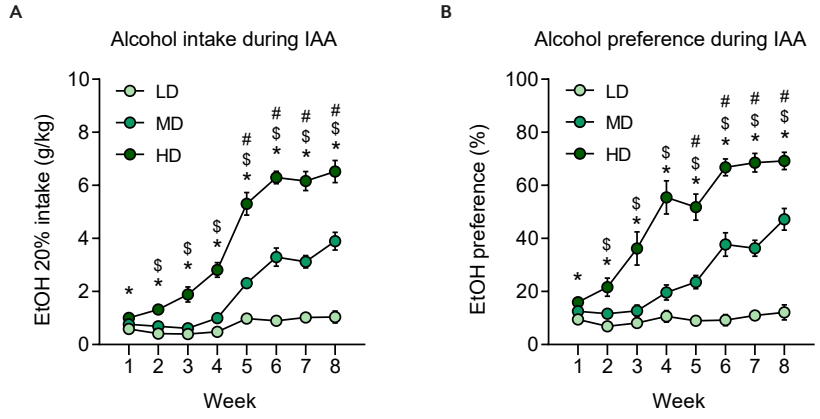

Figure 1. Alcohol intake and preference for LD, MD, and HD during the initial 2 months of IAA, prior to pharmacological treatment. Alcohol intake (A) and preference (B) were higher in HD (n = 12) compared to MD (n = 16), and LD (n = 12), and higher in MD compared to LD. Data are presented as the mean + SEM. * significant differences between HD and LD ($p < 0.05$); $ significant differences between HD and MD ($p < 0.01$); # significant differences between MD and LD ($p < 0.01$).

During treatment with the pharmacological compounds, the significant group differences in alcohol intake remained throughout the course of the study (baclofen: F_{group} (2,35) = 42.927, $p < 0.001$; naltrexone: F_{group} (2,37) = 22.990, $p < 0.001$; N-acetylcysteine: F_{group} (2,37) = 12.704, $p < 0.001$). Moreover, group differences in alcohol intake typically became more pronounced as the session progressed from 2 to 24 hours (baclofen: $F_{time \times group}$ (2,38) = 27.572, $p < 0.001$; naltrexone: $F_{time \times group}$ (2,42) = 20.129, $p < 0.001$; N-acetylcysteine: $F_{time \times group}$ (2,43) = 12.530, $p < 0.001$). Alcohol preference also remained significantly different between groups throughout the experiment (baclofen: F_{group} (2,35) = 47.423, $p < 0.001$; naltrexone: F_{group} (2,37) = 16.249, $p < 0.001$; N-acetylcysteine: F_{group} (2,37) = 7.754, $p = 0.002$).

Baclofen treatment

The effects of baclofen on alcohol intake and preference are depicted in Figure 2. Baclofen significantly decreased alcohol intake (F_{dose} (3,105) = 16.812, $p < 0.001$). This effect was dependent on the time in the session ($F_{dose \times time}$ (4,134) = 3.747,

p = 0.007), but was independent of group, although a trend was observed for an effect of baclofen dependent on group when disregarding timepoints in the session ($F_{dose\ x\ group}$ (6,105) = 2.127, p = 0.056; $F_{dose\ x\ time\ x\ group}$ (8,134) = 0.913, p = 0.505) (Figure 2A). Post hoc analyses showed that alcohol intake was significantly reduced after treatment with the highest dose of baclofen (3.0 mg/kg) when compared to vehicle at all three timepoints (p < 0.001).

Alcohol preference was not significantly affected by baclofen, although a trend was observed for a reduction in alcohol preference (F_{dose} (3,105) = 2.618, p = 0.055). This trend was independent of the time in the session ($_{Fdose\ x\ time}$ (4,127) = 2.034, p = 0.100), and was not dependent on the group ($F_{dose\ x\ group}$ (6,105) = 0.825, p = 0.553; $F_{dose\ x\ time\ x\ group}$ (7,127) = 0.641, p = 0.727) (Figure 2B). Water intake was not significantly altered by baclofen (F_{dose} (3,101) = 2.324, p = 0.082; $F_{dose\ x\ group}$ (6,101) = 0.460, p = 0.830; Supplementary Figure 1A).

Taken together, baclofen selectively reduced alcohol intake across the subgroups.

Naltrexone treatment

The effects of naltrexone on alcohol intake and preference are shown in Figure 3. Naltrexone caused an overall significant decrease in alcohol intake (F_{dose} (2,74) = 3.773, p = 0.028). Post hoc analyses showed that alcohol intake was significantly reduced after treatment with 1.0 mg/kg naltrexone compared to vehicle (p = 0.038). The effects of naltrexone on alcohol intake were independent of the time in the session ($F_{dose\ x\ time}$ (3,99) = 1.448, p = 0.236) and of the group ($F_{dose\ x\ group}$ (4,74) = 0.853, p = 0.496; $F_{dose\ x\ time\ x\ group}$ (5,99) = 0.900, p = 0.490) (Figure 3A).

In contrast to its effects on alcohol intake, naltrexone did not significantly alter alcohol preference in any of the groups at any of the timepoints tested (F_{dose} (2,74) = 0.488, p = 0.616; $F_{dose\ x\ time}$ (3,96) = 0.438, p = 0.697; $F_{dose\ x\ group}$ (4,74) = 0.297, p = 0.879; $F_{dose\ x\ time\ x\ group}$ (5,96) = 1.181, p = 0.324) (Figure 3B). Moreover, water intake was not significantly affected by naltrexone treatment either (F_{dose} (2,74) = 2.320, p = 0.105; $F_{dose\ x\ group}$ (4,74) = 1.643, p = 0.172; Supplementary Figure 1B).

Taken together, naltrexone caused a modest reduction in alcohol intake throughout the session, regardless of the individual baseline levels of alcohol intake.

N-Acetylcysteine treatment

The effects of N-acetylcysteine on alcohol intake and preference are shown in Figure 4. N-acetylcysteine did not significantly affect alcohol intake in any of the groups at any of the timepoints tested (F_{dose} (3,111) = 0.399, p = 0.754; $F_{dose\ x\ time}$ (4,143) = 0.335, p = 0.848; $F_{dose\ x\ group}$ (6,111) = 1.488, p = 0.189; $F_{dose\ x\ time\ x\ group}$ (8,143) = 0.961, p = 0.468) (Figure 4A).

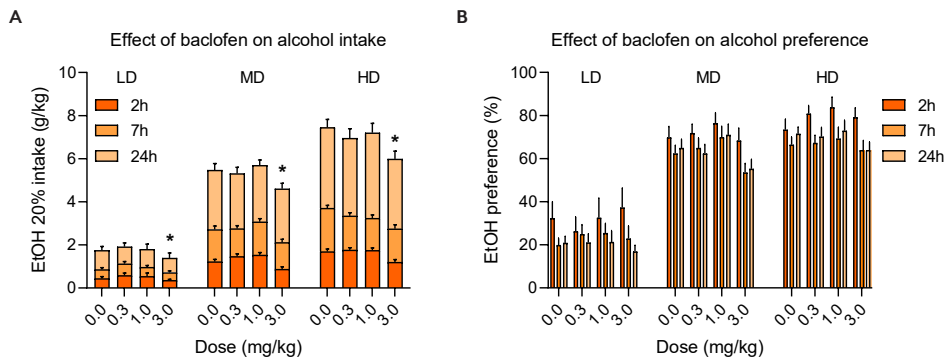

Figure 2. The effects of baclofen on alcohol intake and preference in LD, MD, and HD. Baclofen decreased alcohol intake (A) to a similar extent in LD (n = 10), MD (n = 16), and HD (n = 12). Post hoc analyses revealed that alcohol intake was significantly reduced after treatment with 3.0 mg/kg baclofen compared to vehicle at all three timepoints. Alcohol preference (B) was not affected by baclofen in either group at any timepoint. Data are presented as the mean + SEM. * significantly different from vehicle, p < 0.001.

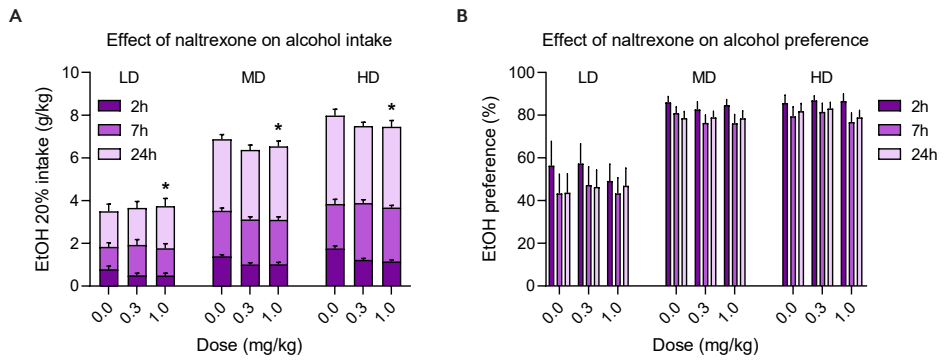

Figure 3. The effects of naltrexone on alcohol intake and preference in LD, MD, and HD. Naltrexone decreased alcohol intake (A) and this effect was independent of group (LD n = 12, MD n = 16, HD n = 12). Post hoc analyses revealed that alcohol intake was significantly reduced after treatment with 1.0 mg/kg naltrexone compared to vehicle. Alcohol preference (B) was not affected by naltrexone in either group at any timepoint. Data are presented as the mean + SEM. * overall differences from vehicle across the drinking session by post hoc pairwise comparisons, p < 0.05.

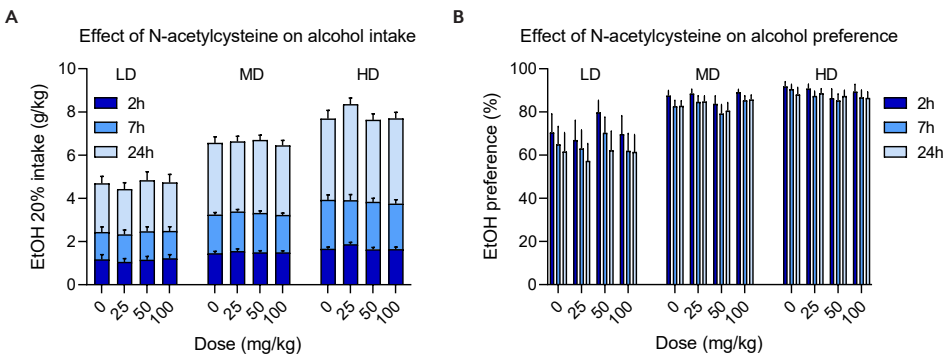

Figure 4. The effects of N-acetylcysteine on alcohol intake and preference in LD, MD, and HD. N-acetylcysteine did not affect alcohol intake (A) in any of the groups (LD n = 12, MD n = 16, HD n = 12) at any of the timepoints. N-acetylcysteine did not affect alcohol preference (B) in any of the groups at any of the timepoints. Data are presented as the mean + SEM.

Similar to its effects on alcohol intake, N-acetylcysteine did not significantly alter alcohol preference (F_{dose} (3,111) = 0.079, p = 0.971; $F_{dose\ x\ time}$ (4,156) = 0.228, p = 0.930; $F_{dose\ x\ group}$ (6,111) = 1.803, p = 0.105; $F_{dose\ x\ time\ x\ group}$ (8,156) = 0.966, p = 0.467) (Figure 4B). Furthermore, water intake was not significantly affected by N-acetylcysteine (F_{dose} (3,111) = 0.168, p = 0.918; $F_{dose\ x\ group}$ (6,111) = 1.292, p = 0.267; Supplementary Figure 1C).

Taken together, N-acetylcysteine did not significantly alter alcohol intake, alcohol preference or water intake.

Discussion

We determined the involvement of GABAergic, opioid, and glutamatergic neurotransmission in alcohol consumption, taking individual baseline differences in alcohol intake into account. To this aim, we assessed the effects of baclofen, naltrexone, and N-acetylcysteine on alcohol consumption in subgroups of rats that drank low, medium, or high levels of alcohol. Treatment with baclofen and naltrexone caused reductions in alcohol intake. By contrast, treatment with N-acetylcysteine did not affect alcohol consumption. Remarkably, the effects of baclofen and naltrexone on alcohol consumption were similar across subpopulations of LD, MD, and HD, indicating that individual differences in alcohol intake are not associated with differences in sensitivity to these drugs. These findings suggest that GABAergic, opioid, and glutamatergic pathways do not contribute to the individual variation in alcohol consumption in Lister Hooded rats under IAA conditions.

Baclofen

The $GABA_B$ receptor agonist baclofen reduced alcohol intake and we observed a trend towards a reduction in alcohol preference after treatment with this compound. Water intake was not affected, suggesting specific effects of baclofen on alcohol consumption. These findings are in line with other preclinical studies that reported a suppressing effect of baclofen on alcohol consumption that was selective for alcohol intake (Colombo et al., 2000; Daoust et al., 1987; Perfumi et al., 2002; Stromberg, 2004; Walker & Koob, 2007). Additionally, baclofen reduced operant responding for alcohol in rats, although reductions in responding for sucrose have also been observed (Anstrom et al., 2003; Janak & Gill, 2003). Moreover, baclofen suppressed operant alcohol self-administration in both alcohol-dependent and nondependent rats with an increased sensitivity to baclofen in the dependent rats (Walker & Koob, 2007). Open-label clinical studies further support the potential of baclofen to reduce alcohol craving and intake in alcohol dependent individuals (Addolorato et al., 2000; Flannery et al., 2004), although results from double-blind, placebo-controlled, randomised clinical trials have been inconsistent. Some studies reported a significantly higher percentage of subjects maintaining total alcohol abstinence (Addolorato et al., 2002, 2007; Müller et al., 2015), whereas others found no effect of baclofen on abstinence and craving (Beraha et al., 2016; Garbutt et al., 2010).

Despite generally consistent outcomes in preclinical studies, a variability in the efficacy of baclofen treatment on alcohol consumption across rat strains has also been reported (Maccioni et al., 2012). Baclofen was found to selectively reduce alcohol intake in Long-Evans rats (Daoust et al., 1987), Wistar rats (Stromberg, 2004), and genetically selected Sardinian alcohol-preferring rats (Colombo et al., 2000; Perfumi et al., 2002). However, baclofen differentially affected operant alcohol self-administration in three different lines of selectively bred, alcohol-preferring rats (i.e. Indiana alcohol-preferring, Sardinian alcohol-preferring, and Alko Alcohol rats) (Maccioni et al., 2012). In this latter study, only the highest dose of baclofen, 3.0 mg/kg, effectively reduced operant alcohol self-administration, which is in line with our findings that 3.0 mg/kg baclofen reduced alcohol intake while lower dosages were not effective.

Although the mechanism by which baclofen exerts its effect on alcohol intake has not been unravelled, it is known that $GABA_B$ receptors are present in the VTA, both on dopamine neuronal cell bodies and on glutamatergic afferent neuron terminals (Bowery et al., 1987; Morales & Margolis, 2017). It has been hypothesised that by activation of these $GABA_B$ receptors, alcohol-stimulated dopamine release is directly and indirectly inhibited (Addolorato & Leggio, 2010; Westerink et al., 1996; Yoshida

et al., 1994). Also, baclofen seems to suppress alcohol-induced dopamine release in the nucleus accumbens shell (Colombo et al., 2004). Another hypothesis of the mechanism of baclofen's effect on alcohol consumption, is that $GABA_B$ receptor stimulation could, in some ways, substitute for the effects of alcohol (Chick & Nutt, 2012; de Beaurepaire, 2018). Alcohol and baclofen can have similar behavioural effects including confusion, feelings of drunkenness, unsteady gait, dizziness, and impairment in attention and memory (de Beaurepaire, 2018; Rigal et al., 2015). Although alcohol and baclofen do not seem to act directly on the same receptors, they both potentiate the actions of GABA, so that $GABA_B$ receptor stimulation through baclofen could partially mimic the actions of alcohol.

The suppressing effect of baclofen on alcohol intake was similar across subpopulations of LD, MD, and HD, suggesting that GABAergic mechanisms do not likely contribute to individual differences in alcohol intake. $GABA_B$ receptor function was shown to be lower in limbic areas of Sardinian alcohol-preferring and Sardinian alcohol-non-preferring rats before exposure to alcohol (Castelli et al., 2005), although these differences disappeared after one month of voluntary alcohol consumption. The prolonged exposure to alcohol that was used in this study, to model the maintenance phase of human AUD, may therefore also explain the lack of differences in sensitivity to baclofen across the subgroups of LD, MD, and HD in the present study. Although subpopulations responded similarly to baclofen in this phase, this therefore does not exclude the possibility that initial differences in $GABA_B$ receptor function might have contributed to individual differences in intake during acquisition of alcohol drinking.

Naltrexone

The opioid receptor antagonist naltrexone caused modest reductions in alcohol intake and did not significantly alter alcohol preference or water intake. Previous preclinical studies have repeatedly shown that naltrexone reduces alcohol consumption in rats in a variety of strains, including Wistar rats (Daoura & Nylander, 2011; Momeni et al., 2015; Steensland et al., 2012), alcohol-preferring rats (Coonfield et al., 2004; Parkes & Sinclair, 2000), and Long Evans rats (Coonfield et al., 2002; Ferraro et al., 2002; Higley & Kiefer, 2006; Hill & Kiefer, 1997; Simms et al., 2008). However, opioid receptor antagonists may not be selective and instead broadly affect consumption and palatability as they have been shown to decrease food intake (Brands et al., 1979; Brown & Holtzman, 1979) and fluid consumption in rats (Hill et al., 2010; Juárez & Barrios De Tomasi, 2008; Simms et al., 2008).

Nevertheless, multiple clinical trials have confirmed that naltrexone produces significant benefits in the treatment of AUD, such as reduced alcohol drinking, craving, and decreased relapse rates (Heinälä et al., 2001; O'Malley et al., 1992; Volpicelli et al., 1992). Because of these beneficial effects, naltrexone was approved by the FDA and the EMA for treatment of AUD. However, there is a large individual variation in the effects of naltrexone in AUD patients (Kiefer et al., 2008; Spanagel & Kiefer, 2008) which has been shown in humans and animals to be related to variations in the μ-opioid receptor gene (OPRM1) (Barr et al., 2010; Bilbao et al., 2015; Henderson-Redmond et al., 2018; Vallender et al., 2010). Specifically, the A118G (Asn40Asp) single nucleotide polymorphism seems to predict treatment response to naltrexone. Carriers with at least one copy of the Asp40 allele respond better to naltrexone than alcohol dependent Asn40 homozygote patients (Anton et al., 2008; Oslin et al., 2003; Ray & Hutchison, 2007) although not all studies agree with these findings (Gelernter et al., 2007).

Although the mechanism is not completely understood, it is hypothesised that naltrexone exerts its effects partly via an interaction with the mesolimbic dopamine system. Alcohol induces a release of β-endorphin, which is an endogenous opioid peptide, in the VTA and nucleus accumbens (Jarjour et al., 2009; Marinelli et al., 2003). Naltrexone is thought to decrease alcohol-induced dopamine release via the blockade of VTA μ-opioid receptors, which otherwise would be activated by endogenous opioid peptides that would suppress the inhibitory GABAergic interneurons (Benjamin et al., 1993; Di Chiara & Imperato, 1988; Gonzales & Weiss, 1998; Johnson & North, 1992; Spanagel et al., 1992; Valenta et al., 2013). As a result, naltrexone is considered to indirectly attenuate alcohol-induced dopamine release. Moreover, naltrexone is thought to exert its effects by dampening the reinforcing properties of alcohol, which are mediated partly through the endogenous opioid system. Selective μ-opioid receptor and δ-opioid receptor antagonists attenuated alcohol-induced place preference and naltrexone rendered ethanol more aversive in rats (Higley & Kiefer, 2006; Matsuzawa et al., 1999). Consistently, naltrexone decreased ratings of liking of alcohol and ratings of desire to drink, both prior to and following alcohol administration in humans (McCaul et al., 2000). Thus, blockade of the μ-opioid receptor through naltrexone largely prevents the activation of this receptor by alcohol-induced endogenous opioids, thereby blunting the positive reinforcing effects of alcohol.

Differences in μ-opioid receptor expression levels have also been described for alcohol-preferring and alcohol non-preferring rats. Alcohol-preferring rats are characterised by higher levels of μ-opioid receptors in the VTA, nucleus accumbens

shell and core, and prefrontal cortex (de Waele et al., 1995; Marinelli et al., 2000; McBride et al., 1998). Others report decreased μ-opioid receptor binding in the hippocampus and amygdala in brain sections of alcohol-preferring rats, whereas the substantia nigra showed higher binding density of the μ-opioid receptors relative to the alcohol non-preferring brain sections (Soini et al., 1998). The present findings do not provide evidence for differences in naltrexone sensitivity across subpopulations of LD, MD, and HD, suggesting that μ-opioid receptors unlikely contribute to the differences in alcohol intake between these subgroups.

N-Acetylcysteine

The cysteine precursor N-acetylcysteine did not affect alcohol intake and preference in the current study. This is in contrast to recent findings showing that N-acetylcysteine treatment reduced alcohol intake and the motivation to consume alcohol in rats (Lebourgeois et al., 2018, 2019; Quintanilla et al., 2016). There were some differences in the methodologies with the approach for the current study, such as in sex, strain, consumption versus operant self-administration, and alcohol pre-exposure that may explain these contrasting effects. Quintanilla and colleagues (2016) exposed female alcohol-preferring rats continuously for 25 days to an alcohol solution and water. N-acetylcysteine (30 mg/kg and 60 mg/kg) was tested either during the acquisition phase or the maintenance phase, whereas we assessed tested N-acetylcysteine only during the maintenance phase. Interestingly, both N-acetylcysteine doses reduced alcohol intake during the maintenance phase, but not during the acquisition phase. The study by Lebourgeois and colleagues (2018) subjected male Long Evans rats to IAA followed by operant alcohol self-administration. Next, the effect of N-acetylcysteine was determined on alcohol self-administration, alcohol seeking during extinction, and motivation to consume alcohol. At a dose of 100 mg/kg, N-acetylcysteine decreased operant alcohol self-administration, alcohol seeking, and motivation to consume alcohol. Similar results were found in another recent study, in which male Wistar rats were subjected to IAA, operant alcohol self-administration, and chronic intermittent ethanol vapor exposure to induce alcohol dependence (Lebourgeois et al., 2019). Thereafter, the effects of N-acetylcysteine on operant alcohol self-administration were determined 8 hours into withdrawal. N-acetylcysteine reduced operant alcohol self-administration and motivation to consume alcohol. Altogether, previous preclinical studies suggest that N-acetylcysteine is effective in reducing alcohol consumption, but this was not found in the current study. Moreover, N-acetylcysteine has been shown to reduce alcohol related behaviours in clinical samples (Back et al., 2016; Squeglia et al., 2016, 2018), emphasising its therapeutic potential.

Limitations and future directions

Together with an earlier study, our findings suggest that individual differences in alcohol intake are not associated with differences in GABA, opioid, glutamate, and dopamine neurotransmission (Spoelder et al., 2016). However, this interpretation should be taken with caution as only a limited number of pharmacological compounds per system were tested and the compounds were all administered systemically.

A further limitation of this study is the gradual increase in alcohol intake that emerged in all groups as the study progressed. Baseline levels of alcohol consumption increased, for example from 2 g/kg/day in the baclofen testing phase to 4 g/kg/day in the N-acetylcysteine testing phase for the LD. To limit the impact of the escalating levels of alcohol intake in all groups on the variability in the data, we employed a block design in which the three compounds were tested consecutively in separate Latin square designs. Although the possibility cannot be ruled out, it is unlikely that this prolonged alcohol exposure before the N-acetylcysteine testing has affected the results from the N-acetylcysteine treatment as previous preclinical studies showed a suppressing effect of N-acetylcysteine on alcohol intake, also after long periods of exposure (Lebourgeois et al., 2019).

Importantly, LD, MD, and HD subgroups remained distinguishable throughout the complete experiment, despite the shift in baseline alcohol intake levels. The HD are thought to display a typical AUD phenotype already after two months of alcohol exposure with more motivation to obtain alcohol and more punishment-resistant alcohol-directed behaviour than LD (Spoelder et al., 2015, 2017). However, the substantial increase in alcohol consumption in LD and MD after prolonged exposure suggests that characteristics of an AUD phenotype might also develop in LD and MD rats, albeit at a slower pace (Spoelder et al., 2017). This implies that, besides excessive amounts of alcohol consumption, cumulative alcohol exposure might be a risk factor for the development of AUD.

Another limitation is that the human AUD pathology was not fully mimicked by our experimental design because we focused on one component, i.e. alcohol consumption. However, AUD entails more than solely alcohol intake and preference as it is characterised by a loss of control and continued alcohol use despite adverse consequences. Therefore, the modest effects in the current study do not exclude the potential value of baclofen, naltrexone, and N-acetylcysteine for the treatment of AUD. Future research should therefore address the pharmacological effects of these compounds on individual differences in other behavioural aspects of AUD, such as relapse or loss of control.

Moreover, other potential valuable approaches are treatments in which (subthreshold) doses of different compounds are combined, for example as was done in recent studies (Israel et al., 2019; Moore et al., 2014; Navarro et al., 2019; Rezvani, 2000; Stromberg, 2004). Since multiple neurochemical systems are involved in alcohol intake, improved efficacy of pharmacotherapy might arise from combining different compounds. Moreover, it is a common strategy in pharmacotherapy for other diseases to simultaneously use multiple drugs that act on different targets. A potential benefit of combination pharmacotherapy is that it may allow for the use of lower doses of each compound, thereby reducing the negative side effects that might lead to lower compliance and higher relapse (Stromberg, 2004).

Conclusion

To conclude, treatment with baclofen and naltrexone reduced voluntary alcohol intake in rats, independent of the individual alcohol intake levels. These findings suggest that $GABA_B$ and μ-opioid receptor mediated neurotransmission contribute to alcohol consumption, but do not explain the degree of individual variation in alcohol intake in our setup. By contrast, N-acetylcysteine did not affect alcohol intake, implying that glutamatergic neurotransmission does not contribute to alcohol intake in our model. Taken together, this study contributes to the understanding of the role of GABA, opioid, and glutamate neurotransmission in alcohol intake.

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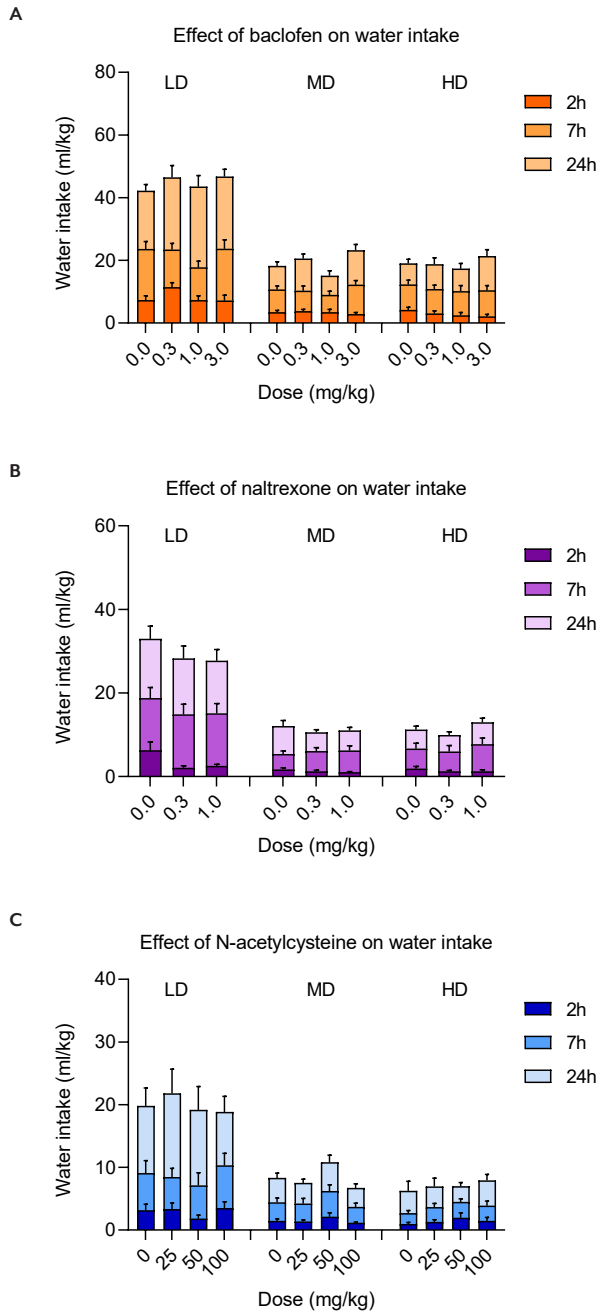

Supplementary Figure 1. The effects of baclofen, naltrexone, and N-acetylcysteine on water intake in LD, MD, and HD. Water intake was not significantly affected by baclofen (**A**), by naltrexone (**B**), or by N-acetylcysteine (**C**) in either group at any timepoint. Data are presented as the mean + SEM.

Chapter 8

Appendix: Involvement of the basolateral amygdala, the orbitofrontal cortex, and the nucleus accumbens in sucrose seeking under threat of adversity in rats

A. Maryse Minnaard[1,2]
Johanna A. S. Smeets[1,2]
Geert M. J. Ramakers[2]
Roger A. H. Adan[2]
Heidi M. B. Lesscher[1]
Louk J. M. J. Vanderschuren[1]

[1] Department of Population Health Sciences, Unit of Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
[2] Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, The Netherlands

Background

Loss of control is a hallmark of substance use disorder (SUD) as is reflected in many of the diagnostic criteria for SUD. In chapter 3, we introduced a novel task to allow for measuring this behavioural feature of SUD. The Seeking under Threat of Adversity (STA) task aimed to better emulate key aspects of addictive behaviour in humans. In chapter 4, the STA task was utilised to investigate the involvement of the prelimbic prefrontal cortex (PrL) in reward seeking under threat of adversity. The effects of pharmacological inactivation of the PrL, through local infusions of a GABA agonist mixture (i.e. baclofen and muscimol), were assessed in rats that were trained to seek alcohol or sucrose rewards. We found that PrL inactivation selectively reduced suppression of alcohol seeking in the face of a threat of punishment.

Neurobiological changes in various other brain regions have been implicated in control over reward seeking and taking, including the basolateral amygdala (BLA), the orbitofrontal cortex (OFC), and the nucleus accumbens (NAc) (e.g. Ambroggi et al., 2008; Jean-Richard-dit-Bressel & McNally, 2016; Kasanetz et al., 2010; Killcross et al., 1997; Orsini et al., 2015; Pelloux et al., 2013; Stuber et al., 2011). The OFC has been associated with behavioural control over reward seeking. Inactivation of the OFC impaired the use of aversive instrumental memory, leading to an increase in punished responding (Jean-Richard-dit-Bressel & McNally, 2016). Contrasting findings were reported in a study where rats appeared more, rather than less, sensitive to punishment after lesioning of the OFC as it decreased risk-taking (Orsini et al., 2015). The PrL and the OFC are part of a larger interconnected network as they receive direct and indirect (via thalamus) projections from brain regions known to be involved with drug reinforcement, such as the BLA and the NAc. In turn, the PrL and the OFC also project to these brain areas (Moorman et al., 2015; Rempel-Clower, 2007). Moreover, the BLA and NAc are implicated in reward-directed behaviour, as lesions of the BLA impaired conditioned punishment (Killcross et al., 1997) as well as punished cocaine seeking (Pelloux et al., 2013). The BLA projection to the NAc is thought to drive reward seeking (Ambroggi et al., 2008; Stuber et al., 2011), and altered plasticity in the NAc has been implicated in resistance to punished cocaine self-administration (Kasanetz et al., 2010). Nevertheless, little is known about the involvement of these and other brain regions in loss of control over substance use.

To explore the involvement of the BLA, OFC, and NAc in sucrose seeking when under threat of adversity, we pharmacologically inactivated each of these brain regions and determined the effects of inactivation on sucrose seeking behaviour in the STA task. To that aim, Lister Hooded rats were trained to respond for sucrose and were tested in the STA task shortly after local infusions of a mixture of baclofen and muscimol.

Methods

A total of 44 adult male Lister Hooded rats (Charles River, Germany), weighing 250-300 grams at the start of the experiments, were used in these experiments. All rats were equipped with 26-gauge bilateral guide cannulas (Plastics One, USA) that were aimed at the BLA (AP -3.4, ML ±5.0, DV -7.3 (0° angle), n = 12), the lateral OFC (AP +2.6, ML ±2.6, DV -3.1 (5° angle), n = 12), or the NAc (AP +1.4, ML ±2.0, DV -5.6 (5° angle), n = 20) with coordinates in mm relative to bregma (Paxinos & Watson, 2004).

All animals underwent the exact same housing, behavioural training, and testing procedures as described in chapter 4. Histological verification and data analysis were performed as outlined in chapter 4.

Data from rats that lost one or both of their cannulas before the experiment was finished or from rats with one or both cannulas placed outside the target area were discarded from all analyses. In total, 6 rats from the BLA group, 4 rats from the OFC group, and 10 rats from the NAc group were excluded.

Results

BLA inactivation

To determine the role of the BLA in behavioural control over sucrose seeking, the BLA was inactivated prior to seeking under threat of adversity (STA) tests. The number of ALPs was significantly lower in tone-shock sessions compared to baseline sessions, independent of baclofen/muscimol treatment (Figure 1A; $F_{sessiontype}$ (1,5) = 7.389, p = 0.042; $F_{treatment}$ (1,5) = 0.015, p = 0.907; $F_{sessiontype\ x\ treatment}$ (1,5) = 0.003, p = 0.960). The number of rewards obtained was not significantly different between tone-shock and baseline sessions, although a trend towards less rewards in the tone-shock session was observed, which was not affected by infusion with baclofen/muscimol (Figure 1B; $F_{sessiontype}$ (1,5) = 6.531, p = 0.051; $F_{treatment}$ (1,5) = 0.625, p = 0.465; $F_{sessiontype\ x\ treatment}$ (1,5) = 0.132, p = 0.732). Moreover, analysis of the suppression ratio data did not reveal significant differences between baclofen/muscimol and saline treatments (Figure 1C-D; t(5) = 0.157, p = 0.881). Taken together, baclofen/muscimol infusions in the BLA did not alter suppression of sucrose seeking in the STA test.

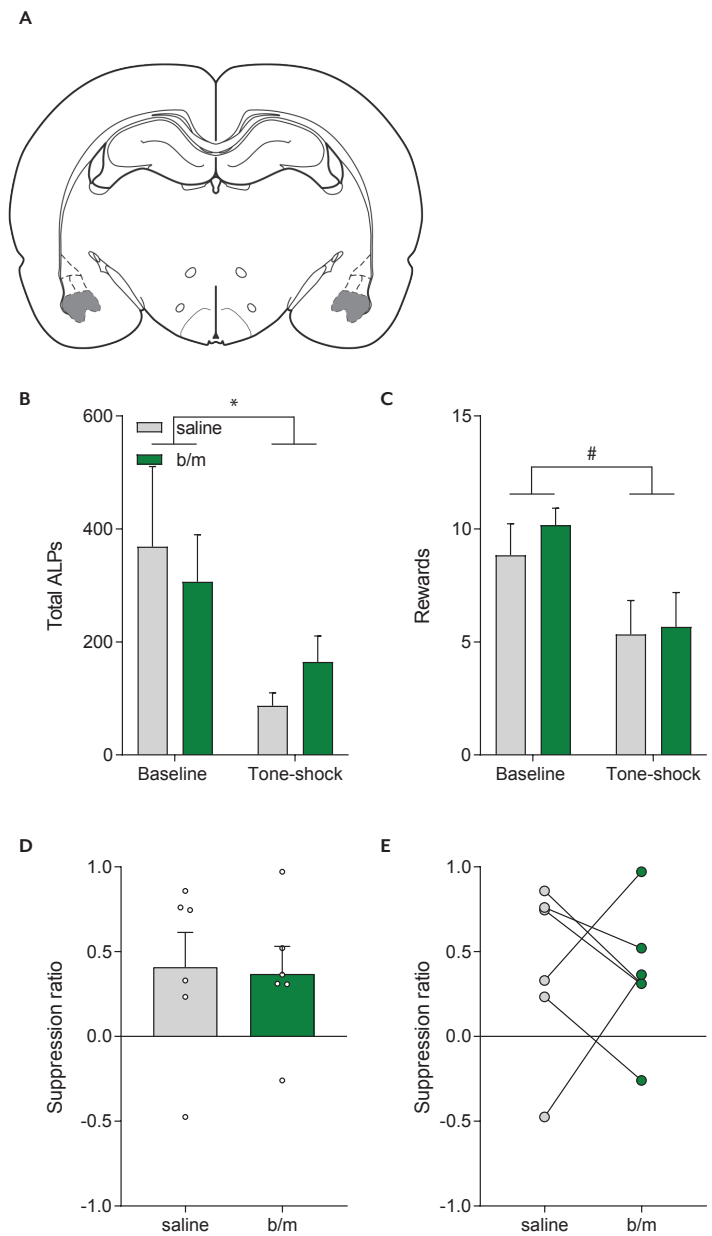

Figure 1. Effects of pharmacological inactivation of the basolateral amygdala (BLA) on sucrose seeking behaviour in the STA task. A. Infusions were administered into the BLA, which is indicated in grey in the coronal brain section. **B.** Total active lever presses (ALPs) made in baseline and tone-shock sessions. **C.** Number of rewards obtained in baseline and tone-shock sessions. **D.** Suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. **E.** Individual suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. Data expressed as mean + SEM. Significant differences ($p < 0.05$) are indicated with *. A trend towards a main effect of session type ($p = 0.051$) is indicated with #.

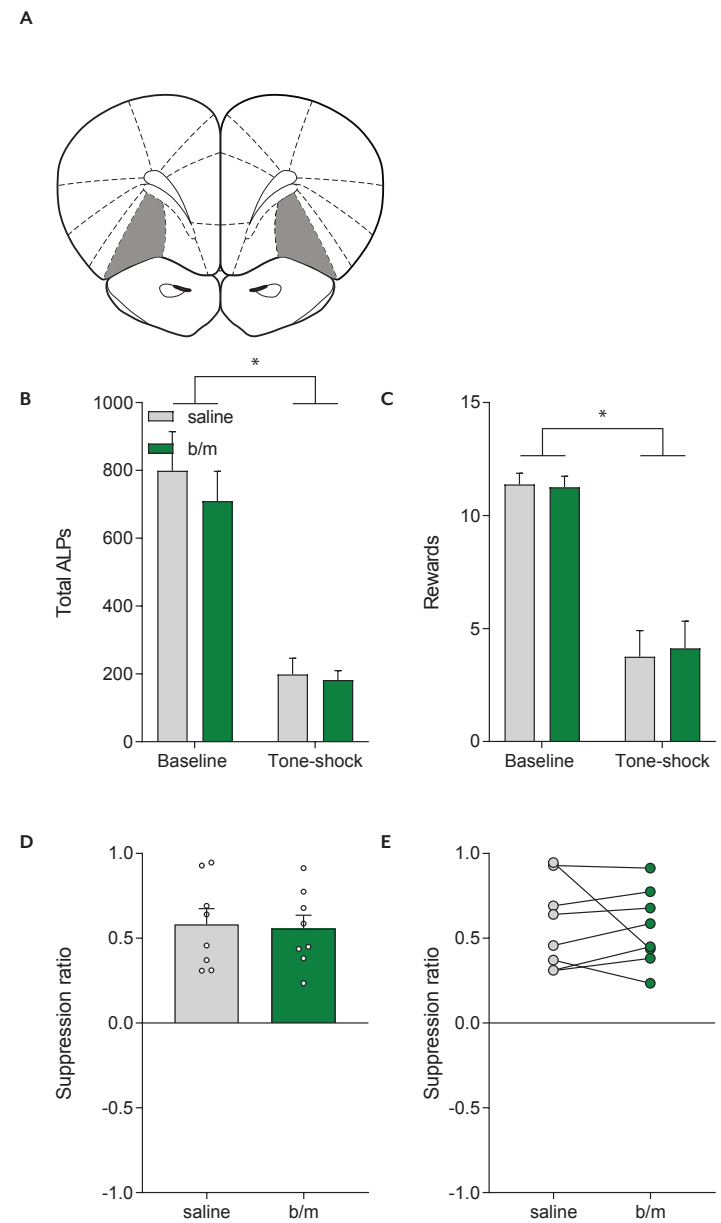

Figure 2. Effects of pharmacological inactivation of the lateral orbitofrontal cortex (OFC) on sucrose seeking behaviour in the STA task. A. Infusions were administered into the OFC, which is indicated in grey in the coronal brain section. **B.** Total active lever presses (ALPs) made in baseline and tone-shock sessions. **C.** Number of rewards obtained in baseline and tone-shock sessions. **D.** Suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. **E.** Individual suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. Data expressed as mean + SEM. Significant differences ($p < 0.05$) are indicated with *.

OFC inactivation

To determine the role of the OFC in behavioural control over sucrose seeking, the OFC was inactivated prior to seeking under threat of adversity (STA) tests. The number of ALPs and rewards obtained were significantly lower in tone-shock sessions when compared to baseline sessions, independent of baclofen/muscimol infusion into the OFC (Figure 2A-B; ALPs: $F_{sessiontype}$ (1,7) = 23.596, p = 0.002; $F_{treatment}$ (1,7) = 0.001, p = 0.980; $F_{sessiontype\ x\ treatment}$ (1,7) = 0.404, p = 0.545. rewards: $F_{sessiontype}$ (1,7) = 32.104, p < 0.001; $F_{treatment}$ (1,7) = 0.111, p = 0.749; $F_{sessiontype\ x\ treatment}$ (1,7) = 0.259, p = 0.626). Moreover, analysis of the suppression ratio data revealed no significant difference between baclofen/muscimol and saline treatments (Figure 2C-D; Z = -0.420, p = 0.674). Taken together, baclofen/muscimol infusions in the OFC did not alter suppression of sucrose seeking.

NAc inactivation

To determine the role of the NAc in behavioural control over sucrose seeking, the NAc was inactivated prior to seeking under threat of adversity (STA) tests. The number of ALPs and obtained rewards were significantly lower in tone-shock sessions compared to baseline sessions, independent of baclofen/muscimol infusions (Figure 3A-B; ALPs: $F_{sessiontype}$ (1,9) = 16.820, p = 0.003; $F_{treatment}$ (1,9) = 0.957, p = 0.354; $F_{sessiontype\ x\ treatment}$ (1,9) = 4.414, p = 0.065. rewards: $F_{sessiontype}$ (1,9) = 16.020, p = 0.003; $F_{treatment}$ (1,9) = 0.067, p = 0.801; $F_{sessiontype\ x\ treatment}$ (1,9) = 1.598, p = 0.238). Subsequent analysis of the suppression ratio data revealed that suppression ratios were significantly higher upon baclofen/muscimol infusions when compared to saline infusions (Figure 3C-D; t(9) = 2.844, p = 0.019). Taken together, baclofen/muscimol infusions in the NAc increased suppression ratios, without affecting the number of ALPs and rewards obtained.

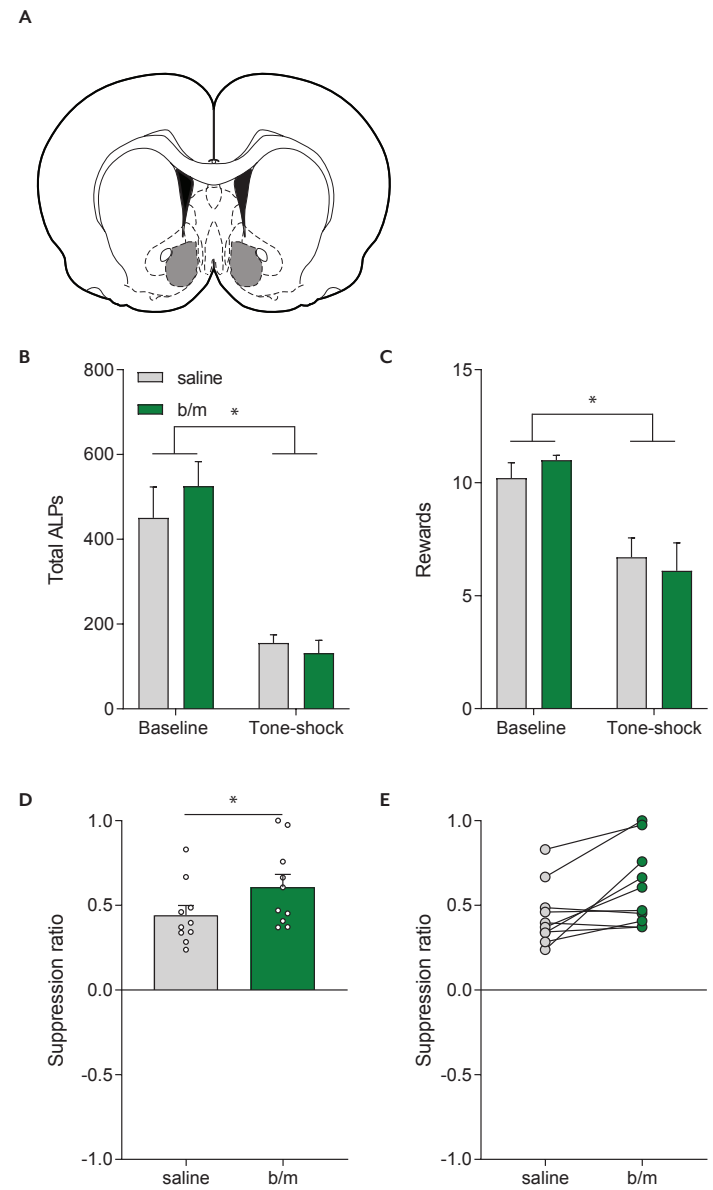

Figure 3. Effects of pharmacological inactivation of the nucleus accumbens (NAc) on sucrose seeking behaviour in the STA task. A. Infusions were administered into the NAc, which is indicated in grey in the coronal brain section. **B.** Total active lever presses (ALPs) made in baseline and tone-shock sessions. **C.** Number of rewards obtained in baseline and tone-shock sessions. **D.** Suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. **E.** Individual suppression ratios of sessions after baclofen/muscimol (b/m) infusions and after saline infusions. Data expressed as mean + SEM. Significant differences (p < 0.05) are indicated with *.

Concluding remarks

The present set of experiments investigated the involvement of the BLA, OFC, and NAc in sucrose seeking when under threat of adversity. No effect of pharmacological inactivation of the BLA was found on group level. However, two kinds of effects were observed when examining the individual datapoints: four rats showed a decrease in suppression ratio while two rats showed a steep increase in suppression ratio upon BLA inactivation. Further investigations using a larger sample size are warranted to clarify these findings. Moreover, pharmacological inactivation of the NAc increased suppression for sucrose. However, this effect seemed to be largely driven by an increase in the number of ALPs in baseline sessions, whereas NAc inactivation seemed to have little effect on the number of ALPs in tone-shock sessions. This observation could reflect a disinhibition of operant responding when no (threats of) negative consequences are present (see e.g. Carlezon & Thomas, 2009; Yun et al., 2004). Furthermore, the infusions in the NAc were not restricted to only the NAc shell or core. Future studies should selectively target these NAc subregions to assess the involvement of the NAc shell and core separately.

Unfortunately, the experiments presented here suffered from a high dropout rate. In total, 20 rats were excluded which limited the number of subjects to 24. To prevent the loss of cannulas, we recommend performing the surgeries shortly before testing in future experiments. Additionally, it may be considered to house the rats in cages with a flat top which they cannot reach with their heads, so that damage to the cannulas can be avoided.

Taken together, our findings show that pharmacological inactivation of the BLA and OFC did not affect sucrose seeking, whereas inactivation of the NAc increased suppression for sucrose. Although inactivation of the NAc lead to higher suppression ratios, the number of seeking responses was not significantly affected. Thus, sucrose seeking under threat of adversity was relatively unaffected in these experiments.

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Chapter 9

General discussion

The overarching aim of this thesis was to gain insight into changes in brain and behaviour that characterise substance use disorder (SUD), by using behavioural models that capture important aspects of addictive behaviour in combination with neuropharmacological interventions in rats. Inspired by existing behavioural models of SUD, we designed and utilised a novel animal model to assess loss of control over substance use. We investigated the interrelation of alcohol use disorder (AUD) associated behaviours. Moreover, we assessed the role of dopaminergic neurotransmission in motivation and reward valuation. We also examined the involvement of GABAergic, opioid, and glutamatergic neurotransmission in individual differences in alcohol consumption. Altogether, the findings in this thesis contribute to the understanding of the underlying behavioural and neurobiological elements associated with SUD. In this final chapter, the main findings will be revisited, thereby providing an overall summary. Subsequently, we interpret our findings in a broader context, consider some methodological issues, discuss the potential implications, and present directions for future research.

1. Summary of main findings

In chapter 2, we reviewed the literature regarding preclinical punishment models of compulsive substance use that employ aversive stimuli. These aversive stimuli can target either touch, gustation, or interoception; widely applied punishers in substance self-administration studies include electric foot shock, quinine, lithium chloride and histamine. In punishment models, the pursuit of rewarding substances is often associated with aversive consequences, and the willingness of animals to endure this adversity to obtain the reward is assessed. Importantly, a reduced sensitivity to punishment in such a context is considered to represent loss of control over substance use. Altogether, studies using these punishment models have substantially moved the preclinical SUD field forward and we expect that further development and optimisation of these models will continue to contribute to our knowledge about the neuronal and behavioural structure of SUD.

In chapter 3, we aimed to develop a novel behavioural task in rats to improve resemblance of addictive behaviour in humans. Inspired by existing punishment models as discussed in chapter 2, we constructed an operant task in which control over reward seeking behaviour was measured under threat of adversity (i.e. the Seeking under Threat of Adversity (STA) task), which was operationalised using a tone cue that predicted a response-contingent probabilistic foot shock punishment. We showed that threat of adversity reduced reward seeking and that a moderate shock intensity was sufficient to consistently suppress seeking behaviour for both alcohol and sucrose when tested repeatedly. Thus, we present the STA task as a novel tool to assess control over substance seeking.

In chapter 4, we aimed to investigate the involvement of the prelimbic prefrontal cortex (PrL) in reward seeking under threat of adversity, applying the STA task as presented in chapter 3. Considering its potential involvement in response inhibition and compulsive reward seeking, the PrL may be particularly important in situations involving behavioural control over reward pursuit. Pharmacological inactivation of the PrL reduced suppression of responding for alcohol, but not for sucrose in the STA task, indicating that activity in the PrL is required for suppression of alcohol seeking under threat of adversity. These findings suggest that reduced neural activity in the PrL promotes persistent alcohol seeking behaviour which may underlie compulsive alcohol use in AUD.

In chapter 5, we aimed to explore the relationship between four behavioural measures that have been associated with AUD: alcohol intake, habit formation, motivation, and punishment sensitivity. We found that most measures were not correlated, except for an association between alcohol intake and aversion-resistant alcohol intake, and no separate clusters were detected in the data. A small group of animals was consistently classified as displaying AUD-like behaviours. The composition of the remaining subpopulation of animals with the highest addiction severity score varied substantially, depending on the combination of behavioural measures that were included in the composition score.

In chapter 6, we aimed to determine the role of mesocorticolimbic dopamine signalling in the relationship between price and consumption of sucrose, to better understand its role in cost-benefit decision making. Stimulation of mesocorticolimbic dopaminergic neurotransmission was found to alter cost-benefit trade-offs in a complex manner. While increasing demand elasticity - which is indicative of a decrease in essential value - of palatable food, stimulation of mesocorticolimbic dopaminergic neurotransmission increased incentive motivation. Together, these findings imply that mesocorticolimbic dopamine signalling differentially influences distinct components of value-based decision making processes.

In chapter 7, we aimed to determine the involvement of GABAergic, opioid, and glutamatergic neurotransmission in individual differences in alcohol consumption. Subgroups of low, medium, and high alcohol drinking rats (LD, MD, and HD) were selected based on alcohol intake using an intermittent-every-other-day two-bottle

choice procedure. The subgroups were treated with baclofen, naltrexone, and N-acetylcysteine, and the effects on alcohol consumption were assessed. We found that treatment with baclofen and naltrexone reduced voluntary alcohol intake, independent of the individual alcohol intake levels. These findings suggest that $GABA_B$ and μ-opioid receptor mediated neurotransmission contribute to alcohol consumption, but do not explain the degree of individual variation in alcohol intake in our setup. By contrast, N-acetylcysteine did not affect alcohol intake, implying that glutamatergic neurotransmission does not contribute to alcohol intake in our model.

In the appendix, we presented a set of experiments in which we aimed to explore the involvement of the basolateral amygdala (BLA), the orbitofrontal cortex (OFC), and the nucleus accumbens (NAc) in sucrose seeking under threat of adversity. For that purpose, we used the STA task in combination with pharmacological inactivation, similar to chapter 4. Pharmacological inactivation of the BLA and OFC did not affect sucrose seeking, whereas inactivation of the NAc increased suppression for sucrose without altering the number of seeking responses. Altogether, sucrose seeking under threat of adversity was relatively unaffected in these experiments, suggesting that behavioural control over sucrose seeking does not depend on neuronal activation in the BLA, the OFC, or the NAc.

2. Reflection on findings

Behavioural mechanisms of substance use disorder

Loss of control as a hallmark of substance use disorder

An important contribution of this thesis is the presentation of a novel behavioural model, i.e. the STA task, for the assessment of loss of control over reward seeking. SUD is characterised by a lack of control over substance use, which is reflected in many of the SUD diagnostic criteria. Loss of control can be considered a collective term, covering a series of processes that are affected in SUD. More specifically, substance abuse is associated with impaired performance and aberrant brain activity in tasks requiring goal maintenance, response inhibition, or cognitive flexibility (Bühringer et al., 2008; Garavan & Stout, 2005; Goldstein & Volkow, 2011; Goschke, 2014).

Interestingly, the STA task revealed differences between control over sucrose and alcohol, in that PrL inactivation reduced alcohol seeking under threat of adversity but did not affect sucrose seeking in the STA task, as described in chapter 4. This may speak for specificity of the STA task, since other studies have shown that PrL inactivation may lead to a more general loss of control over reward seeking (Limpens et al., 2015; Verharen et al., 2019b). In support of this reinforcer specificity, seeking behaviour for sucrose in the STA task was relatively unaffected by inactivation of the BLA, the OFC, and the NAc, although not (yet) conclusive. Follow-up studies in which these brain regions are inactivated prior to testing in the STA task for alcohol may yield further insight into the specific neurobiological mechanisms involved in control over alcohol use.

A particular strength of the STA task is that it allows for repeated testing within subject, thereby eliminating the need for a non-punished control group. Moreover, the task can be varied in many ways by the manipulation of different attributes of the task, such as using different punishment intensities, punishment probabilities, or the duration of the warning tone. These and other variations can alter the severity of the threat, thereby exposing the animals to more or less challenging situations.

In order to evaluate the quality of animal models, a framework of validities can be used (Belzung & Lemoine, 2011; Willner, 1986). This is to assess how well a given animal model, such as the STA model, represents the human disorder and the potential for therapeutic translation. Ideally, animal models of SUD should display face validity, construct validity, and predictive validity, thereby aiming at bridging the gaps between basic animal research and medical practice. Being a novel task inspired by existing models and several of the SUD diagnostic criteria, the STA task indeed aspires to do this. The STA task achieves considerable face validity because the consumption of the alcohol rewards is oral and voluntary, which mimics human alcohol drinking (Jeanblanc et al., 2019). Moreover, the tone cue that conveys the warning of probable punishment aims to create awareness of the risk for negative consequences of further pursuit, similar to humans suffering from AUD. Regarding construct validity, the main constructs of SUD can be considered to be (1) protracted seeking responses that are (2) controlled by stimuli in the environment and (3) eventually become compulsive (4) after protracted exposure to the drug (5) in some vulnerable individuals (Belin-Rauscent et al., 2016). The first and second constructs have been captured by the STA model because animals portray reward seeking responses that are suppressed by a cue that is associated to a negative consequence. Furthermore, predictive validity of the STA model is supported by the findings in chapter 4. Reduced neuronal activity in the prefrontal cortex is thought to play a role in the breakdown of cognitive control over use in SUD. Consistent, PrL inactivation resulted in a reduced suppression of alcohol seeking. Nevertheless, several follow-up experiments should be performed to better grasp construct and predictive validity of this model. For instance, evaluating the effects of medication

treatments that reduce alcohol consumption and craving, such as baclofen, in individual animals that display no suppression of alcohol seeking in the STA task (i.e. reflecting a loss of control) is needed to confirm the predictive validity of this model. Such an approach would relate back to the third construct (i.e. the compulsive aspect) and the fifth construct of the individual vulnerability. Furthermore, prolonged alcohol exposure might be required prior to selecting these individual animals that display no suppression of alcohol seeking in the STA task, which ties in with the fourth construct (i.e. protracted exposure to the drug) of SUD.

Motivation and value in decision making

Disadvantageous decision making is typical for psychiatric disorders such as eating disorders, bipolar disorder, and SUD. We were able to capture distinct components of value-based decision making by using a combination of demand analyses and motivation assessment, as described in chapter 6. Remarkably, we observed differential effects of mesocorticolimbic dopamine neuron stimulation on essential value and incentive motivation. Leaving aside the inherent differences in the schedules of reinforcement, we can speculate that these two measures might tap into different underlying constructs. As discussed in the general introduction, the incentive sensitisation theory posits a separation of the psychological process of motivationally 'wanting' a reward from hedonically 'liking' the same reward (Berridge & Robinson, 2016). Perhaps the stimulation of mesocorticolimbic dopamine signalling intensified the level of wanting the reward, reflected by an increased motivation that was measured in a progressive ratio (PR) schedule of reinforcement, while the pleasurable effects of the reward were deflated, reflected by a reduction in the essential value. Preclinical testing of this hypothesis requires separate assessment of liking and wanting, which is challenging in rats. One approach is to operationalise liking as an enhancement of orofacial reactions to taste that reflect positive hedonic impact (e.g. tongue protrusions and lateral tongue protrusions) without enhancement of neutral or aversive reactions, and wanting as enhanced food intake and eating behaviour (Smith & Berridge, 2007). Local dopamine stimulations combined with the assessment of liking and wanting of sucrose might clarify the differential effects of mesocorticolimbic dopamine neuron stimulation on essential value and incentive motivation.

Behavioural economics models could be used to further investigate the processes that underlie SUD. Since behavioural economical concepts, such as essential value and demand intensity, are less common in preclinical research on SUD, it would be interesting to combine demand analyses with other behavioural tasks, such as the STA task or the assessment of quinine adulterated alcohol consumption. This would give insight into the relationship between SUD associated behaviours, for example whether animals that show aversion-resistant reward seeking also display higher essential value for a reward. For instance, highly impulsive rats have been shown to display more inelastic nicotine demand than low impulsive rats, although this was not observed for alcohol (Diergaarde et al., 2012).

Another interesting direction would be to analyse demand longitudinally. For instance, to measure demand after short and after prolonged drug exposure to assess whether a subgroup of animals can be identified that shows a reduction in demand elasticity over time. Such an approach relates back to the key constructs of SUD that include a progression to compulsive use in some individuals after protracted exposure to the drug (Belin-Rauscent et al., 2016; Vanderschuren & Everitt, 2004). Indeed, procedures with extended access to cocaine have been shown to increase the essential value of cocaine over time (Bentzley et al., 2014; Christensen et al., 2008; James et al., 2019). These animals might also show a transition from casual use to more compulsive use, which might subsequently be assessed in punishment models, as proposed in the previous paragraph.

Habit formation

In this thesis, we investigated different aspects of SUD-like behaviour (chapter 5). We found a distribution of addiction-like behaviour across the population, which is in line with other studies (Deroche-Gamonet et al., 2004; O'Neal et al., 2020). One of the behaviours that was measured was sensitivity to alcohol outcome devaluation, whereby a reduction in sensitivity reflects habit formation. Similar to the other behavioural measures assessed, profound individual differences were observed at each outcome devaluation test. However, this experiment did not provide evidence for increased habitual behaviour after extensive alcohol self-administration training.

Impaired decision making has been associated with SUD, but the exact role of habitual control therein remains highly debated. A prominent theory is that SUD progresses from initial goal directed use to habitual use, eventually leading to compulsive use (Everitt & Robbins, 2005, 2016). Consistent, preclinical studies have shown that prolonged self-administration of nicotine, cocaine, or alcohol can produce habitual control over drug seeking (Clemens et al., 2014; Corbit et al., 2012; LeBlanc et al., 2013; Zapata et al., 2010), but conflicting results have also been reported (Halbout et al., 2016; Samson et al., 2004). It is thought that habitual control arises through repetition and learning, whereby environmental stimuli come to automatically elicit responses that were initially made spontaneously by the animal (Robbins & Costa, 2017). This process leads to a strong association

between stimulus and response, and a dissociation from the value of the outcome. However, there are not many studies that demonstrate a decrease in sensitivity to devaluation as a function of behavioural repetition in humans. Although some studies found overreliance on habit learning in AUD and cocaine dependence or a reduced sensitivity to devaluation after extensive training (Ersche et al., 2016; Sjoerds et al., 2013; Tricomi et al., 2009), recent studies report no evidence for habit formation in human subjects (de Wit et al., 2018; Hogarth et al., 2019; Luijten et al., 2020). Together with our findings, these conflicting reports highlight the complexity of the role of habit learning in SUD.

It is thought that goal directed and habitual action controls are two fundamental strategies that may exist in parallel (Balleine & O'Doherty, 2010). Decision making behaviour may be biased more toward a goal directed control in some situations, while in another context habitual control may dominate. This has been simulated in preclinical experimental setups in which animals were trained to respond for food rewards in two distinct contexts that are thought to require different action control mechanisms (Gremel et al., 2016; Gremel & Costa, 2013; Nordquist et al., 2007; Renteria et al., 2018). One context biased towards goal directed action control by using random ratio schedules of reinforcement, whereas in another context a random interval schedule of reinforcement was used to bias towards habitual action control. This setup allowed for examination of the loss of goal directed action control in one situation, while leaving goal directed control intact in another setting in the same animal.

Given that contrasting action control strategies coexist, it can be hypothesised that these two concurrent systems also occur in distinct behaviours in SUD. However, the involvement of goal directed and habitual processes in behavioural control remains poorly understood. Drug addicted patients procure drugs in creative and flexible ways which might not be governed by habitual control, but drug consumption might simultaneously become habitual (Schreiner et al., 2020; Singer et al., 2018). In other words, drug taking rather than drug seeking might become habitual (Lüscher et al., 2020). Moreover, habitual behaviour can possibly contribute to the shift to compulsive drug pursuit, but it might not be essential. Consistent, it has been shown that cocaine addiction-like behaviours can still emerge in rats even when the development of habitual behaviour was circumvented by requiring rats to solve puzzles in order to obtain cocaine (Singer et al., 2018). Adding further to the complexity is the recent discovery that rats responded more for a nondrug alternative reward than for cocaine, and this choice was unaffected by devaluation of the alternative reward (Vandaele et al., 2019, 2020). This inflexible, habitual responding specifically for choice of a nondrug reward does not seem consistent with theories that suggest that drugs of abuse may contribute to compulsive drug use by promoting habitual drug seeking at the expense of alternative activities. Altogether, more investigation into how decision making processes contribute to SUD and the underlying mechanisms is needed to better understand the role of habits in SUD.

Individual vulnerability to substance use disorder

Many people consume alcohol and other substances of abuse on a regular basis, but only a minority of users ultimately becomes addicted. This individual vulnerability to SUD may be determined by genetic, environmental, or psychological factors. We showed a marked individual variability in various behaviours, such as alcohol consumption levels, habit formation, motivation, and aversion sensitivity in relation to AUD-like behaviour (chapter 5). Previously, we found that individual differences in alcohol intake were associated with motivation and aversion sensitivity, as high drinking animals showed more motivation to obtain alcohol and displayed more punishment-resistant alcohol-directed behaviour than low drinking animals (Spoelder et al., 2015, 2017). In line with this, we found that alcohol consumption was correlated with aversion-resistant alcohol consumption, as described in chapter 5. However, there were no significant associations between any of the other measures, suggesting that the distribution across these behaviours can vary substantially between animals.

To simulate SUD in preclinical studies, efforts have been made to measure behaviours that resemble multiple diagnostic criteria for SUD, such as increased motivation and persistent reward seeking during a signalled period of reward unavailability (Belin et al., 2009; Deroche-Gamonet et al., 2004; Domi et al., 2019; Jadhav et al., 2017; Radke et al., 2017). This integration of several dimensions of addiction-like behaviour has led to the perception of a broad range of severity of drug abuse in rats. Additionally, it allows for the investigation of neurobiological adaptations specifically occurring in the brain of rats developing the behavioural hallmarks of SUD. We investigated whether distinct groups of rats could be identified based on four behavioural measures (i.e. alcohol consumption levels, habit formation, motivation, and aversion sensitivity) in an unbiased approach which revealed no evidence for separate groups. Moreover, the commonly used three-criteria classification was applied, which resulted in the identification of subgroups that showed lower to higher degrees of AUD-associated behaviour. A subgroup of animals displayed consistent AUD-like behaviour, but for a large group of animals, the categorisation varied substantially depending on which behavioural measures were included. These

findings confirm the incoherent variability across the behavioural measures and suggest that the various behavioural components contribute differently to the AUD phenotype across individual animals, which is consistent with the heterogeneity as seen in the human AUD pathology. These findings emphasise the importance of an integrated approach that considers the complexity of SUD, and the need for careful consideration of selection methods based on different behavioural measures.

Brain mechanisms of substance use disorder

Prefrontal cortex

The prefrontal cortex is a crucial node in executive control, which refers to a range of higher cognitive processes including attention, planning, working memory, cognitive flexibility, and response inhibition (Dalley et al., 2004; Miller & Cohen, 2001). SUD is characterised by compromised prefrontal control over substance intake (Dalley et al., 2004; Goldstein & Volkow, 2011; Koob & Volkow, 2016; Volkow & Fowler, 2000). Within the prefrontal cortex, the PrL has been implicated in response inhibition and compulsive drug seeking (Chen et al., 2013; Limpens et al., 2015; Seif et al., 2013; Verharen et al., 2019b). Therefore, we specifically focused on the role of the PrL in loss of control over substance seeking. We found that the PrL plays a role in maintaining control over alcohol reward seeking behaviour, as described in chapter 4.

Although the PrL appears to contribute to control over reward seeking, the underlying processes mediating this effect are currently unknown. The PrL is part of a larger interconnected network, whereby it receives direct and indirect (via thalamus) projections from brain regions known to be involved in drug reinforcement, such as the ventral tegmental area (VTA), the BLA and the NAc. In turn, the PrL also projects to these brain areas via pyramidal glutamatergic neurons (Moorman et al., 2015). The BLA and NAc are implicated in reward-directed behaviour, as BLA inactivation impaired conditioned punishment and disinhibited punished seeking in a conflict task (Killcross et al., 1997; Piantadosi et al., 2017), while the BLA projection to the NAc is thought to drive reward seeking (Ambroggi et al., 2008; Stuber et al., 2011). Moreover, the glutamatergic PrL projections to the NAc have been implicated in reinstatement of (cocaine) reward seeking and inhibition of this pathway was shown to lead to compulsive eating behaviour (Domingo-Rodriguez et al., 2020; Stefanik et al., 2013). Furthermore, a majority of VTA and medial prefrontal cortex neurons respond to punishment risk in a synchronised manner by modulating their firing rates (Park & Moghaddam, 2017).

Considering that the animals face a threat in the STA task, processes underlying fear responding and punishment learning might also be involved. The glutamatergic projection from the BLA to the PrL is known to be critical for driving fear responses and the PrL projection to the rostromedial tegmental nucleus has been shown to contribute to punishment learning (Burgos-Robles et al., 2017; Li et al., 2019). Based on these findings, it could be hypothesised that the PrL is crucial in processing the aversive signal from the BLA and translating it into an initiation of an inhibitory response. Thus, although the exact mechanisms are not fully understood, the PrL is connected to multiple brain regions that have repeatedly been associated with control over (punished) reward seeking.

Mesocorticolimbic dopamine system

The mesocorticolimbic dopamine system, which is the dopamine system originating in the VTA, projecting to the NAc and prefrontal cortex, is prominently involved in reward-directed behaviours (Berridge & Robinson, 1998; Bromberg-Martin et al., 2010; Hamid et al., 2016; Keiflin & Janak, 2015; Robbins & Everitt, 2007; Salamone & Correa, 2012; Schultz, 2016). Dopamine signalling in the NAc is thought to contribute to effort-based choice behaviour (Floresco, 2015; Floresco et al., 2008; Mai et al., 2012; Salamone et al., 2016b, 2016a). Previously, NAc dopamine depletions have been shown to decrease willingness to exert effort for reward, while mesocorticolimbic dopamine stimulation increased motivational behaviour (Aberman & Salamone, 1999; Boekhoudt et al., 2018; Boender et al., 2014; de Jong et al., 2015; Ishiwari et al., 2004). Remarkably, stimulation of mesocorticolimbic dopaminergic neurotransmission was found to increase demand elasticity - which is indicative of a decrease in essential value - of sucrose and increased incentive motivation, as described in chapter 6. These findings imply that mesocorticolimbic dopamine signalling differentially influences distinct components of value-based decision making processes. Perhaps these distinct components can be speculated to relate to the incentive sensitisation theory. The incentive sensitisation theory of SUD posits that 'wanting' may grow over time independently of 'liking' as an individual becomes addicted, because of sensitisation of brain mesolimbic systems (Berridge & Robinson, 2016; Robinson & Berridge, 1993). Functionally, mesolimbic sensitisation renders brain 'wanting' systems hyperreactive to drug cues and contexts, while 'liking' is not thought to increase, and may even decrease. Since quite general dopamine manipulation techniques were used, future research using specific and local dopamine manipulations might clarify how dopamine modulates motivation and value.

Individual variability

Besides showing profound individual variability in various behaviours, we also set out to study these underlying neural processes. Intermittent alcohol access was used to capture the individual variation in alcohol consumption and the involvement of GABA, opioid, and glutamate signalling herein was determined. Together with an earlier study, no evidence was found that GABA, opioid, glutamate and dopamine neurotransmission are associated with the individual variation in alcohol consumption (Spoelder et al., 2016). However, vulnerability to SUD has been associated to several phenotypes including anxiety, sensation seeking, and impulsivity, which are considered risk traits for SUD. Moreover, more than 50% of the vulnerability to SUD can be attributed to genetic factors (Demers et al., 2014; Goldman et al., 2005; Hiroi & Agatsuma, 2005; Verhulst et al., 2015). Therefore, individual variability in SUD might be explained to a great extent by multiple factors, starting on a molecular genetic level and eventually directly or indirectly influencing neurotransmitter signalling cascades.

3. Methodological considerations

Throughout this thesis, we took a comprehensive approach as we aspired to capture multiple aspects of SUD. Our approach included a wide array of behavioural tests to simulate human SUD-related behaviour as accurately as possible in a preclinical context. Nevertheless, any scientific approach has limitations which need to be borne in mind when interpreting and evaluating the findings. We address three critical considerations regarding our methodology.

First, the possible genetic influence on behaviour measured in our experiments is unknown. SUD has a significant genetic component as approximately 40% to 60% of the vulnerability to SUD can be attributed to genetic factors (Demers et al., 2014; Goldman et al., 2005; Hiroi & Agatsuma, 2005). We used an outbred stock of Lister Hooded rats that is genetically undefined, meaning that each animal may be genetically unique. However, the vulnerability for SUD cannot be attributed to a single gene but is thought to result from complex interactions among multiple genes and genetic interactions with environmental influences (Enoch, 2012). Although genetic influences cannot be excluded, we chose to focus on behavioural aspects that are associated with SUD. Importantly, outbred stocks are in fact not necessarily more variable in behaviour than inbred strains, as a recent meta-analysis reported no overall difference in phenotype variability between inbred and outbred animals (Tuttle et al., 2018).

Second, whether the findings can be extrapolated to the female population could be questioned. Sex differences in SUD-like behaviour have been described in humans and in animal models (Becker et al., 2017; Greenfield et al., 2010). For instance, women tend to exhibit a greater rate of escalation of substance use than men (Bobzean et al., 2014). Similarly, female rats acquire substance self-administration more readily than males and escalate their substance use more rapidly than males (Carroll et al., 2002; Jackson et al., 2006; Reichel et al., 2012). We used male rats in all experiments because SUDs generally affect more men than women. Twelve month prevalence for AUD is 17.6% in men and 10.4% in women, and the prevalence for other drug use disorders is 4.9% in men and 3.0% in women (Grant et al., 2015, 2016). Interestingly, it has recently been shown that reward learning, exploration, and motivation subtly fluctuate across the oestrous cycle in rats (Verharen et al., 2019a). As these are behaviours central to SUD, these findings support the relevance of investigations in female samples in SUD research.

Third, the translatability of animal models to humans is important to consider, since animal models of addiction have been criticised (Field & Kersbergen, 2020; Heilig et al., 2019). Indeed, much progress in animal research has been made, for instance by the deconstruction of psychiatric disorders into endophenotypes and by embracing individual variability. Moreover, animal models allow for in-depth functional neurobiological research by using invasive techniques and for tracking the course of SUD from naïve to addicted. Although we acknowledge the substantial translational step between animals and humans, we and others argue that animal models can reproduce key endophenotypes that at least relate to human SUDs and aid in the exploration of why these occur in some subjects but not in others (Deroche-Gamonet, 2020; Perry & Lawrence, 2020). Therefore, animal research remains essential especially for elucidating underlying brain and behavioural mechanisms of SUD (Vanderschuren & Ahmed, 2020).

4. Implications and future prospects

Future directions

The potential to use the STA task is a promising future research direction for investigating brain and behavioural mechanisms implicated in SUD in a preclinical setting. For instance, using the STA task in combination with neuropharmacological interventions will enable us to investigate the role of brain regions implicated in behavioural control in persistent alcohol pursuit. More specifically, regions of interest would be the BLA, the OFC, and the NAc because we showed that seeking behaviour

for sucrose in the STA task was rather unaffected by pharmacological inactivation of these areas. Besides providing insight into the underlying neural mechanisms of persistent alcohol seeking, these experiments could also aid in confirming the reinforcer specificity of the STA task regarding alcohol and sucrose and thereby, the value of the STA task as a behavioural tool in addiction research. Another important future step that should be taken is the study of loss of control over other substances of abuse, such as cocaine. As a first step, rats trained to respond for cocaine should be tested in the STA model to determine the degree of threat that is sufficient to suppress cocaine seeking. Furthermore, investigating the loss of control over opioid seeking would be an interesting avenue and pressing issue, especially in the light of the currently ongoing opioid crisis (Rasmussen et al., 2019; Vadivelu et al., 2018; Volkow & Collins, 2017).

More pathway-specific research would be recommended to follow up on our findings that the PrL is implicated in behavioural control over alcohol seeking. This would extend our knowledge on the neuronal circuits underlying loss of control. For instance, targeting the PrL-NAc pathway or the BLA-PrL pathway through chemogenetic or optogenetic tools to assess its role in loss of control over alcohol seeking would be informative as these pathways have recently been implicated in compulsive reward seeking and driving fear responses, respectively (Burgos-Robles et al., 2017; Domingo-Rodriguez et al., 2020).

The findings in this thesis also acknowledge the importance of considering individual variation in preclinical research. Therefore, it seems a logical next step to investigate the individual variation in loss of control over reward seeking behaviour. One way to do so is to compare high alcohol drinking animals to low alcohol drinking animals by using the STA model, or compare high impulsive rats to low impulsive rats since impulsivity is known to be a risk factor for SUD (Perry & Carroll, 2008; Verdejo-García et al., 2008). Another approach is to investigate whether other SUD-related behaviours are associated with loss of control. This could be achieved by exposing rats to multiple behavioural assessments, such as the PR schedule of reinforcement as an indicator for motivation or the 5-choice serial reaction time task as an indicator for impulsivity, and the STA task. Association analyses of performance in these tasks can reveal whether there is coherence in these behaviours.

Regarding the role of dopamine in motivation and value, further experiments into the underlying neural mechanisms would help to better understand our seemingly paradoxical findings. In chapter 6, the mesocorticolimbic dopamine pathway was targeted, which comprises dopaminergic cells within the VTA that mainly project to the NAc (Morales & Margolis, 2017). Experiments directed at studying the involvement of specific subtypes of dopamine receptors might clarify how this neurotransmitter modulates motivation and value. A way to do this is by assessing the effects of infusion of dopamine receptor agonists and antagonists into the NAc on demand curves and on performance in the PR schedule of reinforcement. Alternatively, chemogenetics could be used to target specific projections from the NAc. Such experiments would also be informative about the involvement of the NAc output pathways in motivation and reward valuation.

Clinical implications

Decades of research into SUD have led to major advances, such as insight into the initial molecular sites of action of substances of abuse, the main components of the reward system in the brain, and the discovery of agents that have been translated into treatments. Nevertheless, therapeutic options for SUD are still limited. A few pharmacotherapeutic options are available, which include disulfiram, naltrexone, and acamprosate as approved medications for the treatment of AUD, but these prove not to be effective for all patients (Heilig et al., 2019; van den Brink, 2012). Similarly, evidence-based psychosocial treatments, such as cognitive-behavioural therapy and contingency management interventions, also have limited effectiveness (Volkow & Boyle, 2018).

Although no novel treatment options were tested, the findings presented in this thesis can contribute to the development of effective pharmacotherapy. One of the promising strategies explores the use of pharmacological compounds to target endophenotypes associated with SUD. Whereas attenuation of drug reward has been a main focus in the past, the importance of executive function and inhibitory cognitive control in SUD is becoming increasingly apparent (Brady et al., 2011). In particular, medications that improve cognitive function could be beneficial in the treatment of SUD because these cognitive enhancers could improve impulse control, planning, and decision making (Volkow & Boyle, 2018). Examples of existing cognitive enhancers are modafinil, atomoxetine, and varenicline but many more compounds could potentially restore executive control over drug use (Brady et al., 2011; Sofuoglu et al., 2013, 2016). Interestingly, preclinical studies so far have shown a dissociation between effects of these compounds on cognition and on relapse (Broos et al., 2015; Gobin & Schwendt, 2020). The effect of these and novel compounds on loss of control over reward seeking could be assessed preclinically using the STA model. Preclinical investigation in the STA model would allow for assessment of the effects on behaviour and could be combined with further research into underlying neurobiological mechanisms, which is less feasible in humans.

The finding that the composition of the subpopulation of animals with the highest 'addiction severity score' varied substantially confirms the clinical heterogeneity among SUD patients. Many factors and complex gene-environment interactions contribute to the development of SUD; this leads to a large heterogeneity in terms of both the symptom dimensions and the severity. Such variety cannot be tackled by a single strategy but requires an individualised approach (Johnson, 2010). For instance, response to pharmacological treatment may be predicted through the use of pharmacogenetic testing (Heilig et al., 2011), which identifies genetic biomarkers that are predictive of individual sensitivity to particular medications. Moreover, combinations of pharmacotherapies and behavioural therapies may be effective in enhancing treatment outcomes (Loftis & Huckans, 2013). Altogether, an individualised strategy is likely to optimise treatment response, and this prompts the need for developing clinical assessments that will allow for treatment tailoring.

5. Concluding remarks

The present thesis contributes to a better understanding of the neurobiological and behavioural changes that characterise SUD, using a combination of behavioural tools and neuropharmacological interventions. First and foremost, we presented and utilised a new behavioural task to assess behavioural control over reward seeking which can be further used in future preclinical research. Second, we revealed differentiating effects on distinct components of decision making and confirmed the role of dopamine in motivation. Moreover, considerable individual differences were found in consumption, motivation, aversion-resistant intake, and habit formation, underscoring the heterogeneous nature of SUD and the importance of implementing individual variation in SUD research. Ultimately, the accumulating knowledge on SUD will aid the development of better treatment options for this harmful, destructive brain disorder.

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Addendum

A

Nederlandse samenvatting

Verslaving aan genotmiddelen is een groot maatschappelijk probleem dat miljoenen mensen treft. Verslaving veroorzaakt veel schade; niet alleen voor de gebruiker zelf, maar ook voor anderen in de samenleving. Bovendien draagt verslaving aanzienlijk bij aan de wereldwijde ziektelast doordat middelenmisbruik een risicofactor is voor allerlei mentale en lichamelijke ziekten. Het doel van het in dit proefschrift beschreven onderzoek was om inzicht te krijgen in veranderingen in hersenen en gedrag die kenmerkend zijn voor verslaving. Hierbij is gebruik gemaakt van gedragsmodellen in ratten, waarmee we belangrijke aspecten van verslavingsgedrag kunnen meten, in combinatie met neuro-farmacologische interventies. Gezamenlijk dragen de bevindingen in dit proefschrift bij aan een beter begrip van de onderliggende gedrags- en neurobiologische processen van verslaving.

Verslaving is een chronisch recidiverende hersenaandoening die wordt gekenmerkt door aanhoudend gebruik van genotmiddelen, ondanks schadelijke gevolgen. Dit kunnen middelen zijn zoals tabak, alcohol, psychostimulantia zoals cocaïne, amfetamine en MDMA, of opiaten zoals heroïne en morfine. De diagnose voor verslaving, ook wel middelengebruiksstoornis (substance use disorder) genoemd, wordt gesteld met behulp van het diagnostisch en statistisch handboek van psychiatrische aandoeningen (DSM-5). Verslaving heeft naast farmacologische kenmerken, zoals tolerantie en het optreden van onthoudingsverschijnselen, ook psychologische en gedragskenmerken, waaronder een verhoogde motivatie voor middelen, escalatie van gebruik en het onvermogen om middelengebruik te beperken. De diagnostische criteria van verslaving weerspiegelen de klinische heterogeniteit van de aandoening, omdat klinische verschijnselen en de hevigheid van de stoornis aanzienlijk verschillen tussen patiënten. Verslaving kan worden gekwalificeerd als licht, matig of ernstig, afhankelijk van het aantal diagnostische criteria waaraan wordt voldaan. Een belangrijk kenmerk van verslaving is het verlies van controle over middelengebruik, zoals blijkt uit de meeste DSM-5-criteria voor verslaving. Dit uit zich bijvoorbeeld in voortdurend middelengebruik in gevaarlijke omstandigheden of aanhoudend gebruik ondanks het besef dat het gebruik problemen met zich meebrengt of verergert. Daarom is de focus op verlies van controle over middelengebruik een relevante invalshoek voor wetenschappelijk onderzoek naar verslaving.

Hoewel veel mensen regelmatig alcohol en andere drugs gebruiken voor recreatieve doeleinden, raakt slechts een klein deel van de gebruikers verslaafd. Desalniettemin is verslaving een groot medisch en maatschappelijk probleem, mede doordat het niet goed behandelbaar is. Momenteel zijn er een aantal behandelingsopties voor verslaving beschikbaar waaronder cognitieve gedragstherapie, medicatie en sociale ondersteuningsstrategieën. De werkzaamheid van deze behandelingen varieert echter van persoon tot persoon en de kans op terugval na een behandeling is groot. Daarom is er dringend behoefte aan betere behandelstrategieën voor personen met een verslaving. Een beter begrip van de neurobiologie die ten grondslag ligt aan verslaving kan daar mogelijk bij helpen.

Hoofdstuk 2 bestaat uit een literatuurstudie naar bestaande preklinische diermodellen van controleverlies over middelengebruik die negatieve prikkels gebruiken. Bij deze diermodellen wordt het nemen van belonende middelen gekoppeld aan negatieve consequenties. De bereidheid van dieren om deze bestraffing te doorstaan om het middel te kunnen krijgen wordt vervolgens gemeten. Belangrijk hierbij is dat een verminderde gevoeligheid voor negatieve prikkels in een dergelijke context wordt beschouwd als een verlies van controle over middelengebruik. Deze negatieve prikkels richten zich op tast, smaak of interoceptie, oftewel zintuiglijke signalen die van binnenuit het lichaam komen. Veel toegepaste negatieve prikkels in onderzoeken naar zelftoediening van belonende stoffen zijn elektrische voetschokken, de bittere smaakstof kinine, lithiumchloride of histamine. In de afgelopen decennia heeft het preklinische verslavingsonderzoek aanzienlijke vooruitgang geboekt door studies die gebruik maken van dergelijke diermodellen. Deze diermodellen worden voortdurend verder ontwikkeld zodat ze het klinische beeld van verslaving steeds beter weerspiegelen. Het verwerken van diagnostische criteria van verslaving, zoals aanhoudend gebruik ondanks het besef dat het gebruik problemen met zich meebrengt, in de modellen kan de validiteit verbeteren. Verdere ontwikkeling en optimalisatie van deze diermodellen zal blijven bijdragen aan de kennis over de neuronale en gedragsstructuur van verslaving.

Hoofdstuk 3 beschrijft de ontwikkeling van een nieuwe gedragstaak voor ratten die tracht specifieke aspecten van menselijk verslavingsgedrag na te bootsen. Deze gedragstaak bevat namelijk respons-afhankelijke negatieve consequenties met een zekere waarschijnlijkheid. Immers, de negatieve consequenties van middelenmisbruik zijn doorgaans het directe gevolg van de handelingen van de gebruiker (respons-afhankelijk), maar niet consistent of onvermijdelijk (waarschijnlijk). Geïnspireerd door bestaande gedragsmodellen, zoals besproken in **hoofdstuk 2**, hebben we een operante gedragstaak ontworpen waarin controle over het zoekgedrag naar beloningen (alcohol en suiker) werd getest door middel van het risico op negatieve consequenties (de 'Seeking under Threat of Adversity' (STA) taak). Eerst trainden we de ratten om op een pedaal te drukken voor een beloning. Vervolgens lieten we

een waarschuwingssignaal horen aan de dieren, namelijk een pieptoon. Deze toon was een teken dat blijven drukken op de pedaal mogelijk bestraft zou worden. Bij elke pedaaldruk gedurende de pieptoon was er een 25% kans op een milde voetschok. Op deze wijze werden de dieren geconfronteerd met een gesignaleerde dreiging van straf waarop ze hun gedrag konden aanpassen. We vonden dat de dreiging van straf het responderen voor beloningen verminderde en dat een matige schokintensiteit voldoende was om het zoekgedrag voor zowel alcohol als suiker consistent te onderdrukken wanneer de dieren herhaaldelijk getest werden. Dit maakt de STA-taak geschikt als nieuw instrument om toe te passen in vervolgonderzoek naar de controle over middelengebruik.

In **hoofdstuk 4** hebben wij vervolgens deze STA-taak toegepast om de betrokkenheid van de prelimbische prefrontale cortex (PrL) bij het zoeken naar beloningen onder dreiging van straf te onderzoeken. Gezien de mogelijke betrokkenheid van dit hersengebied bij het onderdrukken van impulsen en het dwangmatig zoeken naar beloningen, zou de PrL een cruciale rol kunnen spelen in verlies van controle over middelengebruik. We vonden dat farmacologische inactivatie van de PrL de onderdrukking van het responderen voor alcohol verminderde in de STA-taak, maar niet voor suiker. Met andere woorden, inactiveren van de PrL maakte dat de dieren bleven drukken op de pedaal die gekoppeld was met het verkrijgen van alcohol (maar niet suiker), ondanks de dreiging van een negatieve consequentie. Dit suggereert dat hersenactiviteit in de PrL vereist is voor het kunnen onderdrukken van het zoeken naar alcohol onder dreiging van straf. Deze bevindingen wijzen erop dat dwangmatig alcoholgebruik tijdens alcoholverslaving veroorzaakt kan worden door een verminderde neurale activiteit in de PrL. De prefrontale cortex zou daarom een interessant doelwit kunnen zijn voor de behandeling van verslaving om de controle terug te krijgen.

Hoofdstuk 5 beschrijft onderzoek naar de verbanden tussen vier gedragsmaten die zijn geassocieerd met alcoholverslaving, namelijk: alcoholinname, gewoontevorming, motivatie voor alcohol en de mate waarin alcoholgebruik gevoelig is voor negatieve prikkels. Deze gedragsmechanismen spelen een rol bij alcoholverslaving en het doel was om de onderlinge relatie tussen deze maten in kaart te brengen. Onze verwachting was dat er een subpopulatie van ratten kon worden geïdentificeerd die kenmerken van alcoholverslavingsgedrag zou vertonen. De samenhang tussen de vier gedragsmaten werd gemeten door correlaties en een statistische analyse die aangeeft of de data uniform en willekeurig worden verdeeld of dat er afzonderlijke clusters van dieren in te detecteren zijn. Verder berekenden we verslavingsscores voor elk dier en keken we naar de consistentie van de groep dieren die een hoge verslavingsscore hadden. Het bleek dat de meeste gedragsmaten niet gecorreleerd waren met elkaar, behalve alcoholinname en aversie-resistente alcoholinname waarbij een hogere alcoholinname samenhing met een hogere aversie-resistente alcoholinname. Ook werden er geen afzonderlijke clusters van dieren in de data gedetecteerd. Verder liet een kleine groep dieren gedrag zien dat wijst op alcoholverslaving. Deze dieren scoorden consistent hoog op combinaties van gedragsmaten voor verslavingsgedrag. Daarnaast was er nog een groep dieren die ook hoog scoorde op verslavingsgedrag, maar de samenstelling van deze groep was afhankelijk van de gedragsmaten die werden meegenomen. De variabele samenstelling van de subpopulatie met een hoge verslavingsscore suggereert dat de vier gedragscomponenten in verschillende mate bijdragen aan het verslavingsachtige beeld bij individuele dieren. Dit komt overeen met de heterogeniteit van verslaving bij mensen. De bevindingen benadrukken dat de individuele variatie en heterogeniteit van groot belang zijn in gedragscomponenten die samen de essentie vormen van verslaving in preklinische studies.

Hoofdstuk 6 beschrijft onderzoek naar de rol van de signaalstof dopamine bij het afwegen van kosten en baten. Vrijwel alle genotmiddelen hebben een effect op het beloningssysteem in de hersenen: ze leiden allemaal tot een verhoogde afgifte van de signaalstof dopamine. Dopamine is betrokken bij belonings- en cognitiegerelateerde processen. Waardetoekenning van beloningen beïnvloedt beslissingen over de afweging van kosten en baten. De precieze rol van dopamine hierbij is nog niet precies bekend. Daarom onderzochten we de rol van dopamine neurotransmissie bij het toekennen van waarde aan beloningen. Hierbij lag de focus op de rol van het mesocorticolimbische dopamine systeem bij het bepalen van het verband tussen prijs en consumptie van suiker. Ook keken we naar de motivatie voor suiker. Dit werd gedaan door te meten hoe vaak de dieren bereid waren om op een pedaaltje te drukken voor suiker, als ze voor elke volgende portie suiker vaker op het pedaaltje moesten drukken. Stimulatie van mesocorticolimbische dopamine signalering bleek de kosten-baten afwegingen op een complexe manier te veranderen. Enerzijds nam de consumptie sterker af naarmate de prijs toenam. Dit werd weerspiegeld in een stijging van de elasticiteit van de vraag naar suiker, wat een indicatie is voor een afname van de waarde. Anderzijds verhoogde stimulatie van mesocorticolimbische dopaminerge neurotransmissie de motivatie voor suiker. Deze bevindingen laten zien dat mesocorticolimbische dopamine signalering diverse effecten kan hebben, afhankelijk van het aspect van het besluitvormingsproces.

In **hoofdstuk 7** onderzochten we de betrokkenheid van andere signaalstoffen, namelijk GABA, opioïden en glutamaat, bij individuele verschillen in alcoholgebruik. Bij dit onderzoek hebben we gebruik gemaakt van het feit dat wij een sterke variatie zien in

de mate van alcoholinname tussen individuele ratten, waarbij ratten die veel alcohol drinken ook gedrag laten zien dat op verslaving lijkt. In deze studie werd een groep ratten herhaaldelijk blootgesteld aan alcohol. Ze kregen deze alcohol om de dag aangeboden in hun thuiskooi voor een periode van twee maanden. Vervolgens werden subgroepen van ratten geselecteerd die weinig, gemiddeld en veel alcohol dronken. De subgroepen werden behandeld met de farmaca baclofen, naltrexon en N-acetylcysteïne en de effecten op alcoholinname werden gemeten. Baclofen stimuleert de $GABA_B$ receptoren en naltrexon bindt zich aan opioïdreceptoren, waardoor het effect van opioïden wordt geblokkeerd. N-Acetylcysteïne is een stof die kan worden omgezet naar cysteïne, wat een aminozuur is dat betrokken is bij glutamaterge neurotransmissie. We ontdekten dat behandeling met baclofen en naltrexon, maar niet met N-acetylcysteine, de vrijwillige alcoholinname verminderde, onafhankelijk van de individuele mate van alcoholinname. Deze bevindingen suggereren dat door $GABA_B$ en μ-opioïde receptoren gemedieerde neurotransmissie bijdraagt aan alcoholgebruik, maar dat ze de mate van individuele variatie in alcoholinname in deze studieopzet niet verklaren.

Hoofdstuk 8 is een bijlage, die een reeks experimenten beschrijft waarin we de betrokkenheid van de basolaterale amygdala (BLA), de orbitofrontale cortex (OFC) en de nucleus accumbens (NAc) onderzochten bij het responderen voor suiker onder dreiging van negatieve consequenties. Hiervoor werd de STA-taak gebruikt in combinatie met farmacologische inactivatie van deze hersengebieden, zoals ook toegepast in **hoofdstuk 4**. Eerder onderzoek heeft aangetoond dat neurobiologische veranderingen in de BLA, de OFC en de NAC mogelijk betrokken zijn bij de controle over het zoeken naar en het nemen van beloningen. In het huidig onderzoek bleek dat farmacologische inactivatie van de BLA en de OFC geen invloed had op het responderen voor suiker onder dreiging, terwijl inactivatie van de NAc de onderdrukking van suiker verhoogde zonder het aantal responsen significant te veranderen. Al met al was het zoekgedrag naar suiker onder dreiging van straf relatief onaangetast in deze experimenten, wat suggereert dat gedragscontrole over het zoeken naar suiker niet afhankelijk is van neuronale activatie in de BLA, de OFC of de NAc.

Samengevat geeft het onderzoek beschreven in dit proefschrift inzicht in gedrags- en neurobiologische processen die ten grondslag liggen aan verslaving. In het bijzonder hebben we een nieuwe gedragstaak gepresenteerd en gebruikt om gedragscontrole over het zoeken naar beloningen te beoordelen, die verder kan worden toegepast in vervolgonderzoek. Uiteindelijk zal meer kennis over de onderliggende mechanismen van verslaving bijdragen aan de ontwikkeling van effectievere behandelingen voor deze aandoening.

Curriculum Vitae

Anne Maryse Minnaard was born on the 10th of October, 1991 in Bodegraven, The Netherlands. She obtained her Athenaeum degree and International Baccalaureate Certificate English at the Scala College in Alphen aan den Rijn in 2009.

In the same year, Maryse moved to Amsterdam and attended Amsterdam University College, where she followed a Liberal Arts and Sciences curriculum with a major in psychology and a minor in health and biomedical sciences. In 2012, she obtained her Bachelor of Arts degree *cum laude*.

Thereafter, Maryse was enrolled in the research master Neurosciences at the VU University in Amsterdam. She conducted a research internship at the VU University with Prof. Dr. Taco de Vries and Dr. Leontien Diergaarde. Here, she studied the effects of a serotonin receptor agonist on cocaine and sucrose demand elasticity and intensity in rats. Her next internship was at the Alzheimer Center and the department of Nuclear Medicine & PET Research at the Amsterdam UMC with Prof. Dr. Bart van Berckel and Dr. Marissa Zwan. Here, she investigated the relationship of neurodegenerative biomarkers in dementia.

After obtaining her Master of Science degree, Maryse started her PhD project with Prof. Dr. Louk Vanderschuren and Dr. Heidi Lesscher at the Utrecht University, and Prof. Dr. Roger Adan and Dr. Geert Ramakers at the UMC Utrecht Brain Center in 2015. Together with Janna Smeets, she investigated the mechanisms underlying loss of control over substance use, the results of which are presented in this thesis.

In September 2020, Maryse started as a Data Science Trainee at the ACM (Authority for Consumers & Markets), the NZa (Dutch Healthcare Authority), and the AFM (Authority for the Financial Markets).

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