

WHAT MAKES US DRINK?

Alcohol consumption in the rat in connection with reward and cognition

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colofon

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WHAT MAKES US DRINK?

Alcohol consumption in the rat in connection with reward and cognition

Waarom drinken we?

Alcohol consumptie door ratten in relatie tot beloning en cognitie (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 17 maart 2016 des middags te 12.45 uur

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Voor mijn ouders

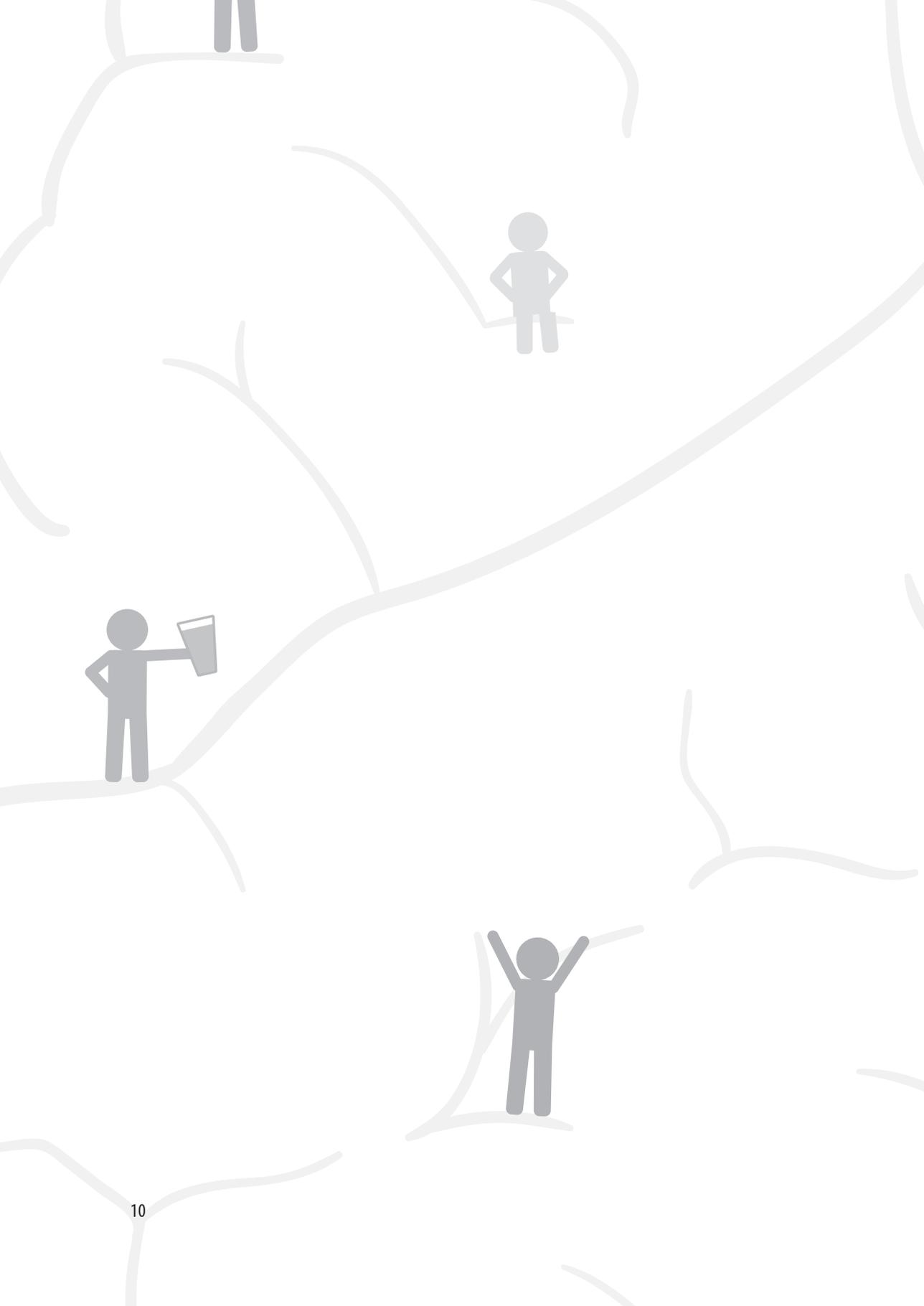
***My liver may be fucked
but my heart is honest
and my words are true
like the sky is blue***

- Passenger

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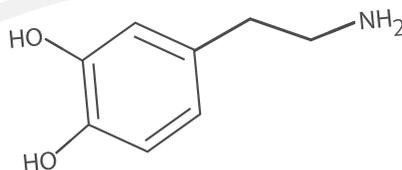
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CHAPTER 1

GENERAL INTRODUCTION

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ALCOHOL USE DISORDER

Alcohol use disorder (AUD) is a chronic relapsing brain disorder, characterized by persistent and excessive alcohol use with the explicit knowledge of its negative consequences (McLellan et al., 2000; American Psychiatric Association 2013). Alcohol is, together with tobacco, the most widely used substance of abuse worldwide. AUD is associated with medical problems, loss of productivity, crime, accidents and risky behaviours (Ericksen and Trocki 1992; Wechsler et al., 2000; Volkow and Li 2005; Cherpitel et al., 2012). As a result, AUD is considered to be the most harmful and costly form of substance abuse for individuals and society overall (Uhl and Grow 2004; Nutt et al., 2010). Interestingly, a large geographical variation exists in adult alcohol consumption per capita (Fig. 1). The highest levels of alcohol consumption are reported in Europe, Russia, Argentina and Australia (WHO 2011). In Europe, in 2009, 76% of the people of 15 years and older occasionally drank alcohol,

Figure 1

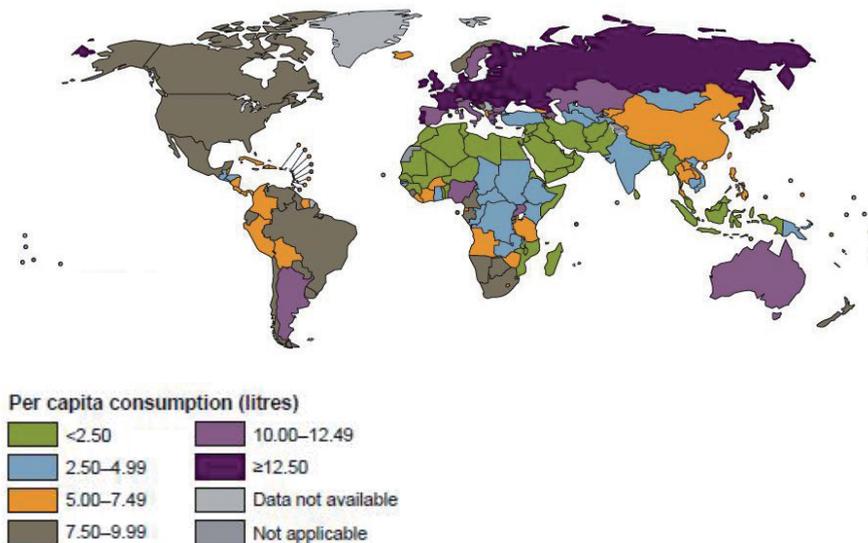


Figure 1. Total consumption in litres of pure alcohol per capita in adults (>15 years of age). The data represent estimates of 2005 using average recorded alcohol consumption in 2003–2005 and unrecorded alcohol consumption in 2005 (WHO 2011).

with large differences across European countries (WHO 2011; Van Laar et al., 2014). Results derived from prospective and longitudinal studies revealed that alcohol consumption peaks after adolescence, between 18-25 years of age (Labouvie et al., 1997; Arnett 2000; Costanzo et al., 2007; Sloan et al., 2011).

It has been estimated that 76 million people suffer from AUD worldwide (WHO 2011; United Nations Office on Drugs and Crime 2012). The age of onset of AUD is mostly seen in the late teens or early to mid-20s, descending to more severe forms of AUD in late 30s (American Psychiatric Association 2013). In the Netherlands, 478.000 people were diagnosed with an AUD between 2007-2009 according to the DSM-5 criteria (Van Laar et al., 2014). In the United States, the 12-month prevalence is estimated to be 4.6% among 12-17-year-olds and 8.5% among adults of 18 and older. AUD is more often diagnosed in men (12.4%) than women (4.9%) (American Psychiatric Association 2013).

AUD can be divided into two subtypes (Cloninger 1987; Babor et al., 1992). Type 1 AUD is characterized by a relatively late onset with few premorbid comorbidities. Type 2 AUD is a more severe form of AUD, characterized by an early onset. Type 2 AUD has been associated with multi-substance dependence, antisocial behaviours, aggression and impulsive behaviours (Cloninger et al., 1988; Hallman et al., 1996; Basiaux et al., 2001; Finn et al., 2002; Hiroi and Agatsuma 2005; Dom et al., 2006; Perry and Carroll 2008; Van Laar et al., 2014). Moreover, Type 2 AUD is also thought to have a genetic component (Le Foll et al., 2009).

The diagnosis of AUD is based on 11 criteria that can be categorized as impaired control, social impairment, risky use and pharmacological criteria (Table 1). The DSM-5 addresses each class of substances (e.g. alcohol, cannabis, opioids, stimulants) as a separate substance use disorder, but these substance use disorders are diagnosed based on highly comparable criteria. AUD can vary from mild to severe, whereby AUD is considered mild in case of 2-3 criteria are met, moderate when 4-5 criteria are met and severe when 6 or more criteria are fulfilled.

Importantly, the majority of the DSM-5 criteria for AUD reflect loss of control over alcohol use. However, current therapeutic strategies are mainly aimed at attenuating the subjective effect of alcohol or craving for alcohol (van den Brink 2012). Pharmacotherapies may therefore prolong the time before

Table 1

Diagnostic Criteria of Alcohol Use Disorder according to DSM-5

A problematic pattern of alcohol use leading to clinically significant impairment or distress, as manifested by at least two of the following, occurring within a 12 month period:

Impaired control

1. Alcohol is often taken in larger amounts or over a longer period than was intended.
 2. There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.
 3. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
 4. Craving, or a strong desire or urge to use alcohol.
-

Social impairment

5. Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
 6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
 7. Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
-

Risky use

8. Recurrent alcohol use in situations in which it is physically hazardous.
 9. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
-

Pharmacological criteria

10. Tolerance, as defined by either of the following:
 - a. A need for markedly increased amounts of alcohol to achieve intoxication or desired effect.
 - b. A markedly diminished effect with continued use of the same amount of alcohol.
 11. Withdrawal, as manifested by either of the following:
 - a. The characteristic withdrawal syndrome for alcohol (refer to criteria A and B of the criteria set for alcohol withdrawal)
 - b. Alcohol (or a closely related substance, such as benzodiazepine) is taken to relieve or avoid withdrawal symptoms.
-

patients relapse, but continued absence of AUD remains difficult to achieve (Watkins et al., 2003). It has therefore been suggested that the control over alcohol consumption should be the focus in addiction therapies (Wolffgramm et al., 2000; Lesscher and Vanderschuren 2012; Hopf and Lesscher 2014). Understanding the behavioural and neural mechanisms underlying AUD, including the mechanisms that drive the descent from controlled alcohol use into loss of control over alcohol use, may therefore facilitate the development of novel treatments. In **Chapters 2 and 3** of this thesis, we describe animal models that capture loss of control over alcohol seeking and consumption, which will allow for the in-depth analysis of the neurobiological and behavioural mechanisms of AUD.

AUD often co-occurs with other mental disorders (Goldman et al., 2005). In adolescents, conduct disorder often co-occurs with AUD (Fein et al., 2004; Compton et al., 2005; Goldstein et al., 2007). In adults, AUD is frequently accompanied by depression, anxiety, schizophrenia, bipolar disorder, Attention-Deficit/Hyperactivity Disorder (ADHD) and insomnia (Weiss and Rosenberg 1985; Khan et al., 2005). In some cases, the mental disorder precedes the development of AUD and therefore may be considered a risk factor for AUD. For example, it has been reported that patients with a mental disorder may use substances of abuse for reasons of self-medication, e.g. to relieve states of stress or anxiety (Kushner et al., 2000; Bolton et al., 2006, 2009; Robinson et al., 2009). In addition, ADHD, which is characterized by a high level of impulsivity, has been identified as a risk factor for substance use disorders (Wilens and Upadhyaya 2007; Maxwell 2013; Reinhardt and Reinhardt 2013). Conversely, exposure to alcohol has also been reported to enhance the development of mental disorders (Kushner et al., 2000). Thus, there may be a bi-directional relationship between AUD and other mental disorders. Examples of factors that contribute to the risk for AUD will be addressed in the following section.

INDIVIDUAL VULNERABILITY TO ALCOHOL USE DISORDER: ROLE OF IMPULSIVITY, DECISION MAKING AND CUE REACTIVITY

Many people consume alcohol on a regular basis with occasional feelings of intoxication, but only a minority (3-5%) ever develop AUD (Anthony et al., 1994; Costanzo et al., 2007; Staff et al., 2010; United Nations Office on Drugs and Crime 2012). Although prolonged exposure to excessive amounts of alcohol

increases the chance of developing AUD, excessive alcohol consumption is not always necessary for AUD to emerge because some individuals show symptoms of AUD after limited exposures (Hiroi and Agatsuma 2005). Multiple factors, e.g. genetics, personality characteristics, developmental and environmental influences as well as neurobiological factors, have been related to the susceptibility for AUD (Pihl and Peterson 1995; Bates and Labouvie 1997; Littlefield et al., 2009; Ramchandani et al., 2011; Enoch 2012; Jurk et al., 2015). Forty to sixty percent of the variance in the risk for AUD is estimated to be explained by genetic influences (Cloninger 1987; Han et al., 1999; Hicks et al., 2004; Goldman et al., 2005; Hiroi and Agatsuma 2005; Agrawal and Lynskey 2008; Enoch 2013). However, it is important to note that any one gene variation is likely to explain just 1-2% of the risk for AUD (Hiroi and Agatsuma 2005), suggesting that AUD is a multi-genetic disorder (Sweitzer et al., 2012). Instead of attempting to identify genetic risk factors for AUD, it may therefore be more valuable to focus on endophenotypes associated with AUD, i.e. behavioural or neural characteristics that are abundant in people at risk (e.g. first-degree relatives) for AUD (Gottesman and Gould 2003). For example, individuals with lower self-control or high levels of impulsivity and 'novelty seeking', possibly reflecting impairments of inhibitory mechanisms in the brain, are thought to be predisposed to develop AUD (Cloninger 1987). Important studies in this respect are those by Ersche et al., that indicate that not only patients with substance use disorders display inhibitory control problems, but also their siblings without substances use disorders (Ersche et al., 2010, 2013).

Impulsivity

Impulsivity is considered an important factor in AUD, especially in Type 2 AUD (Perry and Carroll 2008; Dalley et al., 2011; Voon et al., 2013). Impulsive behaviours can be defined as '*Actions which are poorly conceived, prematurely expressed, unduly risky or inappropriate to the situation and often result in undesirable consequences*' (Durana and Barnes 1993). Impulsivity is a heterogeneous construct that can be broadly divided into *impulsive action* and *impulsive choice* (Evenden 1999; Chamberlain and Sahakian 2007; Pattij and Vanderschuren 2008; Dalley et al., 2011). The neural mechanisms regulating these types of impulsivity are different (Pattij and Vanderschuren 2008), emphasizing the importance of distinguishing them (Broos et al., 2012). *Impulsive actions*, or 'motor impulsivity', may be reflected by premature responses, i.e. the inability to withhold responding until an instruction stimulus is presented, or the failure to cancel a response once it has been initiated

(Eagle and Baunez 2010). *Impulsive choice* can be operationalized by impaired *delay discounting*, i.e. the tendency to choose a small immediate reward over a large delayed reward or *effort discounting*, i.e. the proclivity to choose for small rewards that require little effort over a large reward that demands more effort. In impulsive choice tasks, the delays or efforts are gradually increased within a session to determine a cut-off point (Rachlin et al., 1991; Beck and Triplett 2009). Besides several objective methods, described below, impulsivity can be assessed in humans using questionnaires, such as the Barratt Impulsiveness Scale (BIS-11) (Patton et al., 1995; Dalley et al., 2011). Importantly, these retrospective questionnaires and the behavioural measures of impulsivity usually do not correlate very well. This may be related to insufficient objectivity or detail in the questionnaires (Dalley and Roiser 2012) or hint at differences in the underlying behavioural and neural constructs.

There is emerging evidence for impulsivity as a risk factor for AUD. Impulsive action has been reported to be increased in abstinent AUD patients (Voon et al., 2013) as well as after chronic alcohol exposure in rodents (Walker et al., 2011; Irimia et al., 2013). Greater impulsive choice in the delay discounting task has been observed among individuals with a positive family history for AUD (Petry et al., 2002; Acheson et al., 2011; Mitchell 2011). Hence, impulsive choice and impulsive action may be a consequence of prolonged alcohol abuse (Dom et al., 2006; Perry and Carroll 2008; Salgado et al., 2009; Dalley et al., 2011), but may also occur as a pre-existing personality trait that increases the risk for AUD (Goudriaan et al., 2007; Marczinski et al., 2007; Courtney et al., 2012). In other words, the relationship between AUD and impulsivity may be bidirectional (Verdejo-Garcia et al., 2008), but this directionality is difficult to assess in the human population. Interestingly, alcohol naïve rats and mice that have been selectively bred for their high level of alcohol consumption (Sinclair et al., 1989; Colombo et al., 1995; Li and McBride 1995; Le et al., 2001; Crabbe et al., 2009) show an enhanced impulsive choice in comparison to their non-preferring counterparts (Wilhelm and Mitchell 2008; Oberlin and Grahame 2009; Beckwith and Czachowski 2014; Perkel et al., 2015). Moreover, in a prospective study using outbred rats, it was observed that a higher degree of impulsive choice predicted a higher level of alcohol intake (Poulos et al., 1995), although this was not replicated in a more recent study (Stein et al., 2015). However, the question whether a period of voluntary alcohol intake affects impulsive action and impulsive choice remains unanswered. Therefore, we investigated the effects of alcohol on both types of impulsive behaviour in **Chapters 4 and 5** of this thesis.

Decision making

AUD has been associated with risky decision making, whereby most studies show a higher preference for disadvantageous, risky choices in AUD patients (Bechara et al., 2001; Loeber et al., 2009; Salgado et al., 2009; Kim et al., 2011), although less risky decision making in AUD patients has also been reported (Ashenhurst et al., 2011). Risky decisions may be operationalized as favoring large rewards that are uncertain, or may entail loss or punishment over smaller, safe or certain rewards. The discrepancies in the relation of risky decision making with AUD may be due to the decision making task used. Examples of these tasks are the Game of Dice Task (Brand et al., 2005), the Balloon Analogue Risk Taks (BART) (Lejuez et al., 2002), Cambridge Gambling Task (Rogers et al., 1999) and the Iowa Gambling Task (IGT) (Bechara et al., 1994). While some of these tasks measure general decision making, such as the IGT, other tasks are more related to risk, such as the BART. Interestingly, both in humans and in rodents, a subgroup of healthy individuals display a disadvantageous decision making profile (Bechara and Damasio 2002; Dunn et al., 2006; Rivalan et al., 2009), comparable to patients suffering from psychiatric disorders such as ADHD, aggressive personality disorders or substance use disorders. Hence, this suggests that this subgroup of individuals may possess characteristics that might make them vulnerable to develop a substance use disorder. Moreover, the rats that display risky and disadvantageous decision making were shown to be hypersensitive to rewards (Rivalan et al., 2009). Therefore, individual differences in decision making may contribute to the development of substance use disorders.

Acute alcohol challenges in impulsivity and decision making tasks revealed mixed effects in healthy controls. In some cases, alcohol increased impulsivity and sub-optimal decision making, while other studies showed the opposite or no effects (George et al., 2005; Ramaekers and Kuypers 2006; Perry and Carroll 2008; MacKillop et al., 2011; Bidwell et al., 2013; Caswell et al., 2013). These findings extend to rodents, where acute alcohol treatment has been shown to have no or very little effect on decision making and impulsivity (Tomie et al., 1998a; Bizarro et al., 2003; Olmstead et al., 2006; Oliver et al., 2009; Mitchell et al., 2011; Semenova 2012; Pena-Oliver et al., 2014; Spoelder et al., 2015a). These findings suggest that alcohol in itself may not be responsible for the impairments in decision making and impulse control observed in AUD patients. However, it has been reported that acute alcohol exposure in heavy drinkers resulted in less behavioural control in comparison to light drinkers.

Moreover, heavy drinkers reported to feel more stimulated after alcohol, which may contribute to their risk to develop AUD (Marczinski et al., 2007; King et al., 2011; Reed et al., 2012). Hence, the effect of alcohol may be differentially perceived in individuals at risk for AUD. In **Chapter 4** we therefore investigated how acute and repeated alcohol exposure affects decision making and in **Chapter 5** we investigated the relationship between individual vulnerability to AUD with decision making, impulsive action and impulsive choice.

Cue reactivity

Repeated pairing of alcohol consumption with alcohol-related stimuli is thought to endow these stimuli with motivational significance or incentive salience. As a result, these alcohol-associated cues come to elicit approach behaviour and become powerful drivers of alcohol seeking (Tiffany 1990; Robinson and Berridge 1993, 2001; O'Brien et al., 1998; Field and Cox 2008; Tomie et al., 2008; Tomie and Sharma 2013; Everitt and Robbins 2015). Indeed, encounters with drug-associated cues can instigate craving and relapse (O'Brien et al., 1998; Carter and Tiffany 1999; Tiffany and Conklin 2000; Shaham et al., 2003). The importance of substance-associated cues has therefore been emphasized in several theories of substance use disorders (Stewart et al., 1984; Robinson and Berridge 1993; Tomie 1996; Everitt and Robbins 2015). Reward-associated conditioned stimuli are thought to have three fundamental properties: they are '1: attractive and attention grabbing, drawing individuals into close proximity with it; 2: in itself desirable, in the sense that they can reinforce novel actions to obtain them; 3: can evoke a conditioned motivational state capable of both instigating reward-seeking behaviour, and invigorating ongoing behaviour' (Everitt et al., 2001; Cardinal et al., 2002; Milton and Everitt 2010). Interestingly, individual variation between animals and humans exists in the attribution of incentive salience to reward-associated cues (Zener 1937; Brown and Jenkins 1968; Wilcove and Miller 1974; Burns and Domjan 1996; Tomie et al., 2000; Cole and Adamo 2005; Tomie et al., 2012), i.e. some individuals approach and manipulate the cue, so called 'sign-trackers', whereas other individuals approach the location of reward delivery, so called 'goal-trackers' (Fig. 2).

Individual variation in conditioned approach behaviour in rats has recently been explored in a series of studies utilizing a Pavlovian conditioning procedure, in which a brief presentation of a localizable cue that can be manipulated, such as a lever, is paired with the delivery of a reward in a different location (Flagel et al., 2007, 2009; Meyer et al., 2012). Importantly,

Figure 2

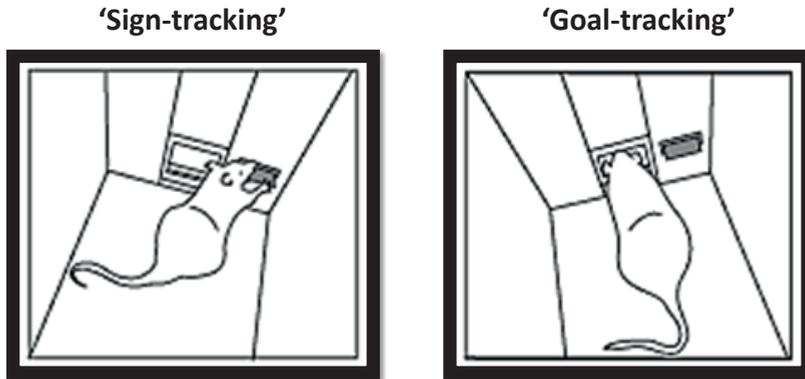


Figure 2. Illustration of the sign-tracking and goal-tracking conditioned response. During the presentation of the reward-predictive cue, sign-trackers approach the cue, suggesting that the cue has developed into an incentive motivational stimulus. Goal-trackers show a conditioned response directed at the food magazine, indicating that the cue merely functions as a predictor for an upcoming reward.

both sign- and goal-tracking individuals display a conditioned response, but show approach behaviour towards different identities (i.e. cue vs goal) (Flagel et al., 2009; Robinson and Flagel 2009). In sign-trackers, the reward-predictive cue develops into an incentive motivational stimulus, whereas for goal-trackers the cue is merely a predictor of the upcoming reward. Interestingly, even when contact with the reward-predicting cue leads to a negative contingency, i.e. no reward delivery, sign-trackers still continue to approach the reward-predictive cue (Williams and Williams 1969; Killeen 2003). Sign- and goal-tracking in rats was originally described in rats that were selectively bred as high- or low responders (bHR-bLR) to a novel environment, meaning that the bHR show a larger increase in locomotor activity in a novel environment compared to bLR (Flagel et al., 2010, 2011). Subsequently, sign- and goal-tracking phenotypes have also been observed in outbred populations of rodents (Flagel et al., 2007, 2011, 2014; Meyer et al., 2012; Fitzpatrick et al., 2013; Spoelder et al., 2015b). The individual differences in rats in their tendency to acquire a sign- or goal tracking conditioned response have been related to the vulnerability to addiction (Flagel et al., 2010, 2014; Saunders and Robinson 2013; Tomie and Sharma 2013). Indeed, the bHR were shown to be more susceptible to self-administration of amphetamine, cocaine, nicotine, morphine and alcohol (Ambrosio et al., 1995; Piazza et al., 2000; Suto et al., 2001; Nadal et al., 2002). In addition, bHR exhibit a higher tendency to seek rewards,

show higher motor impulsivity and a greater tendency for reinstatement of cocaine seeking (Flagel et al., 2014). In more recent studies, it was shown that conditioned reinforcers are more effective in maintaining self-administration in sign-trackers than in goal-trackers. Moreover, the conditioned reinforcers instigate reinstatement in sign-trackers (Saunders and Robinson 2010, 2011). Acute alcohol administration in rats has been shown to result in a stronger bias towards the reward-predicting cue, illustrating that alcohol by itself, by way of its pharmacological effect, may augment the sign-tracking conditioned response (Tomie et al., 1998b). Moreover, alcohol can both reliably function as an effective conditioned and unconditioned stimulus (Tomie and Sharma 2013). However, the relationship of the attribution of incentive salience to reward related cues with alcohol self-administration remains largely unknown.

These behaviours displayed by rats may have face validity for responsivity to drug-associated cues in humans. Several studies have examined individual differences in approach tendencies to reward-predicting stimuli in humans (Field et al., 2005; Palfai 2006; Thewissen et al., 2007; Field and Cox 2008; Van Gucht et al., 2008; Wiers et al., 2009; Christiansen et al., 2012). Interestingly, individuals with a 'reward-seeking' personality allocated more visual resources to reward-predicting stimuli (Hickey et al., 2010). Also, human studies have been directed at examination of the association between alcohol and approach tendencies to cues. For example, heavy drinkers and individuals with high levels of alcohol craving show an enhanced approach behaviour to alcohol-related pictures, as well as other appetitive stimuli, but not to general positive or neutral stimuli (Field et al., 2005; Wiers et al., 2009). An association in the other direction has also been reported; approach behaviour towards alcohol cues predicted a higher alcohol consumption (Palfai 2006; Christiansen et al., 2012). Hence, the causality or the bidirectionality of this association is incompletely understood and underlying neurobiological mechanisms are unknown. Therefore, in **Chapters 5 and 7**, we investigated the relationship between alcohol consumption and the attribution of incentive salience to a reward-predictive cue.

ADOLESCENCE AS A PERIOD OF ENHANCED ADDICTION VULNERABILITY

In humans, adolescence, i.e. the period between puberty and adulthood, is characterized by an increase in risky and impulsive behaviours, including

experimentation with substances of abuse (Beck and Treiman 1996; Caudill and Kong 2001; Kuposov et al., 2002; Gardner and Steinberg 2005; Casey and Jones 2010; Larsen et al., 2010). For example, adolescents have difficulty in resisting temptations and often make more impulsive choices, as measured in the delay discounting task (Steinberg et al., 2009). Moreover, functional neuroimaging studies revealed that adolescents show an exaggerated response of the ventral striatum to stimuli that signal reward, in comparison to children and adults (Delgado et al., 2000; Ernst and Paulus 2005; Galvan et al., 2006, 2007; Geier et al., 2010).

Alcohol is the most commonly used substance of abuse by adolescents. Adolescents often consume alcohol in an uncontrolled manner (Windle et al., 2008; Witt 2010). It has been suggested that adolescents may be less sensitive to the negative sedative and 'hangover' effects of alcohol in comparison to adults, while strong positive influences such as social facilitation are experienced, which therefore encourages further alcohol use (Doremus et al., 2003; Spear 2011, 2014). Of importance, adolescent alcohol use increases the likelihood of developing AUD in adulthood (Hingson et al., 2006; Dawson et al., 2008; Blomeyer et al., 2013). In rodents, it has been shown that even a very brief experience with alcohol during adolescence, but not during adulthood, results in subsequent higher alcohol intake in adulthood (Fabio et al., 2014). Importantly, several studies emphasized that the adolescent brain is particularly vulnerable to alcohol-induced functional changes (Monti et al., 2005; Zeigler et al., 2005; Crews et al., 2007; Pascual et al., 2009; Philpot et al., 2009; Guerri and Pascual 2010; Squeglia et al., 2012; Schindler et al., 2014). The mesolimbic dopamine system (which will be described in more detail below), for example, undergoes major developmental changes during adolescence (Chambers et al., 2003). Thus, it has been shown that the density of dopamine D1 and D2 receptors in the striatum peaks during early adolescence (Seeman et al., 1987; Andersen et al., 2000). The use of alcohol during adolescence may therefore exacerbate an already enhanced ventral striatal response, resulting in augmented reward-related learning processes that impact, for example, decision making (Kelley et al., 2004; Goudriaan et al., 2007; Casey and Jones 2010). Indeed, animal studies have shown that adolescent alcohol use, but not adult alcohol use, promotes risky decision making during adulthood which is associated with perturbation in the mesolimbic dopamine system (Nasrallah et al., 2009, 2011; Clark et al., 2012; Schindler et al., 2014). However, although adolescents as a group may be more sensitive to reward and prone

to risky behaviour, there are marked individual differences in these traits. It remains a challenge to identify which individuals are more vulnerable to descend into maladaptive risk taking activities (Gardner and Steinberg 2005). Interestingly, it has been shown that the striatal response to reward-predicting cues in adolescents positively correlates with a sensation-seeking personality and self-reported excitement (Bjork et al., 2008). In **Chapter 7**, we investigated the consequences of adolescent alcohol exposure on approach behaviour to reward-predicting cues and concurrent dopamine release in the ventral striatum, providing a potential mechanism for the well-documented vulnerability of individuals with early-life alcohol use for AUD in adulthood.

Social experiences during childhood and adolescence are of major importance for behavioural development, since they seem to serve as practice scenarios in order to obtain the necessary competencies to become socially capable adults (Vanderschuren et al., 1997; Nelson et al., 2005; Blakemore 2008; Cacioppo and Hawkley 2009). Disruptions in early social experiences can lead to long-lasting neurobiological changes, rendering an altered behavioural and cognitive repertoire later in life (Cacioppo and Hawkley 2009; Baarendse et al., 2013). Perceived social isolation by humans has been shown to be a risk factor for impaired executive functioning, increased negative perceptions and an enhanced propensity to consume alcohol (Bonin et al., 2000; Cacioppo and Hawkley 2009; Alwan et al., 2011; Whelan et al., 2014). Like humans, young animals also show an abundance of social play behaviour. Social play behaviour is a highly vigorous, characteristic form of social interaction that is thought to facilitate behavioural and cognitive development (Panksepp 1981; Vanderschuren et al., 1997; Bell et al., 2010). Animal studies support the role of social development in the sensitivity to consume alcohol, whereby in general, higher levels of alcohol consumption are observed in rats that were reared in isolation (Schenk et al., 1990; Hall et al., 1998; Lodge and Lawrence 2003; Roman et al., 2005; Cruz et al., 2008; Nylander and Roman 2013; Whitaker et al., 2013). Interestingly, social play behaviour is assumed to be modulated through neural systems that also mediate the rewarding effects of alcohol, which suggests a critical role for social play behaviour in the development of the brain reward system (Trezza et al., 2010; Siviý and Panksepp 2011). In **Chapter 6**, we investigated the consequences of social play deprivation, i.e. a social isolation period for a short time during development when social play behaviour is particularly prominent, on alcohol consumption and reinforcement in adulthood.

THE MESOLIMBIC DOPAMINE SYSTEM

To understand the development and maintenance of AUD, it is critical to investigate the underlying neurobiological mechanisms. There is a considerable amount of literature to suggest that different types of substances of abuse, as well as their associated reward-predicting stimuli, activate comparable neural systems. In the last decades, the role of the mesolimbic dopamine system has been a major topic of investigation in, among others, substance use disorders and other forms of motivated behaviour (Everitt and Robbins 2005; Kalivas 2008; Pierce and Vanderschuren 2010; Salamone and Correa 2012; Everitt and Robbins 2015). Most substances of abuse, including alcohol, enhance activity of the mesolimbic dopamine system in both humans and animals (Di Chiara and Imperato 1986, 1988; Wise and Rompre 1989; Weiss et al., 1993; Boileau et al., 2003; Doyon et al., 2003; Urban et al., 2010), thereby affecting motivation and goal-directed behaviour (Everitt and Robbins 2005; Salamone et al., 2007; Salamone and Correa 2012; Floresco 2015). This is of great interest, because the primary mechanisms of action varies for the different substances of abuse. Alcohol is of particular interest in this respect, because there is no single primary target of alcohol; its effects are mediated through, among others, NMDA, GABA-A, glycine, 5-HT₃ and nicotinic ACh receptors (Mehta and Ticku 1988; Lovinger et al., 1989; Aguayo 1990; Simson et al., 1991; Wang et al., 1994; Yu et al., 1996; Kobayashi et al., 1999; Lovinger 1999; Mihic 1999; Narahashi et al., 1999; Vengeliene et al., 2008; Spanagel 2009; Barker and Taylor 2014; Korpi et al., 2015).

The mesolimbic dopamine system comprises dopamine neurons that originate in the ventral tegmental area (VTA) in the midbrain. The axonal processes of these dopaminergic neurons terminate in several brain regions including the nucleus accumbens (NAcc), amygdala, hippocampus, prefrontal cortex and the olfactory tubercle (Fallon and Moore 1978a, b; Fallon et al., 1978; Swanson 1982; Oades and Halliday 1987; Heimer et al., 1991; Ikemoto 2007). The NAcc receives the most dense dopamine projections from the VTA and it has bidirectional connections with many other brain regions in the mesolimbic dopamine system (Mogenson et al., 1980; Kelley and Domesick 1982; Chang and Kitai 1985; Hurley et al., 1991; Berendse et al., 1992; Sesack and Pickel 1992; Pennartz et al., 1994; Nicola et al., 2000; Zahm 2000; Carelli and Wightman 2004; Fields et al., 2007). Therefore, it has been suggested that the NAcc acts as a 'limbic-motor' interface, whereby information about emotions and cognitive

processes is integrated to gain access to motor output systems of the brain (Mogenson et al., 1980; Pennartz et al., 1994; O'Donnell 2003; Roesch et al., 2009; Cacciapaglia et al., 2011; Floresco 2015).

With regard to the effects of alcohol on the mesolimbic dopamine system, it has been shown that acute alcohol administration stimulates the spontaneous activity of dopaminergic neurons in the VTA (Gessa et al., 1985; Brodie et al., 1990, 1999; Brodie and Appel 2000; Grace 2000), increasing the release of dopamine in its terminal regions, such as the NAcc (Di Chiara and Imperato 1986, 1988). Moreover, it has been shown that rats directly self-infuse alcohol into the VTA (Gatto et al., 1994; Rodd-Henricks et al., 2000; Rodd et al., 2004). Because of the presumed importance of the mesolimbic dopamine system in substance use disorders, including AUD, we studied the involvement of dopamine in alcohol consumption, reinforcement and Pavlovian conditioned approach behaviour in **Chapters 7, 8 and 9**.

The role of dopamine in reward-related behaviours

Dopaminergic neurotransmission within the NAcc has been implicated in many reward-related functions, most prominently motivation, goal-directed behaviour, incentive salience and reward-driven learning (Berridge 2007; Robbins and Everitt 2007; Schultz 2007; Willuhn et al., 2010; Salamone and Correa 2012; Floresco 2015). Motivated behaviour takes places in different phases. First, the individual approaches or seeks the reinforcer, which is described as appetitive or preparatory behaviour. Subsequently, the individual may gain access to the reinforcer and consumes it (Salamone and Correa 2012). Importantly, research suggests that mesolimbic dopaminergic neurotransmission is especially important during the first, appetitive phase of motivated behaviour. Indeed, after dopamine depletions or pharmacological inhibition of dopamine neurotransmission, the core aspects of consuming the reward remain unaffected, while instrumental behaviour is reduced (Salamone and Correa 2012). It is important to note that dopaminergic neurotransmission is also involved in processing aversive stimuli and dysphoric motivational states (Young 2004; Jensen et al., 2007; Menon et al., 2007; Anstrom et al., 2009; Baliki et al., 2010; Schultz 2010; Lammel et al., 2011; Lemos et al., 2012), but it is currently unknown whether there are separate dopamine neurons that respond to appetitive and aversive stimuli.

Several distinct, but not necessarily exclusive theories have been proposed to explain the involvement of mesolimbic dopamine in reward-related behaviour (Schultz 1998; Berridge 2001; Robinson and Berridge 2001; Cardinal et al., 2002; Di Chiara 2002; Salamone and Correa 2002; Berridge 2007; Yin et al., 2008). It has been proposed that phasic dopamine release can act as a 'reward prediction error' signal which is necessary for learning stimulus-reward associations (Schultz et al., 1997; Waelti et al., 2001; Bayer and Glimcher 2005; Tobler et al., 2005; Day et al., 2007). It has also been argued that the role of dopamine in reward is the attribution of incentive salience to cues that signal an upcoming reward (Berridge and Robinson 1998; Berridge 2007). Because the predictive and motivational properties of reward-associated cues are usually acquired together, it has been difficult to distinguish these theories.

Dopamine receptor subtypes

There are five subtypes of dopamine receptors, which are grouped in two families: the dopamine D1-like (D1 and D5) and D2-like (D2, D3, D4) receptor subtypes. The dopamine D1 and D2-like receptors were initially defined on the basis of their distinct transduction mechanisms and pharmacological profiles (Spano et al., 1978; Keabian and Calne 1979). Dopamine D1 receptors stimulate adenylyl cyclase activity and produce the second messenger molecule cyclic-AMP, whereas dopamine D2 receptors inhibit this second messenger system. It has been observed that the expression of dopamine D2 receptors in limbic areas is reduced in AUD patients (Hietala et al., 1994; Volkow et al., 1996, 2002; Tupala et al., 2001, 2003) as well as in alcohol-preferring rodents (Stefanini et al., 1992; McBride et al., 1993; Zhou et al., 1995; Bice et al., 2008). In addition, both the dopamine D1 and D2 receptor seem to be involved in alcohol consumption and reinforcement (Linseman 1990; Silvestre et al., 1996; Files et al., 1998; Cohen et al., 1999; Melendez et al., 2005; Ding et al., 2015). However, the relative contributions of the different dopamine receptor subtypes in AUD remains incompletely understood, and it is unknown whether individual susceptibility to AUD is related to differences in dopamine signaling. Therefore, in **Chapter 9**, we assessed the effects of selective dopamine D1 and D2 receptor agonists and antagonists on alcohol consumption in rats that display individual variation in alcohol consumption.

Dissociable roles for the sub-regions of the striatum

The striatum is a heterogeneous brain region that comprises several sub-regions based on their anatomical connectivity and behavioural functions (Voorn

et al., 1989, 2004; Heimer et al., 1991; Brog et al., 1993; Pennartz et al., 1994; Groenewegen et al., 1999; Zahm 1999, 2000), viz. ventral regions including the NAcc shell and core, and dorsal regions, including the putamen or dorsolateral striatum (DLS) and caudate or dorsomedial striatum (DMS). The shell and core sub-regions of the NAcc (Heimer et al., 1991; Groenewegen et al., 1999), have been shown to possess different functional properties and roles in reward-related behaviour (Zahm 1999; Di Chiara 2002; Voorn et al., 2004; Yin et al., 2008). For example, the shell may mediate hedonic states and the reinforcing properties of natural rewards and substances of abuse (Ikemoto et al., 1997a; Pecina and Berridge 2000; Rodd-Henricks et al., 2002; Engleman et al., 2009). Therefore, the NAcc shell may be of importance in the initiation of reward-seeking behaviour. Conversely, the NAcc core appears to play a prominent role in conditioning processes and the regulation of motor activity (Ito et al., 2004; Day et al., 2007; Ikemoto 2007; Flagel et al., 2011) and it may therefore play a role in the acquisition and maintenance in reward-seeking behaviour. Recent findings in animal studies suggest a regional specificity of the actions of alcohol in the striatum (Jeanblanc et al., 2009; Wang et al., 2010; Chen et al., 2011; Corbit et al., 2012; Adermark et al., 2013; Fanelli et al., 2013; Logrip et al., 2015). The involvement of dopamine in the ventral striatum in alcohol reinforcement has been demonstrated by local infusions of dopamine receptor agonists and antagonists (Hodge et al., 1992, 1997; Rassnick et al., 1992; Samson et al., 1993; Czachowski et al., 2001; Samson and Chappell 2004) and lesions of the ventral striatal dopamine system (Rassnick et al., 1993; Ikemoto et al., 1997b). In a recent study, it was shown that dopamine receptors in the NAcc shell, the ventral pallidum and the medial prefrontal cortex, but not the NAcc core, are involved in mediating the reinforcing effects of alcohol infused into the VTA (Ding et al., 2015). Moreover, it has been shown that alcohol increases extracellular dopamine in the NAcc during experimenter administered alcohol (Di Chiara and Imperato 1986; Yoshimoto et al., 1992), during alcohol self-administration, and during an anticipatory period before alcohol self-administration (Weiss et al., 1993; Melendez et al., 2002; Doyon et al., 2003; Doyon et al., 2005).

The DLS has been implicated in the development of habit formation (Packard and Knowlton 2002; Yin et al., 2004, 2008; Balleine and O'Doherty 2010; Furlong et al., 2014). Moreover, the DLS is thought to modulate compulsive drug seeking after extended substance abuse exposure (Vanderschuren and Everitt 2004; Belin et al., 2009b; Pierce and Vanderschuren 2010; Zapata et al., 2010; Everitt and Robbins 2015). Indeed, the DLS is highly sensitive to alcohol cues in

alcohol dependent patients (Grusser et al., 2004; Wilson et al., 2004; Vollstadt-Klein et al., 2010; Sjoerds et al., 2013), and it has recently, been shown to be involved in habitual alcohol seeking (Corbit et al., 2012, 2014). In addition, the DLS has recently also been implicated in the primary reinforcing properties of substances of abuse (Veeneman et al., 2012, 2015; Willuhn et al., 2012). The DMS, on the other hand, is important for the implementation of planned actions, i.e. goal-directed behaviour. It has been proposed that the different sub-regions of the striatum are hierarchically organized, whereby each sub-region functions as an intermediary in the hierarchy and transfers information to the next level (Haber et al., 2000; Yin et al., 2004, 2008; Everitt and Robbins 2005; Belin and Everitt 2008; Belin et al., 2009b). This model predicts that the striatal sub-regions modulate different stages of instrumental learning. Indeed, numerous studies have shown that under certain conditions, for example upon extended substance use or overtraining, the control of actions can shift from the goal-directed DMS system to the habit-directed DLS system (Hikosaka et al., 1989; Delgado et al., 2004; Everitt and Robbins 2005; Samejima et al., 2005; Belin et al., 2009a; Thorn et al., 2010; Zapata et al., 2010; Corbit et al., 2012; Murray et al., 2012; Pierce et al., 2012; DePoy et al., 2013; Barker and Taylor 2014; Everitt and Robbins 2015), albeit that it is not clear whether these striatally-modulated forms of learning occur in series or in parallel.

In contrast to the significant effort which has been devoted to identifying the role of dopamine in the different striatal sub-regions in the reinforcing properties of stimulants, like cocaine, it is unknown whether dopamine in the sub-regions of the ventral and dorsal striatum show differential effects on alcohol-motivated behaviour. Therefore, in **Chapter 8** of this thesis, we systematically assessed the role of dopamine in the different striatal sub-regions on operant alcohol reinforcement.

ANIMAL MODELS AND EXPERIMENTAL TECHNIQUES

The use of animal models has greatly contributed to our understanding of AUD (McBride and Li 1998; Sanchis-Segura and Spanagel 2006; Panlilio and Goldberg 2007; Vengeliene et al., 2009; Pautassi et al., 2010; Crabbe et al., 2011, 2014; Lesscher and Vanderschuren 2012; Vanderschuren and Ahmed 2013; Hopf and Lesscher 2014; Belin et al., 2015; Belin-Rauscent et al., 2015). Animal models provide a valuable means to investigate characteristic symptoms of substance use disorders, including reinforcing properties of substances,

reinstatement (relapse) to substance use, loss of control over substance use and individual vulnerability for substance use disorders (Ahmed and Koob 1998; Shaham et al., 2003; Deroche-Gamonet et al., 2004; Ahmed and Koob 2005; Vanderschuren and Everitt 2005; Belin et al., 2008, 2009a; Bossert et al., 2013). The great advantages of using animal models are that 1) very specific aspects of AUD can be studied in isolation under controlled genetic and environmental influences, 2) the causality of associations between certain factors and AUD can be studied, and 3) the underlying neurobiological mechanisms can be investigated. A potential drawback of animal models is that the complicating (for example, societal and familial) interactions associated with the AUD are difficult to incorporate. In the following sections, I will briefly introduce the experimental paradigms available to assess AUD-like behaviour, with a focus on the methods used in the current thesis.

Alcohol access paradigms

During the last decades, animal models for alcohol ingestion and AUD have been improved substantially. In voluntary consumption models, animals, usually rodents, typically receive access to an alcohol solution in their home cage. Rats have either continuous alcohol access (CAA) or intermittent alcohol access (IAA). Interestingly, IAA promotes higher levels of voluntary alcohol intake compared to CAA, without the need of initiation procedures such as sucrose fading (Wise 1973; Rhodes et al., 2005; Simms et al., 2008; Hwa et al., 2011; Hayton et al., 2012; Lesscher et al., 2012). Importantly, intermittent or limited access to alcohol has been shown to induce a transition from moderate to escalated intake and compulsive alcohol consumption, which are critical features of the development of AUD (Hopf et al., 2010; Lesscher et al., 2010). Profound individual differences in alcohol intake have been reported in rodents (Simms et al., 2008; Hwa et al., 2011; Sabino et al., 2013), whereby animals that drink the largest amounts of alcohol display measurable levels of intoxication (Murphy et al., 1986; McBride and Li 1998; Crabbe et al., 2009). To study high alcohol intake and its determinants, several strains of rats or mice, that have been selectively bred for high or low alcohol intake or preference have been generated (Bell et al., 2006; Ciccocioppo et al., 2006; Colombo et al., 2006; Crabbe et al., 2006; Overstreet et al., 2006; Quintanilla et al., 2006; Sommer et al., 2006). In the present thesis, we did not make use of these selectively bred alcohol-preferring and non-preferring rodents, but instead used an outbred strain which displays large individual differences in behaviour. We chose this approach because we intended not to primarily focus on genetic factors but

instead on behavioural and cognitive factors that may contribute to AUD-like behaviour.

Models to assess reinforcement, motivation and compulsive alcohol use

Reinforcement is defined operationally as 'an increase in the probability or frequency of a particular behaviour upon presentation of a given stimulus or response as a consequence of this behaviour' (Skinner 1938). Therefore, reinforcement requires a behavioural response, that is amenable to experimental analyses.

Alcohol reinforcement is typically measured in an operant conditioning chamber, where the animal (rat or mouse) is required to perform an action (e.g. a nose poke or a lever press) in order to gain access to a reward. The alcohol reward is usually presented as a small amount of alcohol (e.g. 0.1 ml/reward) which can be consumed orally, although intravenous and intra-gastric routes are also used (Gonzales et al., 2004). Typically, there are two levers or nose poke holes in the chamber. One lever is designated as the active lever, responding on which results in the presentation of alcohol, and the other is designated as the inactive lever, responding on which has no programmed consequences. Importantly, different phases and aspects of alcohol self-administration can be studied in the operant paradigm, such as acquisition, maintenance, motivation, escalation, extinction and reinstatement. The most commonly used schedules of reinforcement include the fixed ratio-, progressive ratio-, second-order-, seeking-taking, and random interval schedules of reinforcement. The fixed ratio, random interval and progressive ratio schedules of reinforcement, used in this thesis, will be briefly explained here. Under fixed ratio schedules, the animal is required to make a fixed number of responses in order to obtain the reward, thereby providing a direct relationship between the response rate and reward delivery. Under a random interval schedule, the first active lever press initiates the random interval during which both levers stay extended and lever pressing is without consequences until the random interval elapses. After completion of the random interval, an active lever press results in the delivery of alcohol. Random interval schedules induce a high and constant number of responses and they are therefore suitable to assess the impact of pharmacological or environmental stimuli on alcohol seeking in a within-session design as well as in extinction. Under a progressive ratio schedule of reinforcement, the animal has to make an increasing number of responses

according to a linear or exponential formula for each subsequent reward. The so called 'break-point' during the progressive ratio schedule of reinforcement refers to the highest response requirement that the animal achieves before the session elapses or before responding ceases. Hence, determination of the break-point provides a measure of the incentive motivational value of the reinforcer (Katz 1990; Markou et al., 1993; Richardson and Roberts 1996; Arnold and Roberts 1997).

Continued substance use despite the knowledge of adverse consequences is a hallmark of substance use disorders (American Psychiatric Association 2013). During the past decade, animal models have been developed and improved to capture compulsive alcohol use, operationalized as continued alcohol use despite aversive consequences. In these models, lithium chloride-induced illness, mild electric shocks or quinine adulteration serve as punishments (Spanagel 2009; Vengeliene et al., 2009; Barker and Taylor 2014; Hopf and Lesscher 2014). In **Chapter 2** we used a quinine adulteration procedure and in **Chapter 3** we measured conditioned suppression of alcohol seeking; where rats were confronted with a tone that was previously associated with unpredictable mild electric footshocks. Quinine is a bitter tastant, which induces taste aversion in rodents. Therefore, continued alcohol intake, despite it being adulterated with quinine has been interpreted as loss of control over alcohol use (Wolffgramm and Heyne 1991; Spanagel and Holter 1999; Wolffgramm et al., 2000; Turyabahika-Thyen and Wolffgramm 2006; Vengeliene et al., 2009; Hopf et al., 2010; Lesscher et al., 2010; Loi et al., 2010). Mild electric footshocks have been used in several ways to assess compulsive substance use in rodents. For example, rats were required to cross an electrical barrier (Jenkins et al., 1926) in order to obtain access to a substance of abuse (Cooper et al., 2007), to continue to respond for substances of abuse despite the risk for a footshock (Deroche-Gamonet et al., 2004; Pelloux et al., 2007; Jonkman et al., 2012a; Marchant et al., 2013; Seif et al., 2013), or to become insensitive to a warning signal, e.g. a tone, that was previously paired with a footshock during substance seeking (Vanderschuren and Everitt 2004; Limpens et al., 2014).

Importantly, in previous studies, only a minority of rats that received chronic exposure to cocaine continued to seek or take the drug despite negative consequences (Deroche-Gamonet et al., 2004; Pelloux et al., 2007; Belin et al., 2008; Jonkman et al., 2012b). This observation is of interest because this subgroup may be representative for the subset of humans who develop

a substance use disorder after the consummation of substances of abuse. Moreover, the subgroup of rats which continued to seek cocaine despite adverse consequences exhibited high levels of impulsivity and novelty seeking and showed a higher motivation to obtain cocaine and a reduced ability to seek cocaine despite signaled unavailability (Deroche-Gamonet et al., 2004; Belin et al., 2008, 2009a; Kasanetz et al., 2013). Individual differences in the sensitivity to shock-induced punishment during alcohol reinforcement and quinine-resistant intake of alcohol has also been observed (Wolffgramm et al., 2000; Fachin-Scheit et al., 2006; Turyabahika-Thyen and Wolffgramm 2006; Seif et al., 2013). In **Chapters 2 and 3**, we investigated how individual differences in alcohol consumption relate to subsequent 1) resistance to quinine adulteration and 2) conditioned suppression of alcohol seeking.

Models to assess decision making and impulsivity in rodents

To advance our knowledge on the underlying neurobiological mechanisms concerning impulsivity and decision making, several rodent models have been developed (Eagle and Baunez 2010; de Visser et al., 2011; Winstanley 2011; Robbins 2002). A great advantage of these rather complex models is the ability to assess multiple aspects of cognitive performance. The models are often strikingly similar to their human equivalent and therefore potentially possess a high face validity.

A frequently used task to measure continuous attention and motor impulsivity in rodents is the 5-choice serial reaction time task (5CSRTT) (Robbins 2002), which is derived from the continuous performance task in humans (Rosvold et al., 1956). In the human version, the subject is asked to pay continuous attention to a computer screen in which a target stimulus is infrequently presented within a sequence of stimuli and to respond upon the presentation of the stimulus. In the rodent version of this task, the animal is also required to pay attention to an array of five holes in which in one of the holes a stimulus light will appear. In order to receive a reward, usually a sucrose pellet, the rat is trained to poke its nose in the illuminated hole in order to receive a sucrose pellet (Fig. 3). When the rat is not responding within a certain amount of time, this is scored as an omission, and when the rat pokes his nose in a non-illuminated hole this is scored as an incorrect response. Interestingly, when the animal makes a nose poke response before the stimulus light is presented, i.e. during the inter-trial interval (ITI), this is registered as a premature response, reflecting impulsive action. In the studies described in **Chapters 4 and 5**

of this thesis, an omission, incorrect or premature response are signaled to the animal as a mistake by the illumination of the house light in the operant chamber for 5 sec (Fig. 3). In **Chapters 4 and 5**, the animals were essentially trained in the 5CSRTT, where they learn to make a correct nose poke response, i.e. a nose poke response in the illuminated hole, prior to training for more complex decision making tasks.

Decision making: the rat gambling task (rGT)

One widely used task that mimics the complexity of daily life decision making, is the Iowa Gambling Task (IGT), which combines several factors that guide decision making, including the unpredictability of reward and punishment, the weighing of a short-term small reward vs. a long-term large reward, and the necessity to exert behavioural control in order to maximize long-term gains (Bechara et al., 1994). The IGT was originally developed to assess specific cognitive impairments of patients with damage to the ventromedial part of the prefrontal cortex (Bechara et al., 1994), but has since been used to determine decision making deficits in several mental disorders as well, including substance use disorder (Cavedini et al., 2002; Ernst and Paulus 2005; Goudriaan et al., 2005; Sevy et al., 2007). In this task, participants play a card game in which they have to choose cards from four decks, which differ in the probability and magnitude of monetary gains and losses. Participants are instructed to gain as much (hypothetical) money as possible, but do not have prior knowledge about the task contingencies. Unbeknownst to the participants, the two decks that initially appear most attractive (by producing higher gains), are the least profitable in the long run, since they also produce higher losses. The optimal choice strategy is therefore to select cards from the two advantageous decks with small gains and small penalties, as opposed to the two disadvantageous decks with larger gains but also heavy long-term losses. Hence, one critical feature of the IGT is the risk of losing, which is defined as loss of accumulated gains by making an unfavorable bet, and is distinct from failing to win. Healthy human subjects have shown a shift in decision making strategy from primarily explorative at the beginning of the task (when the win/loss contingencies at each deck are still unknown), towards exploitative over the course of the task (when the task contingencies become known).

Recently, new rodent models based on the same principle have been developed (van den Bos et al., 2006; Pais-Vieira et al., 2007; Rivalan et al., 2009; Zeeb et al., 2009; de Visser et al., 2011). As for the IGT, maximal gains are obtained

Figure 3

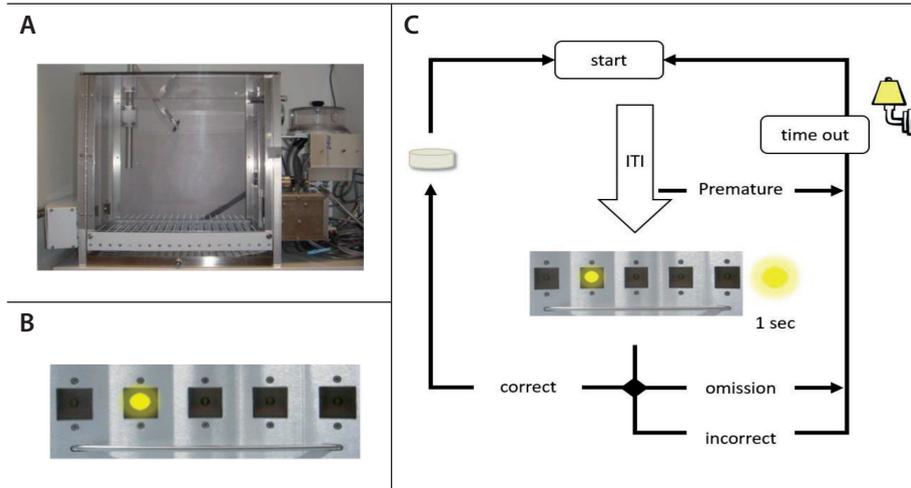
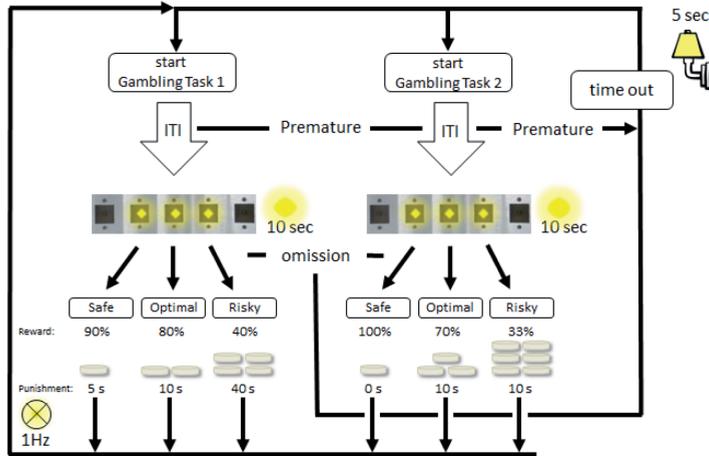


Figure 3. (A): Picture of an operant conditioning chamber used to assess the cognitive abilities of rodents. On the left side is a curved wall with five nose poke apertures and on the right side there is a dispenser which delivers the sucrose pellet in the magazine. (B): Illustration of the illumination of a stimulus light in one of the five apertures. (C): Example of the trial structure of the 5CSRTT. After a trial has started, the animal has to withhold responding during the inter-trial interval (ITI) until one of the stimulus lights is illuminated for 1 sec. A correct response will result in the delivery of a sucrose pellet. A premature response, an omission and an incorrect response results in a time-out period of 5 sec in which the house light will be illuminated. Thereafter, a new trial starts.

by choosing small reward options and avoiding the tempting risky response options which provide large rewards, but are also associated with a higher probability and magnitude of punishment. In these animal models, money is replaced by palatable food to function as the reward. However, the replacement of money with food, complicates the modelling of loss during the IGT since sugar pellets are immediately eaten. Hence, it is impossible to take them away during a loss trial and therefore the final outcome can never be an absolute resource deficit, which is theoretically possible in the human version (de Visser et al., 2011). Of all the different rGT versions developed for rodents, the rGT developed by Zeeb et al. (2009) probably signals loss in a way that is most comparable to the human situation. Here, loss is signaled by a time-out period during which no food pellets can be earned in a task with limited play time, thereby restricting maximization of earnings after making a disadvantageous choice. Moreover, this rGT version is the first that enables concurrent, but dissociated, assessment of decision making and motor impulsivity (measured as premature responses: Robbins 2002; Zeeb et al., 2009). In **Chapters 4 and 5**,

Figure 4

A



B

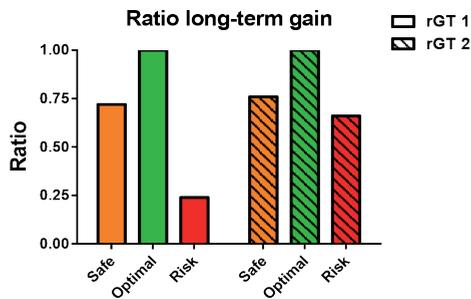


Figure 4. (A): Example of the trial structure of the rGT. Both rGT versions measured omission and premature responses in the same way; these responses resulted in a time-out period of 5 sec. In both versions, after the ITI of 5 sec, the middle three nose poke apertures were illuminated for 10 sec. The feedback provided to the animal was different between the safe, optimal and risky option, which also differed between rGT versions. The displayed percentages reflect the probability of reward, the pellets reflect the number of pellets received when the rat gets rewarded, and the punishment reflects the length of the time-out period (in sec), during which the light in the chosen nose poke aperture flashes with a frequency of 1 Hz. (B): Displays the ratio of long-term gain for each option.

we used modified versions of the rGT developed by Zeeb et al. (2009). Our rGT version was designed to have one optimal choice and two suboptimal choice options (i.e., safe and risky). As such, the safe choice, with a high probability of reward but a small reward size is nonetheless suboptimal, and may reflect loss-averse decision making. The optimal choice has a lower probability of reward

Figure 5

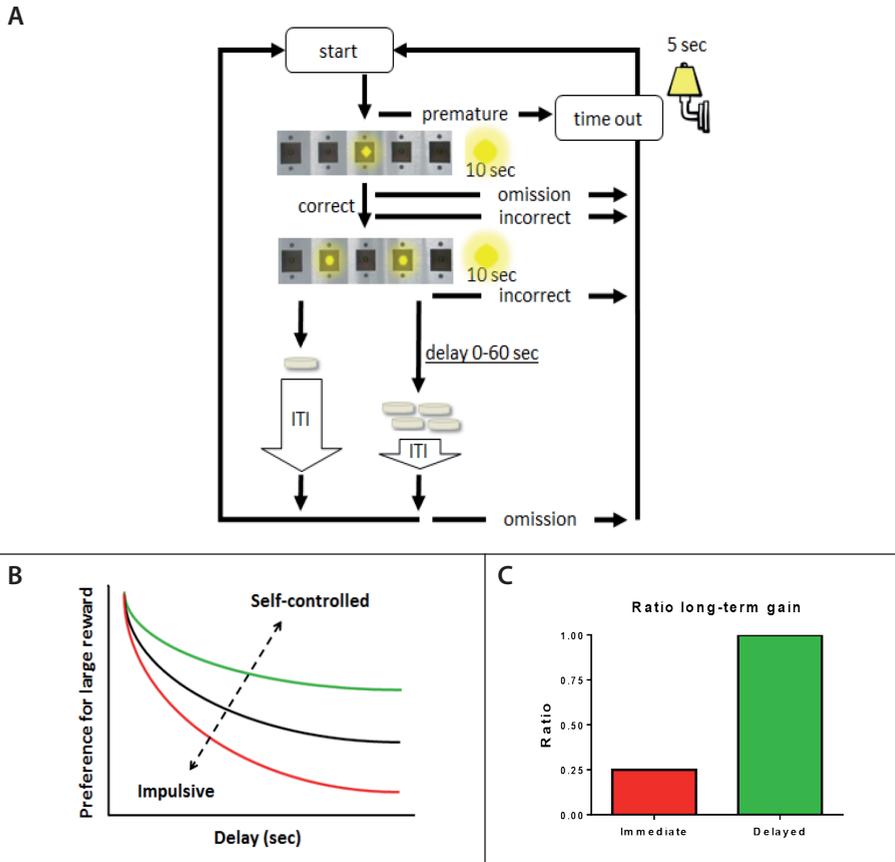


Figure 5. (A): Example of the trial structure of the DRT. The animal is required to first make a nose poke response in the middle illuminated response hole. Thereafter, the two nose poke apertures adjacent to the middle aperture will illuminate and the animal can make a choice: receiving one pellet immediately or receiving 4 pellets after a pre-set delay. Importantly, due to the adjustable duration of the ITI, the total length of each trial is the same, irrespective of the choice. Comparable to the initial nose poke training in the 5CSRTT, a premature response, an omission or a incorrect response results in a time-out period of 5 sec in which the house light will be illuminated for 5 sec. (B): Illustration of the preference for the large delayed reward depending on the delay prior to the large reward. (C): Displays the ratio of long-term gain for each option.

but yields a larger reward compared to the safe choice, resulting in a largest gain in the long run. The risky choice yields the highest reward, but the reward probability is low and the punishment is relatively large, which therefore yields the lowest gain in the long run compared to the safe and optimal choice. Risky choice behaviour may therefore be related to a decision making strategy

based on a high sensitivity to reward or a low sensitivity to punishment. We investigated the effects of acute and repeated alcohol treatment on two rGT versions in **Chapter 4**. The three choices of the two versions differed in the probability of receiving the reward, the reward size (i.e. number of sucrose pellets) and the punishment magnitude (Fig. 4).

Impulsive choice: delayed reward task (DRT)

In the DRT, subjects are asked to make a choice between a small immediate reward or a larger delayed reward (Ainslie 1975; Rachlin et al., 1991; Kirby and Marakovic 1996). Similar to the rGT, human subjects are usually rewarded with (hypothetical) money (Johnson and Bickel 2002) and rodents with food (Evenden and Ryan 1996). In the DRT, the delay and magnitude of the small immediate reward are usually kept constant, whereas the delay for the large reward increased over the session. Delay discounting is based on the assumption that the value of a reward declines with increasing delay. Hence, humans and animals will come to forgo the large delayed reward in favour of the smaller but immediate reward as the delay to the large reward increases. A steep discounting curve, usually a hyperbolic function, has been labeled as impulsive, whereas a shallow curve implies self-control. In some versions of the DRT, the delay for the large reinforcer is adjusted based on the subject's previous choices in order to determine the indifference point for each subject independently (Richards et al., 1999). The indifference point reflects the situation in which the immediate and delayed reward options appeal equally to the subject; it therefore represents the subjective value of the delayed option. The rodent version of the DRT is fairly similar to the human version (Evenden and Ryan 1996; Cardinal et al., 2000, 2006). In **Chapter 5**, we used a DRT paradigm in which the delays for the large reward are increased within the session in 5 blocks of 10 choice trials (van Gaalen et al., 2006; Baarendse and Vanderschuren 2012). Importantly, the total trial time for the immediate or delayed reward options are kept similar. Therefore constantly choosing the large delayed reward will result in the highest gain (Fig. 5).

***In vivo* micro-infusions and fast-scan cyclic voltammetry**

One of the great advantages of using animal models is that invasive techniques allow for in-depth functional neurobiological research. In **Chapters 7 and 8** we studied the involvement of the mesolimbic dopamine system in reward sensitivity by intracerebral infusion of a dopamine receptor antagonist and fast-scan cyclic voltammetry (FSCV). FSCV is an electrochemical chemical

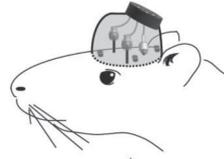
detection method that can be used to monitor sub-second changes in concentrations of electroactive molecules (Phillips et al., 2003; Wheeler and Carelli 2009) (Fig. 6). The monoamine neurotransmitters, including dopamine, can undergo oxidation-reduction reactions, that is, they can lose and gain electrons depending on the surrounding voltage (Robinson et al., 2003; Heien et al., 2005). A triangular waveform voltage is applied to a carbon fiber electrode implanted into a dopamine-rich brain region of an animal (Clark et al., 2010). During each waveform application, dopamine molecules in the vicinity of the electrode will undergo electrolysis and produce a current, which can be detected by the electrode. The flow of electrons between the dopamine molecule and the microelectrode is measured as current and is directly proportional to the concentration of dopamine molecules that were oxidized. In order to monitor sub-second changes in phasic dopamine release, this waveform application is repeated every 100 milliseconds, yielding a 10 Hz sampling rate.

Figure 6

A



B



C

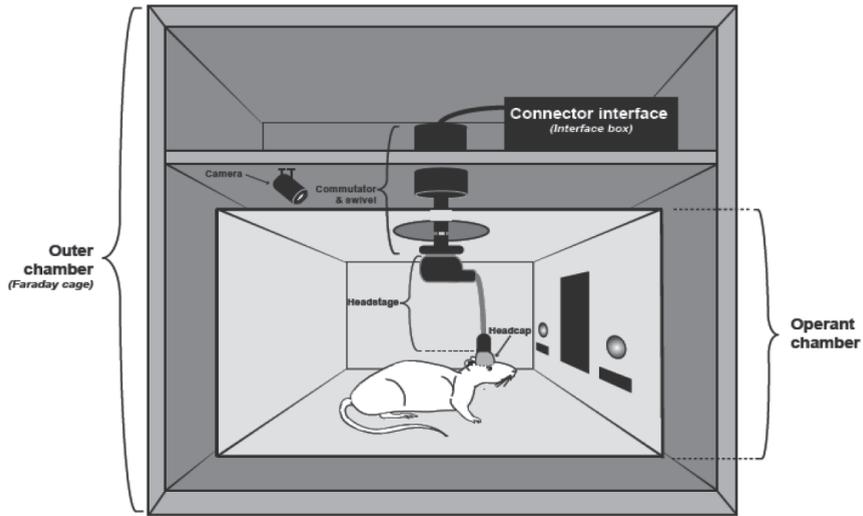
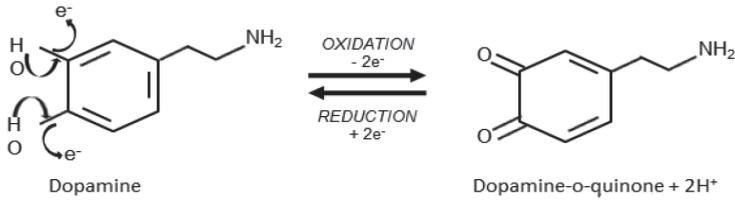


Figure 6

D



E

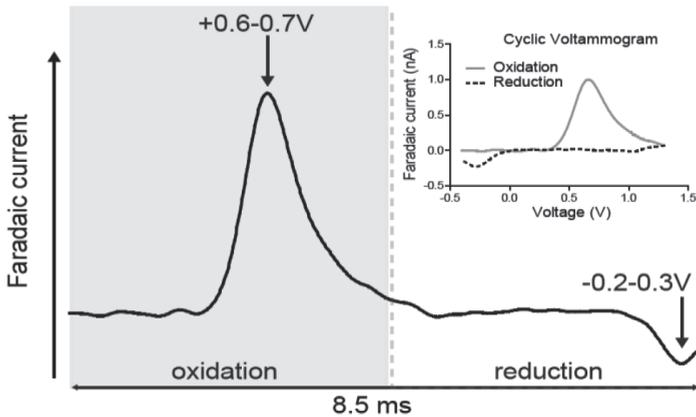


Figure 6. (A): Illustration of the chronically implantable carbon fiber microelectrode with a tip of 150 μm . (B): The different components (i.e. chronic microelectrodes, reference electrode, screws, wires) which are encased in dental cement to form a headcap with only one connector exposed on top. (C): The operant chamber set-up during FSCV. (D): During the application of the triangular waveform, dopamine transforms into dopamine-o-quinine via the liberation of two electrons (oxidation) and back to dopamine via the gain of two electrodes (reduction). (E): The flow of electrons generated through oxidation and reduction is measured as faradaic current at the carbon fiber microelectrode. The graph demonstrates the electrochemical signature of dopamine in which the peak dopamine oxidation usually occurs at +0.6-0.7V and the peak reduction at -0.2-0.3V. Inset: Cyclic voltammogram of dopamine, current vs voltage plot.

AIMS AND OUTLINE OF THE THESIS

In this chapter, I have introduced several concepts and aspects that are relevant for understanding the mechanisms that underlie AUD. It is important to note that AUD is a complex disorder, in which personality characteristics such as impulsivity, cue reactivity and sociability play an important role. The overarching aim of this thesis is to provide insight into how these different factors contribute to individual vulnerability of AUD.

In **Chapter 2**, we investigated individual variation in voluntary alcohol consumption in rats using continuous and intermittent alcohol access paradigms. Consequently, we related these individual differences to alcohol reinforcement and motivation and inflexible alcohol use, i.e. continued alcohol consumption despite an aversive bitter taste.

In **Chapter 3**, the development of compulsive characteristics of alcohol seeking was examined using a conditioned suppression model of alcohol seeking after limited and extended alcohol use, as well as in selected groups of low and high alcohol drinking rats.

In **Chapter 4**, the effects of acute and repeated alcohol administration were assessed in two modified versions of the rodent gambling task, that differ in the net gain and the punishment magnitude associated with the different response options. Using this approach, we were able to investigate whether alcohol influences the feedback regarding punishment or reward, or both. Moreover, the effect of subsequent alcohol challenges were tested in vehicle and alcohol pre-treated rats.

In **Chapter 5**, we investigated the relationship between individual variability in alcohol consumption with impulsivity, decision making and Pavlovian conditioned approach behaviour. Moreover, we assessed whether alterations in Pavlovian conditioned approach behaviour are the cause or consequence of alcohol consumption. In addition, the effects of acute alcohol exposure on decision making in the rodent gambling task and the delayed reward task were assessed in selected subgroups of low and high alcohol drinking rats.

In **Chapter 6**, the long-term consequences of a short period of social isolation during adolescence, essentially depriving rats from social play behaviour, on voluntary alcohol consumption and reinforcement in adulthood were assessed. In **Chapter 7**, we investigated whether adolescent alcohol use alters incentive learning processes in adulthood through a perturbation of the mesolimbic dopamine system. To this end, we investigated stimulus-evoked

phasic dopamine neurotransmission in the NAcc core using FSCV during the acquisition, maintenance, extinction, and reacquisition of a Pavlovian conditioned approach procedure in adult rats with a history of adolescent alcohol consumption.

In **Chapter 8**, the role of dopamine in ventral and dorsal striatal sub-regions in alcohol reinforcement was investigated under a fixed-ratio 1 and a progressive ratio schedule of reinforcement.

In **Chapter 9**, we investigated the effectiveness of selective dopamine D1 and D2 receptor agonists and antagonist in altering voluntary alcohol intake in selected low and high alcohol drinking rats.

In **Chapter 10**, the results presented in this thesis are discussed and the findings are related to translational and clinical implications.

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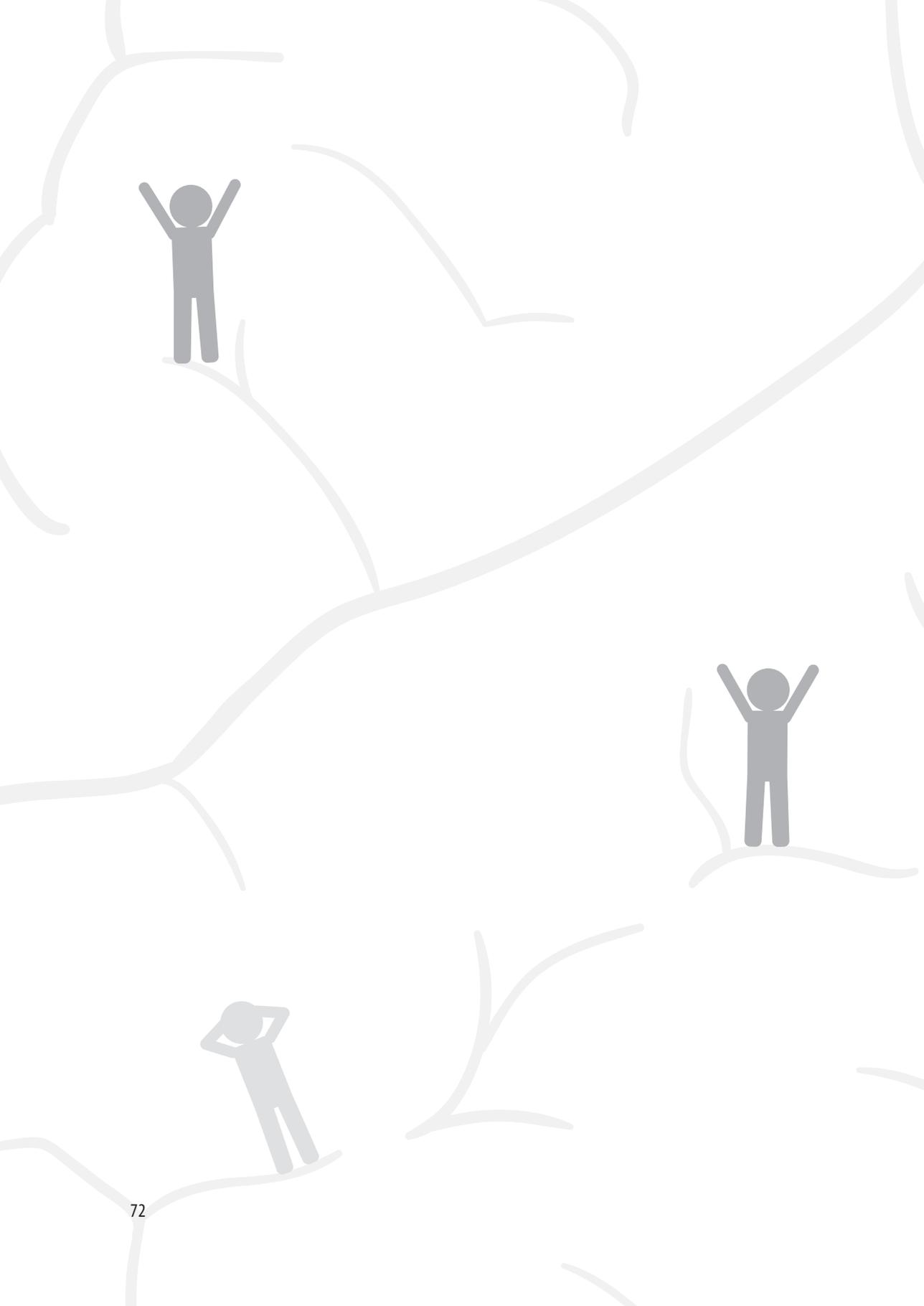
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CHAPTER 2

INDIVIDUAL VARIATION IN ALCOHOL INTAKE PREDICTS REINFORCEMENT, MOTIVATION, AND COMPULSIVE ALCOHOL USE IN RATS

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ABSTRACT

Alcohol is one of the most commonly used psychoactive substances. Prolonged alcohol use can result in alcohol use disorder (AUD), characterized by excessive and compulsive alcohol consumption. Importantly, however, the development of AUD only happens in a minority of individuals who consume alcohol. To understand the individual vulnerability for AUD, models that capture both the individual variability in alcohol consumption and the transition from casual to compulsive alcohol use are essential. Individual variability in voluntary alcohol intake and the preference for alcohol were assessed under continuous alcohol access (CAA) and intermittent-every-other-day alcohol access (IAA) schedules in the home cage using outbred Lister Hooded rats. Subsequently, the reinforcing properties of alcohol were tested in an operant setting. In subsequent experiments we performed a quinine adulteration experiment to assess inflexible alcohol consumption and blood alcohol levels (BAL) were assessed after voluntary alcohol consumption. We found marked individual differences in alcohol consumption and preference under both access schedules, whereby subgroups of high and low alcohol drinking rats (HD; LD) could be identified. HD with IAA increased their alcohol intake over days in the first month, whereas LD did not. Moreover, when alcohol access time was extended from 7h/day to 24h/day for rats with IAA, alcohol intake profoundly increased in HD with IAA, whereas LD with IAA maintained low levels of alcohol intake. Furthermore, HD earned more alcohol than LD under both fixed ratio and progressive ratio schedules of reinforcement. We further found that HD continued their intake of a quinine-adulterated alcohol solution to a larger extent than LD and HD showed higher BAL after thirty minutes of alcohol consumption. These profound individual differences in alcohol intake, reinforcement, motivation and AUD-like behaviour provide a promising tool to unravel the neurobehavioural underpinnings of individual vulnerability for AUD.

INTRODUCTION

With approximately two billion current users worldwide, alcohol is among the most widely used substances of abuse (Anderson 2006; WHO 2011). Prolonged alcohol use can result in alcohol use disorder (AUD), a chronic relapsing disorder that is characterized by excessive alcohol intake and a compulsive engagement in alcohol use (American Psychiatric Association 2013). Importantly, the development of AUD happens in a subpopulation of 3-5% of people who consume alcohol, affecting 76 million people worldwide (Anderson 2006; Rehm et al., 2009; WHO 2011; Effertz and Mann 2013). This individual variability in the development of AUD is considered to result from an interaction between prolonged alcohol use, genetic predisposition, and psychosocial, cognitive and environmental risk factors (Chassin et al., 2002; Anderson 2006; Goudriaan et al., 2011; Enoch 2013). Given its medical, societal and economic burden (Effertz and Mann, 2013) and the limited number of effective treatment strategies for AUD (van den Brink 2012; Pierce et al., 2012), it is critical to investigate the mechanisms that underlie individual vulnerability for AUD.

An increasing number of preclinical models have been developed to assess AUD-like behaviour in rodents (Wolffgramm and Heyne 1991; Simms et al., 2008; Crabbe et al., 2009; Lesscher et al., 2009). Rodents voluntarily consume more alcohol in paradigms with intermittent alcohol access (IAA) or repeated alcohol deprivations, compared to models with continuous alcohol access (CAA) (Wise 1973; Simms et al., 2008; Loi et al., 2010; Hwa et al., 2011; Cippitelli et al., 2012; Sabino et al., 2013). Moreover, IAA induces a transition from moderate to escalated alcohol intake, a critical feature of AUD. Another important hallmark of human AUD is the continued use of alcohol despite adverse consequences (American Psychiatric Association 2013); this has been captured in preclinical models of continued use in the face of adversity in which (conditioned) footshocks, bitter taste or lithium chloride-induced sickness serve as aversive stimuli (Turyabahika-Thyen and Wolffgramm 2006; Hopf et al., 2010; Chen et al., 2013; Vanderschuren and Ahmed 2013; Hopf and Lesscher 2014). For example, rats and mice with extended exposure to IAA develop resistance to quinine-modulation of alcohol intake, indicative of inflexible alcohol consumption (Wolffgramm and Heyne 1991; Hopf et al., 2010; Lesscher et al., 2010).

Individual differences in alcohol use have been documented in human and preclinical studies (Chassin et al., 2002; Goudriaan et al., 2007; Simms et al., 2008; Hwa et al., 2011; Hayton et al., 2012; Sabino et al., 2013) and several rodent lines have been bred for their differences in alcohol consumption (Sinclair et al., 1989; Colombo et al., 1995; Li and McBride, 1995; Le et al., 2001; Crabbe et al., 2009). For example, studies have examined whether individual differences in anxiety-related behaviours in outbred populations predict high alcohol consumption, or vice versa (Spanagel et al., 1995; Hayton et al., 2012; Bahi 2013; Sharko et al., 2013). However, individual differences in alcohol consumption have not been related to individual differences in alcohol reinforcement and AUD-like behaviour in outbred rodents. In the present study, we therefore assessed individual differences in alcohol intake in outbred rats under IAA and CAA conditions. Subsequently, we assessed whether individual variability in alcohol intake relates to operant alcohol self-administration, as well as the resistance to quinine modulation of alcohol intake. Knowledge about alcohol reinforcement and AUD-like behaviour in selected high versus low alcohol drinking rats will facilitate the investigation of the neurobehavioural mechanisms underlying the individual risk for AUD.

MATERIALS AND METHODS

Animals

Male Lister Hooded rats, obtained from Harlan (Horst, The Netherlands; Experiment 1) or Charles River (Sulzfeld, Germany; Experiment 2 and 3), weighing 220-250g (~7-9 weeks old) on arrival in our laboratory were used. Rats were housed individually under controlled temperature and humidity conditions and a reversed 12h light/dark cycle (lights off 7.00 AM) with *ad libitum* access to water and chow. Rats were acclimatized to the housing conditions for two weeks and were weighed and handled at least once per week. All experiments were approved by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op de dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Experiment 1

Intermittent alcohol access (IAA) versus continuous alcohol access (CAA)

Rats were given either continuous (n=20) or intermittent (n=20) access to alcohol and water in a two-bottle choice setup in the home cage. For CAA,

alcohol was presented for 24h/day, 7 days a week for 2 consecutive months. For IAA, alcohol was presented three days a week (Monday-Wednesday-Friday) for 7h/day between 9.00 AM and 16.00 PM (i.e., during the dark phase) in the first month; access was extended to 24h/day in the second month (Fig. 1). On alcohol drinking days, the rats were presented with two bottles, fitted with stainless-steel dual ball bearing drinking spouts, containing 20% alcohol (v/v) (Klinipath, The Netherlands) or water. Bottles were weighed before and after each session. In addition, the bottles of rats with CAA were weighed on Monday-Wednesday-Friday after 7h of access in the first month, to compare their intake with rats with IAA. Alcohol intake and preference were calculated per rat per session and averaged per week, i.e. 3 sessions per week for IAA and 7 sessions per week for CAA, or per month, i.e. 12 sessions for IAA and 28 sessions for CAA. Alcohol was freshly diluted with tap water once per week to a final concentration of 20% (v/v). Bottle positions were switched between sessions (IAA) or days (CAA) to avoid side bias. After two months, the rats were divided into low, medium, and high alcohol drinking rats. In order to select rats that consistently consumed low or high levels of alcohol throughout the experiment, rats were ranked from low to high based on the animals' average alcohol intake per week and were assigned ranking scores. These weekly ranking scores were summed over the two months of the experiment to calculate a total ranking score. This was performed separately for the IAA and CAA groups. Rats within the lower and upper 25% of the total ranking score were designated as low and high alcohol drinking rats (LD; HD), respectively. The median 50% of the population (medium drinking rats; MD) were used in other experiments (not presented here).

Alcohol self-administration under FR and PR schedules of reinforcement

HD and LD were subsequently trained and tested in operant conditioning chambers (29.5 cm L, 24 cm W 25 cm H; Med Associates, Georgia, VT, USA), situated in light- and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8 cm wide retractable levers, placed 11.7 cm apart and 6 cm from the grid floor. A cue light (28 V, 100mA) was present above each lever, a liquid dipper was in a magazine between the levers and a house light (28 V, 100mA) was located on the opposite wall. The position of the active and inactive levers was counterbalanced between rats. Pressing the active lever raised the dipper cup containing alcohol (0.1 ml, 20% v/v), illuminated the cue light above the active lever and switched off

the house light. Access to alcohol was terminated 10 sec after a head entry into the magazine, the cue light was turned off and after a 5 sec interval a new trial started. Pressing the inactive lever was recorded, but had no programmed consequences. The rats were tested 5 days/week. Alcohol consumption during operant behaviour was calculated by weighing the alcohol container underneath the dipper cup before and after each 60 min session; the alcohol solution was refreshed before each session of each rat. Experimental events and data recording were controlled using MED-PC for Windows.

Rats were habituated to the operant chamber for two 30-min sessions during which 15 alcohol rewards were freely available every other minute. Thereafter, the rats were trained under a fixed ratio 1 (FR1) schedule of reinforcement. As soon as the animals had acquired responding (i.e. at least 10 rewards in three subsequent sessions under the FR1 schedule), the response requirement was increased to FR2, FR5 and FR10 during which the animals had to earn at least 10 rewards for 2-3 sessions before progressing to the next FR or progressive ratio (PR) schedule. Based on the results of previous studies, a linear PR schedule of reinforcement was used, in which 2 (PR2, i.e. 2, 4, 6, 8, 10, etc.) and subsequently 4 (PR4; i.e. 4, 8, 12, 16, 20, etc.) additional lever presses were required for each subsequent reward (Ritz et al., 1994; Brown et al., 1998; Rodd et al., 2003). Responding under the PR schedules was deemed stable when there was <25% variation in reward deliveries over three subsequent sessions. The breakpoint under the PR schedules was defined as the maximum number of presses performed in the last, successfully completed ratio in either the 1h session or when no reward had been obtained in 20 min. Responding for alcohol was analyzed in 10 min bins to investigate the pattern of responding during the operant session.

Quinine avoidance and sucrose preference tests

To assess taste sensitivity of the HD and LD, the rats received two-bottle choice tests. For sucrose preference, the rats were offered one bottle containing tap water and one bottle with graded concentrations of sucrose (0 - 5% w/v) in tap water for 2h. For quinine avoidance, the rats were presented with one bottle containing tap water and one bottle with graded concentrations of quinine (0 – 1.0 g/L; Sigma-Aldrich, Germany) in tap water for 24h and measurements were taken after 2h and 24h. The bottles were weighed prior to and after each session that started at 9:00 AM. Each concentration was offered for two consecutive days and bottle positions were switched between sessions to

avoid side bias. Sucrose preference and quinine avoidance was calculated as the percentage of sucrose/quinine consumption of the total fluid intake.

Experiment 2

Quinine modulation of alcohol intake

The effects of quinine adulteration were assessed as previously described (Lesscher et al., 2010) in a second group of HD (n=16) and LD (n=16). Subgroups were selected as described above. These rats received IAA for two months, subsequently served as subjects in a decision making task where they received IAA for 2 h access/session, and were thereafter re-exposed to IAA with 24 h access/session for 8 weeks before the onset of the quinine modulation experiment (Fig.1). The alcohol solution was adulterated with increasing concentrations of quinine (0 – 1.0 g/L). Each concentration was tested once, bottle positions were switched between sessions to avoid side bias and bottles were weighed after 24h.

Experiment 3

Blood alcohol levels (BAL)

BALs following voluntary alcohol intake were determined in a third group of HD (n=12) and LD (n=12) (Fig.1). Subgroups were selected as described above, with the exception that these animals were exposed to graded alcohol concentrations in the IAA paradigm over 10 consecutive weeks: 2 weeks 5% v/v (7h/day), 2 weeks 10% v/v (7h/day) and 20% v/v (3 weeks 7h/day and 3 weeks 24h/day). Blood samples were collected from the lateral tail vein, immediately after 30 min access to alcohol (20% v/v) in the home cage, into EDTA coated capillary tubes (Sarstedt, Numbrecht, Germany) and immediately stored on ice. Blood samples were spun at 3000 rpm for 20 min (at 4°C) and plasma was stored at -20°C until blood alcohol analysis. BALs (mg/dl) were determined using an NAD-ADH reagent kit (Sigma-Aldrich, Schnellendorf, Germany) and a standard curve for quantitation (Lesscher et al., 2009).

Statistical analysis

Two rats failed to maintain responding for alcohol during operant training, blood collection was unsuccessful for four rats and three rats had one unreliable measurement of water/alcohol consumption during the quinine adulteration experiment; these rats were excluded from the concerning analyses. For analyses of operant behaviour, the alcohol intake, number of lever presses and breakpoints were averaged over the three sessions during

which the rat reached the response criteria as described for Experiment 1. Data were analyzed using one-, two and three-way repeated-measures ANOVA's with time, quinine and sucrose concentrations as within-subject variables and alcohol access condition (IAA vs CAA) and/or subgroup (HD vs LD) as between-subject variables. Each parameter was tested for normality with a Kolmogorov-Smirnov test. Mauchly's test of sphericity was used to test if variances of the differences between treatment levels were equal. If the assumption of sphericity was violated, degrees of freedom were corrected using Huynh-Feldt estimates of sphericity. When appropriate, *post hoc* analyses were conducted using Student's t-tests and paired t-tests. A non-parametric Mann-Whitney U test for group comparisons was used when a certain variable was not normally distributed. The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean \pm SEM. Statistical analyses were conducted using SPSS 20.0 for Windows.

RESULTS

Experiment 1

Home cage alcohol intake and preference - IAA versus CAA

Alcohol intake and preference changed over the course of the first 4 weeks, but differently for rats with IAA and CAA (intake: $F_{(3,105)\text{week} \times \text{access}} = 14.56$, $p < 0.001$; preference: $F_{(3,114)\text{week} \times \text{access}} = 7.17$, $p < 0.001$) (Fig. 2A-B). Rats with CAA consumed more alcohol compared to rats with IAA during the first two weeks, but both groups consumed similar levels of alcohol in weeks 3 and 4. Because rats with IAA had access to alcohol for 7 h/day, and rats with CAA for 24 h/day, we also measured alcohol intake and preference for the rats with CAA over the first 7h of each session on Monday-Wednesday-Friday, in parallel to the IAA group. During these 7 hours, rats with IAA consumed a similar amount of alcohol in weeks 1-2 but consumed more alcohol than rats with CAA in weeks 3-4 ($F_{(3,114)\text{week} \times \text{access}} = 6.42$, $p < 0.001$) (Fig. 2A-B). Moreover, rats with IAA showed a higher preference for alcohol in weeks 2-4 ($F_{(3,114)\text{week} \times \text{access}} = 6.67$, $p < 0.001$) compared to rats with CAA. Rats with IAA consumed more alcohol ($F_{(1,38)\text{access}} = 8.17$, $p = 0.007$) and showed a greater preference for alcohol ($F_{(1,38)\text{access}} = 7.52$, $p = 0.009$) during the second month, when both groups had access to alcohol for 24h/day (Fig. 2A-B).

Figure 1

Procedural Timeline of the Experiments

Experiment 1

Home-cage consumption		FR1, FR2, FR5, FR10 PR2, PR4	Sucrose and quinine sensitivity in water
IAA: 7h/day 3 days /wk n=20	IAA: 24h/day 3 days /wk n=20	IAA: HD n=5 LD n=5	IAA: HD n=5 LD n=5
CAA: 24h/day 7 days /wk n=20	CAA: 24h/day 7 days /wk n=20	CAA: HD n=5 LD n=5	CAA: HD n=5 LD n=5
4 weeks	4 weeks	8 weeks	3 weeks

Experiment 2

Home-cage consumption		rGT (not in this manuscript) & Home-cage consumption	Home-cage consumption	Quinine modulation
IAA: 7h/day 3 days /wk n=64	IAA: 24h/day 3 days /wk n=64	IAA: 2h/day 3 days /wk HD n=16 LD n=16	IAA: 24h/day 3 days /wk HD n=16 LD n=16	HD n=16 LD n=16
4 weeks	4 weeks	11 weeks	8 weeks	3 weeks

Experiment 3

Home-cage consumption				BAL measurements
IAA: 7h/day 3 days /wk	IAA: 7h/day 3 days /wk	IAA: 7h/day 3 days /wk	IAA: 24h/day 3 days /wk	HD n=12 LD n=12
5% alcohol n=48	10% alcohol n=48	20% alcohol n=48	20% alcohol n=48	
2 weeks	2 weeks	3 weeks	3 weeks	1 day

Figure 1. Subgroups of HD and LD (25% of upper and lower part of distribution) were selected based on alcohol intake in the home-cage during 8 weeks (Experiment 1 and 2) or 10 weeks (Experiment 3).

Individual differences in home cage alcohol consumption

We observed marked individual differences in alcohol intake and preference between the animals, which were most pronounced in rats subjected to IAA. The alcohol intake of rats with IAA in Exp. 1. ranged from 0.64-2.32 g/kg/7h (mean±SEM: 1.39±0.10) and 0.50-4.84 g/kg/24h (mean±SEM: 1.93±0.27), whereas the alcohol intake of rats with CAA ranged from 1.33-2.23 g/kg/24h (mean±SEM: 1.65±0.06) and 0.52-1.99 g/kg/24h (mean±SEM: 1.09±0.12) in the first and second month, respectively. Analyses of the alcohol intake and preference of the HD, MD and LD confirmed differences between the selected subgroups in rats with IAA ($F_{(2,17)group} = 30.60, p < 0.001$; $F_{(2,17)group} = 20.69, p < 0.001$,

respectively) and CAA ($F_{(2,17)group} = 24.50, p < 0.001$; $F_{(2,17)group} = 25.40, p < 0.001$, respectively) (Fig. 2C-F). When comparing the alcohol intake of the first month to the second month, the subgroups with IAA responded differently to the increase in alcohol access duration (7h/day to 24h/day) ($F_{(2,17)month \times group} = 12.70, p < 0.001$); HD with IAA increased their intake when access time was extended while the LD and MD subgroups did not (Fig. 2C). Alcohol preference was not changed in HD with IAA while MD and LD showed a reduction in alcohol preference upon the increment in session duration ($F_{(2,17)month \times group} = 8.14, p = 0.003$) (Fig. 2D). Rats with CAA showed a trend for differential alcohol consumption between subgroups over time ($F_{(2,17)month \times group} = 3.14, p = 0.069$) and overall alcohol intake declined in the second month ($F_{(1,17)month} = 48.62, p < 0.001$) (Fig. 2E). The alcohol preference of rats with CAA declined over time in LD but not in MD and HD ($F_{(2,17)month \times group} = 4.61, p = 0.025$) (Fig. 2F). There were no differences in total fluid intake between the subgroups with IAA ($F_{(2,17)group} = 0.33, p = 0.726$), but total fluid intake was different between CAA subgroups ($F_{(2,17)group} = 4.54, p = 0.026$); HD consumed less fluid than MD ($p < 0.05$) (data not shown).

Alcohol self-administration under FR and PR schedules of reinforcement

After two months of home cage alcohol consumption under IAA or CAA conditions, HD and LD were trained to self-administer alcohol. LD required more FR training sessions (15 ± 1.0) to fulfill the response requirements to proceed to the PR schedules than HD (11 ± 0.5) ($F_{(1,14)group} = 6.70, p = 0.021$), independent of access condition (IAA or CAA, $F_{(1,14)access} = 1.14, p = 0.304$) (data not shown). Responding under the FR1 ($F_{(1,14)access} = 0.14, p = 0.712$; $F_{(1,14)access \times group} = 2.07, p = 0.172$), PR2 ($F_{(1,14)access} = 0.35, p = 0.567$; $F_{(1,14)access \times group} = 0.42, p = 0.530$) and PR4 schedules ($F_{(1,14)access} = 0.04, p = 0.849$; $F_{(1,14)access \times group} = 0.21, p = 0.654$), as well as breakpoint under the PR2 and PR4 schedules (PR2: $F_{(1,14)access} = 0.35, p = 0.566$; PR4: $F_{(1,14)access} = 0.08, p = 0.783$) and the alcohol consumed during the operant sessions (FR1: $F_{(1,14)access} = 0.33, p = 0.575$; PR2: $F_{(1,14)access} = 0.15, p = 0.704$; PR4: $F_{(1,14)access} = 0.36, p = 0.559$) did not differ between CAA and IAA rats (data not shown), data from these groups were therefore collapsed. Under an FR1 schedule of reinforcement, HD made more active lever presses than LD ($F_{(1,16)group} = 6.54, p = 0.021$). Responding declined in a similar manner for both HD and LD during the session ($F_{(3,40)group \times time} = 0.74, p = 0.515$) (Fig. 3A). Likewise, under a PR2 schedule of reinforcement, HD showed higher response levels than LD ($F_{(1,16)group} = 7.44, p = 0.015$), and lever pressing

Figure 2

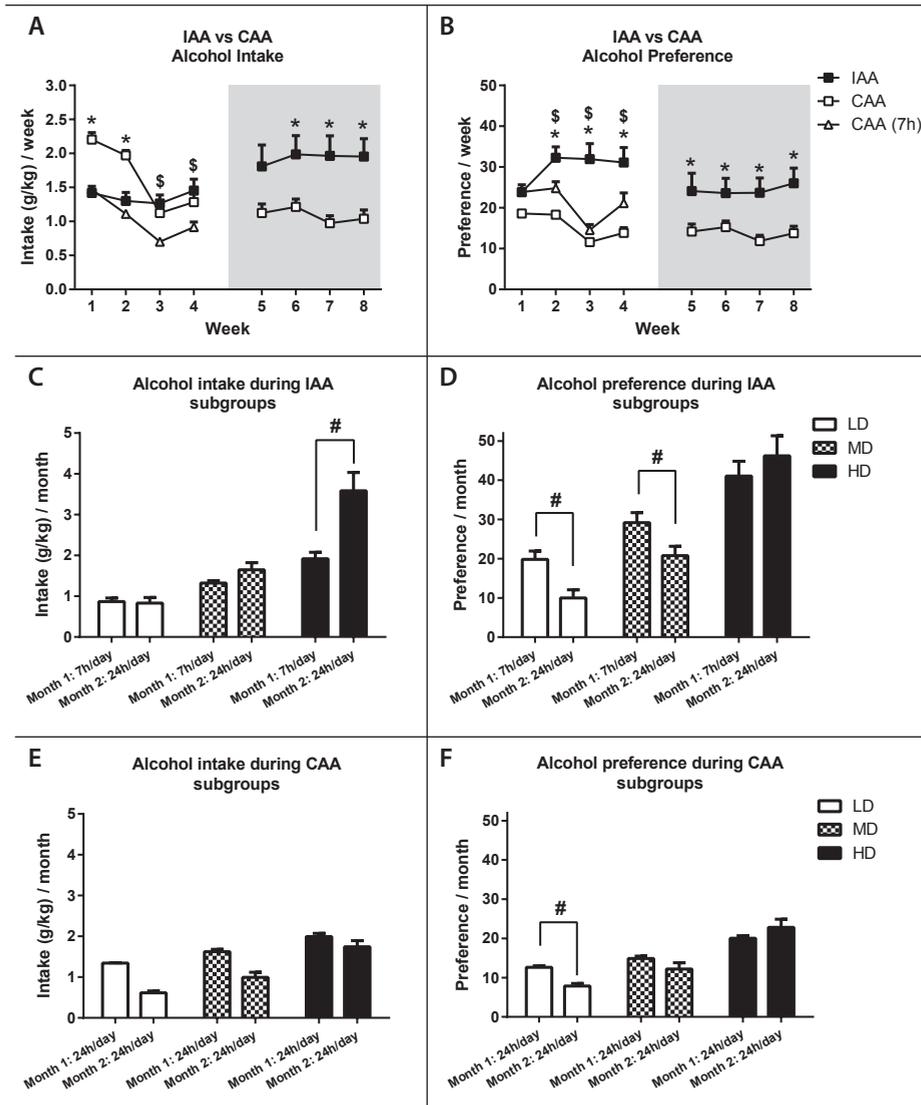


Figure 2. Alcohol intake and preference during IAA versus CAA in the home-cage. (A-B) Alcohol intake (A) and preference (B) differed between rats with IAA and CAA, in both the first month (white area) and second month (grey area). (C-D) HD with IAA increased their alcohol intake when access to alcohol was extended (C) and retained similar alcohol preference over two months (D). (E-F) All subgroups with CAA reduced their alcohol intake from the first to the second month (E) but HD and MD retained a similar alcohol preference over both months (F). Data are shown as mean + SEM average alcohol intake and preference per week (A-B) or month (C-F). * Significant differences between IAA and CAA (24h measurement); \$ Significant differences between IAA and CAA (7h measurement) (*post hoc* Student's *t*-tests, $p < 0.05$). # Significant differences within the subgroup (*post hoc* paired *t*-tests, $p < 0.05$).

Figure 3

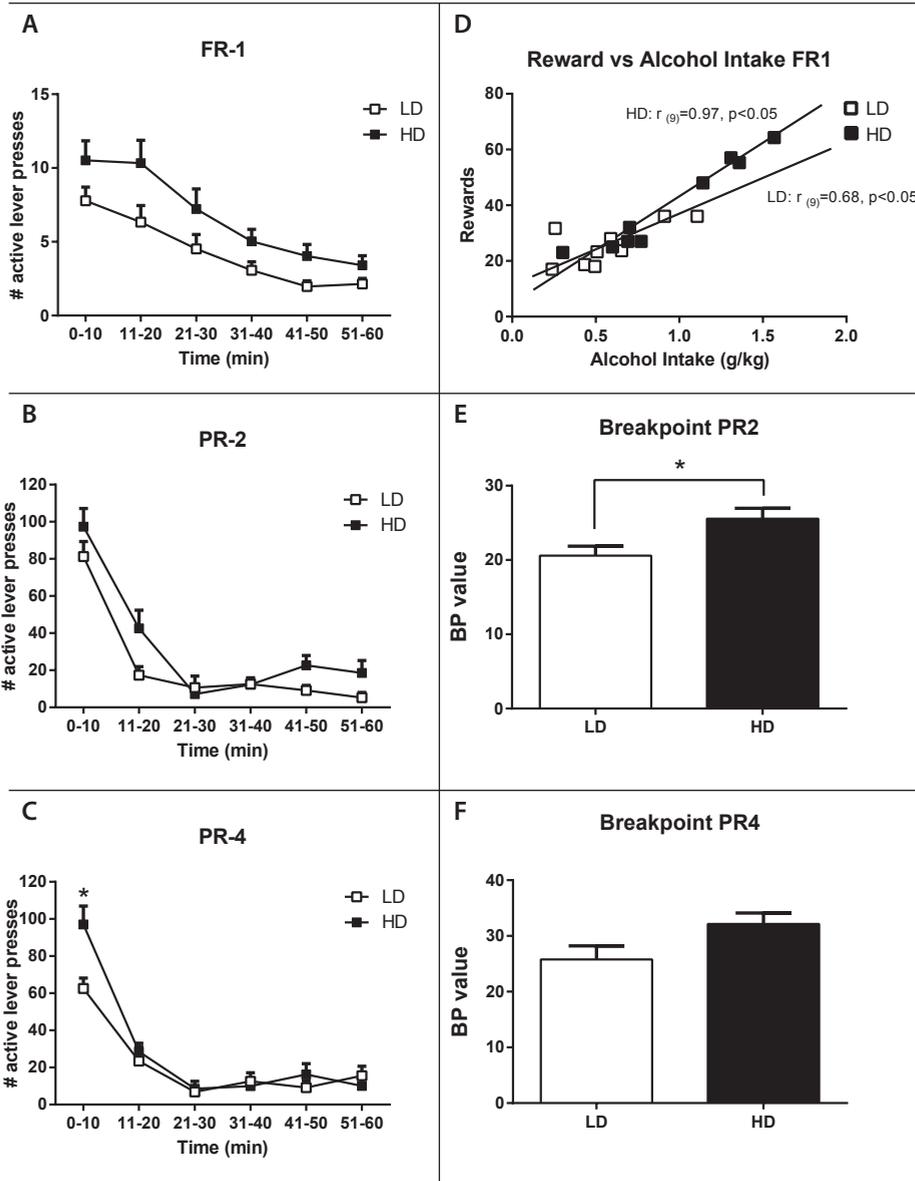


Figure 3

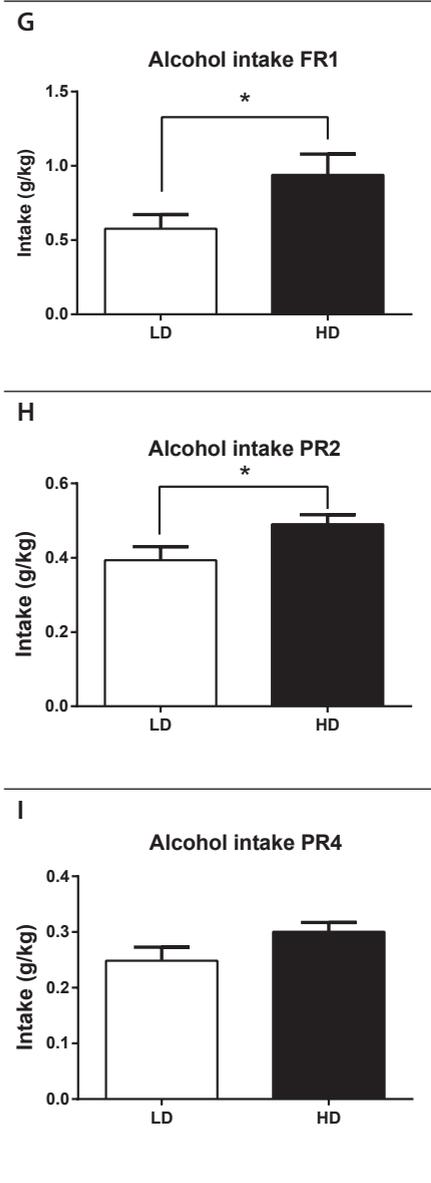


Figure 3. Operant responding for alcohol by LD and HD under FR1 and PR schedules of reinforcement. (A-C) Number of active lever presses under the FR1 and PR schedules (D) The number of earned rewards correlated with alcohol intake (g/kg) during the FR1 sessions. (E-F) HD showed a higher breakpoint during the PR2 schedule, and a trend towards a higher breakpoint under the PR4 schedule. (G-I) HD consumed more alcohol than LD under the FR1 and PR2 schedules, but not under the PR4 schedule. Data are shown as mean + SEM. * Significant between-subgroup differences (*post hoc* Student's t-tests, * $p < 0.05$).

Table 1

Sucrose preference and quinine avoidance (%) in HD and LD. Volumes were measured 2 or 24h after presentation of the bottles.

Group		Substance:	Sucrose			
		Concentration (%)	0	0.1	1.0	5.0
		w/v):				
HD	2h		50.1 ± 5.1	49.9 ± 3.3	81.3 ± 2.7*	95.1 ± 0.8*
LD	2h		46.2 ± 4.1	55.5 ± 3.0	78.0 ± 4.2*	95.1 ± 0.9*
		Substance:	Quinine			
		Concentration	0	0.1	0.3	1.0
		(g/L):				
HD	2h		52.7 ± 4.2	37.0 ± 3.4*	30.6 ± 2.8*	31.7 ± 4.7*
LD	2h		52.9 ± 2.6	40.2 ± 1.7*	32.2 ± 2.7*	34.2 ± 2.5*
HD	24h		54.2 ± 3.3	19.0 ± 1.1*	16.3 ± 1.0*	15.6 ± 0.8*
LD	24h		52.9 ± 3.8	18.4 ± 1.0*	14.1 ± 0.8*	14.9 ± 0.7*

Values represent mean ± SEM. * Significantly different from 0 % w/v or 0 g/L (paired t-test), $p < 0.05$. Previous IAA or CAA in the home-cage did not interact with the subgroups; therefore the results of the subgroups with previous IAA or CAA were pooled.

declined in a similar manner for both groups ($F_{(4,69)\text{group} \times \text{time}} = 1.65$, $p = 0.167$) (Fig. 3B). Analysis of the PR4 data revealed an interaction between group and session time ($F_{(5,80)\text{group} \times \text{time}} = 4.32$, $p = 0.002$) but no main effect of HD versus LD ($F_{(1,16)\text{group}} = 2.32$, $p = 0.148$). *Post hoc* analyses showed that HD made more lever presses compared to LD in the first 10 min of the task ($p < 0.05$) (Fig. 3C). HD reached higher breakpoints than LD under the PR2 schedule of reinforcement ($F_{(1,16)\text{group}} = 6.85$, $p = 0.019$), and there was a trend towards a higher breakpoint in HD under the PR4 schedule ($F_{(1,16)\text{group}} = 4.08$, $p = 0.060$) (Fig. 3E-F). The number of rewards obtained correlated with the amount of alcohol consumed for the FR1 schedule of reinforcement (Fig. 3D), but not for PR2 and PR4 schedules (data not shown). Analysis of alcohol intake during operant self-administration showed higher alcohol intakes in HD vs LD under the FR1 and PR2 schedule (FR1: $F_{(1,16)\text{access}} = 4.56$, $p = 0.049$; PR2: $F_{(1,16)\text{access}} = 4.74$, $p = 0.045$) but not for the PR4 schedule ($F_{(1,16)\text{access}} = 3.15$, $p = 0.095$) (Fig. 3G-I). Active lever press and reward collection latencies did not differ between groups on any of the reinforcement schedules (Supplementary Table 1).

Figure 4

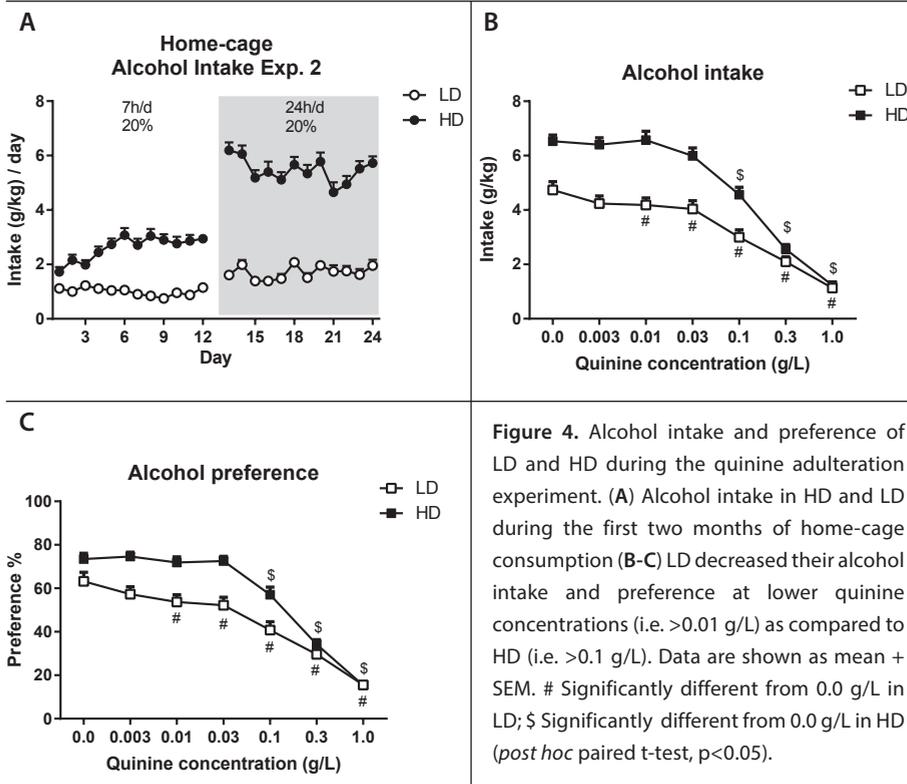


Figure 4. Alcohol intake and preference of LD and HD during the quinine adulteration experiment. (A) Alcohol intake in HD and LD during the first two months of home-cage consumption (B-C) LD decreased their alcohol intake and preference at lower quinine concentrations (i.e. >0.01 g/L) as compared to HD (i.e. >0.1 g/L). Data are shown as mean + SEM. # Significantly different from 0.0 g/L in LD; \$ Significantly different from 0.0 g/L in HD (*post hoc* paired t-test, $p < 0.05$).

Sucrose preference and quinine avoidance

To rule out the possibility that the differential alcohol intake and motivation in HD and LD is a result of altered taste sensitivity, the animals were subsequently tested for sucrose preference and quinine avoidance in a two-bottle choice test. Sucrose preference did not differ between HD and LD ($F_{(6,95)\text{concentration} \times \text{group}} = 0.42, p = 0.856$) nor between rats with previous IAA and CAA exposure ($F_{(3,95)\text{concentration} \times \text{access}} = 0.44, p = 0.713$) (Table 1). Quinine aversion was not different for HD and LD (after 2h: $F_{(6,102)\text{concentration} \times \text{group}} = 0.46, p = 0.835$; after 24h: $F_{(3,59)\text{concentration} \times \text{group}} = 1.25, p = 0.300$) (Table 1). Quinine aversion was comparable for rats with previous IAA or CAA exposure after 2h exposure ($F_{(3,102)\text{concentration} \times \text{group}} = 1.35, p = 0.262$), although there was a significant interaction with the subgroups after 24h exposure ($F_{(2,59)\text{concentration} \times \text{group}} = 3.41, p = 0.049$). However, *post hoc* tests only revealed a significant difference between access groups for the 0 g/L quinine concentration ($p < 0.05$).

Experiment 2

Quinine modulation of alcohol intake

To determine whether HD show inflexible alcohol consumption, i.e. continued intake of an aversive, quinine-containing alcohol solution, we performed a quinine adulteration experiment in a separate group of rats with a history of IAA. Analysis of the alcohol intake of LD and HD in the first two months with IAA indicated that HD increased their alcohol over days in the first month with 7h alcohol access/day, while LD did not ($F_{(7,207)\text{day} \times \text{group}} = 8.73, p < 0.001$) (Fig. 4A). Consistent with the first experiment, HD increased their alcohol intake to a larger extent than LD when comparing alcohol intake between the first and second month ($F_{(1,30)\text{month} \times \text{group}} = 95.13, p < 0.001$). Subgroup differences in alcohol intake persisted during the subsequent 2h IAA sessions ($F_{(1,30)\text{group}} = 70.11, p < 0.001$), as well as during 24h IAA re-exposure ($F_{(1,30)\text{group}} = 46.59, p < 0.001$), prior to the start of the adulteration experiment (data not shown).

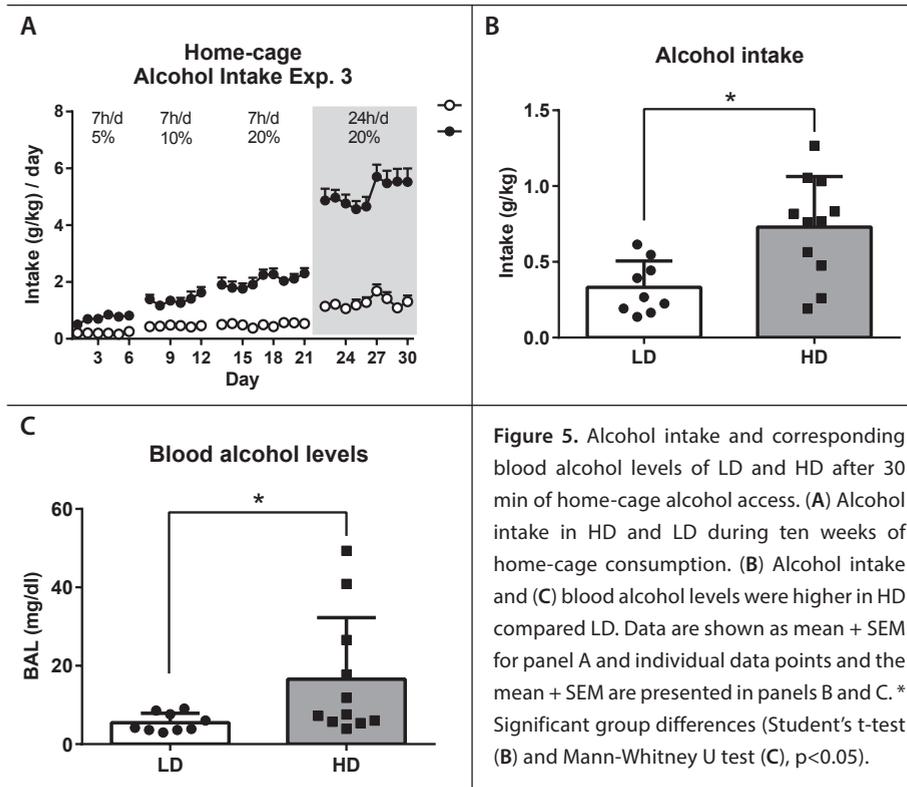
Analysis of the quinine adulteration data showed a significant interaction between quinine concentration and subgroup for both alcohol intake ($F_{(6,155)\text{concentration} \times \text{group}} = 11.31, p < 0.001$), and alcohol preference ($F_{(6,162)\text{concentration} \times \text{group}} = 4.84, p < 0.001$), indicative of a differential sensitivity to quinine adulteration in HD and LD (Fig. 4B-C). LD decreased their alcohol intake and preference at quinine concentrations of 0.01 g/L and higher ($p < 0.03$), whereas HD only decreased their alcohol intake and preference at tenfold higher quinine concentrations (i.e. 0.1 g/L and higher, $p < 0.002$). During the experiment, HD retained higher levels of alcohol intake and alcohol preference compared to LD (intake: $F_{(1,27)\text{group}} = 30.99, p < 0.001$, preference: $F_{(1,27)\text{group}} = 23.32, p < 0.001$) at all, except for the two highest, quinine concentrations.

Experiment 3

BAL in HD and LD

BAL after alcohol consumption were assessed in a third group of LD and HD. HD increased their alcohol intake to a larger extent compared to LD upon the increment in alcohol concentration during the 7h sessions ($F_{(10,217)\text{day} \times \text{group}} = 6.25, p < 0.001$) (Fig. 5A). Similar to the previous experiments, the alcohol intake of the 20% alcohol concentration increased to a larger extent in HD as compared to LD as session duration increased from 7h to 24h/day ($F_{(7,151)\text{day} \times \text{group}} = 16.41, p < 0.001$) (Fig. 5A). HD showed a higher alcohol intake and preference than LD during the 30 minutes of alcohol access before blood sampling ($t_{(18)\text{intake}} = 3.23, p = 0.005$; $t_{(18)\text{preference}} = 3.10, p = 0.006$) (Fig 5B), which also resulted in higher BALs in

Figure 5



HD compared to LD ($U = 23$, $p = 0.046$) (Fig. 5C). Moreover, BALs correlated with alcohol intake ($r_{(18)} = 0.60$, $p = 0.005$).

DISCUSSION

In this study, we observed marked individual differences in voluntary alcohol intake and preference in outbred Lister Hooded rats. The subgroup of the HD with IAA escalated their alcohol intake upon extension of the alcohol access duration. Moreover, HD showed greater alcohol reinforcement and motivation to obtain alcohol and they continued to consume alcohol despite an aversive taste to a greater extent than LD. These findings show that high alcohol drinking rats develop compulsive characteristics of alcohol use, a hallmark of AUD in humans.

Chronic versus intermittent alcohol exposure in rodents

The IAA paradigm produces higher levels of alcohol intake in rodents in comparison to CAA (Wise 1973; Simms et al., 2008; Loi et al., 2010; Hwa et al., 2011; Cippitelli et al., 2012; Sabino et al., 2013). Consistent with these studies, we found greater alcohol intake in rats with IAA compared to CAA. Rats with CAA reduce their alcohol intake after two weeks of alcohol access, which has been observed previously (Cippitelli et al., 2012), but not consistently so (Wise 1973; Colombo et al., 1995; Loi et al., 2010; Sabino et al., 2013). It is assumed that intermittent exposure to alcohol increases the rewarding properties of alcohol, which may facilitate the development of AUD (Brown et al., 1998; Rodd et al., 2003; O'Dell et al., 2004). We observed no differences between rats with previous home cage IAA or CAA in operant responding for alcohol, which may be explained by the fact that during operant self-administration for = 5 days/week, rats were exposed to similar amounts of alcohol, thereby reducing potential group differences over sessions. Moreover, cumulatively, the rats with CAA have consumed more alcohol during home cage alcohol access compared to rats with IAA, which may explain why the motivation to obtain alcohol was not different for rats with IAA and CAA.

Individual differences in alcohol intake and reinforcement

In this study, we consistently observed a high degree of individual variability in alcohol intake in outbred Lister Hooded rats, which was more pronounced under IAA than under CAA conditions. These individual differences in alcohol intake were highly consistent across three batches of animals from two different vendors (Supplementary Table 2). To assess individual differences within a population of Lister Hooded rats, rats were classified in subgroups of LD and HD based on their alcohol consumption in the home cage. A potential limitation of this approach is that by excluding the MD, the data may not be subjected to linear regression analyses. Nevertheless, the differences in alcohol reinforcement, motivation and loss of control over alcohol use between the selected subgroups provide valuable information about individual differences in the risk for AUD.

During the first month with 7h IAA sessions, the HD gradually increased their alcohol intake over time, whereas the LD did not. Moreover, subsequent increases in alcohol access duration from 7h/day to 24h/day in the second month, led to a larger increase in alcohol intake in HD compared to LD. Thereafter, in agreement with other IAA studies, alcohol intake stabilized, which suggests that animals titrate their alcohol consumption to a preferred level of intoxication (Simms et

al., 2008; Loi et al., 2010; Cippitelli et al., 2012; Sabino et al., 2013). Individual differences in alcohol intake in outbred rodent populations have been related to certain behavioural factors (e.g. anxiety and decision making) (Spanagel et al., 1995; Hayton et al., 2012; Bahi 2013; Sharko et al., 2013; McMurray et al., 2014), but have not directly been related to alcohol reinforcement and AUD-like behaviours. Interestingly, the current data show that rats which have been selected on their high alcohol intake in the home cage (HD) obtained more rewards during FR1 and PR schedules of reinforcement than LD. Furthermore, the HD reached the response criteria to continue to PR schedules faster than the LD, illustrating their increased sensitivity to the reinforcing effects of alcohol. HD made more active lever presses during the entire FR1 and PR2 session, while under the PR4 schedule of reinforcement, the HD performed more active responses in the first 10 minutes of the session. These data suggest that the animals adjust the response requirement to the alcohol reward, which influences their responding for alcohol during the session, indicating that the animals primarily lever press in the beginning of the session where the response requirement is lower compared to the later stages of the PR session. The positive relationship between alcohol consumption in the home cage and alcohol reinforcement in an operant setting has been previously reported in animals selectively bred for high or low alcohol consumption (Ritz et al., 1994; Files et al., 1997; Samson et al., 1998; Vacca et al., 2002). However, it has also been shown that there is not a complete overlap between the genes that contribute to differences in home cage consumption and alcohol reinforcement (Ritz et al., 1994; Samson et al., 1998). In sum, the individual differences in alcohol intake observed in this study are related to the reinforcing properties of alcohol and may mimic the diversity in the propensity for alcohol consumption in humans, supporting the validity of our approach as a rodent model for AUD (Hill et al., 2000; Chassin et al., 2002; Tucker et al., 2003; Goudriaan et al., 2007).

Importantly, we observed higher blood alcohol levels (BAL) after 30 minutes of alcohol consumption in HD as compared to LD, which corresponded with the alcohol intake. The BALs after 30 minutes of voluntary alcohol consumption were comparable to the average blood alcohol levels described by other studies using similar IAA procedures (Simms et al., 2008; Loi et al., 2010; Cippitelli et al., 2012; Sabino et al., 2013), where most rats show BAL between 20 and 40 mg/dl, with a few animals reaching blood alcohol levels up to 80mg/dl.

Aversion-resistant alcohol intake

In general, loss of control over substance use emerges upon extended and excessive substance use (American Psychiatric Association 2013) and this loss of control over substance use has been modelled in rodents (Wolffgramm and Heyne 1991; Ahmed and Koob 1998; Deroche-Gamonet et al., 2004; Vanderschuren and Everitt 2004; Turyabahika-Thyen and Wolffgramm 2006; Pelloux et al., 2007). For example, the continued use of alcohol in conflict situations, by adulterating the alcohol solution with quinine or by concurrently providing an attractive alternative, is considered to reflect the compulsive motivation for alcohol that is observed in humans with AUD (Vengeliene et al., 2009; Hopf and Lesscher 2014). In the present study, we observed that HD exhibited a greater aversion-resistance in alcohol intake compared to LD after a total of 6-7 months of IAA exposure, indicative of less flexible alcohol consumption in HD. This is in line with previous studies that reported quinine-resistant motivation for alcohol after at least 3-4 months of alcohol consumption (Wolffgramm and Heyne 1991; Hopf et al., 2010). The current findings are in agreement with those of Turyabahika-Thyen and Wolffgramm (2006), who reported that individual rats which displayed continued intake of bitter-tasting alcohol solutions, had, in retrospect, previously consumed more alcohol compared to rats that showed flexible, quinine-sensitive, alcohol intake. Together, these findings reveal individual differences in rats in susceptibility to inflexible alcohol consumption, a hallmark of AUD.

The HD and LD did not differ in taste sensitivity for quinine or sucrose, which is in agreement with previous comparisons between selected high versus low or alcohol experienced versus non-experienced rats (O'Dell et al., 2004; Turyabahika-Thyen and Wolffgramm 2006; Hopf et al., 2010; Loi et al., 2010). Importantly, this makes it less likely that differences in taste sensitivity between the subgroups explain the differences in alcohol intake, alcohol reinforcement and flexibility of alcohol intake between HD and LD.

Further investigation is required to discern whether the enhanced motivation to obtain alcohol and the development of aversion-resistant alcohol intake in HD is the consequence of the amount of alcohol the HD consumed, their innate susceptibility for AUD-like behaviour or an interaction between these factors. Previous studies have, for example, shown that the development of addiction-like behaviour in a subgroup of animals, after extended cocaine self-administration, was not related to the amount of cocaine the animals had self-administered

(Deroche-Gamonet et al., 2004; Pelloux et al., 2007; Chen et al., 2013). The HD in our study, however, increased their alcohol intake and, correspondingly, show a higher motivation to respond for alcohol and a greater aversion-resistance in alcohol intake, which suggests that there is a predisposition to develop AUD-like behaviour in this subgroup of animals.

Concluding remarks

Our results indicate that a subgroup of Lister-hooded outbred rats escalate their alcohol consumption during home cage IAA. These individual variations concur with differences in alcohol reinforcement, motivation and AUD-like behaviour. The behavioural characteristics of these high alcohol drinking rats – escalated and compulsive alcohol use – captures key aspects of AUD. Therefore, the current model provides a framework for more in-depth analyses of the neurobehavioural mechanisms underlying individual vulnerability to AUD, which may facilitate the development of novel behavioural and pharmacological interventions for this devastating condition.

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SUPPLEMENTARY MATERIALS

Table S1

Average reward collection and active lever press latencies during the 1h self-administration session.

		IAA vs CAA	HD vs LD		Mean (sec) ± SEM
Reward collection latency (sec)	FR 1	$F_{(1,14) \text{ access}} = 0.03, p=0.877$	$F_{(1,14) \text{ subgroup}} = 2.44, p=0.140$	HD	14.14 ± 9.49
		$F_{(1,14) \text{ access} \times \text{subgroup}} = 1.38, p=0.260$		LD	31.15 ± 16.74
	PR 2	$F_{(1,14) \text{ access}} = 0.45, p=0.515$	$F_{(1,14) \text{ subgroup}} = 0.60, p=0.451$	HD	0.69 ± 0.07
		$F_{(1,14) \text{ access} \times \text{subgroup}} = 0.21, p=0.654$		LD	0.61 ± 0.05
	PR 4	$F_{(1,14) \text{ access}} = 0.91, p=0.355$	$F_{(1,14) \text{ subgroup}} = 0.79, p=0.388$	HD	0.63 ± 0.04
		$F_{(1,14) \text{ access} \times \text{subgroup}} = 0.45, p=0.515$		LD	0.58 ± 0.05
Active lever press latency (sec)	FR 1	$F_{(1,14) \text{ access}} = 0.02, p=0.885$	$F_{(1,14) \text{ subgroup}} = 3.52, p=0.082$	HD	58.81 ± 11.46
		$F_{(1,14) \text{ access} \times \text{subgroup}} = 0.01, p=0.910$		LD	80.49 ± 7.91
	PR 2	$F_{(1,14) \text{ access}} = 0.07, p=0.800$	$F_{(1,14) \text{ subgroup}} = 0.27, p=0.614$	HD	112.70 ± 16.44
		$F_{(1,14) \text{ access} \times \text{subgroup}} = 0.01, p=0.928$		LD	117.29 ± 35.42
	PR 4	$F_{(1,14) \text{ access}} = 1.08, p=0.315$	$F_{(1,14) \text{ subgroup}} = 0.62, p=0.455$	HD	125.21 ± 20.72
		$F_{(1,14) \text{ access} \times \text{subgroup}} = 0.23, p=0.641$		LD	167.76 ± 31.30

Latencies were LOG transformed prior to statistical analysis (one-way ANOVA's).

Table S2

Alcohol intake and preference of the used cohorts.

Experiment	Vendor		Intake (g/kg)		Alcohol preference	
			7h sessions	24h sessions	7h sessions	24h sessions
1 (n=20)	Harlan	Mean ± SEM	1.39 ± 0.10	1.93 ± 0.27	29.81 ± 2.35	24.38 ± 3.52
		Min	0.64	0.50	12.14	5.94
		Max	2.32	4.84	52.40	59.00
2 (n=64)	Charles River	Mean ± SEM	1.68 ± 0.09	3.62 ± 0.19	29.61 ± 1.58	38.97 ± 2.03
		Min	0.64	0.88	10.22	9.70
		Max	4.16	7.91	65.23	78.93
3 (n=48)	Charles River	Mean ± SEM	1.32 ± 0.10	3.14 ± 0.25	39.58 ± 2.64	39.06 ± 2.94
		Min	0.30	0.59	8.95	5.87
		Max	2.49	6.55	74.03	75.63

Univariate analyses of the average alcohol intake and preference per month with vendor and subgroup as factors indicated that the alcohol intake and preference was not different between vendors during the during 7h sessions (intake: $F_{(1,125) \text{ vendor}} = 1.89$, $p=0.171$; preference: $F_{(1,125) \text{ vendor}} = 2.64$, $p=0.107$). During the 24h sessions, however, rats of Charles River showed a higher alcohol intake and preference compared to the rats from Harlan (intake: $F_{(1,124) \text{ vendor}} = 41.57$, $p<0.001$; preference: $F_{(1,124) \text{ vendor}} = 28.55$, $p<0.001$). More importantly, the vendor location did not interact with the selected subgroups, neither during the 7h sessions (intake: $F_{(2,125) \text{ vendor} \times \text{subgroup}} = 1.69$, $p=0.188$; preference: $F_{(2,125) \text{ vendor} \times \text{subgroup}} = 1.82$, $p=0.167$), nor the 24h sessions (intake: $F_{(2,124) \text{ vendor} \times \text{subgroup}} = 2.55$, $p=0.082$; preference: $F_{(2,124) \text{ vendor} \times \text{subgroup}} = 2.05$, $p=0.134$).

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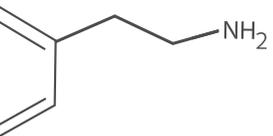
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CHAPTER 3

LOSS OF CONTROL OVER ALCOHOL SEEKING IN RATS DEPENDS ON INDIVIDUAL VULNERABILITY AND DURATION OF ALCOHOL CONSUMPTION

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Submitted



ABSTRACT

Alcohol use disorder (AUD) is a chronic, relapsing brain disorder, characterized by excessive alcohol use and persistent alcohol seeking despite explicit knowledge of its negative consequences. Importantly, AUD develops after chronic excessive alcohol use in a subgroup of individuals who drink alcohol, suggesting that AUD results from an interaction between individual vulnerability and prolonged exposure to alcohol. The present study investigated conditioned suppression of alcohol seeking to assess the contribution of prolonged exposure to alcohol and individual levels of alcohol intake to loss of control over alcohol use. First, the optimal shock intensity to induce conditioned suppression of alcohol seeking was determined after 2 months of intermittent alcohol access (IAA) in the home-cage. Next, to investigate the impact of prolonged alcohol exposure, conditioned suppression was reassessed after 2 more months of IAA. To determine the influence of individual levels of alcohol intake on loss of control over alcohol seeking, conditioned suppression was assessed in subgroups of low (LD) and high (HD) alcohol drinking rats, that were discerned based on their individual levels of alcohol consumption during 2 months of IAA. Our results showed that conditioned suppression of alcohol seeking was reduced after 4 months of IAA when compared to 2 months of IAA. Moreover, unlike the LD, the HD were resistant to conditioned suppression of alcohol seeking, although both groups showed comparable expression of conditioned fear. These findings show that the development of loss of control over alcohol seeking, a key characteristic of AUD in humans, is dependent on both the extent of alcohol exposure and the individual's propensity to consume alcohol. Studying the neurobiological mechanisms of conditioned suppression may shed light on the mechanisms underlying loss of control over alcohol use, which is essential for the development of innovative treatments for AUD.

INTRODUCTION

Alcohol is among the most widely used substances of abuse worldwide (Anderson 2006; WHO 2011). The prevalence of alcohol use disorder (AUD) among adults is 3-5% (Anderson 2006; Rehm et al., 2009; WHO 2011), implying that AUD only occurs in a minority of users. Importantly, this modest percentage of alcohol users with AUD still amounts to a large number of people, i.e. over 200 million worldwide (Anderson 2006; Rehm et al., 2009; WHO 2011; Effertz and Mann 2013; Gowing et al., 2015).

Loss of control over use is a key characteristic of substance use disorders, including AUD (American Psychiatric Association 2013). To understand the underlying neural mechanisms, an increasing number of preclinical models of loss of control over substance use have been developed. For example, rodents show continued substance use or seeking despite adverse consequences, operationalized as resistance of self-administration to punishment, such as lithium-induced malaise, mild electric footshocks or footshock-associated stimuli (for review see Lesscher and Vanderschuren, 2012; Vanderschuren and Ahmed, 2013; Hopf and Lesscher, 2014). Continued substance seeking despite the presentation of footshocks or footshock-associated stimuli has been demonstrated after self-administration of cocaine (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt 2004; Pelloux et al., 2007, 2015; Belin et al., 2008, 2009, 2011; Jonkman et al., 2012a,b; Chen et al., 2013). For orally ingested substances, such as alcohol, the taste of the substance solution can be rendered aversive with quinine. Interestingly, after excessive or prolonged alcohol consumption, resistance to quinine adulteration has been demonstrated in mice and rats (Wolffgramm 1991; Wolffgramm and Heyne 1995; Wolffgramm et al., 2000; Hopf et al., 2010; Lesscher et al., 2010; Seif et al., 2013, 2015; Spoelder et al., 2015). However, except for one study (Seif et al., 2013), insensitivity of alcohol seeking to footshock (-associated stimuli) has not been demonstrated.

Considering that only a small proportion of the individuals that use alcohol develop AUD, it is important to understand the factors that determine the transition from recreational, controlled to compulsive, uncontrolled alcohol use. Previous studies suggest that the extent of exposure to alcohol or cocaine is a key factor in this process (Wolffgramm 1991; Wolffgramm and Heyne 1995; Wolffgramm et al., 2000; Deroche-Gamonet et al., 2004; Vanderschuren and

Everitt 2004; Pelloux et al., 2007, 2015; Belin et al., 2008; 2009; 2011; Hopf et al., 2010; Lesscher et al., 2010; Jonkman et al., 2012b; Chen et al., 2013). Hopf and colleagues showed that rats become resistant to quinine- and footshock-modulation of alcohol self-administration after 3-4 months of alcohol consumption (Hopf et al., 2010; Seif et al., 2013, 2015). Similarly, rats display reduced suppression of cocaine seeking upon presentation of footshock-associated cues after prolonged cocaine exposure (Vanderschuren and Everitt 2004; Limpens et al., 2014b). In addition, it has been demonstrated that loss of control does not inevitably occur in all animals that take alcohol or cocaine (Deroche-Gamonet et al., 2004; Pelloux et al., 2007, 2015; Belin et al., 2008; 2009; 2011; Chen et al., 2013; Spoelder et al., 2015), indicating that individual vulnerability factors, such as impulsivity (Belin et al., 2008) contribute to the development of substance use disorders as well. Indeed, we have recently shown that individual differences in alcohol consumption in rats predict resistance to quinine modulation of alcohol consumption (Spoelder et al., 2015).

The aim of this study was to assess the role of both prolonged alcohol consumption and individual differences in alcohol consumption in loss of control over alcohol seeking. For this purpose, we used a conditioned suppression setup (Kearns et al., 2002; Vanderschuren and Everitt 2004; Limpens et al., 2014a, b) in Lister Hooded rats, which we recently found to display substantial individual differences in alcohol intake (Spoelder et al., 2015). Optimal parameters to induce conditioned suppression of alcohol seeking were first determined. Next, we re-tested conditioned suppression of alcohol seeking after two more months of alcohol consumption. Furthermore, control over alcohol seeking was compared for subgroups of high and low alcohol drinking rats. We hypothesized that prolonged alcohol exposure results in reduced suppression of alcohol seeking and that the rats which consume high levels of alcohol are more prone to lose control over alcohol seeking compared to low alcohol drinking rats.

MATERIALS AND METHODS

Animals

Adult male Lister Hooded rats, obtained from Charles River (Sulzfeld, Germany) were housed individually under controlled temperature and humidity conditions and a reversed light/dark cycle (lights off 7.00 AM –lights on

7.00 PM) with ad libitum access to water and chow. Rats were acclimatized to the housing conditions for two weeks upon arrival in our laboratory and were weighed and handled at least once per week. All experiments were approved by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op de dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Voluntary home cage alcohol consumption

The rats were given access to 20% (v/v) alcohol (Klinipath, The Netherlands) and water in a two-bottle choice setup in the home cage with intermittent alcohol access (IAA) for three days a week (Monday-Wednesday-Friday) as previously described (Spoelder et al., 2015). In the first month, alcohol was presented for 7h/day between 9.00 AM and 16.00 PM (i.e., during the dark phase) and access to alcohol was subsequently extended to 24h/day in the following month(s). Alcohol intake and preference were calculated per rat per session and averaged per week. To select rats that consistently consumed low or high levels of alcohol throughout the experiment, rats were ranked from low to high based on the rats' average alcohol intake per week and were assigned ranking scores. These weekly ranking scores were then summed to calculate a total ranking score per rat which was used to divide rats in subgroups (Spoelder et al., 2015). For *Experiment 1*, 32 medium alcohol drinking rats (MD) were selected from a population of 64 rats using a quartile split to assess the impact of prolonged alcohol consumption on loss of control over alcohol seeking. For *Experiment 2*, that was designed to assess the relationship between individual levels of alcohol consumption and loss of control over alcohol seeking, 16 low alcohol drinking rats (LD) and 16 high alcohol drinking rats (HD) were selected from 48 rats (from two batches) using a tertile split. Ranking was performed separately for the three batches in this study.

Alcohol self-administration

The rats were trained and tested in operant conditioning chambers (29.5 cm L, 24 cm W 25 cm H; Med Associates, Georgia, VT, USA), situated in light- and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8 cm wide retractable levers, placed 11.7 cm apart and 6 cm from the grid floor. A cue light (28 V, 100mA) was present above each lever, a liquid dipper was in a magazine between the levers and a house light (28 V, 100mA) and a 85 dB, 2900 Hz tone generator were located on the opposite wall. The position of the active and inactive levers was counterbalanced

between rats. For all schedules of reinforcement, pressing the active lever once (during fixed ratio (FR)1 sessions) or after a random interval (RI) has elapsed (during RI sessions) raised the dipper cup containing alcohol (0.1 ml, 20% v/v), illuminated the cue light above the active lever and switched off the house light. Access to alcohol was terminated 10 sec after a head entry into the magazine, the cue light was turned off and after a 5 sec interval a new trial started. Pressing the inactive lever was recorded, but had no programmed consequences. The alcohol solution was refreshed before each session.

The rats were tested 3 days/week (Monday-Wednesday-Friday) and were first trained under a FR1 schedule of reinforcement. After three FR1 sessions, a RI schedule of reinforcement was implemented. In RI sessions, the first active lever press initiated the RI during which both levers remained extended. Lever pressing during the RI was recorded but was without consequences. After completion of the RI, an active lever press resulted in the delivery of alcohol. The rats were tested in RI sessions with increasing average interval durations (3 x RI 5 sec, 3 x RI 15 sec, 2-3 x RI 30 sec and 2-3 x RI 60 sec); these sessions were 30 min in duration. Finally, the rats were trained under a RI 120 sec schedule for five 60 min sessions. Stable responding was defined as <25% variation in active responses during the RI in the first 15 min of the last three RI 120 sec sessions. Experimental events and data recording were controlled using MED-PC for Windows.

Conditioned suppression of alcohol seeking

The footshock conditioning procedures as well as the conditioned suppression test for alcohol seeking behaviour were comparable to the procedures previously described for cocaine and sucrose (Vanderschuren and Everitt 2004; Limpens et al., 2014a, b). The rats were assigned to groups that either underwent fear conditioning, with conditioned stimulus (CS)-footshock pairings (CS+), or underwent control conditioning (CS-). Group assignments were based on the rats' mean seeking responses per minute during the first 15 min of the last three RI 120 sec sessions, so that the CS+ and CS- groups had equal mean seeking rates prior to conditioning. In *Experiment 1*, different shock intensities were used to determine the optimal shock intensity for conditioned suppression of alcohol seeking, as we previously did for cocaine and sucrose (Limpens et al., 2014b). To that aim, based on their baseline RI 120 sec responding, MD were assigned to one of four CS groups, and received either no footshocks (N = 10: CS-) or one of the three different shock

intensities (N = 7: 0.35 mA CS+, N = 8: 0.40 mA CS+, N = 7: 0.45 mA CS+). In *Experiment 2*, the rats were either fear-conditioned with 0.40 mA footshocks (LD: N = 8; HD: N = 8) or were used as controls and underwent control conditioning (LD: N = 8; HD: N = 8). Acquisition of the CS-shock association was established in chambers (conditioning chambers) different from operant self-administration chambers (SA chambers). To habituate the rats to the conditioning chambers, they were pre-exposed to the chambers for 30 min on three days, in between the RI 120 sec test sessions. The CS-shock conditioning session started with a 5 min period in which only the house light was illuminated, followed by two periods of 10 min during which a 85 dB, 2900 Hz tone (separated by an inter-trial-interval of 10 min) was constantly presented. During the 10 min tone presentations, 10 unpredictable, scrambled footshocks (1 sec duration) were delivered, resulting in 20 shocks in total for each CS+ rat. The second 10 min tone presentation was followed by a 5 min period with no tone presentation, before the conditioning session was completed. Rats in the CS- control group were subjected to the same procedure, except that they did not receive footshocks.

After conditioning, the rats received two additional RI 120 sec training sessions. Subsequently, conditioned suppression of alcohol-seeking behaviour was assessed in the SA chambers. The house light was illuminated throughout the conditioned suppression test. Two min after the start of the session, the levers were extended and remained extended throughout the 12 remaining min of the test. Two-minute intervals in which the tone CS was presented (CS-ON interval) were alternated with two-minute intervals where the tone CS was absent (CS-OFF interval). Alcohol seeking was examined in extinction, i.e. responding on the levers was recorded, but had no programmed consequences. To avoid altered responding due to the lack of (smell of) alcohol, the cup containing 20% alcohol (v/v) was present underneath the liquid dipper, similar to actual alcohol self-administration sessions.

After the conditioned suppression test, the MD in *Experiment 1* received 24h IAA for another two months and were subsequently re-trained under the FR1 (1x), RI 30 sec (1x), RI 60 sec (2x), RI 120 sec schedules (5x). In between the RI 120 sec sessions, the rats were again habituated to the conditioning chamber for three days. Subsequently, the rats were re-conditioned using the same CS-conditioned footshock intensity or control procedure they were exposed to before, received two RI 120 sec sessions and were re-tested for conditioned suppression.

Conditioned freezing

After completion of the conditioned suppression test, conditioned freezing to the footshock-associated tone was determined in LD and HD from Experiment 2. Therefore, one week after the conditioned suppression test, the rats underwent fear conditioning (CS+) or control conditioning (CS-); rats were assigned to the same group as previously. Fear conditioning procedures were similar as described in the previous section. On the subsequent day, freezing behaviour, defined as the absence of any movement other than breathing (Blanchard and Blanchard 1969; Bouton and Bolles 1980; LeDoux et al., 1984), was video-taped in the same conditioning chamber during the first 2 min after placement in the chamber, without the CS+ tone presentation and during the subsequent 2 min with the CS+ tone presentation. The frequency and duration of freezing behaviour, was scored from DVD-taped behaviour using Observer software by an observer who was blind to the treatment groups (Noldus, Wageningen, NL).

Statistical analysis

All data were analyzed by one, two, three or four-way repeated measures ANOVA with CS group (Exp. 1: CS-, 0.35 mA, 0.40 mA or 0.45 mA; Exp. 2: CS+ and CS-) or group (LD or HD) as the between-subjects variables and the average alcohol intake and preference per month, the IAA access duration (2 or 4 months), interval (CS ON and OFF 2 min periods) and tone (No-tone vs Tone) as the within-subjects variables. Mauchly's test of sphericity was used to test if variances of the differences between treatment levels were equal. If the assumption of sphericity was violated, degrees of freedom were corrected using Huynh-Feldt estimates of sphericity to more conservative values. Corrected degrees of freedom are presented rounded to the nearest integer. When appropriate, post hoc analyses were conducted using Student's t-tests or pairwise Bonferroni comparisons. Each parameter was tested for normality with a Kolmogorov-Smirnov test. In case the behavioural parameters were not normally distributed, data was square root transformed (active responses in conditioned suppression test and freezing behaviour) or log transformed (latency data) prior to statistical analyses, which resulted in normal distribution of the data in all cases. The threshold for statistical significance was set at $p < 0.05$. All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, N.Y. USA). The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean \pm SEM. Graphs were made using Graphpad Prism 6.

Figure 1

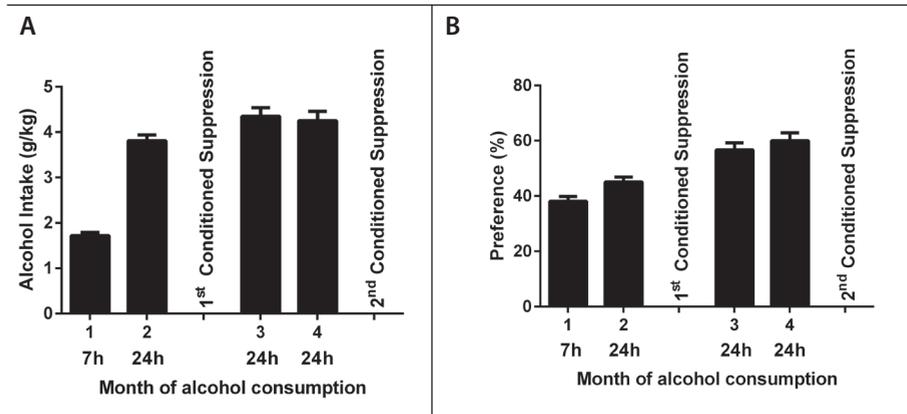


Figure 1. Alcohol consumption in the home cage during the two months preceding each conditioned suppression test. (A) Alcohol intake increased upon extension of the access duration from 7h to 24h, increased further after the first conditioned suppression test but remained stable between the third and fourth month of alcohol exposure. (B) Alcohol preference increased upon extension of the access duration from 7h to 24h and continued to increase over time. Data are shown in mean + SEM.

RESULTS

Experiment 1: Effect of prolonged alcohol consumption on conditioned suppression of alcohol seeking

Alcohol intake and self-administration

Alcohol intake and preference increased over the course of IAA (Intake: $F_{(2,58)\text{month}} = 96.9, P < 0.001$; Preference: $F_{(2,58)\text{month}} = 33.5, P < 0.001$). *Post hoc* pairwise comparisons showed that alcohol intake increased when access time was extended from 7h in the first month to 24h in the second month ($P < 0.001$), increased further after the initial conditioned suppression test ($P < 0.01$), and remained stable during the last two months of IAA (Fig. 1A). A near-significant trend towards an increase in alcohol preference was apparent upon extension of the access time from the first to the second month of IAA ($P = 0.055$). Alcohol preference continued after the initial conditioned suppression test from the second to the third month ($P < 0.001$) but remained unchanged from the third to the fourth month of IAA (Fig. 1B). Importantly, the CS groups did not differ in alcohol intake or alcohol preference on any of the time points tested ($F_{(3,28)\text{CS group}} = 0.53, \text{N.S.}$; $F_{(6,58)\text{month} \times \text{CS group}} = 0.90, \text{N.S.}$) (data not shown).

The CS groups (CS-, 0.35 mA, 0.40 mA and 0.45 mA) responded equally during baseline RI 120 sec sessions prior to the first and second fear conditioning session ($F_{(3,28)CS\ group} = 0.13$, N.S.). For this analysis, the data for the first 15 min of each RI 120 sec session, corresponding to the duration of the conditioned suppression test, were considered. Responding prior to the second conditioned suppression test was lower when compared to the first test ($F_{(1,28)time} = 9.5$, $P < 0.01$), independent of the CS group ($F_{(3,28)time \times CS\ group} = 2.7$, N.S.) (data not shown).

Conditioned suppression of alcohol seeking - active responses

Analysis of the number of active responses during the conditioned suppression test after limited alcohol exposure revealed significant effects of fear conditioning on alcohol seeking ($F_{(3,28)CS\ group} = 11.6$, $P < 0.001$), which were dependent on the interval (CS ON/OFF) ($F_{(13,118)CS\ group \times interval} = 4.0$, $P < 0.001$) (Fig. 2A). *Post hoc* pairwise comparisons revealed that, relative to the CS- group, responding was significantly reduced in the 0.35 mA ($P < 0.05$) and the 0.40 mA CS group ($P < 0.001$), with a trend for the 0.45 mA CS group ($P = 0.082$). Further comparisons per interval confirmed significant conditioned suppression of alcohol seeking during the first tone presentation in all CS+ groups ($P < 0.001$). However, conditioned suppression was only persistent throughout the session in the 0.40 mA CS group ($P < 0.05$).

After extended alcohol exposure, there was an interval-dependent effect of fear conditioning on alcohol seeking ($F_{(3,28)CS\ group} = 2.1$, N.S.; $F_{(12,112)interval \times CS\ group} = 4.0$, $P < 0.001$) (Fig. 2B). *Post hoc* pairwise comparisons of the three CS+ groups to the CS- group revealed no significant differences in alcohol seeking between any CS+ group and the CS- group. Further comparisons per interval revealed suppression of alcohol seeking only during the first CS ON interval ($P < 0.001$ for the 0.35 mA and 0.40 mA group; $P < 0.05$ for the 0.45 mA group).

Conditioned suppression of alcohol seeking – latency to first active response

Analysis of the latency to make the first active response per CS ON/OFF interval during the conditioned suppression test after limited alcohol exposure, revealed a significant difference between the conditioning groups ($F_{(3,28)CS\ group} = 20.4$, $P < 0.001$), independent of the session interval ($F_{(15,140)interval \times CS\ group} = 0.99$, N.S.) (Fig. 2C). *Post hoc* pairwise comparisons

Figure 2

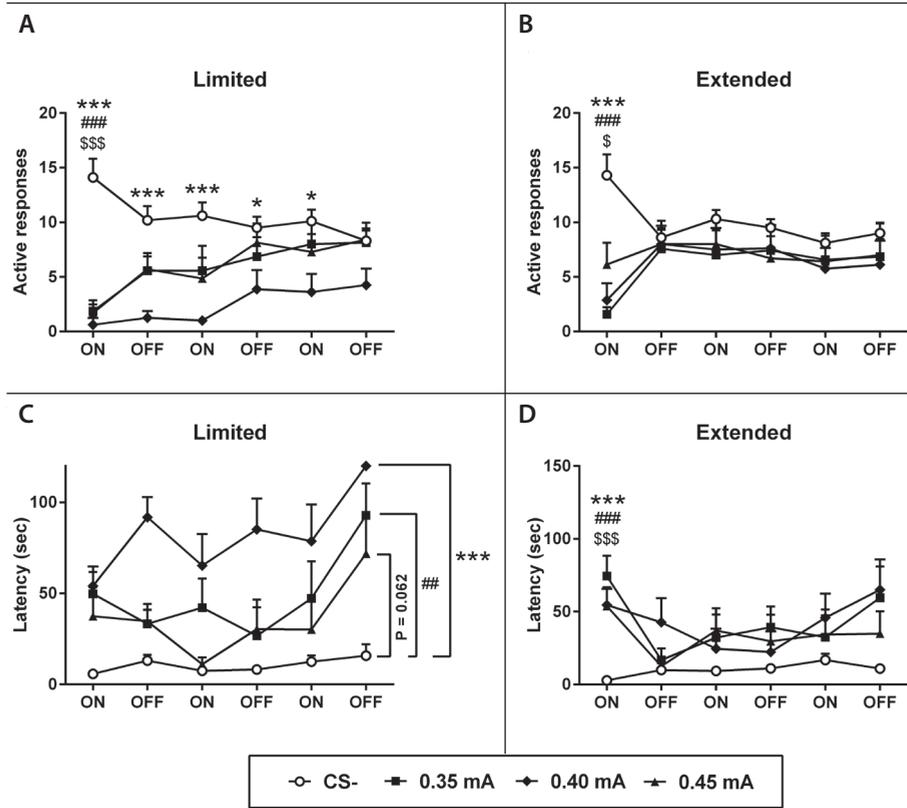


Figure 2. Conditioned suppression of alcohol seeking after limited (2 months; A, C) and prolonged alcohol consumption (4 months; B, D). (A-B) Number of active responses during consecutive CS ON and CS OFF intervals in rats conditioned with different footshock intensities (0.35 mA, 0.40 mA and 0.45 mA) after limited (A) or extended (B) alcohol consumption. (C-D) Latencies to the first active response during the CS ON and CS OFF intervals in rats conditioned with different footshock intensities (0.35 mA, 0.40 mA and 0.45 mA) after limited (C) and extended (D) alcohol consumption. Data are presented as mean + SEM. *and *** indicate a significant difference between the 0.35 mA group and the CS- group (*post hoc* pairwise comparisons $P < 0.05$ and $P < 0.001$, respectively). ## and ### Indicate a significant difference between the 0.40 mA group and the CS- group (*post hoc* pairwise comparisons, $P < 0.01$ and $P < 0.001$, respectively). \$ and \$\$\$ Indicate a significant difference between the 0.45 mA group and the CS- group (*post hoc* pairwise comparisons $P < 0.05$ and $P < 0.001$, respectively).

Figure 3

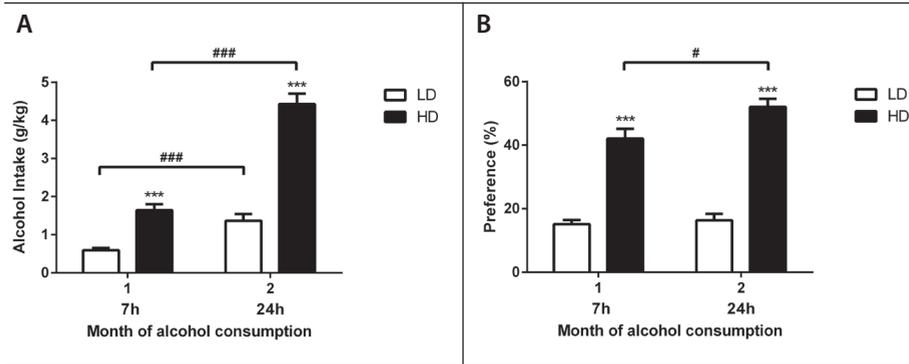


Figure 3. Alcohol consumption in the home cage preceding the conditioned suppression test in LD and HD. (A) Alcohol intake was higher in HD and increased to a greater extent in HD compared to LD upon the extension of the alcohol access duration from 7h/day in the first month to 24h/day in the second month. (B) Alcohol preference was higher in HD and increased in the second month in HD only. Data are presented as mean + SEM. *** Significant difference between LD and HD (*post hoc* student's t-test, $P < 0.001$). # and ### Significant difference between the first month (7h sessions) and second month (24h sessions) of alcohol consumption (*post hoc* student's t-test, $P < 0.05$ and $P < 0.001$, respectively).

showed that the active response latency was increased, relative to the CS- group, in the 0.35 mA and 0.40 mA groups ($P < 0.01$ and $P < 0.001$, respectively) with a trend for 0.45 mA group ($P = 0.062$).

The latency to the first active response was enhanced in the conditioned groups during the conditioned suppression test after extended alcohol exposure, as evident from an overall effect of the CS group ($F_{(3,28)CS\ group} = 4.5$, $P < 0.05$), which was dependent on the session interval ($F_{(15,140)interval \times CS\ group} = 4.0$, $P < 0.001$) (Fig. 2D). *Post hoc* pairwise comparisons revealed significantly increased latencies for the 0.35 mA and 0.40 mA groups ($P < 0.05$), but not for the 0.45 mA group. Subsequent comparisons per interval showed that the active response latency was only enhanced during the first CS ON interval ($P < 0.001$ for all intensities tested).

Experiment 2: Individual differences in alcohol consumption and conditioned suppression of alcohol seeking

Alcohol intake and self-administration

HD showed higher alcohol intake and preference than LD ($F_{(1,24)group} = 139.3$, $P < 0.001$ and $F_{(1,24)group} = 127.6$, $P < 0.001$) (Fig. 3). Consistent with our previous

Figure 4

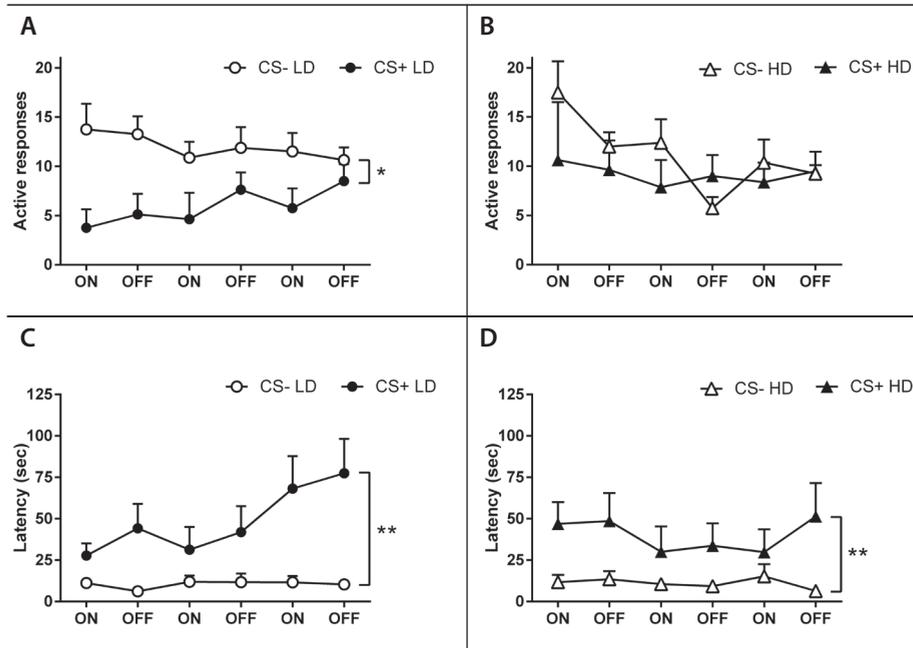


Figure 4. Conditioned suppression of alcohol seeking in LD and HD. (A-B) Number of active responses during consecutive CS ON and CS OFF intervals. The LD show conditioned suppression of alcohol seeking, as reflected by reduced number of active responses made by the CS+ compared to the CS- group (A). In contrast, the HD show no significant conditioned suppression, i.e. the number of active responses upon CS presentation was not different between the HD CS+ and CS- group (B). (C-D) Both the LD and HD CS+ subgroups showed a longer latency to the first active response, when compared to their respective CS- controls. Data are presented as mean + SEM. * and ** Significant difference between CS- and CS+ groups (ANOVA; $P < 0.05$ and $P < 0.01$, respectively).

studies (Spoelder et al., 2015), the augmented alcohol intake when access to alcohol was increased from 7h/day in the first month to 24h/day in the second month was more pronounced in HD compared to LD ($F_{(1,24)\text{month} \times \text{group}} = 67.9$, $P < 0.001$). Moreover, the preference for alcohol increased with extended access time in HD but not in LD ($F_{(1,24)\text{month} \times \text{group}} = 9.3$, $P < 0.01$). There were no differences in alcohol intake and preference between the CS- and CS+ groups (intake: $F_{(1,24)\text{month} \times \text{group} \times \text{CS group}} = 0.51$, N.S. and $F_{(1,24)\text{group} \times \text{CS group}} = 1.9$, N.S.; preference: $F_{(1,24)\text{month} \times \text{group} \times \text{CS group}} = 1.02$, N.S. and $F_{(1,24)\text{group} \times \text{CS group}} = 1.99$, N.S.).

Analysis of the RI 120 sec sessions showed that the HD made more active responses during the first 15 min of the last three RI 120 sec sessions than LD (38.8 ± 3.5 versus 26.1 ± 2.9 , respectively; $F_{(1,32)\text{group}} = 7.5$, $P < 0.05$). Importantly,

there were no differences between CS- and CS+ groups ($F_{(1,32)CS\ group} = 0.0$, N.S. and $F_{(1,32)group \times CS\ group} = 0.45$, N.S.) in baseline responding under the RI 120 sec schedule of reinforcement (data not shown).

Conditioned suppression of alcohol seeking

Presentation of the footshock-associated CS reduced the number of active responses in the LD ($F_{(1,12)CS\ group} = 9.0$, $P < 0.05$) independent of session interval ($F_{(1,60)interval \times CS\ group} = 2.1$, N.S.) (Fig. 4A). By contrast, the number of active lever presses was not changed by the footshock CS in HD ($F_{(1,12)CS\ group} = 0.76$, N.S., $F_{(1,60)interval \times CS\ group} = 1.6$, N.S.) (Fig. 4B).

The latency to make the first active lever press was higher for both LD and HD CS+ rats, when compared to their CS- controls (LD: $F_{(1,12)CS\ group} = 14.8$, $P < 0.01$; HD: $F_{(1,12)CS\ group} = 12.3$, $P < 0.01$) (Fig. 4C and 4D). The increased latency to make the first active response was independent of session interval (LD: $F_{(1,60)interval \times CS\ group} = 0.49$, N.S.; HD: $F_{(1,60)interval \times CS\ group} = 0.52$, N.S.).

Conditioned freezing

Analysis of the freezing behaviour of the rats during 2 min before (no tone) and during CS (tone) presentation revealed that the CS+ conditioned LD and HD spent significantly more time freezing compared to the CS- controls (LD: $F_{(1,12)CS\ group} = 59.4$, $P < 0.001$; HD: $F_{(1,12)CS\ group} = 71.9$, $P < 0.001$) (Fig. 5). Moreover, fear conditioning was augmented upon presentation of the tone for HD ($F_{(1,12)tone \times CS\ group} = 20.8$, $P < 0.001$), with a trend for LD ($F_{(1,12)tone \times CS\ group} = 4.6$, $P = 0.053$). Separate analyses of freezing behaviour prior to and during tone presentation revealed augmented context- and CS-induced freezing in the CS+ group compared to the CS- group in both LD (tone OFF: $F_{(1,15)CS\ group} = 23.1$, $P < 0.001$; tone ON: $F_{(1,15)CS\ group} = 70.1$, $P < 0.001$) and HD (tone OFF: $F_{(1,15)CS\ group} = 29.0$, $P < 0.001$; tone ON: $F_{(1,15)CS\ group} = 94.5$, $P < 0.001$).

DISCUSSION

In the present study, we investigated the role of prolonged alcohol consumption and individual differences in alcohol consumption in the development of loss of control over alcohol seeking. To that aim, we investigated conditioned suppression as a measure of control over alcohol seeking, either in moderate alcohol drinking rats after a limited and prolonged alcohol drinking history or in preselected groups of rats displaying high and low levels of alcohol

Figure 5

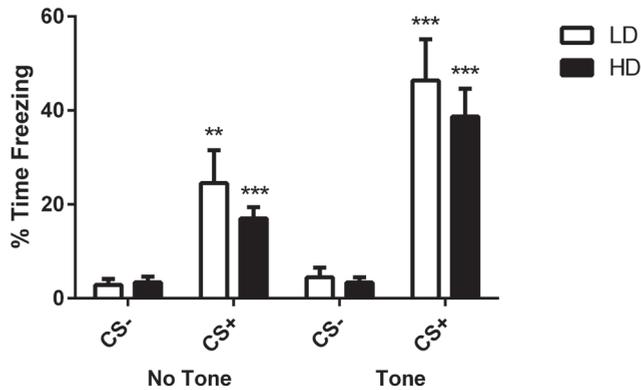


Figure 5. Freezing behaviour in LD and HD during the 2 min before (No Tone) and during 2 min presentation of the footshock-associated CS+ (Tone) period in the conditioning chamber. The LD and HD CS+ groups showed significant context- and CS-induced freezing, when compared to their respective CS- control groups. Data are presented as mean + SEM. ** and *** Significant difference between CS+ and CS- groups within LD and HD groups (*post hoc* student's t-tests; $P < 0.01$ and $P < 0.001$, respectively).

consumption. Consistent with our hypothesis, we observed resistance to conditioned suppression of alcohol seeking (1) in rats with a protracted alcohol drinking history and (2) in selected high alcohol drinking rats. These findings show that loss of control over alcohol use is dependent on both the extent of alcohol exposure and the individual's propensity to consume alcohol. Individuals that display high levels of alcohol consumption are therefore at increased risk for AUD, but individuals that show lower levels of alcohol consumption may also lose control over their alcohol consumption with prolonged and cumulating exposure to alcohol.

Conditioned suppression of alcohol seeking: role of shock intensity

To optimize the assessment of conditioned suppression, we first determined the effects of different footshock intensities on the degree of conditioned suppression of alcohol seeking. For the present study, we only included intensities with which we expected to observe conditioned suppression on the basis of our previous assessment of conditioned suppression of cocaine and sucrose seeking (Limpens et al., 2014b). Indeed, all three intensities used to condition the medium alcohol drinking rats (0.35 mA, 0.40 mA and 0.45 mA) resulted in conditioned suppression of alcohol seeking. Although the degree

of suppression did not vary considerably between the three intensities, we found the suppression of alcohol seeking at the 0.40 mA intensity most robust. Importantly, using this intensity, the difference in conditioned suppression between rats with limited and extended alcohol exposure was most pronounced. Therefore, the 0.40 mA intensity was chosen to study the relation between individual differences in alcohol consumption and their degree of control over alcohol use.

Loss of control over alcohol seeking after extended alcohol use

Prolonged and excessive substance use are considered critical factors in the development of substance use disorders, including AUD (Ahmed, 2012; Vanderschuren and Ahmed, 2013; Piazza and Deroche-Gamonet, 2013). Indeed, extended cocaine self-administration has been shown to result in loss of control over cocaine seeking, as is evident from resistance to both suppression of punished cocaine seeking (Pelloux et al., 2007, 2015; Jonkman et al., 2012b) and conditioned suppression of cocaine seeking (Vanderschuren and Everitt 2004; Limpens et al., 2014a, b). Hopf et al. showed that rats develop resistance to quinine adulteration and suppression of punished alcohol seeking, indicative of loss of control over alcohol use, after 3-4 months of IAA (Hopf et al., 2010; Seif et al., 2013). We here extend these findings by showing that moderate alcohol drinking rats are sensitive to conditioned suppression of alcohol seeking after 2 months of alcohol consumption under IAA conditions, but that conditioned suppression of alcohol seeking substantially declines after 2 more months of IAA. Importantly, the decrease in conditioned suppression after 4 months of IAA was not accompanied by an increase in responding for alcohol under the RI 120 sec schedule. This suggests that reduced control over alcohol seeking, apparent as lower sensitivity to threat or punishment, is not the result of an increased incentive value of alcohol. Interestingly, in *Experiment 2* (see below), HD did respond more for alcohol under the RI 120 sec schedule than LD, consistent with our previous finding that HD show higher incentive motivation for alcohol (Spoelder et al., 2015). During the test for conditioned suppression, however, the CS- groups of HD and LD responded at comparable levels (see Fig. 4A-B). Indeed, other preclinical studies have shown that increased motivation for substances and loss of sensitivity to punishment can occur independently (Vanderschuren and Everitt 2004; Hopf et al., 2010) or sequentially (Deroche-Gamonet et al., 2004), indicating that these key criteria for substance use disorders (American Psychiatric Association, 2013) are neurally and behaviourally different expressions of addiction. A potential

limitation of the current approach is that the conditioned suppression tests after 2 and 4 months of IAA were performed within the same group of rats, so that repeated testing may have contributed to the reduction in conditioned suppression that we observed after 4 months of IAA. However, Vanderschuren and Everitt (2004) reported comparable resistance to conditioned suppression of cocaine seeking in rats after extended cocaine exposure when tested once or repeatedly, comparable to the rats in this study. Furthermore, conditioned suppression of sucrose seeking was shown to be unaffected by repeated conditioning and testing (Limpens et al., 2014b). Therefore, the resistance to conditioned suppression of alcohol seeking after 4 months, compared to 2 months of IAA is unlikely to be the result of repeated testing. Rather, these findings further emphasize the importance of the degree of exposure to substance of abuse, including alcohol, for the transition to full-blown substance use disorder.

Individual differences in alcohol consumption and loss of control

Our data show that individual differences in alcohol consumption predict the loss of control over alcohol seeking. Our recent studies have demonstrated a high degree of individual variability in alcohol consumption in outbred Lister Hooded rats (Lesscher et al., 2015; Spoelder et al., 2015). Subgroups of high alcohol drinking rats (HD) and low alcohol drinking rats (LD) can be discerned based on their voluntary alcohol consumption under IAA conditions. The present findings demonstrate that HD are more resistant to conditioned suppression of alcohol seeking than LD. Together with our previous report that HD are less sensitive to quinine-adulterated alcohol (Spoelder et al., 2015), these findings indicate that HD show substantially reduced control over alcohol use. Importantly, aversive taste and footshock risk comprise different sensory modalities that are also conceptually different, in that the former is directly associated with alcohol ingestion, whereas the latter entails the threat of a highly unpleasant tactile stimulus (Hopf and Lesscher, 2014). The adverse consequences of human alcohol ingestion often does not coincide with actual alcohol consumption. Therefore, the relevance of taste aversion resistance, where the bad taste of a quinine adulterated alcohol solution accompanies each drinking bout, has been questioned for human AUD, although AUD patients are known to ingest non-beverage, taste-aversive alcohol solutions (e.g. Soo Hoo et al., 2003; Leon et al., 2007). By contrast, the warning signal in conditioned suppression, i.e. the footshock associated tone, represents anticipation of adverse consequences and is not directly aligned in time with

alcohol consumption. Seif et al. (2013) previously described both footshock- and quinine resistance in rats after 3-4 months of IAA, which was promoted by a similar corticostriatal circuit. Here we extend these findings, by showing that HD display resistance to both taste and footshock warning adversities (Spoelder et al., 2015). Together, these findings suggest a common mechanism that mediates the resistance to divergent negative consequences of alcohol drinking that characterizes AUD.

To exclude the possibility that the reduced conditioned suppression in HD reflected impaired fear conditioning, we tested LD and HD for conditioned freezing to the context and tone that were associated with the footshock (Vanderschuren and Everitt 2004). The LD and HD responded equally to both the fear conditioning context and the footshock-associated tone, thus ruling out the possibility that the relative resistance to conditioned suppression of alcohol seeking observed in the HD was merely the result of impaired fear conditioning. The HD showed this characteristic of AUD already after 2 months of alcohol consumption, whereas LD and MD displayed substantial suppression of alcohol seeking after 2 months of IAA. However, the MD do develop loss of control after having consumed alcohol for a total duration of 4 months. These findings further emphasize the notion that the development of AUD, and loss of control over alcohol use in particular, is dependent both on the extent of alcohol exposure, i.e. the individual level of alcohol consumption, and the duration of alcohol exposure. There is substantial individual variability in the risk for AUD in humans. The notion that individual variation in the development of loss of control over alcohol and cocaine seeking emerges in animal models (current study and: Deroche-Gamonet et al., 2004; Pelloux et al., 2007, 2015; Belin et al., 2008, 2009, 2011; Chen et al., 2013; Spoelder et al., 2015) therefore substantiates the relevance of these animal models for addiction. However, this study also shows that not merely a high degree of alcohol consumption, displayed by a subgroup of animals, but also the cumulative degree of exposure to alcohol is an important determinant for the development of AUD. This suggests that not only individuals who consume excessive amounts of alcohol are at risk for AUD, but that also extended consumption of lower levels of alcohol may result in AUD.

To conclude, the present study demonstrates a behavioural characteristic of loss of control over alcohol seeking in rats that is dependent on the extent and duration of voluntary alcohol consumption. Despite their high prevalence

and cost to society, treatment options for AUD are limited in number and efficacy (O'Brien, 2008; Koob et al., 2009; van den Brink, 2012; Pierce et al., 2012). Moreover, the available treatments are directed at reducing reward or relapse (van den Brink, 2012), but are not directed at restoring control over behaviour. The individual variation in alcohol consumption that predicts the degree of conditioned suppression of alcohol seeking provides an important tool to assess the neurobiological mechanisms that determine loss of control over alcohol use, which may contribute to the development of innovative treatments for AUD and other forms of addiction.

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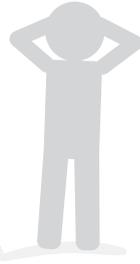
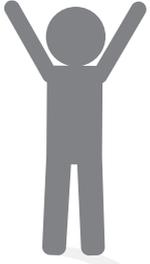
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CHAPTER 4

ALTERED PERFORMANCE IN A RAT GAMBLING TASK AFTER ACUTE AND REPEATED ALCOHOL EXPOSURE

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ABSTRACT

A bidirectional relationship between alcohol use disorder (AUD) and deficits in impulse control and decision making has been suggested. However, the mechanisms by which neurocognitive impairments predispose to, or result from AUD remain incompletely understood. To gain more insight in the effects of alcohol exposure on decision making and impulse control. We used two modified versions of the rat gambling task (rGT) that differ in the net gain and the punishment magnitude associated with the different response options. In Experiment 1, we assessed the effects of acute alcohol treatment (0 - 0.8 g/kg) on rGT performance. In Experiment 2, we determined the effects of alcohol on rGT acquisition (15 sessions, 0.6 g/kg). Next, these animals were challenged with alcohol (0 - 1.0 g/kg) prior to rGT sessions. Acute alcohol treatment suppressed baseline performance in both rGT versions but only modestly altered decision making. Treatment with alcohol during acquisition increased risky choices in the rGT version that involved larger punishment and blunted the reduction in win-shift behaviour during acquisition in both rGT versions. Moreover, rats treated with alcohol during acquisition showed an increase in premature and perseverative responding upon subsequent alcohol challenges (0 – 1.0 g/kg) and were less sensitive to the behavioural suppressant effects of alcohol. Our results show that repeated alcohol exposure alters decision making during rGT acquisition, and reduces the ability to adjust choice behaviour on the basis of feedback. In addition, repeated alcohol exposure unmasks its behavioural disinhibitory effects in the rGT. Impaired responsiveness to feedback and behavioural disinhibition may contribute to the development of AUD.

INTRODUCTION

Alcohol is one of the most widely abused substances worldwide and the problems associated with alcohol use disorder (AUD) pose a major burden to our society (World Health Organization 2011). Estimates are that 76 million people worldwide suffer from AUD (United Nations Office on Drugs and Crime 2012) and AUD is among the most financially costly of all major neuropsychiatric disorders (Effertz and Mann 2013). An important characteristic of AUD is compromised impulse control and decision making, which has been implicated in the development and maintenance of the disorder, as well as the likelihood of relapse (Garavan and Stout 2005; De Wit 2009; Rogers et al., 2010; Dalley et al., 2011; Fineberg et al., 2014). Thus, personality traits such as impulsivity, i.e. the tendency to act without consideration of possible consequences, and suboptimal decision making are alleged risk factors for AUD (Dom et al., 2006; Johnson et al., 2008; de Wit 2009; Dalley et al., 2011; Goudriaan et al., 2011; King et al., 2011). Conversely, AUD patients show maladaptive decision making and reduced impulse control (Bechara et al., 2001; Salgado et al., 2009; Kim et al., 2011; MacKillop et al., 2011; Tomassini et al., 2012; Voon et al., 2014). Hence, impaired decision making and impulsive behaviour appear both to predispose to, and be a consequence of AUD, but cause and effect in the relationship between neurocognitive impairments and AUD can be difficult to disentangle in human studies (Ersche et al., 2013). Preclinical studies that afford more control over experimental subjects and conditions, may therefore aid to understand the relationship between alcohol use, decision making and impulsive behaviour (Tomie et al., 1998; Mitchell et al., 2011; Walker et al., 2011; Irimia et al., 2013), to contribute to the prevention and treatment of AUD (Marhe et al., 2014).

The Iowa gambling task (IGT) is widely used to assess decision making in humans. This task combines several factors that guide everyday decision making, including probabilistic reward and punishment and the necessity to exert behavioural control in order to maximize long-term gains (Bechara et al., 1994). During task acquisition, when the contingencies of the response options are being learned, choice behaviour changes from exploratory sampling to more exploitative advantageous decision making. However, despite the hypothesized relationship between AUD and impaired decision making, studies on the effects of acute exposure to alcohol on decision making have not been conclusive. Acute alcohol exposure studies in humans have reported

an increase in disadvantageous choices (Lane et al., 2004; George et al., 2005), but also unaltered decision making (Ramaekers and Kuypers 2006). In a rodent version of the IGT (rat gambling task (rGT); Zeeb et al., 2009) and a comparable risky decision making task (Simon et al., 2009), acute alcohol exposure did not change decision making (Mitchell et al., 2011; Peña-Oliver et al., 2014). Importantly, in these rodent models, task acquisition takes several weeks of training, whereas in human studies, acquisition and performance of the IGT is usually examined in a single session. Hence, to investigate the effects of alcohol under choice uncertainty in a rodent model, alcohol should preferably be administered during task acquisition when the animals are learning the response-outcome associations.

Here, we aimed to gain further insight into the effects of alcohol exposure on decision making by examining the effects of single and repeated alcohol treatment in two modified versions of the rGT (Zeeb et al., 2009). The animals were offered three choice options, labelled safe, optimal and risky, whereby the task versions were set up such that the choice contingencies differ in the magnitude and probability of reward delivery and punishment. By comparing the effects of alcohol on choice behaviour in the two rGT versions we investigated whether alcohol affects decision making by changing the responsiveness to reward or punishment or if alcohol evokes risky behaviour. First, we assessed the acute effects of alcohol on stable choice behaviour in the two rGT versions. Next, the effects of repeated administration of alcohol on task acquisition were determined, followed by alcohol challenge sessions after choice behaviour had stabilized. Based on previous findings (Mitchell et al., 2011; Peña-Oliver et al., 2014), we hypothesized that acute alcohol administration has limited effects on decision making in animals that show stable choice behaviour, whereas repeated alcohol administration during task acquisition results in disadvantageous decision making, similar to what has been shown in human studies (Lane et al., 2004; George et al., 2005). In addition, we also assessed the effects of alcohol on other behavioural parameters, i.e. choice latencies, omissions, perseverative responses and responses during the inter-trial interval (ITI), i.e. a premature response, which is considered to be a measure of motor impulsivity (Robbins 2002; Pattij and Vanderschuren 2008). Previous studies in humans and rodents have described that alcohol exposure results in impaired inhibitory control, especially after repeated binge-like alcohol exposure (Easdon and Vogel-Sprott 2000; Marczyński et al., 2007; Irimia et al., 2013; Sanchez-Roige et al., 2014). Hence, we expected

to find differential effects of acute and repeated alcohol treatment on these behavioural parameters, especially on motor impulsivity (Bizarro et al., 2003; Peña-Oliver et al., 2009; Walker et al., 2011; Semenova 2012; Irimia et al., 2013).

MATERIALS AND METHODS

Animals

Male Lister Hooded rats (Charles River, Germany) weighing 220-250 g at the start of experimental training were used. The rats were housed in groups of 3-4 rats/cage under controlled temperature and humidity conditions and a reversed light/dark cycle (lights on 7.00 AM – lights off 7.00 PM) with *ad libitum* access to water and chow. After 2-week acclimatization to the housing conditions, the rats were gradually restricted to 5 g chow/100 g body weight/day, which maintained them at 90% of their free-feeding weight. Body weights were monitored weekly and the animals were briefly restrained during the weighing procedure, to habituate them to the injection procedure. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in agreement with Dutch Laws (Wet op de Dierproeven 1996) and European regulations (Guideline 86/609/EEC).

Apparatus

Training and testing was conducted in operant conditioning chambers, illuminated by a white house light, in ventilated sound-attenuating cubicles (Med Associates, St. Albans, VT, USA). Chambers were equipped with an array of five holes in a curved wall, each with an infrared detector and a stimulus light. Sucrose pellets (TestDiet, UK) could be delivered at the opposite wall via a dispenser. The apparatus was controlled using MEDPC software version 1.17 (Med Associates).

Behavioural Procedures

During training, the animals were required to make a nose-poke in the illuminated aperture to obtain sucrose pellets, as described previously (Zeeb et al., 2009; Baarendse et al., 2013). Subsequently, the rats were trained in one of two rGT versions (GT1 or GT2), both with three choices, termed safe, optimal and risky (Table 1). Since choice for one of the response options (choice P3) in the 4 choice rGT has been shown not to change over time (Zeeb et al., 2009; Baarendse et al., 2013), we modified the task by excluding this response option. The rGT versions were designed to have one optimal choice

and two sub-optimal choice options (i.e., safe and risky). Feedback about the contingencies of the three different choices was provided in the form of the number of sucrose pellets received, the probability of receiving the reward, and punishment magnitude. Punishment consisted of a time-out period, which reduced the available session time and resulted in a lower net gain. In GT1, the net gain was different between all three choices, the gain being highest for the optimal choice > safe choice (72% of optimal) > risky choice (24% of optimal). In order to investigate the effect of reward size and probability in the risky choice separately from the lower net gain, we designed GT2 such that the punishment time out of the risky choice was relatively short, making the net gain of the risky choice (66% of optimal) more comparable to the net gain of the safe choice (76% of optimal). Thus, in both rGT versions, the risky choice provides the animal with a high number of sucrose pellets in case the animal is rewarded. However, the negative consequence, i.e. the punishment time-out, is higher in GT1 compared to GT2, resulting in a threefold lower net gain in GT1. Moreover, in GT2, the safe choice consists of a non-probabilistic choice (1 pellet with 100% reward probability) resulting in a larger difference in probability between the safe and optimal choice. The spatial location of the three choices was counterbalanced across subjects in the two rGT versions and remained the same for each animal over the course of the experiment. The middle three response holes of the five hole array were used. The order of the options from left to right in version A was: risky – optimal - safe and in version B: safe – risky – optimal. The animals were tested for 30 min per session, 5-6 days/week.

The task design and trial structure were as previously described (Zeeb et al., 2009; Baarendse et al., 2013). Briefly, a trial started with a 5 sec ITI, followed by illumination of one (during forced choice sessions) or three (during free choice sessions) stimulus lights for 10 sec. A response in an illuminated hole turned off the stimulus light(s), and led to either reward or punishment. During forced choice sessions, only one stimulus light was illuminated in a pseudorandom order to ensure that all animals had equal experience with the contingencies of the three choice options. A nose poke response in a non-illuminated aperture (i.e. incorrect response), a failure to respond within 10 sec (i.e. omission), or a premature response resulted in a 5 sec time-out period, signaled by illumination of the house light. Nose poke responses in the stimulus holes during punishment were scored as perseverative responses, but these had no scheduled consequences.

Table 1

Choice contingencies of the safe, optimal, or risky choice in rGT version 1 (GT1) and rGT version 2 (GT2)

rGT	Choice	#Pellets	Chance %	Punishment time-out (sec)	Theoretical gain	Ratio of long-term gain
GT1	Safe	1	90	5	294	0.72
	Optimal	2	80	10	411	-
	Risky	4	40	40	99	0.24
GT2	Safe	1	100	0	360	0.76
	Optimal	3	70	10	473	-
	Risky	6	33	10	310	0.66

No. of pellets: the number of sucrose pellets the animal receives when rewarded; chance %: the chance to receive a reward; duration of the punishment time-out; theoretical gain: number of pellets that would be obtained if this option was chosen exclusively, which provides an objective value for each response option. Theoretical gain is calculated as $((1800 \text{ s of session duration} / ((5 \text{ s ITI} + (\text{chance of punishment} \times \text{punishment time-out in seconds}))) \times (\text{chance of reward} \times \text{number of pellets}))$. Ratio of long-term gain: number of sucrose pellets, which could be theoretically obtained from that response option, divided by the theoretical number of sucrose pellets of the optimal choice.

Experiments

Experiment 1 was designed to compare the development of choice behaviour over sessions in the two rGT versions and to assess the effects of acute alcohol treatment on stable responding in both rGT versions. The rats were first tested for five free choice sessions, to investigate if the animals would develop a choice preference by spontaneously sampling the choices. As we noted that not all rats explored all three choices, we subsequently introduced five forced choice sessions to ensure that all animals had equal experience with the contingencies of the three options before they were tested for another twenty-one free choice sessions. Subsequently, the animals were treated with five doses of alcohol (0 - 0.8 g/kg) prior to rGT testing.

In Experiment 2, we determined the effect of treatment with a moderate dose of alcohol (0.6 g/kg) on the acquisition of GT1 and GT2. The dose of 0.6 g/kg was chosen because it was the lowest dose that showed significant effects on behaviour in the rGT without causing profound depressant effects in Experiment 1 (see Table 2). This experiment started with five forced choice sessions to ensure that the effects of alcohol or vehicle on the development of choice behaviour were not skewed by rats not knowing all three choice contingencies. Subsequently, the animals were subjected to fifteen free choice

sessions prior to which they received alcohol or vehicle injections. Thereafter, the animals were left undisturbed for two days to ensure complete washout of the alcohol. Subsequently, the animals were trained for ten more sessions without any treatment. Finally, three days after the last non-treatment session, all animals were challenged with five doses of alcohol (0 - 1.0 g/kg).

Drugs

Alcohol (99.5%, Klinipath, The Netherlands) was diluted with saline to a concentration of 10% alcohol (v/v). Injection volumes were adjusted to the body weight and the required dose of alcohol. The alcohol solutions were pre-heated to 32°C by placing the syringes on a heating pad to prevent possible decreases in body temperature after injection of substantial volumes, particularly at the highest alcohol doses. Vehicle (i.e., saline) injection volumes were equivalent to the volume required for an injection of the 0.6 g/kg alcohol dose. Drug solutions were freshly prepared daily and administered intraperitoneally (IP) 15 min prior to behavioural testing. Prior to injections, the rats were habituated twice to the injection procedure. The different alcohol challenge doses were administered according to a Latin square design with a three day cycle for each dose; i.e. a baseline session, followed by the alcohol treatment session and a washout day during which the animals remained in their home cage. During the fifteen acquisition sessions with alcohol or vehicle treatment in Experiment 2, all animals received injections prior to rGT training on Monday, Tuesday, Thursday and Friday. The animals remained in their home cage on Wednesdays and during the weekend in order to minimize irritation of the peritoneal cavity which is potentially caused by repeated injection of the alcohol solution.

Blood alcohol levels

In a separate group of animals, we determined blood alcohol levels (BAL) after an IP injection of 0.6 and 1.2 g/kg alcohol. Thirty min after injection, blood samples were collected from the lateral tail vein in EDTA coated capillary tubes (Sarstedt, Numbrecht, Germany) and immediately stored on ice. In addition, to explore the metabolism of alcohol over time, animals were treated with 0.6 g/kg for blood sampling at 5, 10, 15, 30, 60 and 120 min after injection. Blood samples were spun at 3000 rpm for 20 min (at 4°C) and plasma was stored at -20°C until blood alcohol analysis. BAL (mg/dl) were determined using an NAD-ADH reagent kit (Sigma-Aldrich, Schnellendorf, Germany) and a standard curve for quantitation.

Data analysis

All statistical analyses were conducted using SPSS 20.0 for Windows. Statistical analyses were performed using one-, two-, and three-way repeated-measures ANOVA's with choice, session and dose as within-subject variables and treatment group (alcohol or vehicle) and/or rGT version (GT1 and GT2) as between-subject variables. Trial-by-trial analysis was performed to assess the shifts in choice behaviour between subsequent trials. Depending on whether the animal received a reward or a punishment, it can make the same choice on the subsequent trial or shift towards another choice option, resulting in 4 different possibilities (i.e. win-stay, win-shift, lose-stay, lose-shift) per choice option (safe, optimal or risky), resulting in 12 different possibilities in total. Because not all of these possibilities occurred in each session for each individual animal, the data of the trial-by-trial analyses per choice were averaged over 5 sessions to obtain reliable data points for each animal, calculated as a percentage. For example, lose-shift behaviour after a risky choice was calculated by dividing the number of shifts upon a loss on the risky choice by the total number of losses on the risky choice, multiplied by 100. In addition, we analyzed the percentage of shifts towards another choice option after rewarded and punished trials, regardless of which option was chosen. The total percentage of win-shifts was calculated by dividing the number of win-shifts by the total number of wins during the session, multiplied by 100; the total percentage of lose-shifts was calculated analogously. The data was tested for normality with a Kolmogorov-Smirnov test. When data were not normally distributed, data was square root transformed for count data (e.g. premature responses) and log transformed for latency data, which resulted in normal distribution of the data in all cases. Choice behaviour data and the trial-by-trial data, expressed as percentages, were arcsine transformed. Mauchly's test of sphericity was used to test if variances of the differences between treatment levels were equal. If the assumption of sphericity was violated, degrees of freedom were corrected using Huynh-Feldt estimates of sphericity to more conservative values. Corrected degrees of freedom are presented rounded to the nearest integer. Paired t-tests were used as *post hoc* analyses to compare a drug dose with vehicle. Behavioural parameters of both rGT versions were pooled in case the rGT version did not interact with alcohol treatment effects (i.e., absence of rGT version*dose/session interaction). The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean \pm SEM. Graphs were made using Graphpad Prism 6.

RESULTS

Choice behaviour in GT1 and GT2

Over the course of the first five free choice sessions in Experiment 1, animals changed their choice behaviour in a version-dependent manner ($F_{(8,165) \text{ GT} \times \text{choice} \times \text{session}} = 2.33, p < 0.05$) (Fig. 1). Animals in GT1 (Fig. 1a) initially preferred both the safe and optimal choice above the risky choice ($p < 0.05$), whereas animals in GT2 (Fig. 1b) preferred the safe choice above the optimal and risky choice ($p < 0.05$). Analyses of the 21 free choice sessions that followed the five forced choice sessions indicated that animals developed a preference for the optimal choice ($F_{(16,465) \text{ choice} \times \text{session}} = 9.50, p < 0.05$), which can be expected on the basis of the theoretical gain that is highest for the optimal choice. The overall choice pattern in these 21 free choice sessions did not differ between the two rGT versions ($F_{(16,465) \text{ choice} \times \text{GT} \times \text{session}} = 0.64, \text{NS}$) (Fig. 1).

Blood alcohol levels

Thirty min after injection of 0.6 or 1.2 g/kg alcohol, BAL amounted to 77 ± 3 and 179 ± 1 mg/dl, respectively, which is near and above the legal alcohol limit of 80 mg/dl (Fig. 2a). Investigation of the BAL over time after injection of 0.6 g/kg alcohol, showed maximal BAL with least variation between animals at 15-30 min post-injection. BAL approached zero after 120 min (Fig. 2b).

Acute effects of alcohol on rGT performance

Alcohol changed choice behaviour ($F_{(6,173) \text{ choice} \times \text{dose}} = 2.33, p < 0.05$) independent of rGT version ($F_{(6,173) \text{ choice} \times \text{dose} \times \text{GT}} = 0.95, \text{NS}$) (Fig. 3). Alcohol reduced the percentage of optimal choices ($F_{(3,93) \text{ dose}} = 3.10, p < 0.05$) and *post hoc* analyses revealed a decrease in optimal choices at the doses of 0.2 g/kg, 0.4 g/kg and 0.8 g/kg ($p < 0.05$). Alcohol did not affect the percentage of safe choices ($F_{(2,75) \text{ dose}} = 1.93, \text{NS}$), nor did it alter the percentage of risky choices ($F_{(4,124) \text{ dose}} = 1.82, \text{NS}$) (Fig. 3). Treatment with alcohol reduced the number of choices (0.8 g/kg), premature (0.4 – 0.8 g/kg) and perseverative responses (0.6 – 0.8 g/kg) and increased the number of omissions (0.6 – 0.8 g/kg), choice latencies (0.6 – 0.8 g/kg) and collect latencies (0.6 – 0.8 g/kg) (Table 2).

Effect of alcohol on rGT acquisition

In Experiment 2, rats were repeatedly treated with alcohol (0.6 g/kg) or vehicle prior to the first fifteen free-choice acquisition sessions in the rGT. Analysis of the choice behaviour in these sessions revealed a significant interaction between

Figure 1

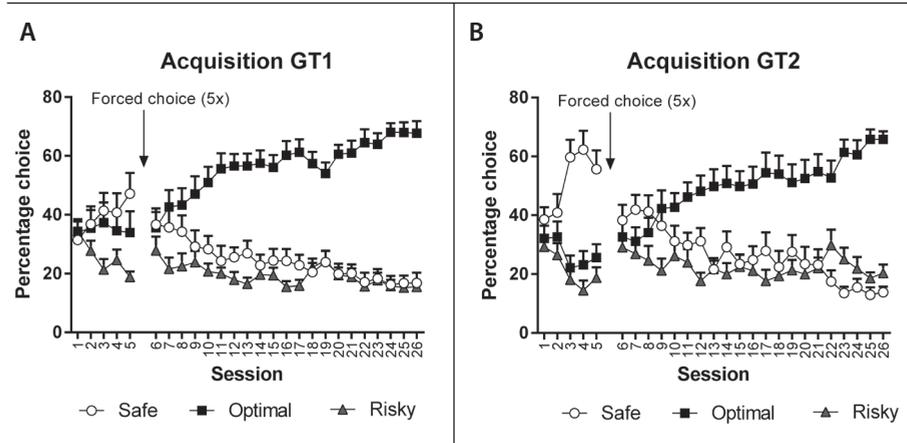


Figure 1. Acquisition of choice behaviour in GT1 (A) and GT2 (B). Choice behaviour during the first five free-choice sessions differed between the two gambling tasks, in that rats showed a higher preference for the safe choice in GT2. Moreover, while animals in GT1 preferred the safe and optimal choice above the risky choice, animals in GT2 preferred the safe choice above the optimal and risky choice. Following five forced-choice sessions, rats in both rGT versions developed a preference for the optimal choice, which became more pronounced with increased training. Data are shown as the mean percentage choice+SEM.

Figure 2

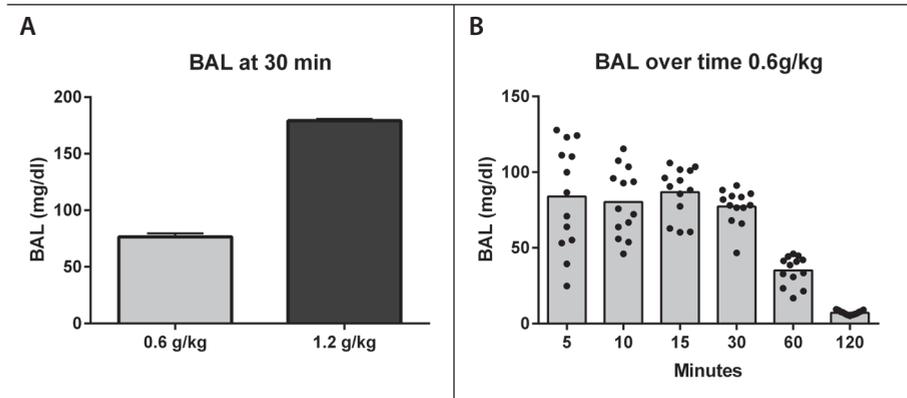


Figure 2. Blood alcohol level (BAL) after an IP alcohol injection. The BAL was assessed in a separate group of animals at 30 min after IP injection of 0.6 and 1.2 g/kg alcohol (A). Investigation of the BAL over time after an injection with 0.6 g/kg alcohol showed maximal BAL with least variation at 15–30 min postinjection (B). Data are shown as the mean+SEM (a) or as mean and individual data points (B).

Figure 3

Effect of acute alcohol on choice behaviour

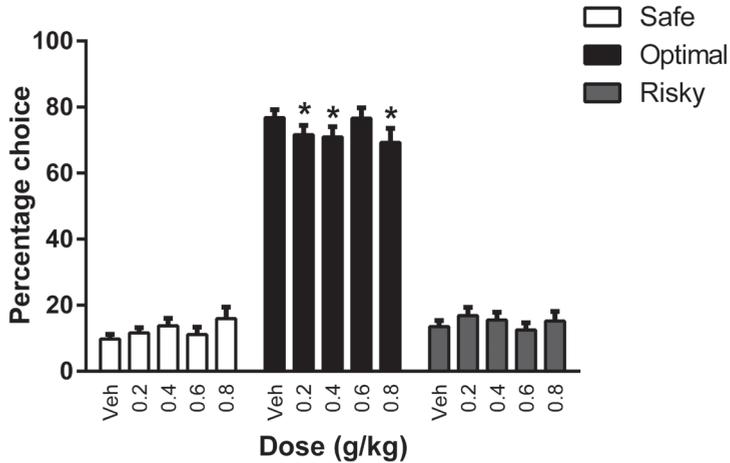


Figure 3. The effect of acute alcohol treatment on stable choice behaviour in the rGT (experiment 1). Alcohol significantly reduced the percentage of optimal choices. This effect of alcohol was independent of GT version. Hence, the data from both rGT versions were collapsed. Data are shown as the mean percentage choice±SEM. *Different from vehicle treatment (*post hoc* paired t test, $p < 0.05$).

Table 2

Effects of alcohol on behaviour in the rGT

Variable	Dose effect	Vehicle	0.2 g/kg	0.4 g/kg	0.6 g/kg	0.8 g/kg
Number of choices	$F_{(3,100)} = 6.362, p < 0.05$	65.75±3.29	63.00±2.85	60.34±3.00	61.22±2.67	50.13*±4.13
Premature responses	$F_{(4,120)} = 25.349, p < 0.05$	18.47±1.39	17.53±1.46	15.53*±1.19	9.38*±1.21	6.28*±0.92
PersevP	$F_{(4,120)} = 15.017, p < 0.05$	21.28±2.03	22.00±2.46	17.44±2.55	15.16*±2.15	9.28*±1.60
Omissions	$F_{(3,99)} = 9.495, p < 0.05$	24.69±4.01	26.97±3.43	26.50±3.77	33.72*±3.38	46.06*±5.03
Choice latency	$F_{(4,120)} = 10.998, p < 0.05$	3.53±0.19	3.50±0.19	3.59±0.17	4.01*±0.18	4.16*±0.13
Collect latency	$F_{(4,120)} = 3.553, p < 0.05$	2.12±0.15	2.48±0.24	2.69±0.31	2.83*±0.27	2.82*±0.23

Data are presented as means±SEM. F values represent the main effect of alcohol dose (repeated-measures ANOVA). *Post hoc* analyses were performed by paired t tests, comparing alcohol doses to vehicle. Data from both rGT versions were pooled because the rGT version did not interact with the effects of alcohol (i.e., there were no GT×dose interactions). *Different from vehicle, $p < 0.05$. PersevP perseverative responses during a punishment trial.

Figure 4

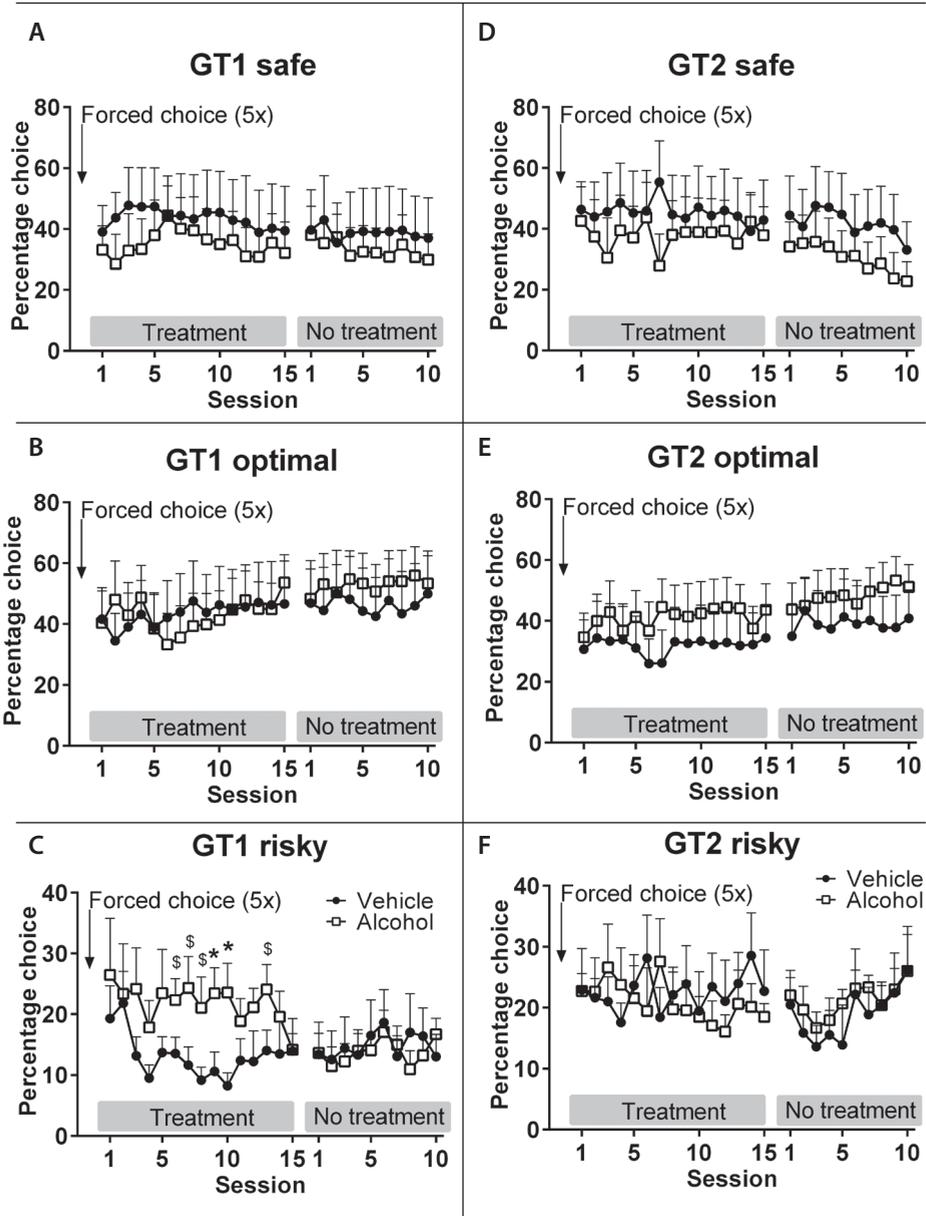


Figure 4. The effects of repeated alcohol (0.6 g/kg) or vehicle administration on the acquisition of choice behaviour in the rGT, followed by ten sessions without treatment. Repeated alcohol administration during rGT acquisition increased risky choices in GT1 (c). Data are shown as the mean percentage choice+SEM. *Different from vehicle-treated animals (*post hoc* Student's *t* test, $p < 0.05$), \$ $p < 0.062$ compared to vehicle-treated rats.

treatment, rGT version and session ($F_{(11,294)} \text{ treatment} \times \text{GT} \times \text{session} = 2.00$, $p < 0.05$) (Fig. 4). Separate analyses per rGT version showed an effect of alcohol on choice pattern in GT1 (Fig. 4a-c) ($F_{(7,100)} \text{ treatment} \times \text{session} = 2.87$, $p < 0.05$), but not in GT2 (Fig. 4d-f) ($F_{(14,196)} \text{ treatment} \times \text{session} = 0.75$, NS). To further explore the effects of alcohol on choice behaviour in GT1, we performed analyses per choice and found that alcohol-treated animals showed a higher percentage of risky choices ($F_{(1,14)} \text{ treatment} = 5.74$, $p < 0.05$), but made a comparable percentage of safe ($F_{(1,14)} \text{ treatment} = 0.25$, NS) and optimal choices ($F_{(1,14)} \text{ treatment} = 0.01$, NS) compared to vehicle-treated animals. *Post hoc* analyses of the risky choice data showed that alcohol-treated animals made more risky choices during sessions 6-10 of alcohol treatment in comparison to vehicle-treated animals (Fig. 4c).

Subsequently, to assess the persistence of the effects of alcohol treatment, choice behaviour was determined for another 10 sessions after cessation of alcohol treatment. In contrast to the treatment period, we did not find an interaction between treatment, rGT version and session on choice behaviour during this stage of the experiment ($F_{(8,364)} \text{ treatment} \times \text{GT} \times \text{session} = 1.17$, NS) (Fig. 4). Importantly, in these 10 sessions without treatment, there was a significant interaction between choice and session ($F_{(13,592)} \text{ choice} \times \text{session} = 3.22$, $p < 0.05$), which was independent of treatment ($F_{(13,592)} \text{ choice} \times \text{session} \times \text{treatment} = 0.71$, NS) or rGT version ($F_{(13,592)} \text{ choice} \times \text{session} \times \text{GT} = 0.86$, NS). Subsequent analyses per choice indicated that the percentage of safe choices decreased over sessions ($F_{(7,205)} \text{ session} = 4.18$, $p < 0.05$), whereas the percentage of optimal choices did not change ($F_{(7,198)} \text{ session} = 1.14$, NS). The percentage of risky choices changed over sessions ($F_{(8,224)} \text{ session} = 2.52$, $p < 0.05$), but *post hoc* analysis revealed no differences with the first post-treatment session.

The pattern of effects on choice behaviour during alcohol treatment, in which alcohol increases risky choice only in the rGT version in which risky choice entailed greater punishment, suggests that alcohol reduces the ability to adjust behaviour after punishment. To further investigate this interpretation, we performed trial-by-trial analyses of choice behaviour to assess whether alcohol-treated animals respond differentially to positive (win) or negative feedback (loss), by shifting towards a different choice option on the next trial. We observed an interaction between treatment and rGT version in the percentage of lose-shifts following a risky choice ($F_{(1,28)} \text{ treatment} \times \text{GT} = 4.37$, $p < 0.05$) as well as a main effect of rGT version ($F_{(1,28)} \text{ GT} = 5.34$, $p < 0.05$) (Fig. 5a-b). Subsequent analyses indicated that alcohol-treated animals in GT1 tended

Figure 5

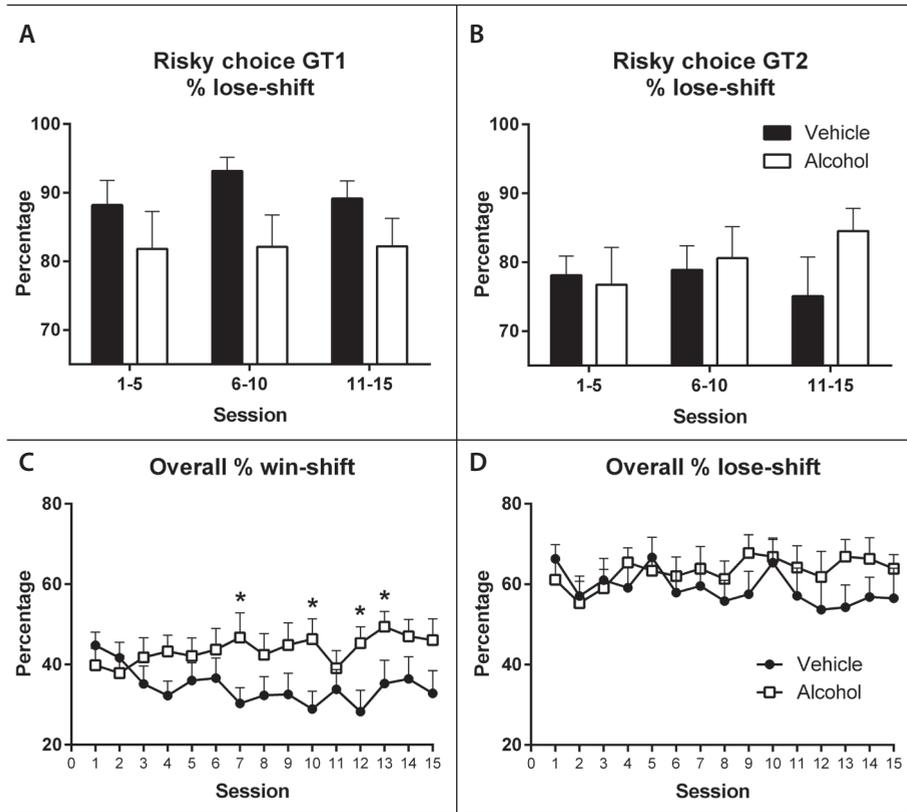


Figure 5. The effects of repeated alcohol (0.6 g/kg) or vehicle administration on the percentage of shifts toward another choice after being rewarded or punished. Repeated alcohol administration during rGT acquisition tended to decrease lose-shift behaviour after punishment on the risky choice in GT1 (A), but not in GT2 (B). Regardless of choice or GT version, vehicle-treated animals showed reduced win-shift behaviour over sessions, whereas alcohol-treated animals did not (C). The percentage of lose-shifts was not different over sessions or between treatment groups (D). Data are presented in bins of five sessions (A, B) or sessions (C, D) and are shown as the mean+SEM percentage of lose shift and win-shift behaviour. *Different from vehicle-treated animals (*post hoc* Student's t test, $p < 0.05$).

to perform less lose-shifts after punishment on the risky choice option ($F_{(1,14) \text{ treatment}} = 4.10, p = 0.062$) (Fig. 5a), whereas alcohol did not affect lose-shift behaviour in GT2 ($F_{(1,14) \text{ treatment}} = 0.88, \text{NS}$) (Fig. 5b). The trial-by-trial analyses after feedback for the safe and optimal choice did not reveal significant differences between treatment groups or rGT version (data not shown). Analyses of the total percentage of shifts after a reward, regardless of which option was chosen, showed an interaction between treatment and

Table 3

Effects of repeated treatment with 0.6 g/kg alcohol or vehicle during acquisition (15 sessions), followed by ten sessions without treatment on behaviour in the rGT.

Variable	Treatment Session 1-15					Post-treatment Session 16-25				
	Effect	Treatment	Effect	Treatment	Effect	Treatment	Effect	Treatment	Effect	Treatment
Number of choices GT1	$F_{(1,28)} = 9.115, p < 0.05$	Vehicle	$F_{(1,28)} = 0.690, NS$	Vehicle	$F_{(1,28)} = 5.577, p < 0.05$	Vehicle	$F_{(1,28)} = 0.426, NS$	Vehicle	$F_{(1,28)} = 5.577, p < 0.05$	Vehicle
		Alcohol		Alcohol		Alcohol		Alcohol		Alcohol
Mean	74.20±5.50	74.73±6.86	64.65±4.19	77.78±5.93	74.73±5.22	68.25±4.71	77.63±4.77	78.41±5.13	81.13±3.25	81.52±2.76
SD	6.65±4.19	6.25±3.87	5.56±4.03	5.18±4.95	5.58±5.09	5.78±4.71	5.56±4.03	5.18±4.95	5.58±5.09	5.78±4.71
Number of choices GT2	$F_{(1,28)} = 2.014, NS$	Vehicle	$F_{(2,45)} = 3.552, p < 0.05$	Vehicle	$F_{(1,28)} = 0.003, NS$	Vehicle	$F_{(1,28)} = 0.472, NS$	Vehicle	$F_{(1,28)} = 0.003, NS$	Vehicle
		Alcohol		Alcohol		Alcohol		Alcohol		Alcohol
Mean	11.71±1.58	11.71±1.58	13.76±1.50	12.89±2.26	11.46±1.54	11.91±1.59	11.71±1.58	11.71±1.58	11.46±1.54	11.91±1.59
SD	10.48±1.67	10.48±1.67	13.61±2.72	19.61*±3.85	11.46±1.54	11.91±1.59	10.48±1.67	10.48±1.67	11.46±1.54	11.91±1.59
Premature responses	$F_{(1,28)} = 20.825, p < 0.05$	Vehicle	$F_{(1,14)} = 6.695, p < 0.05$	Vehicle	$F_{(1,28)} = 6.695, p < 0.05$	Vehicle	$F_{(1,28)} = 2.415, NS$	Vehicle	$F_{(1,28)} = 6.695, p < 0.05$	Vehicle
		Alcohol		Alcohol		Alcohol		Alcohol		Alcohol
Mean	40.18±7.48	40.18±7.48	26.23±3.67	30.40*±5.06	23.15±3.21	23.38±2.70	40.18±7.48	40.18±7.48	23.15±3.21	23.38±2.70
SD	68.25±18.30	68.25±18.30	59.25#±8.33	41.13±3.30	33.00±3.16	36.00±4.38	68.25±18.30	68.25±18.30	33.00±3.16	36.00±4.38
Perseverp GT1	$F_{(1,28)} = 6.052, p < 0.05$	Vehicle	$F_{(1,14)} = 0.537, NS$	Vehicle	$F_{(1,28)} = 3.263, p = 0.082$	Vehicle	$F_{(1,28)} = 0.217, NS$	Vehicle	$F_{(1,28)} = 3.263, p = 0.082$	Vehicle
		Alcohol		Alcohol		Alcohol		Alcohol		Alcohol
Mean	24.88±4.47	24.88±4.47	20.50±3.57	22.25±4.70	17.25±3.51	22.86±3.06	24.88±4.47	24.88±4.47	17.25±3.51	22.86±3.06
SD	24.50±5.17	24.50±5.17	16.50±3.22	16.25±3.02	18.25±3.39	23.88±6.07	24.50±5.17	24.50±5.17	18.25±3.39	23.88±6.07
Perseverp GT2	$F_{(1,28)} = 6.624, p < 0.05$	Vehicle	$F_{(1,28)} = 0.053, NS$	Vehicle	$F_{(1,28)} = 3.263, p = 0.082$	Vehicle	$F_{(1,28)} = 0.217, NS$	Vehicle	$F_{(1,28)} = 3.263, p = 0.082$	Vehicle
		Alcohol		Alcohol		Alcohol		Alcohol		Alcohol
Mean	18.80±3.37	18.80±3.37	18.55±3.51	14.33±2.89	19.43±3.66	20.08±3.66	18.80±3.37	18.80±3.37	19.43±3.66	20.08±3.66
SD	18.79±7.14	18.79±7.14	12.13±3.46	15.90±4.57	17.18±3.80	16.80±3.62	18.79±7.14	18.79±7.14	17.18±3.80	16.80±3.62
Omissions GT1	$F_{(1,28)} = 6.624, p < 0.05$	Vehicle	$F_{(1,28)} = 1.580, NS$	Vehicle	$F_{(1,28)} = 0.821, NS$	Vehicle	$F_{(1,28)} = 1.204, NS$	Vehicle	$F_{(1,28)} = 0.821, NS$	Vehicle
		Alcohol		Alcohol		Alcohol		Alcohol		Alcohol
Mean	32.38±6.69	32.38±6.69	24.28±5.82	28.85±4.82	25.05±4.75	23.10±5.13	32.38±6.69	32.38±6.69	25.05±4.75	23.10±5.13
SD	26.18±5.88	26.18±5.88	28.70±5.12	36.13±6.81	37.91±6.60	31.43±7.67	26.18±5.88	26.18±5.88	37.91±6.60	31.43±7.67
Omissions GT2	$F_{(1,28)} = 2.482, NS$	Vehicle	$F_{(1,28)} = 1.580, NS$	Vehicle	$F_{(1,28)} = 0.821, NS$	Vehicle	$F_{(1,28)} = 1.204, NS$	Vehicle	$F_{(1,28)} = 0.821, NS$	Vehicle
		Alcohol		Alcohol		Alcohol		Alcohol		Alcohol
Mean	3.49±0.16	3.49±0.16	3.20±0.17	3.10±0.19	3.37±0.19	3.35±0.19	3.49±0.16	3.49±0.16	3.37±0.19	3.35±0.19
SD	3.31±0.20	3.31±0.20	2.90±0.19	2.67±0.18	3.09±0.19	3.04±0.23	3.31±0.20	3.31±0.20	3.09±0.19	3.04±0.23

Table 3

Continued

Variable	Treatment Session 1-15				Post-treatment Session 16-25					
	Effect rGT	Effect Treatment	Treatment	1-5	6-10	11-15	Effect rGT	Effect Treatment	Treatment	
Collect latency GT1	F _{(1,27) rGT} = 16.942, p < 0.05	F _{(2,28) treatment rGT x session} = 2.891, p = 0.079	Vehicle	3.16 ± 0.33	2.58 ± 0.26	2.41* ± 0.24	F _{(1,28) rGT} = 3.622, p = 0.067	Vehicle	2.78 ± 0.40	2.57 ± 0.23
			Alcohol	2.74 ± 0.44	2.48 ± 0.28	1.80 ± 0.13		Alcohol	2.27 ± 0.40	2.40 ± 0.30
Collect latency GT2	F _{(1,54) treatment x rGT x session} = 5.500, p < 0.05	F _{(2,28) treatment rGT x session} = 2.733, p = 0.084	Vehicle	3.14 ± 0.38	3.75 ± 0.73	4.86 ± 0.94		Vehicle	2.61 ± 0.51	3.22 ± 0.68
			Alcohol	4.00 ± 0.54	6.96 ± 2.63	5.19 ± 2.06	Alcohol	2.90 ± 0.35	4.12 ± 1.21	

Data are presented as means ± SEM in bins of five sessions during the treatment (sessions 1–15) and posttreatment period (sessions 16–25). Data from both rGT versions were pooled in case the rGT version did not interact with alcohol treatment (i.e., no GT × treatment interaction). Separate analyses were performed for the treatment and posttreatment period *p < 0.05, different from the first session bin; #p < 0.05, different between treatments within the session bin. Persevp: perseverative responses during a punishment trial, NS: not significant.

session ($F_{(9,247) \text{ session} \times \text{treatment}} = 2.69, p < 0.05$), independent of rGT version ($F_{(9,247) \text{ session} \times \text{treatment} \times \text{GT}} = 0.76, \text{NS}$), indicating that the vehicle-treated animals showed a reduction of win-shifts over sessions ($F_{(14,196) \text{ session}} = 2.95, p < 0.05$), whereas the alcohol treated animals did not ($F_{(6,79) \text{ session}} = 0.95, \text{NS}$) (Fig. 5c). Lose-shift behaviour, i.e. percentage of shifts after a punishment, regardless of which option was chosen, was not altered by alcohol treatment ($F_{(12,340) \text{ session} \times \text{treatment}} = 0.71, \text{NS}$) (Fig. 5d).

Treatment with alcohol during rGT acquisition increased premature responding over sessions ($F_{(2,44) \text{ treatment} \times \text{session}} = 3.55, p < 0.05$), which was independent of rGT version ($F_{(2,44) \text{ treatment} \times \text{session} \times \text{GT}} = 1.15, \text{NS}$) (Table 3). In addition, alcohol-treated animals made more perseverative responses during punishment trials compared to vehicle-treated animals ($F_{(1,28) \text{ treatment} \times \text{GT}} = 6.05, p < 0.05$), which was apparent in GT1 ($F_{(1,14) \text{ treatment}} = 6.86, p < 0.05$) but not in GT2 ($F_{(1,14) \text{ treatment}} = 0.54, \text{NS}$). After alcohol treatment was discontinued, alcohol-pretreated animals no longer differed from vehicle-pretreated animals in premature or perseverative responding ($F_{(1,28) \text{ treatment}} = 0.47, \text{NS}$; $F_{(1,28) \text{ treatment}} = 1.12, \text{NS}$, respectively). The numbers of choices and omissions were not different between treatment groups ($F_{(1,28) \text{ treatment}} = 0.69, \text{NS}$; $F_{(1,28) \text{ treatment}} = 0.05, \text{NS}$, respectively), but animals in GT1 made significantly more choices and less omissions compared to GT2, during alcohol treatment ($F_{(1,28) \text{ GT}} = 9.12, p < 0.05$; $F_{(1,28) \text{ GT}} = 6.62, p < 0.05$, respectively) and post-treatment ($F_{(1,28) \text{ GT}} = 3.58, p < 0.05$; $F_{(1,28) \text{ GT}} = 3.26, p = 0.08$, respectively). Choice latency declined over sessions during the treatment period ($F_{(2,56) \text{ session}} = 25.09, p < 0.05$), independent of treatment group ($F_{(2,56) \text{ session} \times \text{treatment}} = 1.32, \text{NS}$) or rGT version ($F_{(2,56) \text{ session} \times \text{GT}} = 0.29, \text{NS}$). Collect latency changed over sessions during the treatment period, which was different for alcohol- and vehicle-treated animals as well as for the rGT versions ($F_{(2,54) \text{ treatment} \times \text{GT} \times \text{session}} = 5.50, p < 0.05$). *Post hoc* analyses indicated that rats in GT1 were faster in their reward collection compared to rats in GT2 ($F_{(1,27) \text{ GT}} = 16.94, p < 0.05$), which was independent from treatment ($F_{(1,27) \text{ treatment} \times \text{GT}} = 0.00, \text{NS}$). There were no differences in choice and reward collection latencies in the post treatment period between the treatment groups ($F_{(1,28) \text{ treatment}} = 1.20, \text{NS}$; $F_{(1,28) \text{ treatment}} = 0.52, \text{NS}$, respectively) (Table 3).

Effects of alcohol challenges on rGT performance after alcohol treatment during rGT acquisition

Subsequent to the rGT sessions without alcohol or vehicle treatment, the animals were challenged with alcohol (0.0-1.0 g/kg) to determine whether

Figure 6

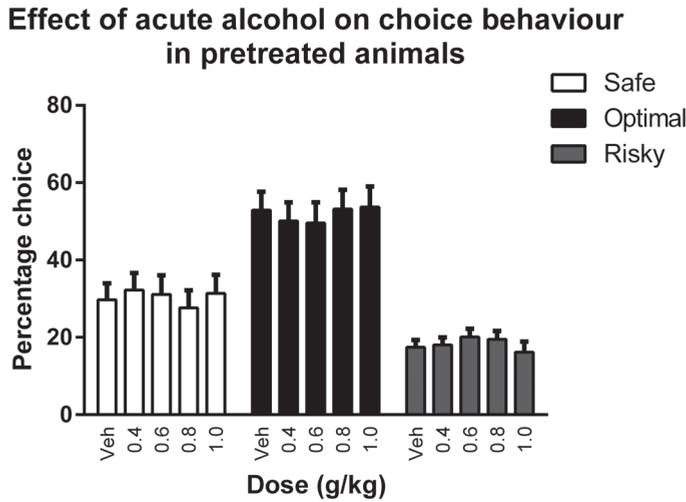


Figure 6. The effects of acute alcohol treatment on stable choice behaviour in the rGT in experiment 2. Alcohol had no effects on choice behaviour, irrespective of pretreatment (alcohol or vehicle) or rGT version. Hence, the data from both pretreatment groups and rGT versions were collapsed. Data are shown as the mean percentage choice+SEM.

alcohol pre-exposure alters the effects of alcohol on rGT performance. Acute alcohol challenges did not change choice behaviour ($F_{(7,190) \text{ dose} \times \text{choice}} = 0.88$, NS), irrespective of whether pretreatment group or rGT version were included as factors ($F_{(7,190) \text{ dose} \times \text{choice} \times \text{pretreatment}} = 0.54$, NS; $F_{(7,190) \text{ dose} \times \text{choice} \times \text{GT}} = 0.93$, NS, respectively) (Fig. 6).

Alcohol challenges differentially altered behaviour in the rGT in alcohol-versus vehicle-pretreated animals (Fig. 7). In animals pretreated with alcohol, alcohol treatment had a biphasic effect, i.e. an increase followed by a decrease as the alcohol dose increased, on the total number of choices, premature and perseverative responses, whereas alcohol decreased these parameters in vehicle-pretreated animals ($F_{(4,120) \text{ dose} \times \text{pretreatment}} = 4.94$, $p < 0.05$; $F_{(3,105) \text{ dose} \times \text{pretreatment}} = 2.70$, $p < 0.05$; $F_{(4,109) \text{ dose} \times \text{pretreatment}} = 3.76$, $p < 0.05$, respectively) (Fig. 7a-c). Alcohol also had a biphasic effect, i.e. a decrease followed by an increase as the alcohol dose increased, on omissions and choice latency in alcohol-pretreated animals, whereas both were increased by alcohol in vehicle-pretreated animals ($F_{(3,96) \text{ dose} \times \text{pretreatment}} = 3.14$, $p < 0.05$; $F_{(4,120) \text{ dose} \times \text{pretreatment}} = 4.24$, $p < 0.05$, respectively) (Fig. 7d,e). Collect latency was not affected by alcohol challenges ($F_{(4,120) \text{ dose} \times \text{pretreatment}} = 0.35$, NS) (Fig. 7f).

Figure 7

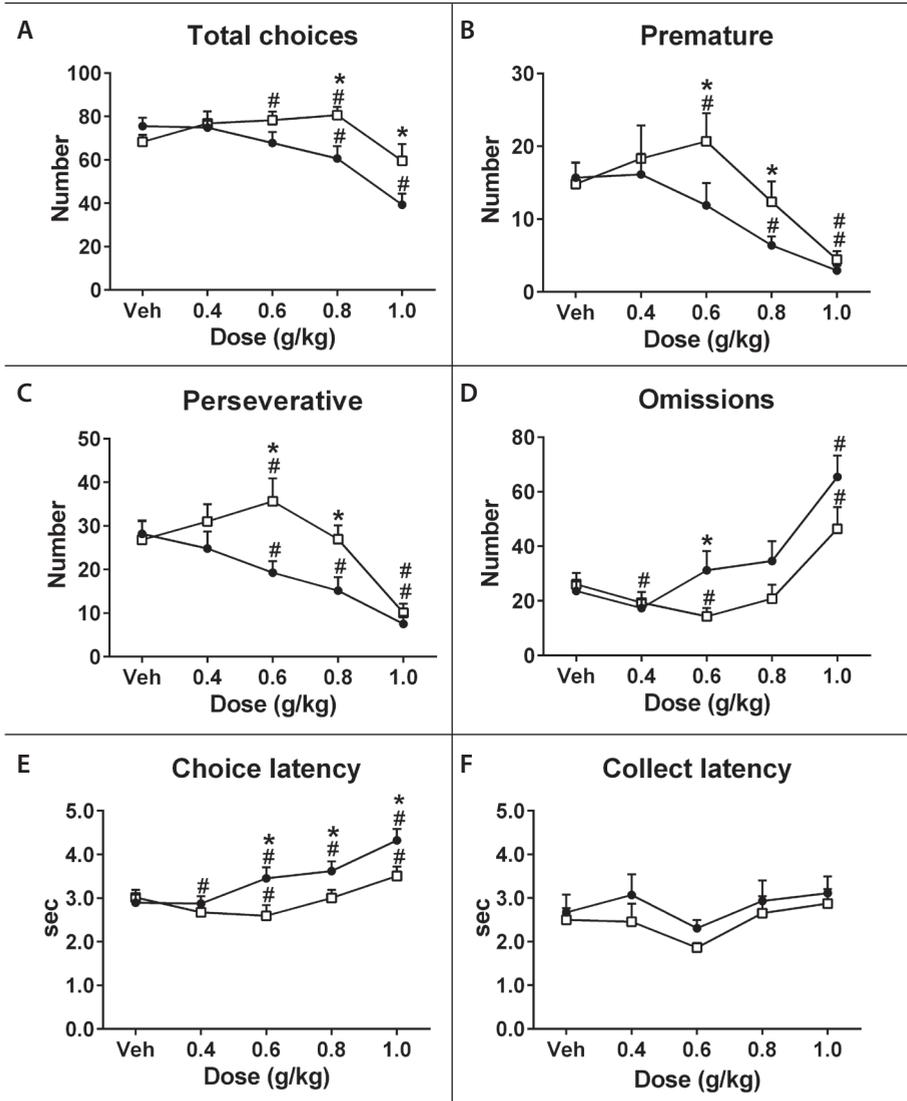


Figure 7. The effects of alcohol on behaviour in the rGT during alcohol challenge sessions in alcohol-pretreated and vehicle-pretreated animals. In vehicle-pretreated animals, alcohol dose-dependently reduced total choices (A), premature responses (B), and perseverative responses (C), and increased omissions (D) and choice latency (E). In contrast, in alcohol-pretreated animals, alcohol had a biphasic effect on total choices, premature responses, perseverative responses (increase followed by decrease as the alcohol dose increased), omissions and choice latencies (decrease followed by increase as the alcohol dose increased). Alcohol pretreatment and alcohol challenges did not affect collect latency (F). The alcohol challenges had similar effects in GT1 and GT2, and the data were therefore pooled. Data are shown as mean+SEM. *Difference between pretreatment groups (*post hoc* Student's t test, $p < 0.05$); #different from vehicle challenge (*post hoc* paired t test, $p < 0.05$).

DISCUSSION

We investigated the effects of alcohol on acquisition and performance in two versions of the rGT that differ in the net gain and the punishment magnitude associated with the different response options. In rats that showed stable rGT performance, alcohol modestly altered choice behaviour, and had behavioural suppressant effects on responding. In contrast, repeated alcohol administration during task acquisition enhanced risk taking in the rGT version in which risky choices entailed long punishment delays (i.e. GT1). Trial-by-trial analyses showed that alcohol-treated rats tended to show less lose-shift behaviour after risky choices in GT1. Regardless of choice or rGT version, the decline in win-shift behaviour during task acquisition was blunted in alcohol-treated rats. Furthermore, pretreatment with alcohol during rGT acquisition caused increases in premature and perseverative responding and a reduction in the behavioural suppressant effects of alcohol upon subsequent alcohol challenge. Together, these results suggest that repeated alcohol administration reduces the ability to use feedback to adjust choice behaviour and unmasks its disinhibitory effects on behaviour.

Alcohol effects on rGT performance

Acute alcohol administration during baseline choice behaviour in the rGT caused a small reduction in the percentage of optimal choices in Experiment 1, but not in Experiment 2, in which animals had been pretreated with alcohol or vehicle during task acquisition. Moreover, even if the vehicle-treated animals in Experiment 2, which are more comparable to the alcohol-naïve animals in Experiment 1, were analyzed separately, no effect of the alcohol challenges on choice behaviour was observed (data not shown). The slight inconsistency in the effect of alcohol on choice behaviour between Experiments 1 and 2 is not likely to result from procedural differences between the experiments. The animals in Experiment 1 were subjected to 5 forced choice sessions after 5 free choice sessions, whereas the animals in Experiment 2 directly received these 5 forced choice sessions after animals reliably acquired nose-poke responding for food. Hence, the lack of these first 5 free choice sessions preceding the forced choice sessions might have influenced the acquisition curve in the second experiment. However, the number of training sessions that the animals received before alcohol challenges was comparable in the two experiments (i.e. 5 forced choice sessions in both experiments and 26 free choice sessions in Experiment 1 versus 25 free choice sessions in Experiment 2). Importantly,

the effects of acute alcohol exposure on other rGT parameters were consistent between both experiments. Together, these results suggest that alcohol has modest effects on established decision making in the rGT.

These findings are in agreement with a recent report (Peña-Oliver et al., 2014) that showed no effect of alcohol on decision making in a mouse version of the IGT. Moreover, in a task where rats were trained to choose between a small food reward and a large food reward that was accompanied by probabilistic footshock, alcohol did not affect choice behaviour (Mitchell et al., 2011). However, the alcohol doses tested here did affect behaviour in the rGT, as they induced decreases in the number of choices, premature and perseverative responses, and increased omissions and latencies, indicating that alcohol has behavioural suppressant effects in the rGT, even if choice behaviour itself is hardly affected.

Human studies on the effects of alcohol on choice behaviour have yielded mixed results. For example, healthy participants showed more risky decision making under the influence of alcohol (Lane et al., 2004; George et al., 2005) but negative results have also been reported (Ramaekers and Kuypers 2006). Of note, in the rGT, animals are typically trained for several weeks until they have established a stable choice pattern and the animals have learned which option results in the highest gain before pharmacological challenges are performed. As a result, the animals have developed a stable choice strategy, which apparently is quite insensitive to alcohol. However, stable choice behaviour in the rGT has previously been shown to be sensitive to pharmacological manipulations, although in most cases other parameters such as premature responses are influenced as well (Zeeb et al., 2009; Baarendse et al., 2013). In human studies, the IGT is performed in one single session, both under uncertain, i.e. when the task contingencies are not fully known, and more certain conditions, i.e. when the choice contingencies become known to the participant. Hence, alcohol treatment during acquisition of the rGT may be more comparable to acute alcohol treatment in the human IGT. That said, because the rGT requires a certain number of training sessions, the animals will receive multiple alcohol treatments, whereas a human study only requires a single treatment with alcohol. Hence, the repeated versus single alcohol administrations might result in different effects on choice behaviour. Moreover, it is likely that different types of memory processes are used within a single session and between sessions. In the human situation, IGT performance relies on working memory

processes, whereas in the rGT both working memory during the session as well as long-term memory between sessions contribute to the animals' choice behaviour. Therefore, we cannot rule out that differential effects of alcohol on these types of memory cause divergent effects in the human IGT and the rGT.

Alcohol effects on rGT acquisition

We found enhanced risky choice behaviour in animals treated with alcohol during task acquisition, selectively in GT1. This indicates that under uncertain conditions, the effects of alcohol on decision making are more pronounced, albeit that they are dependent on the structure of the task. Subsequent to the alcohol treatment sessions, animals were tested for another 10 sessions without treatment. In these sessions, there was no difference in choice behaviour between alcohol- and vehicle-pretreated animals. Thus, although alcohol affected decision making during treatment, it had no lasting consequences for choice behaviour. The most important difference in contingencies for the risky choice option between the two rGT versions is the length of the punishment timeout, which is four times longer in GT1 than in GT2. Whereas vehicle-treated animals showed a gradual reduction in risky choices over test sessions in GT1, the alcohol-treated animals took much longer to adapt their behaviour after punishment after a risky choice. Trial-by-trial analysis revealed that the alcohol-treated animals in GT1 tended to show less lose-shift behaviour after being punished following a risky choice. This was not observed in GT2. Importantly, in both GT1 and GT2, the risky choice provided the animal with a probabilistic large reward. Hence, if alcohol promotes risky behaviour, then risky decision making should have been increased in both rGT versions. In contrast, if alcohol increases reward sensitivity, it is expected that animals make more risky choices in GT2, since the reward magnitude after risky choices is higher in GT2 (6 pellets) than in GT1 (4 pellets). Together, these results suggest that alcohol-induced risky behaviour results from an impaired capacity to adapt choice behaviour on the basis of negative feedback, rather than making animals more risk-prone, or alter their reward sensitivity.

During rGT acquisition, vehicle treated animals showed a steady decline in win-shift behaviour, i.e. less shifts towards another choice option after being rewarded, irrespective of which option was rewarded on the previous trial and irrespective of rGT version. Theoretically, it is expected that the number of shifts after being rewarded declines over sessions as the animals gradually come to show more exploitative decision making over sessions, as has been shown in

human IGT studies (Bechara et al., 1994, 2001). Interestingly, alcohol treatment blunted this decline in win-shift behaviour, suggesting that the alcohol-treated animals are impaired in adjusting their behaviour upon feedback, resulting in reduced shifting from an explorative towards an exploitative decision-making strategy.

Our findings are in line with human studies, in which AUD patients need more trials to shift towards advantageous choices in the IGT (Kim et al., 2011), which may be caused by a reduced sensitivity to losses and a bias towards trials with gains (Gullo and Stieger 2011). Studies using other risky decision making tasks show similar findings, in that AUD patients fail to adjust their behaviour after experiencing negative consequences in the Balloon Analogue Risk Task (Holmes et al., 2009) and the monetary Go/No-Go task (Rossiter et al., 2012). However, comparison of these studies with our data has to be done with caution, as we investigated the acute effect of alcohol on rGT performance and not the long-term effects of alcohol abuse. Nevertheless, studies on acute alcohol exposure in healthy individuals describe comparable findings (George et al., 2005; Loeber and Duka 2009a-b), suggesting that alcohol disrupts the ability to alter behaviour after negative feedback.

Repeated alcohol administration also increased premature responses over sessions (Table 3), showing that repeated alcohol administration caused disinhibitory effects on behaviour. However, these effects on impulsive behaviour occurred later (i.e., sessions 11-15) than the effects of alcohol on risky choice (which was apparent from session 6 onwards), and in both rGT versions. This indicates that repeated alcohol treatment results in impulsive behaviour, but likely through a different mechanism than its effects on decision making. Alcohol treatment may also have affected time perception, so that the long punishment time-out after a risky choice was not perceived as such. Importantly, however, preclinical studies on impulsive choice in delay discounting tasks have shown that alcohol increases choice for a small immediate reward (Tomie et al., 1998; Evenden and Ryan 1999; Olmstead et al., 2006; Wilhelm and Mitchell 2012). Thus, if alcohol alters time perception in a way that a long delay or time-out period is not perceived as such, one would expect alcohol to increase, and not decrease choice for the large delayed reward. Interestingly, in a recent detailed analysis of delay discounting in rats, alcohol did not affect the sensitivity to delay or reward size (Moschak and Mitchell 2013). Moreover, human studies have not shown consistent acute

alcohol effects on delay discounting (Richards et al., 1999; Ortner et al., 2003; Reynolds et al., 2006; Bidwell et al., 2013). We therefore consider it unlikely that alcohol influenced choice behaviour in the rGT as a result of altered time perception.

Differential effects of alcohol treatment in alcohol-pretreated animals and controls

We observed no effect of acute alcohol challenges on decision making in Experiment 2, irrespective of whether animals were pretreated with alcohol or vehicle during rGT acquisition. Hence, previous alcohol treatment did not alter the acute effects of alcohol on choice behaviour. However, animals pretreated with alcohol exhibited behavioural disinhibition upon treatment with low to moderate doses of alcohol, where these doses increased the number of total choices, premature and perseverative responses and decreased omissions and choice latencies. In contrast, vehicle-pretreated animals showed dose-dependent behavioural suppressant effects of alcohol, similar to the acute alcohol challenges in Experiment 1. Thus, behavioural disinhibition in rodents may be unmasked after pretreatment with alcohol, alongside with tolerance to its suppressant effects. The biphasic dose effect of alcohol, where moderate doses induce disinhibition and high doses predominantly result in sedation in both human and animals, is a well-known characteristic of alcohol (Pohorecky 1977). We observed similar biphasic effects of alcohol in alcohol-pretreated animals, but in vehicle-treated animals alcohol had merely behavioural suppressant effects. Previous 5-choice serial reaction time task (5CSRTT) studies have shown that motor impulsivity upon alcohol challenge only increased after multiple cycles of alcohol intoxication and abstinence (Walker et al., 2011; Irimia et al., 2013). In contrast, acute alcohol challenges in otherwise alcohol-naïve animals did not affect impulsive action (Peña-Oliver et al., 2009; Semenova 2012) or reduced impulsivity in the 5CSRTT (Bizarro et al., 2003), the latter being consistent with our findings. Other preclinical studies found that alcohol increases motor impulsivity in naïve animals, but only when a novelty component is introduced (Peña-Oliver et al., 2009; Walker et al., 2011; Irimia et al., 2013). In agreement with the present findings, acute alcohol administration resulted in tolerance to the sedative effects of acute alcohol in adult rats that were pretreated with alcohol during adolescence (Matthews et al., 2008; Semenova 2012). These findings are reminiscent of findings in humans, where heavy alcohol users act more impulsively and report feeling more stimulated after alcohol exposure compared to light users (Marczinski

et al., 2007; King et al., 2011; Reed et al., 2012), which may impair the ability to refrain from drinking. Moreover, people who recently consumed alcohol display less alcohol-induced impairments in motor coordination (Miller et al., 2012). Taken together, acute alcohol challenges after a period of abstinence from repeated alcohol treatment result in disinhibition of behaviour.

Concluding remarks

The experiments described in this study demonstrate augmented risk taking behaviour after alcohol treatment during conditions of uncertainty, e.g. during task acquisition. This may be due to a reduced ability to adjust choice behaviour on the basis of feedback, perhaps resulting from a reduced sensitivity to punishment. Moreover, alcohol pre-exposure unmasks its disinhibitory effects on behaviour. Impaired responsiveness to punishment and behavioural disinhibition may therefore contribute to the development of AUD.

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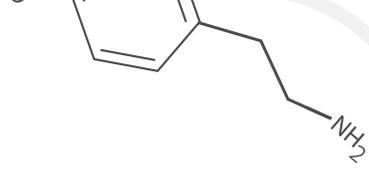
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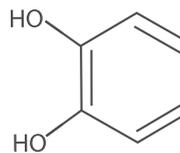
CHAPTER 5

INDIVIDUAL DIFFERENCES IN VOLUNTARY ALCOHOL INTAKE IN RATS: RELATIONSHIP WITH IMPULSIVITY, DECISION MAKING AND PAVLOVIAN CONDITIONED APPROACH BEHAVIOUR

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Submitted



ABSTRACT

Alcohol use disorder (AUD) has been associated with suboptimal decision making and exaggerated impulsivity, but the relationship between AUD and these cognitive deficits is incompletely understood. This study aims to assess whether a period of voluntary alcohol intake differentially affects decision making and impulsive choice in rats that consume low or high amounts of alcohol. Moreover, we investigated the relationship between voluntary alcohol intake and approach behaviour to primary and conditioned rewards. Subgroups of low and high alcohol drinking (LD; HD) rats were identified after 8 weeks of intermittent alcohol access (IAA). These subgroups were subsequently tested in the rodent gambling task (rGT) or the delayed reward task (DRT). Upon stable choice behaviour, the effects of acute systemic alcohol treatment (0 - 1.0 g/kg) on performance in the rGT and DRT were assessed. Pavlovian conditioned approach behaviour was assessed in LD and HD both prior to and after IAA. HD showed more optimal decision making in the rGT. In the DRT, HD showed a transient higher preference for the large delayed reward. In both subgroups, acute alcohol treatment increased optimal decision making in the rGT and increased the preference for the small immediate reward in the DRT. HD showed enhanced sign-tracking behaviour in the Pavlovian conditioned approach task when the test was conducted after IAA. Pavlovian conditioned approach behaviour was however comparable for LD and HD when assessed prior to IAA and both subgroups showed an equivalent increase in sign-tracking behaviour after IAA. HD showed more efficient performance in the rGT and DRT. Moreover, alcohol consumption enhanced approach behaviour to reward-predictive cues, but sign-tracking behaviour does not predict the level of alcohol consumption. Taken together, these findings suggest that high levels of voluntary alcohol intake are associated with enhanced cue- and reward-driven cognitive performance.

INTRODUCTION

Alcohol is consumed by many people on a regular basis, but only a minority (3-5%) of the people that consume alcohol develop an alcohol use disorder (AUD) (Anthony et al., 1994; Costanzo et al., 2007; United Nations Office on Drugs and Crime 2012; American Psychiatric Association 2013). It is therefore of great importance to identify the factors that underlie the individual vulnerability to AUD. Importantly, AUD has been associated with exaggerated levels of impulsivity and suboptimal decision making (Kreek et al., 2005; Perry and Carroll 2008; Redish et al., 2008; de Wit 2009; MacKillop et al., 2011) and an approach tendency towards reward-predicting cues (Field et al., 2005; Wiers et al., 2007; Field and Cox 2008).

Impulsive behaviours, i.e., the tendency to act without consideration of possible consequences, can be categorized into impulsive action and impulsive choice (Evenden 1999; Reynolds et al., 2006; Pattij and Vanderschuren 2008; Eagle and Baunez 2010; Dalley et al., 2011; Winstanley 2011; Hamilton et al., 2015). Both types of impulsivity, as well as suboptimal decision making have been associated with the susceptibility to develop AUD (Bates and Labouvie 1997; Dom et al., 2006; Ernst et al., 2006; Verdejo-Garcia et al., 2008; de Wit 2009; Fernie et al., 2010; Goudriaan et al., 2011; King et al., 2011; Fernie et al., 2013). On the other hand, excessive alcohol use has also been shown to result in exaggerated impulsivity and suboptimal decision making (Vuchinich and Simpson 1998; Petry 2001; Field et al., 2007; Perry and Carroll 2008; Salgado et al., 2009; Kim et al., 2011; MacKillop et al., 2011; Tomassini et al., 2012; Voon et al., 2013), suggesting a complex bidirectionality between impaired impulse control and decision making on the one hand, and AUD on the other. Importantly, alcohol exposure per se may not be responsible for impaired impulse control and decision making in AUD patients. Thus, acute alcohol challenges in healthy controls and rodents have resulted in mixed effects, i.e. either impaired or unaffected decision making, impulsive action and impulsive choice (Evenden and Ryan 1999; Richards et al., 1999; George et al., 2005; Perry and Carroll 2008; MacKillop et al., 2011; Mitchell et al., 2011; Semenova 2012; Caswell et al., 2013; Mejia-Toiber et al., 2014; Pena-Oliver et al., 2014). Interestingly, however, the effect of acute alcohol may be perceived differently in individuals at risk for AUD. Indeed, acute alcohol exposure resulted in less behavioural control in heavy users and alcohol pre-treated rats (Marczinski et al., 2007; Reed et al., 2012; Spoelder et al., 2015b).

It has been shown that substance-predictive cues can acquire conditioned incentive motivational properties that can drive an involuntary conditioned response towards substances of abuse (Stewart et al., 1984; O'Brien et al., 1998; Robinson and Berridge 2001; Shaham et al., 2003; Milton and Everitt 2010; Tomie and Sharma 2013). Interestingly, substantial individual variation between animals and humans exists with regard to the behavioural response to the presentation of reward-predictive cues (Zener 1937; Brown and Jenkins 1968; Wilcove and Miller 1974; Burns and Domjan 1996; Tomie et al., 2000; Cole and Adamo 2005; Stacy and Wiers 2010; Meyer et al., 2012; Tomie et al., 2012). That is, some individuals approach and manipulate the cue, so called 'sign-trackers', whereas other individuals approach the location of reward delivery, so called 'goal-trackers'. In preclinical studies, the rats that showed a tendency to acquire a sign-tracking conditioned response have been characterized as more prone to addictive (Flagel et al., 2007, 2008, 2010; Saunders and Robinson 2010, 2011; Yager and Robinson 2013; Yager et al., 2014) and impulsive behaviour (Flagel et al., 2010; Lovic et al., 2011). There are interesting human parallels to these findings, since heavy alcohol drinking individuals exhibit enhanced approach behaviour to alcohol-related pictures (Field et al., 2005; Wiers et al., 2009) and approach behaviour towards alcohol cues predicts a higher alcohol consumption (Palfai 2006; Fadardi and Cox 2008; Christiansen et al., 2012).

In the present study, we assessed whether individual variability in voluntary alcohol consumption relates to differences in impulsivity, decision making and Pavlovian conditioned approach behaviour. For this purpose, we exploited the substantial degree of individual differences in alcohol intake (Simms et al., 2008; Momeni and Roman 2014; Lesscher et al., 2015; Spoelder et al., 2015a), which we have previously related to the development of compulsive characteristics of alcohol use (Spoelder et al., 2015a). Low and high alcohol drinking rats were compared for decision making in a rat gambling task (rGT) and a delayed reward task (DRT). We hypothesized that the consumption of high amounts of alcohol results in maladaptive decision making and impaired impulse control. In addition, we assessed the effects of acute systemic alcohol challenges on stable choice behaviour in the rGT and DRT in these rats. We hypothesized that low alcohol doses may provoke impulsive action especially in rats with a history of high alcohol consumption. Finally, we compared rats that differ in their degree of alcohol consumption for approach behaviour towards reward-predicting cues, where we predicted that high alcohol consumption induces a sign-tracking phenotype.

MATERIALS AND METHODS

Animals

Two groups (n=64/Experiment) of male Lister Hooded rats (Charles River, Germany), weighing 220-250g (~7-9 weeks old) on arrival were used. The rats were individually housed under controlled temperature and humidity conditions on a reversed 12h light/dark cycle (lights off 7.00 AM) with ad libitum access to water and chow. The rats were acclimatized to the housing conditions for two weeks before experiments commenced and they were weighed and handled at least once per week. The rats were briefly restrained during the weighing procedure, to habituate them to the injection procedure. One week before the start of the operant conditioning experiments, the rats were gradually restricted to 4-5g chow/100g body weight/day, which maintained them at 90% of their free-feeding weight. Two days before operant training, the rats received sucrose pellets (TestDiet, UK) in their home cage to reduce potential food neophobia. Operant behavioural tests were conducted once per day for 5-6 days/week. All experiments were approved by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op de dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Voluntary home cage alcohol consumption

Alcohol access and subgroup selection was performed as previously described (Spoelder et al., 2015a). Briefly, the rats received access to 20% alcohol (v/v from 99.5%, Klinipath, The Netherlands) and water in a two-bottle choice intermittent alcohol access (IAA) setup in the home cage for 7 h/day in the first month and 24 h/day in the second month. Alcohol intake and preference were calculated per rat per session and averaged per week, i.e. 3 sessions per week. In order to select rats that consistently consumed low or high levels of alcohol throughout the experiment, the rats were ranked from low to high based on the rats' average alcohol intake per week and were assigned ranking scores. These weekly ranking scores were then summed to calculate a total ranking score per rat which was used to divide the rats in subgroups. Rats within the lower and upper 12.5% of the total ranking score range were designated as low and high alcohol drinking rats (LD; HD), respectively. The middle 75% were assigned as medium alcohol drinking rats; these were used in other experiments. To demonstrate the maintenance of the LD and HD phenotype at the time of behavioural testing, the rats of

Experiment 1 were subjected to 2h IAA after behavioural testing (between 15.00 PM and 17.00 PM) (Fig. 1).

Apparatus

Training and testing was conducted in operant conditioning chambers, illuminated by a white house light, in ventilated sound-attenuating cubicles (Med Associates, St. Albans, VT, USA). The Pavlovian conditioned approach task was conducted in operant chambers with two 4.8 cm wide retractable levers placed 11.7 cm apart and 6 cm from the grid floor with a magazine between the levers. The chambers used for the rodent gambling task (rGT) and the delayed reward task (DRT) were equipped with an array of five holes in a curved wall, each with an infrared detector and a stimulus light. In these chambers, a magazine was located on the opposite wall. Sucrose pellets could be delivered via a dispenser in the magazine. Nose pokes in the magazine could be detected via the infrared detector. Experimental events and data recording were controlled using MED-PC for Windows.

Habituation and nose poke response training prior to rGT and DRT

For the rGT and DRT experiments, a similar habituation and magazine training procedure was used, as described previously (Baarendse and Vanderschuren 2012; Baarendse et al., 2013; Spoelder et al., 2015b). Briefly, the rats were trained to make a nose poke response in an illuminated response hole to obtain a sucrose pellet for 30 min or 100 trials/session, whichever occurred first. The rats were trained in three stages in which the stimulus duration was reduced from 30 sec, to 20 sec to the final 10 sec. The rats progressed to the next training stage after 30 correct responses. In order to obtain a comparable level of experience in correct performance before the rGT and DRT, the rats that quickly approached the performance criterion were tested 2-3 times/week instead of daily. The training sessions continued until the rats achieved baseline performance, defined by performing $\geq 80\%$ of the trials correctly for 3 consecutive days.

Rat Gambling Task (rGT)

The rGT (Zeeb et al., 2009; Baarendse et al., 2013) was performed as described previously (Spoelder et al., 2015b). Briefly, the rats could choose from 3 options (safe, optimal, risky) in which the safe and risky option resulted in a net gain of 72% and 24% of the optimal option, respectively. The middle three response

holes of the five-hole array were used. The spatial location of the three options was counterbalanced across subjects; these remained the same for each rat over the course of the experiment. During phase 1, the rats were first tested for 10 free choice sessions. To ensure that all rats had equal experience with the contingencies of the three choice options, the rats were subsequently tested during 5 forced choice sessions. In phase 2, the rats first received 5 free choice sessions. Because we observed that several rats had still not explored all three options during these 5 free choice sessions, the following 5 free choice sessions were preceded by 10 min of forced choices. In phase 3, the rats were tested for another 10 free choice sessions which resulted in a stable choice pattern.

A trial started with a 5 sec inter-trial interval (ITI), followed by illumination of one (during forced choice sessions) or three (during free choice sessions) stimulus lights for 10 sec. A response in an illuminated hole turned off the stimulus light(s), and led to either a reward (i.e. sucrose pellets) or punishment (i.e. no reward delivery and time-out period signaled by flashing stimulus light within the chosen hole at 1 Hz). A nose poke response in a non-illuminated aperture (i.e., incorrect response), a failure to respond within 10 sec (i.e., omission), or a response during the ITI (i.e., premature response), resulted in a 5 sec time-out period, signaled by illumination of the house light. Nose poke responses in the stimulus holes during either punishment or reward were scored as perseverative responses, but had no scheduled consequences. The rats were screened for motor impulsivity over the course of the last 10 free choice sessions of the rGT. To achieve this, once per week, on session 23 and 28, the ITI was extended to 7 sec to provoke impulsive behaviour (Dalley et al., 2007).

Delayed Reward Task (DRT)

A detailed description of the DRT procedure has been provided previously (van Gaalen et al., 2006; Baarendse and Vanderschuren 2012). In short, a trial started with a 5 sec ITI whereafter the middle response hole was illuminated for 10 sec. After a response in this hole, the light extinguished and the two response holes adjacent to the middle response hole were illuminated. The DRT session was divided into five blocks of 10 trials. Each block started with two forced choice trials in order to signal the upcoming delay for the subsequent session block. During these forced choice trials either the left or right hole was illuminated in a counterbalanced fashion. For the next 8 trials, both the left and right hole were illuminated and the rats could make a choice. One of the two response holes was rewarded with a small reward (one sucrose pellet)

provided immediately whereas the other response hole was rewarded with a large reward (four sucrose pellets) after a certain delay. The delays for the large reward were assessed in an ascending order within a session per block. The spatial location of the two choices was counterbalanced across subjects and remained the same for each rat over the course of the experiment. As the trial time was fixed, the ITI duration depended on the duration of the delay.

The delays for the large reward were increased over sessions. First, the rats were subjected to 3 sessions with delays for the large reward of 0, 2, 4, 8 and 12 sec (phase 1), followed by 2 sessions with delays of 0, 4, 8, 16, 24 sec (phase 2), 1 session with delays of 0, 8, 16, 32, 48 sec (phase 3) and 6 sessions with the final delays of 0, 10, 20, 40, 60 sec (phase 4). In phase 5, the number of choices were extended from 8 free choices per delay to the final 10 free choices per delay, in which the rats were tested for 16 sessions. Subsequently, the rats were exposed to acute alcohol challenges and six 24 h IAA sessions, after which the rats were again tested on the DRT for 3 sessions (phase 6). In the final 13 sessions, the delay for the large reward was reversed within the session from 60 to 40, 20, 10, 0 sec (phase 7) (Fig. 1). A response in an illuminated hole turned off the stimulus light(s). An incorrect response, an omission or a premature response resulted in a 5 sec time-out period, signaled by illumination of the house light. Nose poke responses in the stimulus holes after making a choice were scored as perseverative responses, but these had no scheduled consequences.

Pavlovian conditioned approach task

Rats were habituated to the chambers for two sessions, during which 50 sucrose pellets were randomly delivered over the course of 25 min with an average inter-reward interval of 30 sec. The Pavlovian conditioned approach procedure was conducted as previously described (Flagel et al., 2011; Spoelder et al., 2015c). Briefly, a trial consisted of the insertion of the left or right lever (counterbalanced between rats) for 8 sec (conditioned stimulus: CS), followed by the response-independent immediate delivery of a sucrose pellet (unconditioned stimulus: US). Cue lights above the lever or within the magazine were not illuminated. The rats were subjected to 25 CS-US presentations in each session, which occurred on a variable inter-trial interval schedule, with on average 90 sec between trials. Lever contacts and food magazine entries during lever presentation were recorded, but had no programmed consequences.

Figure 1

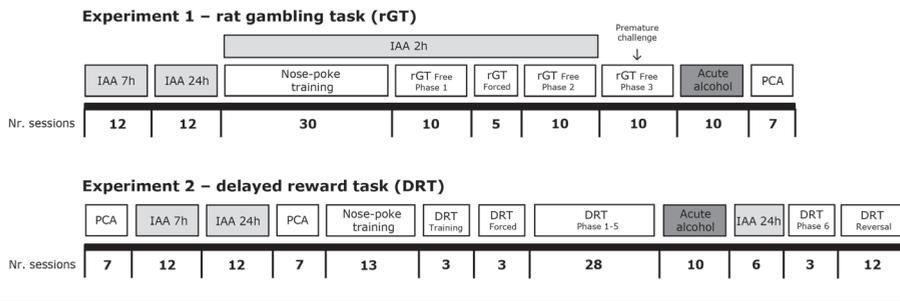


Figure 1. Timeline of the experimental procedures in Experiment 1 and 2. IAA: Intermittent alcohol access, PCA: Pavlovian conditioned approach.

Systemic alcohol injections

Alcohol (99.5%, Klinipath, The Netherlands) was diluted with saline to a concentration of 10% alcohol (v/v). Injection volumes were adjusted to the body weight and the alcohol dose. The syringes were pre-heated to 32°C by a heating pad to prevent possible decreases in body temperature after injection of substantial volumes, particularly at the highest alcohol doses. Vehicle (i.e., saline) injection volumes were equivalent to the volume required for an injection of the 0.6 g/kg alcohol dose. Drug solutions were prepared fresh daily and administered intraperitoneally 15 min prior to behavioural testing. Prior to injections, the rats were habituated twice to the injection procedure. The different alcohol challenge doses were administered according to a within-subjects, Latin square design with a three day cycle for each dose; i.e. a baseline session, followed by an alcohol treatment session and a washout day during which the rats remained in their home cage.

Data analysis

The behavioural measures to assess task performance in the rGT and DRT were calculated as the percentage choice for a certain option, i.e. [number of choices for a certain option / total number of choices × 100]. For the DRT, the area under the curve (AUC) for the overall percentage choice for the large delayed reward was also calculated (Myerson et al., 2001). The allocation of behavioural responses during the Pavlovian conditioned approach task were calculated as a response bias score, i.e. [(lever presses – magazine entries) / (lever presses + magazine entries)], resulting in a number ranging from – 1 (goal-tracking) to + 1 (sign-tracking) (Meyer et al., 2012; Spoelder et al., 2015c). The increase in premature responses upon the extension of the ITI was calculated as a ratio,

i.e. [number of premature responses during long ITI session / the average number of premature responses of the 2 sessions preceding and the 2 sessions following the long ITI session]. Prior to statistical analyses, the number of lever presses and head entries during the Pavlovian conditioned approach task and the number of omissions, premature and preservative responses during the rGT and DRT were square root transformed and the trial initiate-, choice- and collect latency were LOG transformed. Choice behaviour in the rGT, expressed as percentages, was arcsine transformed prior to analyses. Because the nose poke training prior to the rGT and DRT was performed in a similar manner, these data were analyzed together. The data obtained during the Pavlovian conditioned approach task, choice behaviour during the rGT and the AUC in the DRT were analyzed using one-, two- and three-way repeated-measures ANOVA's with choice, session and alcohol exposure as within-subject variables and subgroup (LD; HD) as the between-subject variable. Mauchly's test of sphericity was used to test if variances of the differences between treatment levels were equal. If the assumption of sphericity was violated, degrees of freedom were corrected using Huynh-Feldt estimates of sphericity to more conservative values. Corrected degrees of freedom are presented rounded to the nearest integer. Data of the DRT were analyzed with linear mixed models (Verbeke and Molenberghs 2000), where the delay and group served as variables in the analyses. The data obtained in the rGT and DRT during acute alcohol challenges were also analyzed using linear mixed models. We noticed that the rats were less sedated upon treatment with the second high dose compared to the first (0.8 or 1.0 g/kg) and therefore we included the injection order, together with dose, delay and subgroup as variables in the mixed model analyses. For all mixed model analyses, the covariance structure was explored and modeled appropriately. Student's samples and paired t-tests were used for *post hoc* analyses. All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, N.Y. USA). The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean \pm SEM. Graphs were made using Graphpad Prism 6.

RESULTS

Alcohol consumption during IAA in HD and LD

Alcohol intake and preference increased over the first 4 weeks of IAA for 7h/day in HD, but remained stable in LD (Intake: Exp.1: $F_{(3,42) \text{ week} \times \text{group}} = 15.67$, $p < 0.001$; Exp.2: $F_{(3,42) \text{ week} \times \text{group}} = 8.64$, $p < 0.001$; Preference: Exp.1:

$F_{(3,42) \text{ week} \times \text{group}} = 17.40, p < 0.001$; Exp.2: $F_{(3,39) \text{ week} \times \text{group}} = 4.66, p < 0.01$) (Fig. 2A-D). Upon extension of alcohol access duration to 24 h/day, alcohol intake was increased to a further extent in HD compared to LD (Exp.1: $F_{(1,14) \text{ month} \times \text{group}} = 78.31, p < 0.001$; Exp.2: $F_{(1,14) \text{ month} \times \text{group}} = 12.52, p < 0.005$) (Fig. 2A-B). The preference for alcohol in Exp. 1 increased from the first to the second month in HD but not in LD ($F_{(1,14) \text{ month} \times \text{group}} = 11.89, p < 0.005$), whereas the preference for alcohol in Exp. 2 increased to a similar extent in HD and LD ($F_{(1,14) \text{ month} \times \text{group}} = 0.01, \text{ n.s.}$) (Fig. 2C-D). Alcohol intake and preference during the 4 weeks of alcohol access for 24 h/day remained stable in both groups (Intake: Exp.1: $F_{(3,42) \text{ week} \times \text{group}} = 1.74, \text{ n.s.}$; Exp.2: $F_{(2,33) \text{ week} \times \text{group}} = 1.13, \text{ n.s.}$; Preference: Exp.1: $F_{(3,42) \text{ week} \times \text{group}} = 2.20, \text{ n.s.}$; Exp.2: $F_{(3,42) \text{ week} \times \text{group}} = 1.32, \text{ n.s.}$) (Fig. 2A-D). The total volume intake during the two months was not different between groups

Figure 2

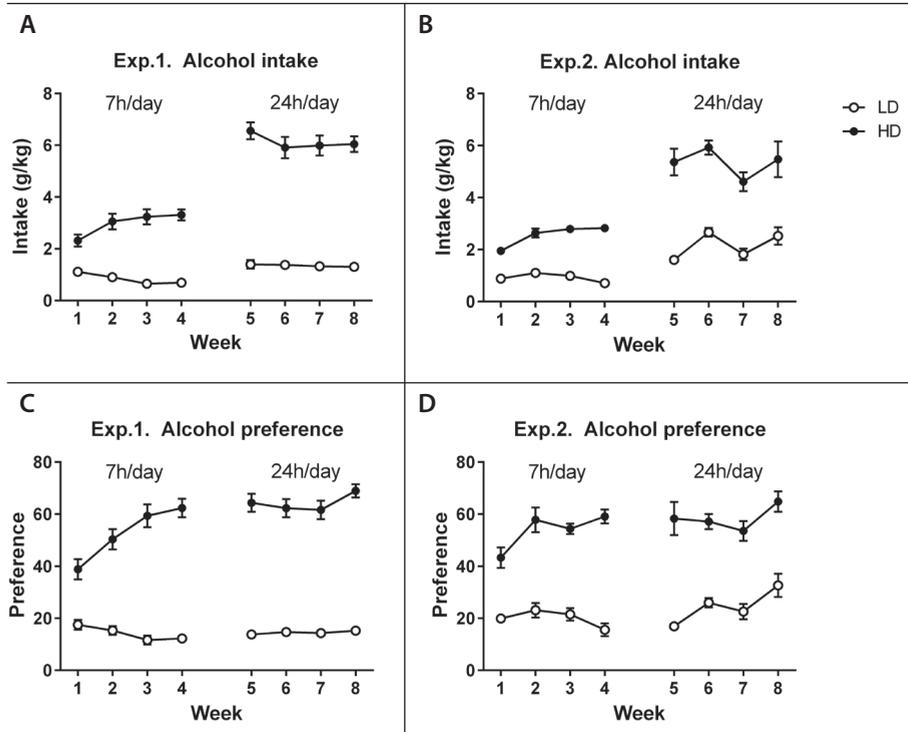


Figure 2. Alcohol consumption and preference for LD and HD during IAA (20%, v/v). Alcohol intake and preference increased in HD during the first 4 weeks of IAA with 7 h access/day but remained low in LD (A-D). HD increased their alcohol intake to a further extent upon session length extension from the first to the second month (A-B). Alcohol intake and preference remained stable during 24 h IAA (A-D). Data are shown as mean \pm SEM.

(Exp.1: $F_{(1,14) \text{ group}} = 0.01$, n.s.; Exp.2: $F_{(1,14) \text{ group}} = 0.56$, n.s.) (data not shown). Importantly, the HD maintained higher levels of alcohol consumption during the 2 h IAA sessions that were incorporated between the rGT tests (intake: LD: 0.77 ± 0.08 , HD: 1.48 ± 0.06 ; $F_{(1,14) \text{ group}} = 51.17$, $p < 0.001$; preference: LD: 39.67 ± 2.73 , HD: 70.14 ± 1.82 ; $F_{(1,14) \text{ group}} = 86.06$, $p < 0.001$) (data not shown).

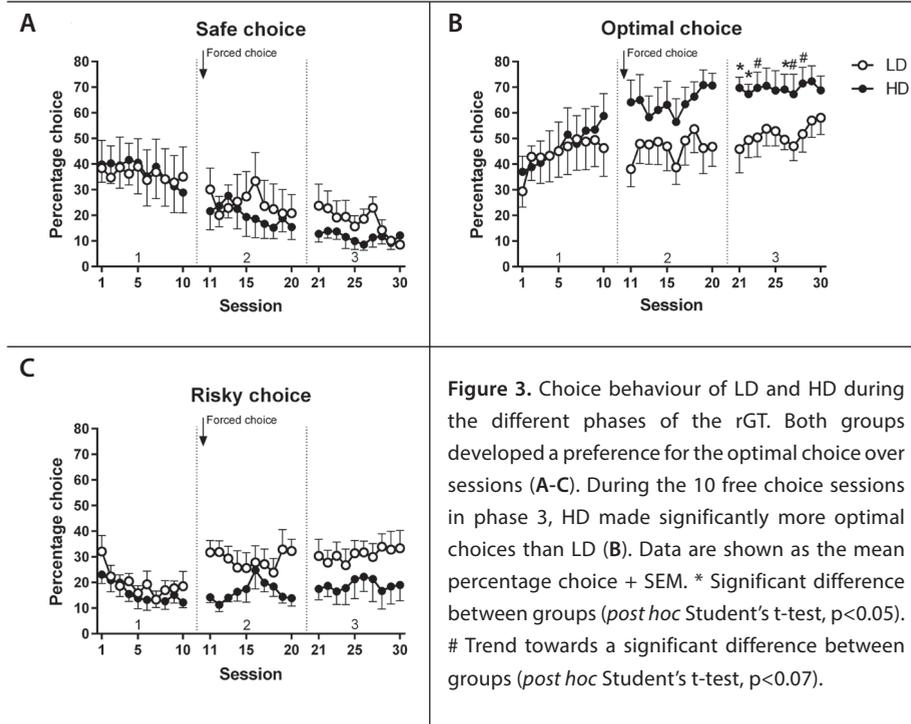
Rat Gambling Task

During the first 10 free choice sessions in phase 1 of the rGT, the rats developed a preference for the optimal option ($F_{(18,252) \text{ choice} \times \text{session}} = 3.18$, $p < 0.001$), independent of group ($F_{(18,252) \text{ choice} \times \text{session} \times \text{group}} = 0.58$, n.s.; $F_{(1,14) \text{ group}} = 0.06$, n.s.) (Fig. 3A-C). Separate analyses per choice indicated that the preference for the safe option did not change ($F_{(9,126) \text{ session}} = 1.50$, n.s.), but the preference for the optimal option increased ($F_{(7,92) \text{ session}} = 3.93$, $p < 0.002$) and the preference for the risky option decreased over sessions ($F_{(9,126) \text{ session}} = 3.86$, $p < 0.002$) (Fig. 3A-C). In the subsequent 10 free choice sessions in phase 2, the difference in preference between the choices was significant ($F_{(2,22) \text{ choice}} = 9.97$, $p < 0.003$) and a similar choice pattern was observed in both groups ($F_{(2,22) \text{ choice} \times \text{group}} = 1.82$, n.s.; $F_{(1,14) \text{ group}} = 0.00$, n.s.) (Fig. 3A-C). In the final phase, the difference in preference for the three options was even further pronounced ($F_{(2,28) \text{ choice}} = 30.02$, $p < 0.001$) and this was different between HD and LD ($F_{(2,28) \text{ choice} \times \text{group}} = 3.37$, $p < 0.05$; $F_{(1,14) \text{ group}} = 5.63$, $p < 0.04$). Separate analyses per choice indicated that HD made more optimal choices than LD ($F_{(1,14) \text{ group}} = 4.92$, $p < 0.05$). However, the groups did not differ in their choice for the safe ($F_{(1,14) \text{ group}} = 1.56$, n.s.) and risky options ($F_{(1,14) \text{ group}} = 2.85$, n.s.) (Fig. 3A-C). During the last 10 free choice sessions, a longer ITI (7 sec) was used during sessions 23 and 28, to provoke an increase in motor impulsivity. We observed a larger increase in premature responses, expressed as a ratio, in HD compared to LD during the first long ITI session (LD: 1.48 ± 0.21 , HD: 2.54 ± 0.34 ; $F_{(1,14) \text{ group}} = 7.01$, $p < 0.02$), but not during the second long ITI session (LD: 2.41 ± 0.54 , HD: 2.52 ± 0.27 ; $F_{(1,14) \text{ group}} = 0.03$, n.s.) (data not shown).

Acute alcohol challenge

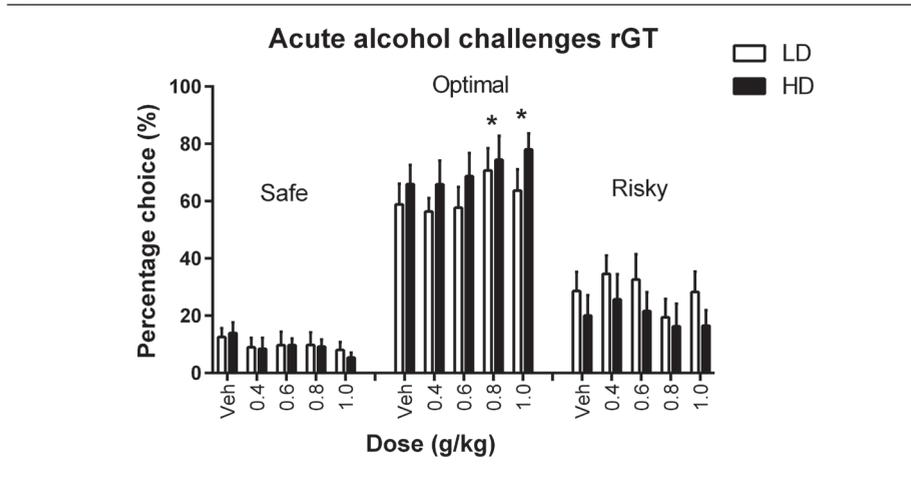
Acute alcohol treatment affected choice behaviour in the rGT ($F_{(8,39) \text{ dose} \times \text{choice}} = 5.31$, $p < 0.001$), in a similar manner in LD and HD ($F_{(8,39) \text{ dose} \times \text{choice} \times \text{group}} = 1.01$, n.s.) (Fig. 4). Subsequent analyses per choice indicated that alcohol treatment dose-dependently increased choice for the optimal option ($F_{(4,78) \text{ dose}} = 4.09$, $p < 0.006$) which was significant after treatment with 0.8 and 1.0 g/kg alcohol. The percentage choice for the safe

Figure 3



5

Figure 4



($F_{(4,83) \text{ dose}} = 2.14$, n.s.) and risky option ($F_{(4,31) \text{ dose}} = 2.15$, n.s.) were not affected by alcohol (Fig. 4). Treatment with alcohol reduced the number of choices, premature and perseverative responses and increased the number of omissions, choice latencies and collect latencies (Table 1). Interestingly, the effects of the highest doses of alcohol (0.8 and 1.0 g/kg) were more pronounced in HD than in LD (Table 1).

Delayed Reward Task

The AUC declined over sessions as the delays were increased during training of phase 1-5 ($F_{(27,378) \text{ session}} = 33.11$, $p < 0.001$), but in a different manner for LD and HD ($F_{(27,378) \text{ session} \times \text{group}} = 2.35$, $p < 0.001$; $F_{(1,14) \text{ group}} = 4.00$, $p = 0.065$) (Fig. 5A). HD showed significantly higher AUC values than LD during phase 1-4 (Fig. 5A). Analyses of choice behaviour over delays in the different phases of the experiment confirmed that the groups differed during phase 1-4, in which HD showed a higher preference for the large delayed reward (phase 1: $F_{(1,20) \text{ group}} = 5.91$, $p < 0.03$; phase 2: $F_{(1,19) \text{ group}} = 7.25$, $p < 0.02$; phase 3: $F_{(1,15) \text{ group}} = 8.23$, $p < 0.02$; phase 4: $F_{(1,16) \text{ group}} = 10.76$, $p < 0.01$), independent of the delays (phase 1: $F_{(4,18) \text{ delay} \times \text{group}} = 2.05$, n.s.; phase 2: $F_{(4,31) \text{ delay} \times \text{group}} = 0.97$, n.s.; phase 3: $F_{(4,29) \text{ delay} \times \text{group}} = 0.71$, n.s.; phase 4: $F_{(4,33) \text{ delay} \times \text{group}} = 1.09$, n.s.) (Fig. 5B). This group difference was no longer present during phase 5 ($F_{(1,58) \text{ group}} = 0.00$, n.s.; $F_{(4,40) \text{ delay} \times \text{group}} = 0.32$, n.s.) (Fig. 5C).

To investigate whether the group differences during phase 1-4 were the residual result of IAA, the rats were re-exposed to 6 IAA sessions and were then re-tested in the DRT (phase 6). During these IAA sessions, HD consumed more alcohol than LD (LD: 1.69 ± 0.34 g/kg/session, HD: 5.20 ± 0.53 g/kg/session; $F_{(1,14) \text{ group}} = 30.36$, $p < 0.001$) and showed a greater preference for alcohol (LD: 24.31 ± 4.31 , HD: 68.86 ± 5.10 ; $F_{(1,14) \text{ group}} = 45.07$, $p < 0.001$). Combined analysis for phase 5 and 6 revealed an interaction between group and phase ($F_{(1,34) \text{ phase} \times \text{group}} = 6.49$, $p < 0.02$), indicating that recent IAA differentially affected choice behaviour in LD and HD (Fig. 5C-D).

Upon reversal of the delays during the session (phase 7), choice for the large delayed reward progressively increased over sessions, towards baseline performance ($F_{(7,100) \text{ session}} = 6.83$, $p < 0.001$; $F_{(7,100) \text{ session} \times \text{group}} = 0.83$, n.s.) (Fig. 5A). Both HD and LD showed a complete reversal of their preference for the large delayed reward in a delay-dependent manner ($F_{(4,32) \text{ delay}} = 94.12$, $p < 0.001$; $F_{(1,16) \text{ group}} = 0.31$, n.s.; $F_{(4,32) \text{ delay} \times \text{group}} = 0.35$, n.s.) (Fig. 5E).

Figure 5

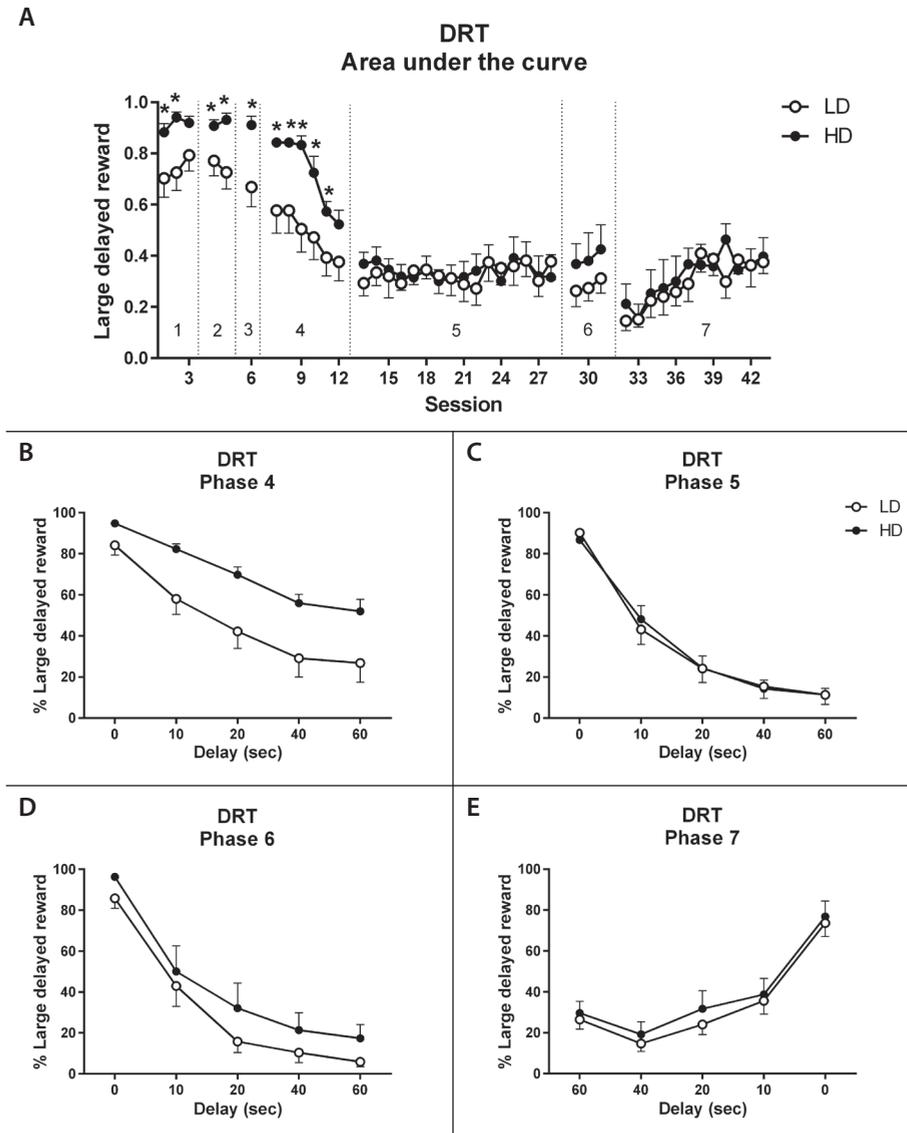


Figure 5. Choice behaviour of LD and HD during the different phases of the DRT. The area under the curve declined over sessions as the delays for the large reward were increased during phases 1-5 (A). The preference for the large delayed reward was higher in HD during phases 1-4 (A-B), but group differences were no longer significant in phase 5 (C). Upon re-exposure to alcohol, the difference between HD and LD re-emerged (A, D). Reversal of the delays (phase 7) did not differentially affect choice behaviour in LD and HD (A, E). B-E depict the averaged choice behaviour across the entire phases. Data are shown as the mean percentage choice + SEM. * Significant difference between groups (*post hoc* Student's t-test, $p < 0.05$).

Acute alcohol challenge

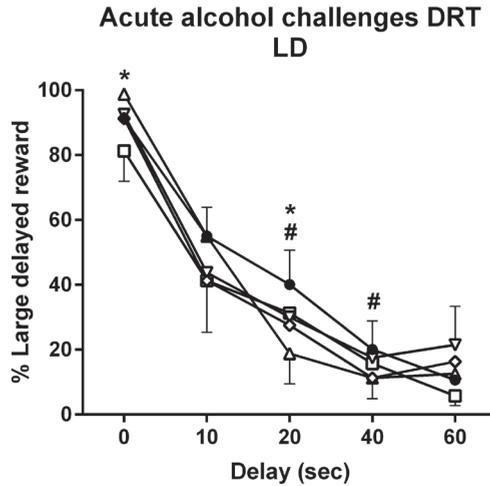
Acute alcohol treatment affected choice behaviour in the DRT ($F_{(4,108) \text{ dose}} = 4.17, p < 0.01$), dependent on the delay for the large reward ($F_{(16,352) \text{ dose} \times \text{delay}} = 2.70, p < 0.001$), but independent of group ($F_{(16,352) \text{ dose} \times \text{delay} \times \text{group}} = 1.54, \text{ n.s.}$) (Fig. 6A-B). Subsequent analyses per delay revealed a significant effect of alcohol during the 0 sec delay ($F_{(4,64) \text{ dose}} = 9.46, p < 0.001$). *Post hoc* analyses indicated that preference for the large reward increased after treatment with 0.6 g/kg alcohol. Alcohol did not affect choice behaviour during the 10 sec delay period ($F_{(4,64) \text{ dose}} = 1.39, \text{ n.s.}$). Alcohol treatment decreased preference for the large delayed reward during the 20 and 40 sec delay period ($F_{(4,28) \text{ dose}} = 3.26, p < 0.03$; $F_{(4,17) \text{ dose}} = 3.75, p < 0.03$, respectively). *Post hoc* analyses indicated that preference for the large reward during the 20 sec delay period decreased after treatment with 0.6 g/kg alcohol. Treatment with 0.4 g/kg alcohol resulted in a trend towards a decrease in the preference for the large reward during both the 20 sec delay period ($p = 0.057$) and 40 sec delay period ($p = 0.064$). During the 60 sec delay, alcohol treatment affected choice behaviour as well ($F_{(4,21) \text{ dose}} = 7.43, p < 0.002$) but no significant *post hoc* differences were observed. Treatment with alcohol reduced the number of initiated trials and perseverative responses and increased the number of omissions and the choice latency. The trial initiation latency and the collect latency were also affected by alcohol but no dose-dependent increase or decrease was observed. Alcohol affected the number of perseverative responses and the choice latency differently between LD and HD, but *post hoc* analyses did not reveal consistent differences between the groups (Table 2).

Pavlovian Conditioned Approach

In the rGT rats (Experiment 1), the number of lever contacts in the Pavlovian conditioned approach task increased over sessions ($F_{(4,50) \text{ session}} = 3.76, p < 0.02$), whereas the head entries into the food magazine during CS presentation remained unchanged ($F_{(5,73) \text{ session}} = 0.73, \text{ n.s.}$) (Fig. 7A-B). The total number of lever contacts was higher in HD than in LD ($F_{(1,14) \text{ group}} = 8.47, p < 0.02$) and this increased to a further extent over sessions ($F_{(4,50) \text{ session} \times \text{group}} = 3.01, p < 0.04$) (Fig. 7A). *Post hoc* analyses indicated that the number of lever presses was higher in HD during sessions 6 and 7. The number of head entries into the food magazine during CS presentation was not different between groups ($F_{(1,14) \text{ group}} = 1.73, \text{ n.s.}$; $F_{(5,73) \text{ session} \times \text{group}} = 1.46, \text{ n.s.}$) (Fig. 7B). As a result, the response bias was higher in HD than in LD ($F_{(1,14) \text{ group}} = 4.98, p < 0.05$), but this did not develop differently in HD and LD ($F_{(4,53) \text{ session}} = 2.53, p = 0.055$; $F_{(4,53) \text{ session} \times \text{group}} = 1.84, \text{ n.s.}$) (Fig. 7C).

Figure 6

A



B

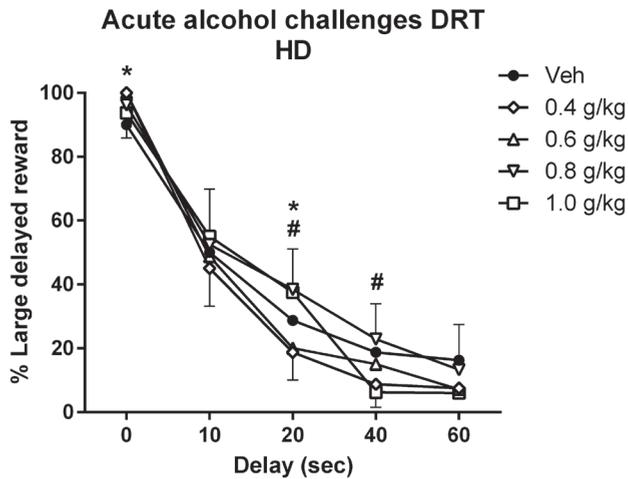


Figure 6. Effects of alcohol treatment on choice behaviour in the DRT. Alcohol affected choice behaviour, depending on the delay for the large reward. During the 0 sec delay, the preference for the large delayed reward increased after treatment with 0.6 g/kg alcohol. Alcohol reduced the preference for the large delayed reward during the 20 sec and 40 sec delay, which was significant after treatment 0.6 g/kg alcohol during the 20 sec delay, and trends were observed after treatment with 0.4 g/kg alcohol during the 20 sec and 40 sec delay. Data are shown as the mean percentage choice + SEM. For reasons of clarity, the results of the LD and HD are shown separately and the SEM are depicted for only the lowest and highest values in the graph. * Significant difference from vehicle for 0.6 g/kg alcohol (*post hoc* paired t-tests, $p < 0.05$), # Trend towards a significant difference from vehicle for 0.4 g/kg alcohol (*post hoc* paired t-tests, $p < 0.065$).

Table 1

Effects of alcohol treatment on behaviour in the rGT.

Variable	Dose effect	Vehicle	0.4 g/kg	0.6 g/kg	0.8 g/kg	1.0 g/kg
Choices	$F_{(4,64) \text{ dose}} = 38.65, p < 0.001$	HD	72.25±7.75	69.38±6.06	62.88±8.23	39.75#±4.25
	$F_{(1,16) \text{ group}} = 3.06, p = 0.086$	LD	70.75±4.86	71.63±2.93	57.38±4.20	62.13±5.63
	$F_{(4,64) \text{ dose} \times \text{group}} = 7.91, p < 0.001$					45.25*±7.01
Omissions	$F_{(4,64) \text{ dose}} = 112.35, p < 0.001$	HD	25.13±5.81	18.13±4.71	21.00±7.54	50.88*±10.08
	$F_{(1,20) \text{ group}} = 1.43, \text{ n.s.}$	LD	14.13±4.58	8.75±2.42	16.13±5.38	29.25±6.60
	$F_{(4,64) \text{ dose} \times \text{group}} = 14.36, p < 0.001$					44.13*±8.77
Premature	$F_{(4,64) \text{ dose}} = 35.50, p < 0.001$	HD	14.25±4.88	14.00±2.69	10.88±4.15	4.00*±1.79
	$F_{(1,8) \text{ group}} = 0.70, \text{ n.s.}$	LD	9.00±2.20	7.00±1.55	6.38±1.34	6.50±1.52
	$F_{(4,64) \text{ dose} \times \text{group}} = 9.32, p < 0.001$					4.25*±1.03
Perseverative	$F_{(4,64) \text{ dose}} = 48.75, p < 0.001$	HD	24.63±5.54	31.00±3.54	22.25±5.98	6.63#*±2.22
	$F_{(1,8) \text{ group}} = 1.06, \text{ n.s.}$	LD	36.75±8.41	40.38±7.68	22.75±3.61	13.00*±2.49
	$F_{(4,64) \text{ dose} \times \text{group}} = 4.25, p < 0.005$					18.00*±4.80
Choice latency (sec)	$F_{(4,17) \text{ dose}} = 59.83, p < 0.001$	HD	3.27±0.48	3.13±0.37	3.69*±0.50	4.25±0.28
	$F_{(1,17) \text{ group}} = 1.49, \text{ n.s.}$	LD	3.07±0.36	3.20±0.34	3.95±0.37	4.01*±0.21
	$F_{(4,17) \text{ dose} \times \text{group}} = 16.10, p < 0.001$					3.79*±0.20
Collect latency (sec)	$F_{(4,64) \text{ dose}} = 11.07, p < 0.001$	HD	2.10±0.32	2.15±0.39	2.23±0.19	2.94*±0.32
	$F_{(1,45) \text{ group}} = 0.01, \text{ n.s.}$	LD	2.63±0.56	2.45±0.42	2.52±0.78	2.53±0.38
	$F_{(4,64) \text{ dose} \times \text{group}} = 5.97, p < 0.001$					2.65±0.41

Data are shown as the mean ± SEM. * Significantly different from vehicle (*post hoc* paired t-test, $p < 0.05$). # Significant difference between HD and LD (*post hoc* Student's t-test, $p < 0.05$).

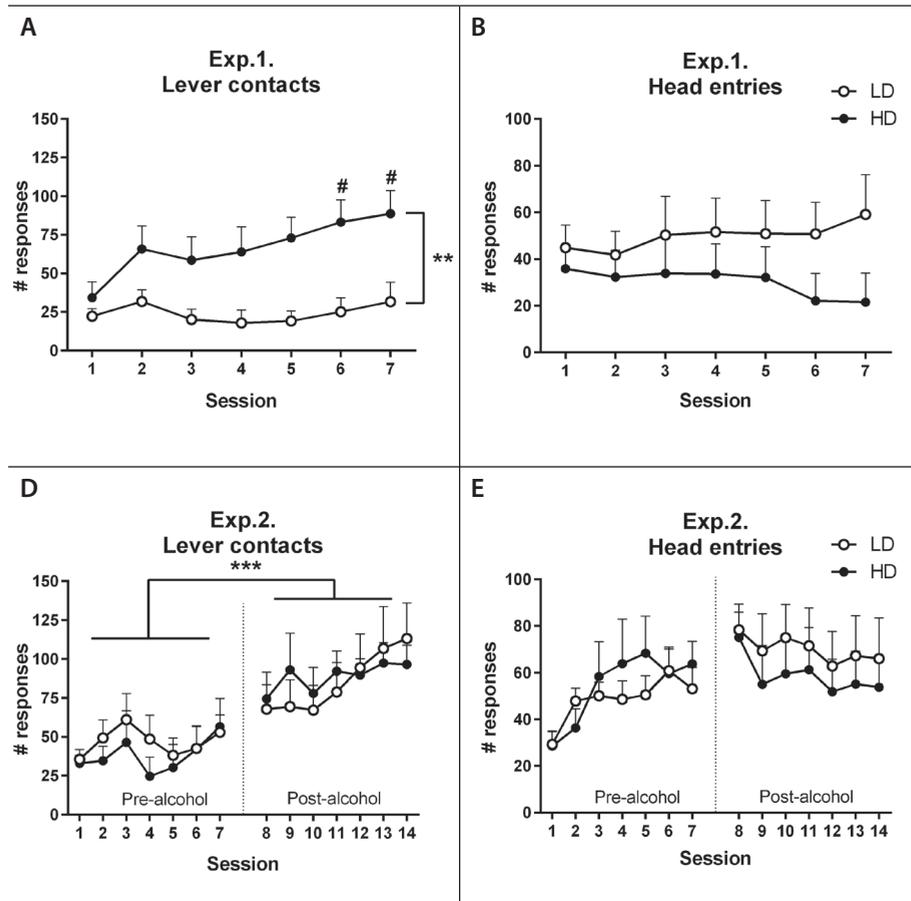
Table 2

Effects of alcohol treatment on behaviour in the DRT.

Variable	Dose effect	Vehicle	0.4 g/kg	0.6 g/kg	0.8 g/kg	1.0 g/kg
Initiated trials	$F_{(4,17) \text{ dose}} = 13.60, p < 0.001$	HD	64.75±0.67	63.13±2.22	60.25±3.53	57.00±4.13
	$F_{(1,14) \text{ group}} = 0.99, \text{ n.s.}$	LD	65.25±0.65	66.13±1.95	63.88±2.58	61.88±3.57
	$F_{(4,17) \text{ dose} \times \text{group}} = 0.30, \text{ n.s.}$					
Omissions	$F_{(4,17) \text{ dose}} = 20.42, p < 0.001$	HD	21.63±9.19	15.88±4.60	38.50*±14.18	43.88*±15.13
	$F_{(1,14) \text{ group}} = 3.17, p = 0.082$	LD	31.00±6.75	29.88±7.14	36.88*±8.71	54.63*±12.08
	$F_{(4,64) \text{ dose} \times \text{group}} = 1.02, \text{ n.s.}$					
Premature	$F_{(4,29) \text{ dose}} = 1.16, \text{ n.s.}$	HD	5.25±1.83	4.63±1.80	3.38±1.25	2.50±0.94
	$F_{(1,14) \text{ group}} = 2.17, \text{ n.s.}$	LD	6.63±2.35	6.25±1.33	4.50±1.25	5.38±1.65
	$F_{(4,29) \text{ dose} \times \text{group}} = 1.47, \text{ n.s.}$					
Perseverative	$F_{(4,64) \text{ dose}} = 4.90, p < 0.003$	HD	22.38±5.35	31.13±4.45	23.25±5.46	16.63±6.20
	$F_{(1,23) \text{ group}} = 0.15, \text{ n.s.}$	LD	24.13±6.32	22.63±5.08	20.75±6.17	26.50±7.88
	$F_{(4,64) \text{ dose} \times \text{group}} = 2.77, p < 0.04$					
Trial initiate latency (sec)	$F_{(4,64) \text{ dose}} = 3.39, p < 0.015$	HD	3.29±0.39	2.96±0.33	3.29±0.37	3.53±0.25
	$F_{(1,14) \text{ group}} = 0.26, \text{ n.s.}$	LD	3.88±0.18	3.57±0.18	3.74±0.15	3.72±0.12
	$F_{(4,64) \text{ dose} \times \text{group}} = 1.42, \text{ n.s.}$					
Choice latency (sec)	$F_{(4,64) \text{ dose}} = 14.16, p < 0.001$	HD	0.43±0.06	0.45±0.05	0.56*±0.07	0.51*±0.06
	$F_{(1,8) \text{ group}} = 0.01, \text{ n.s.}$	LD	0.38±0.03	0.47±0.08	0.48±0.04	0.65*±0.09
	$F_{(4,64) \text{ dose} \times \text{group}} = 3.61, p < 0.02$					
Collect latency (sec)	$F_{(4,13) \text{ dose}} = 6.19, p < 0.01$	HD	2.86±0.48	2.59±0.45	4.08±1.25	3.08±0.36
	$F_{(1,15) \text{ group}} = 1.74, \text{ n.s.}$	LD	4.09±1.33	2.87±0.32	3.22±0.30	3.96±0.62
	$F_{(4,13) \text{ dose} \times \text{group}} = 2.70, p = 0.079$					

Data are shown as the mean ± SEM. * Significantly different from vehicle (post hoc paired t-tests, $p < 0.05$).

Figure 7



To assess whether this difference in approach behaviour between HD and LD was a cause for or a consequence of the different amounts of alcohol consumed by HD and LD, we assessed Pavlovian conditioned approach behaviour prior to and after IAA in the DRT rats (Experiment 2; Fig. 1). We observed that IAA influenced the number of lever presses ($F_{(1,14) \text{ IAA}} = 27.41, p < 0.001$) in a similar manner in HD and LD ($F_{(1,14) \text{ IAA} \times \text{group}} = 1.40, \text{ n.s.}$) (Fig. 7D). The number of lever presses increased in a different manner before and after IAA ($F_{(3,45) \text{ IAA} \times \text{session}} = 3.19, p < 0.03$), independent of group ($F_{(3,45) \text{ IAA} \times \text{session} \times \text{group}} = 1.24, \text{ n.s.}$). Separate analyses before IAA indicated an increase in the number of lever presses over sessions ($F_{(3,49) \text{ session}} = 7.66, p < 0.001$), independent of group ($F_{(1,14) \text{ group}} = 0.12, \text{ n.s.}$; $F_{(3,49) \text{ session} \times \text{group}} = 1.16, \text{ n.s.}$). Similar effects were observed after IAA ($F_{(4,55) \text{ session}} = 3.95, p < 0.01$; $F_{(1,14) \text{ group}} = 0.07, \text{ n.s.}$; $F_{(4,55) \text{ session} \times \text{group}} = 0.86, \text{ n.s.}$) (Fig. 7D). The head entries into the food magazine

Figure 7

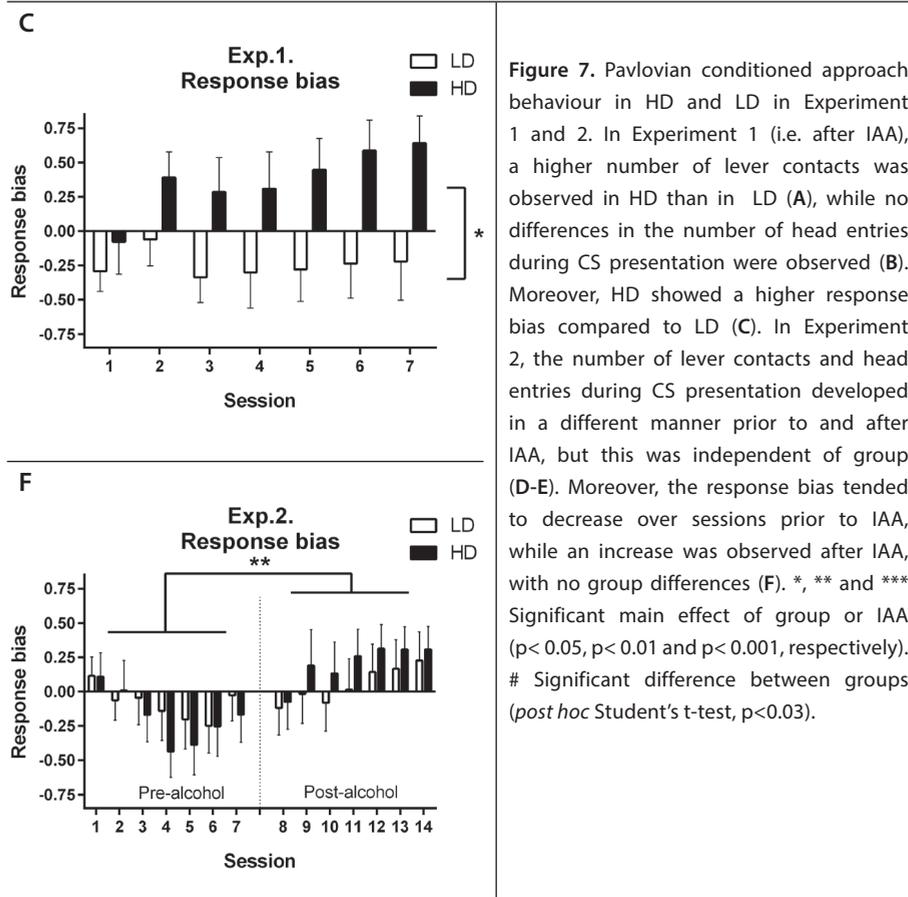


Figure 7. Pavlovian conditioned approach behaviour in HD and LD in Experiment 1 and 2. In Experiment 1 (i.e. after IAA), a higher number of lever contacts was observed in HD than in LD (A), while no differences in the number of head entries during CS presentation were observed (B). Moreover, HD showed a higher response bias compared to LD (C). In Experiment 2, the number of lever contacts and head entries during CS presentation developed in a different manner prior to and after IAA, but this was independent of group (D-E). Moreover, the response bias tended to decrease over sessions prior to IAA, while an increase was observed after IAA, with no group differences (F). *, ** and *** Significant main effect of group or IAA ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). # Significant difference between groups (*post hoc* Student's t-test, $p < 0.03$).

during CS presentation developed in a different manner before and after IAA ($F_{(4,61) \text{ IAA} \times \text{session}} = 7.78$, $p < 0.001$), independent of group ($F_{(4,61) \text{ IAA} \times \text{session} \times \text{group}} = 0.76$, n.s.) (Fig. 7E). Separate analyses before IAA indicated an increase in the number of head entries into the food magazine during CS presentation ($F_{(4,55) \text{ session}} = 3.95$, $p < 0.01$), independent of group ($F_{(1,14) \text{ group}} = 0.06$, n.s.; $F_{(4,55) \text{ session} \times \text{group}} = 0.86$, n.s.), whereas a decrease was observed after IAA exposure ($F_{(3,45) \text{ session}} = 2.95$, $p < 0.04$), independent of group ($F_{(1,14) \text{ group}} = 0.34$, n.s.; $F_{(3,45) \text{ session} \times \text{group}} = 0.43$, n.s.) (Fig. 7E). IAA influenced the response bias ($F_{(1,14) \text{ IAA}} = 8.47$, $p < 0.02$) in a similar manner in HD and LD ($F_{(1,14) \text{ IAA} \times \text{group}} = 2.96$, n.s.) (Fig. 7F). The response bias followed a different pattern over sessions before and after IAA ($F_{(4,49) \text{ IAA} \times \text{session}} = 6.67$, $p < 0.001$), independent of group ($F_{(4,49) \text{ IAA} \times \text{session} \times \text{group}} = 0.93$, n.s.). Separate analyses before IAA indicated a tendency towards a decrease in the response bias over sessions

($F_{(3,46) \text{ session}} = 2.52, p=0.065$), independent of group ($F_{(1,14) \text{ group}} = 0.22, n.s.$; $F_{(3,46) \text{ session} \times \text{group}} = 0.73, n.s.$). However, after IAA, a clear increase in the response bias over sessions was apparent ($F_{(3,44) \text{ session}} = 7.55, p<0.001$), independent of group ($F_{(1,14) \text{ group}} = 0.41, n.s.$; $F_{(3,44) \text{ session} \times \text{group}} = 1.01, n.s.$) (Fig. 7F).

DISCUSSION

In the present study, we investigated the effects of voluntary alcohol intake on choice behaviour in the rGT and the DRT. HD showed more optimal choice behaviour in both the rGT and DRT. Moreover, sign-tracking behaviour was enhanced in HD and sign-tracking behaviour was enhanced after IAA. Acute alcohol exposure increased the preference for the optimal choice in the rGT and increased impulsive choice in the DRT, but this occurred in a similar manner in HD and LD. These findings highlight the association between alcohol use, the behavioural responses to conditioned rewards, impulsivity and decision making and suggest that a high alcohol consumption phenotype relates to enhanced cue- or reward-driven cognitive performance.

During nose poke training prior to the rGT and DRT, we observed a higher percentage of correct responses in HD than in LD. Moreover, we observed a higher percentage choice for the optimal option in HD in the rGT. Together, these data indicate improved cognitive performance in HD, perhaps because HD were more engaged and focused during the tasks. Impairments in decision making and exaggerated levels of impulsivity have generally been observed in severe AUD patients, and to a lesser extent in, for example, binge drinkers and heavy drinkers (Vuchinich and Simpson 1998; Bechara et al., 2001; Petry 2001; Fein et al., 2004; Field et al., 2007; Johnson et al., 2008; Loeber et al., 2009; Salgado et al., 2009; Claus et al., 2011; Gullo and Stieger 2011; MacKillop et al., 2011; Reed et al., 2012; Le Berre et al., 2014). Because HD display key characteristics of AUD, i.e. increased motivation for obtain alcohol and loss of control over alcohol use (Spoelder et al., 2015a), we hypothesized that HD would show suboptimal decision making. However, HD performed better in both the rGT and DRT. Interestingly, several studies are in line with our findings. For example, no differences or even less risky decision making have been observed in AUD patients in the Balloon Analogue Risk Task (Ashenhurst et al., 2011; Claus and Hutchison 2012). Similarly, several preclinical studies showed that alcohol exposure during adulthood did not affect decision making or actually increased cognitive performance (DePoy et al., 2013; Mejia-Toiber et

al., 2014; Schindler et al., 2014). Taken together, these findings show that HD display behavioural characteristics of AUD, but these characteristics are not necessarily paralleled by impaired decision making.

The better performance of HD during nose poke training and the rGT, as well as the increase in impulsive actions in HD, lead us to think that HD might attribute more value to primary or conditioned rewards. In fact, it has been proposed that poorly controlled alcohol drinking may be due to an involuntary sign-tracking conditioned response, resulting in increased consumption of alcohol when confronted with alcohol-related cues (Olmstead et al., 2006; Tomie and Sharma 2013). Interestingly, we observed enhanced conditioned approach behaviour towards a reward-predictive cue in HD compared to LD in the Pavlovian conditioned approach task. In a follow-up of this finding, we showed that LD and HD did not differ in their approach behaviour prior to IAA; the rats all showed a tendency towards goal-tracking behaviour at this stage. A previous study also reported goal-tracking in alcohol naïve rats, which was more pronounced in alcohol-preferring rats (Pena-Oliver et al., 2015). After IAA, all rats were again tested in the Pavlovian conditioned approach task, where they showed increased sign-tracking but this was independent of their level of alcohol consumption. This observation extends previous work that reported increases in sign-tracking behaviour after a period of alcohol exposure (McClory and Spear 2014; Spoelder et al., 2015c). The absence of a group difference in approach behaviour after IAA in Experiment 2 is in apparent contrast to the initial findings, and this is likely related to the fact that the rats in Experiment 2 had already been tested in the Pavlovian conditioned approach task prior to IAA. Human studies have, likewise, reported an association between an approach tendency towards reward-predicting cues and individual levels in alcohol consumption (Field and Cox 2008; Stacy and Wiers 2010). Interestingly, in a recent study, it was shown that the relationship between automatic alcohol approach tendencies and alcohol consumption was not dependent on the level of impulsivity, as measured by the Barratt Impulsiveness Scale (BIS-11), the DRT and a Go/No-Go Task, indicating that the multiple components of impulsivity and the automatic approach tendencies explain a unique variance in alcohol consumption (Christiansen et al., 2012). Taken together, these results show that HD attribute more value to reward-associated cues. Moreover, the enhanced sign-tracking conditioned response in HD is not a trait effect, but is rather the consequence of their high levels of alcohol intake.

Enhanced motor impulsivity, measured by premature responses, has also been observed in AUD patients (Voon et al., 2013) and binge drinkers (Sanchez-Roige et al., 2014). In the rGT, we observed a transient increase in the number of premature responses in HD compared to LD during challenge sessions with a long ITI, while the groups did not differ in impulsive action during baseline sessions with a 5 sec ITI. It has been reported that potential group differences in impulsive action can be enlarged under unexpected and challenging task conditions, such as increasing the ITI between sessions (Dalley et al., 2007; Baarendse and Vanderschuren 2012; Sanchez-Roige et al., 2014). Indeed, previous studies showed that only challenges with long ITI or variable ITI's induced increases in impulsive action after acute or chronic treatment with alcohol (Oliver et al., 2009; Walker et al., 2011; Irimia et al., 2013).

We observed lower impulsive choice behaviour in HD in the DRT. The HD showed a higher preference for the delayed reward during the initial phases of the DRT experiment where the delay for the large reward was relatively short. However, when we increased the delays for the large reward to 60 sec, choice behaviour was no longer different between LD and HD. Upon the reversal of the delays within the session, both subgroups adapted their choice behaviour in a comparable manner. Hence, it is not likely that these findings reflect perseverative responding in HD. The observed transient effect in impulsive choice may be related to the time period between IAA and the DRT test phases. Indeed, upon re-exposure to alcohol for six IAA sessions between phase 5 and 6, an enhanced preference for the large delayed reward tended to emerge again in HD. Another possibility is that the group differences are only observed when 'short' delays for the large reward were used. We observed that the variability in choice behaviour between rats declined as the delays were further increased to a final 60 sec, as has been observed by others as well (Flagel et al., 2010). Interestingly, in line with the current findings, sign-trackers showed enhanced impulsive action, but reduced impulsive choice (Flagel et al., 2010; Lovic et al., 2011). However, contrasting findings have also been reported. Several studies reported that alcohol-naïve alcohol-preferring rats and mice show enhanced impulsive choice behaviour in the DRT compared to their non-preferring counterparts (Wilhelm and Mitchell 2008; Oberlin and Grahame 2009; Beckwith and Czachowski 2014; Perkel et al., 2015), although this finding was not observed by others (Wilhelm et al., 2007; Wilhelm and Mitchell 2012). The delays used in these studies (8, 16 and 25 sec) are in the range of the delays we used during the early phases of the DRT in the current

study (12, 24 and 48 sec). Together, these findings show that HD display lower impulsive choice behaviour, an effect that may be masked at larger delays.

Acute alcohol treatment improved decision making in the rGT in LD and HD. This observation is in contrast to previous findings, showing impaired or unaltered decision making upon acute alcohol exposure in humans and rodents (Lane et al., 2004; George et al., 2005; Ramaekers and Kuypers 2006; Mitchell et al., 2011; Pena-Oliver et al., 2014; Spoelder et al., 2015b). Alcohol-induced perseverance in responding may have increased the percentage choice for the already preferred option in this study, although it remains unclear why this would have occurred after IAA and not in alcohol-naïve or in rats that were pre-exposed to alcohol by injections (Spoelder et al., 2015b). Acute alcohol exposure increases impulsive choice by increasing the preference for the small immediate reward in both subgroups, which is in line with previous studies (Poulos et al., 1995; Tomie et al., 1998; Evenden and Ryan 1999; Olmstead et al., 2006; Wilhelm and Mitchell 2012). These findings also corroborate with increased impulsive choice upon acute alcohol exposure in heavy alcohol drinking individuals compared to light drinkers (Marczinski et al., 2007; King et al., 2011; Reed et al., 2012).

To conclude, the current results show a relationship between the level of alcohol consumption and decision making, impulsivity and Pavlovian conditioned approach behaviour. HD perform better than LD in both the rGT and DRT, allowing them to maximize their gains. In addition, HD show an increased incentive salience to a food-predicting cue, which was the result of alcohol rather than a pre-existing trait. Together, these findings provides novel insight into the underlying mechanisms for individual differences in alcohol consumption that is propelled by more efficient cue- and reward-driven learning processes.

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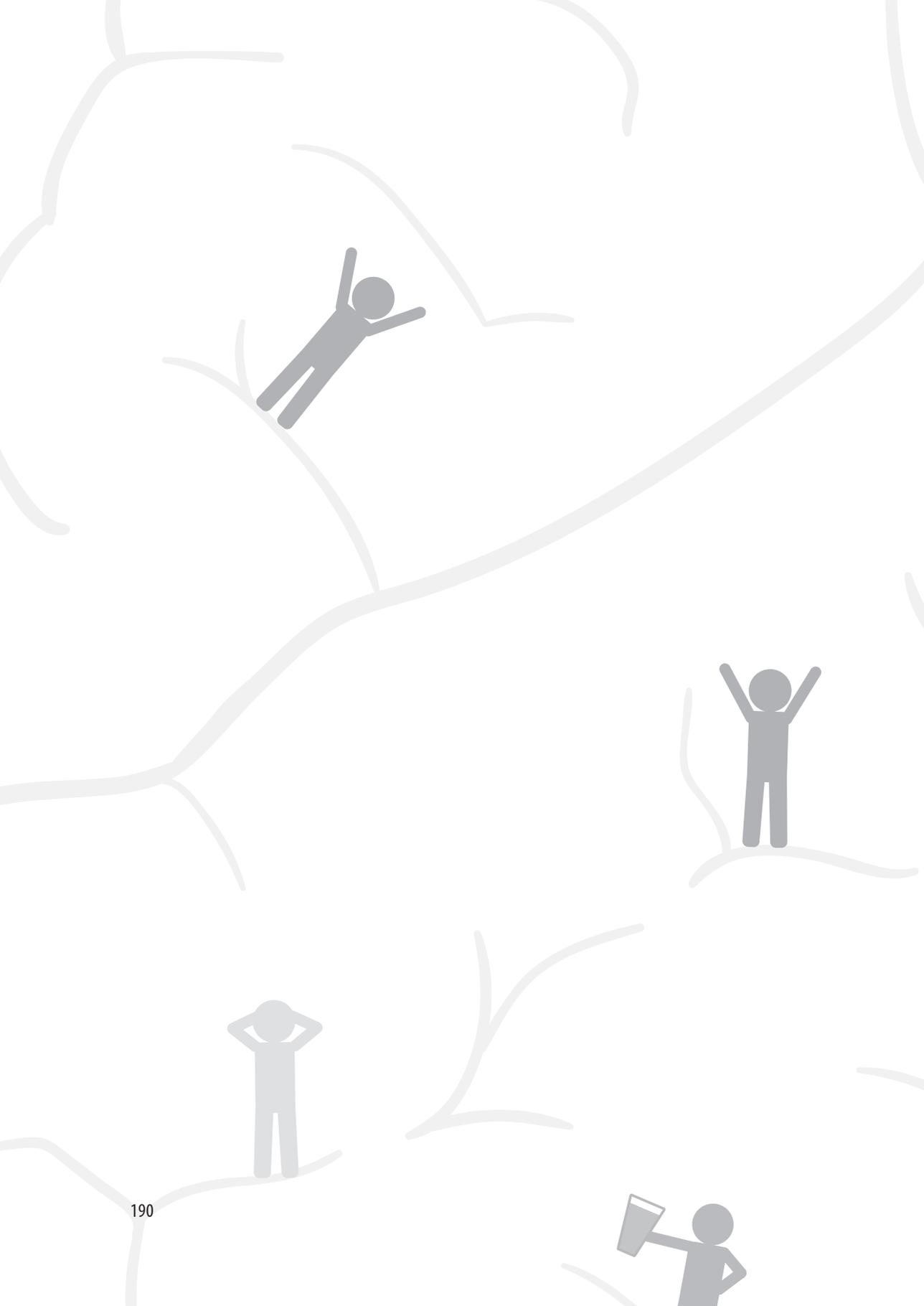
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CHAPTER 6

EARLY SOCIAL ISOLATION AUGMENTS ALCOHOL CONSUMPTION IN RATS

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ABSTRACT

There is a substantial degree of individual vulnerability for alcohol use disorder (AUD), as only a subpopulation of individuals who regularly consume alcohol develop AUD. It is therefore of great importance to understand the factors and mechanisms that contribute to the individual risk for AUD. In this regard, social influences, in particular during development, may be relevant for AUD, since disruptions in early social experiences are associated with an increased risk for AUD. Social play, the most prominent form of social behaviour displayed by young mammals, is rewarding and thought to be important for social, emotional and cognitive development. Recent studies suggest that early social isolation, effectively depriving animals from social play, increases the risk for addictive behaviour. The aim of this study was therefore to explore the long-term consequences of early social isolation on alcohol consumption and motivation for alcohol. To that aim, rats were socially isolated from postnatal days 21-42, followed by four weeks of social housing, and voluntary alcohol consumption and operant responding for alcohol were determined in adulthood. We observed enhanced levels of alcohol consumption in adulthood in previously isolated rats, while operant responding for alcohol was not altered. The impact of early social isolation was independent of the individual variation in alcohol consumption. These data indicate that social isolation, during a developmental period when social play is highly abundant, enhances the propensity to consume alcohol in adulthood. This implies that early social experience may be a protective factor against excessive alcohol use.

INTRODUCTION

Alcohol use disorder (AUD) is a chronic relapsing disorder that affects approximately 76 million people worldwide, thus representing a formidable medical and socioeconomic problem for our society (Rehm et al., 2009; WHO 2011; American Psychiatric Association 2013). Importantly, there is a substantial degree of variability in the vulnerability for developing AUD. That is, most individuals consume alcohol in a controlled manner, but a subpopulation of 3-5% of individuals who regularly consume alcohol come to develop AUD (WHO 2011). Considering that treatment strategies for addiction are currently limited in number and efficacy (O'Brien, 2008; Koob et al., 2009; van den Brink 2012; Pierce et al., 2012), understanding the factors and mechanisms that contribute to the individual variability in the propensity for AUD is essential to prevent and treat this devastating disorder.

Alcohol is often consumed in a social context. In fact, social context and peer norms are important determinants of alcohol consumption (e.g. Perkins 2002; Homish and Leonard 2008; Lau-Barraco et al., 2012). Recent animal studies also provide support for social influences on alcohol consumption. For example, voles show greater preference for alcohol when housed in pairs and they adjust their alcohol consumption in the presence of lower alcohol drinking cagemates (Anacker et al., 2011a; 2011b). Conversely, early social insults have been shown to enhance the propensity to consume alcohol and other drugs of abuse, suggesting that a dysfunctional social context during development comprises a risk factor for AUD (Bonin et al., 2000; Alwan et al., 2011; Stickley et al., 2013; Whelan et al., 2014). Furthermore, social disorders in childhood and adolescence, in particular disruptive behaviour disorders, are associated with a greater incidence of AUD (Compton et al., 2005; Goldstein et al., 2007). There is also ample evidence from animal studies supporting a critical role of social development in addiction sensitivity. For example, peer rearing in non-human primates consistently leads to elevated alcohol consumption in adulthood when compared to mother-reared conspecifics (Higley et al., 1991; Fahlke et al., 2000; Huggins et al., 2012). In agreement with these findings, maternal separation in rodents is known to induce persistent increases in alcohol consumption in adulthood (Roman et al., 2005; Cruz et al., 2008; Nylander and Roman 2013). Furthermore, post-weaning isolation rearing in rodents has repeatedly been shown to result in augmented alcohol consumption and operant responding for alcohol in adulthood (Ellison 1981; Schenk et al.,

1990; Wolffgramm 1990; Hall et al., 1998; Lodge and Lawrence 2003; Advani et al., 2007; Deehan et al., 2007; McCool and Chappell 2009; Sanna et al., 2011; Chappell et al., 2013; Butler et al., 2014).

Social play behaviour is the most prominent form of social behaviour displayed by young mammals (Panksepp et al., 1984; Vanderschuren et al., 1997; Pellis and Pellis 2009). Social play behaviour is rewarding, as demonstrated using place conditioning, operant tasks and T-maze tasks (Mason et al., 1962; Humphreys and Einon 1981; Calcagnetti and Schechter 1992; for review see Trezza et al., 2011). Importantly, social play behaviour is modulated through neural systems that also mediate the rewarding effects of substances of abuse (Trezza et al., 2010; Siviý and Panksepp 2011). This suggests a critical role of social play in the development of brain reward circuitry, which may determine an individual's sensitivity to addictive behaviour. Interestingly, acute treatment with alcohol enhances social play behaviour (e.g. Varlinskaya et al., 2001; Varlinskaya and Spear 2002; Trezza et al., 2009) and the sensitivity to the facilitating effects of acute alcohol on social play behaviour seems to influence the amount of alcohol consumption during adolescence in a sex dependent manner (Varlinskaya et al., 2015). Post-weaning social isolation rearing has been shown to enhance alcohol consumption in rats and mice (Ellison 1981; Schenk et al., 1990; Wolffgramm, 1990; Hall et al., 1998; Lodge and Lawrence 2003; Advani et al., 2007; McCool and Chappell 2009; Sanna et al., 2011; Chappell et al., 2013; Butler et al., 2014). However, these animals were reared in isolation from weaning onward, which leaves the question whether deprivation of social play behaviour contributed to the increase in alcohol consumption. In fact, animals that were socially isolated only during the period in development when social play is most abundant (i.e. postnatal day (PND) 21-42, Panksepp 1981), thus effectively depriving them of social play, show enhanced sensitivity for cocaine self-administration and amphetamine- and alcohol-induced conditioned place preference in adulthood (Whitaker et al., 2013; Baarendse et al., 2014). Taken together, these studies suggest that social play behaviour is essential for the adaptive development of brain reward mechanisms, such that deprivation of social play may increase the risk for later addictive behaviour (Trezza et al., 2014). However, the importance of social play behaviour for the sensitivity or resilience to alcohol consumption is unknown. The aim of this study was therefore to explore the long-term consequences of early social isolation on alcohol consumption and motivation for alcohol. Therefore, we socially isolated rats during PND21-42, and determined alcohol consumption

and operant responding for alcohol during adulthood. We hypothesized that early social isolation would lead to enhanced levels of alcohol consumption in adulthood and increased operant responding and motivation for alcohol.

MATERIALS AND METHODS

Subjects

Male Lister Hooded rats (Charles River, Germany) arrived in litters of six to eight pups at an age of 14 days with a nursing mother. The rats were housed with food and water *ad libitum* under controlled conditions ($20\pm 2^{\circ}\text{C}$ and 50–70% humidity) in a reversed 12-h day/night cycle (lights on, 7 p.m.). Experimental procedures were approved by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op de dierenproeven, 1996) and European regulations (Guideline 86/609/EEC).

Early social isolation and alcohol consumption

As in our previous studies (Baarendse et al., 2013; 2014), the rats were weaned and housed either socially in groups of four rats per cage (SOC) or individually (ISO) at 21 days of age. Half of the rats of each litter was assigned to the SOC group and the other half to the ISO group, in a semi-randomized manner. The rats of the ISO group were re-socialized, i.e. housed together with another previously isolated animal, on day 43. At that time, the animals in the SOC groups were also housed in pairs. After four weeks of social housing, all animals were individually housed for subsequent alcohol consumption experiments two weeks later. The experiment was performed in two batches (N = 24 and N = 48).

For alcohol consumption experiments, we adopted the intermittent every-other-day alcohol access model (Wise, 1973; Simms et al., 2008). Two bottles, fitted with stainless-steel dual ball bearing drinking spouts were placed on the home cage, one bottle contained alcohol (20%, v/v) (Klinipath, The Netherlands) and the other contained water. The positions of the bottles were switched between sessions to avoid the development of side preference. During three consecutive weeks, the rats were given 7-hour concurrent access to alcohol and water on Monday, Wednesday and Friday, during the dark phase of the day-night cycle. Subsequently, during another three consecutive weeks, the rats were given 24-hour concurrent access to alcohol and water, again on Monday, Wednesday and Friday, starting at the beginning of the dark phase of

the day-night cycle. The bottles were weighed before and after each alcohol access period to determine the amount of alcohol and water the animals consumed. Alcohol intake (g/kg), alcohol preference (percentage alcohol volume relative to total volume consumed) and total fluid intake (ml/kg) were calculated per rat per session. Subsequently, the alcohol intake, preference and total fluid intake were averaged across sessions, into values representing average alcohol intake, preference and total fluid intake over the 7h or 24h alcohol consumption sessions, respectively. The rats were divided into Low, Medium and High alcohol drinking rats based on their average alcohol intake in g/kg; this division was made within each social housing group (SOC or ISO). The rats were assigned ranking scores (i.e. 1, 2, 3, 4 etc.; corresponding to the number of rats in the group, in this case 1-12 for both the SOC and ISO group in batch 1 and 1-24 for both the SOC and ISO group in batch 2) based on their average alcohol intake per week. Then, to calculate a total ranking score, the weekly ranking scores were summed across the six weeks of alcohol consumption in order to select rats with a consistent low or high level of alcohol intake. Rats within the lower, middle and upper 33% of the rank list were designated as Low, Medium and High alcohol drinking rats, respectively.

Operant Responding for Alcohol

After two months of alcohol consumption, all rats of the first batch (N = 12 for SOC and ISO) and half of the rats of the second batch (N = 12 for SOC and ISO) were trained to respond for alcohol in operant conditioning chambers. The other half of the second batch were used for pharmacological studies (not in this manuscript). The rats were trained to self-administer alcohol in operant chambers (29.5 cm L, 24 cm W 25 cm H; Med Associates, Georgia, VT, USA) that were enclosed in light- and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8 cm wide retractable levers, the levers were placed 11.7 cm apart and 6 cm from the grid floor. A liquid dipper within a recessed magazine was situated between the levers. A cue light was present above each lever (28 V, 100mA) and a house light (28 V, 100mA) was located on the opposite wall. The position of the active and inactive levers was counterbalanced between rats. Pressing the active lever raised the dipper cup containing alcohol (0.1 ml, 20% v/v), illuminated the cue light above the active lever and switched off the house light. Access to alcohol was terminated 10 sec after detection of a head entry into the magazine, the cue light was turned off, and after a 5 sec interval a new trial started. Pressing the inactive lever was recorded, but had no programmed consequences. To

limit alcohol evaporation, the container was filled with fresh alcohol solution before each session. Experimental events and data recording were controlled using MED-PC for Windows.

As soon as the animals had acquired responding, defined as less than 25% variation in active lever presses over 3 consecutive sessions under the fixed ratio (FR) 1 schedule of reinforcement, the response requirement was increased to an FR2, then to an FR5 and finally to an FR10 schedule of reinforcement, with the requirement that each rat had to earn at least 10 rewards for 2 sessions before progressing to FR5 and FR10, respectively. Subsequently, the rats had to earn at least 10 rewards for 3 sessions during FR10 training before progressing to the progressive ratio (PR) schedules of reinforcement. These requirements were set to assure that the rats understood the task contingencies and performed at least 100 presses under an FR10 to assess a reliable motivation during PR sessions. Once the rats completed FR10 training, a linear PR schedule of reinforcement was introduced, in which 2 (PR2, i.e. 2, 4, 6, 8, 10, etc.) and subsequently 4 (PR4; i.e. 4, 8, 12, 16, 20, etc.) additional lever presses were required for each subsequent reward. This PR paradigm, rather than the commonly used exponential increase in the response requirement (Richardson and Roberts, 1996) was chosen based on the results of previous studies which showed that 1) alcohol non-preferring rats have low breakpoints; 2) the required workload should be increased, however, before the sedative effects of alcohol begin to interfere with operant performance; 3) because alcohol is delivered in relatively small sizes (0.1ml/reinforcement) with a slow absorption rate (Hodos 1961; Ritz et al., 1994; Rodd et al., 2003). Responding was deemed stable when there was <25% variation in reward deliveries over three subsequent sessions. Two rats from the SOC group did not reach stable responding on the PR2 schedule of reinforcement and therefore did not proceed to PR4. The rats were tested for 3 days/week (Monday, Wednesday and Friday), and sessions lasted for 30 min, except for the PR4 schedule of reinforcement which lasted 60 min. The breakpoint was defined as the maximum number of presses performed in the last, successfully completed ratio in either the 1 h session or when no reward had been obtained in 20 min, whichever came first.

Data Analysis

The alcohol consumption data were averaged across the 7h and 24h sessions, respectively, and analyzed by two-way ANOVA with group (SOC and ISO) and

subgroup (Low, Medium, High) as the between-subjects factors. For analyses of the operant self-administration data, the number of lever presses (FR1) and breakpoints (PR2 and PR4) were averaged over the first three sessions in which the rat acquired the response criteria as described above. These data were also analyzed by two-way ANOVA with group (SOC and ISO) and subgroup (Low, Medium, High) as the between-subjects factors. Post hoc analyses were performed when appropriate using two-tailed t-tests. Differences between pairs of means were considered significant at $\alpha < 0.05$. SPSS 22.0 (SPSS Inc., Chicago, Illinois, USA) was used for data analysis. Data are presented as mean \pm SEM.

RESULTS

Analysis of the alcohol consumption data revealed a large variation in alcohol intake in the population of the rats. Inspection of the individual levels of alcohol intake revealed a shift towards higher levels of alcohol intake across the range of alcohol consumption during the 7h and 24h access sessions for the ISO rats when compared to the SOC animals (Fig. 1).

The animals were assigned to subgroups of Low, Medium and High alcohol drinking rats. Analysis of the 7 hour alcohol consumption data showed that the subgroups (Low, Medium and High) differed in their level of alcohol intake ($F_{(2,71) \text{ subgroup}} = 106.5, P < 0.001$). Moreover, early social isolation increased the level of alcohol intake ($F_{(1,71) \text{ group}} = 7.6, P < 0.01$), but there was no differential effect of early social isolation in the subgroups of rats ($F_{(2,71) \text{ group} \times \text{subgroup}} = 1.1, \text{N.S.}$) (Fig. 2A). The Low, Medium and High alcohol drinking rats also differed in their preference for alcohol over water ($F_{(2,71) \text{ subgroup}} = 64.2, P < 0.001$) and preference for alcohol was significantly higher in the ISO group ($F_{(1,71) \text{ group}} = 7.5, P < 0.001$), but this effect was not subgroup dependent ($F_{(2,71) \text{ group} \times \text{subgroup}} = 0.137, \text{N.S.}$) (Fig. 2B). There were no differences between the groups or the subgroups in the total volume consumed by the rats in the 7 hour sessions ($F_{(2,71) \text{ subgroup}} = 0.90, \text{N.S.}; F_{(1,71) \text{ group}} = 0.47, \text{N.S.}; F_{(2,71) \text{ subgroup} \times \text{group}} = 1.3, \text{N.S.}$) (Fig. 2C).

In line with the analysis of the 7 hour consumption data, analysis of the 24 hour alcohol consumption data confirmed that the amount of alcohol consumed by Low, Medium and High alcohol drinking rats was significantly different ($F_{(2,71) \text{ subgroup}} = 109.8, P < 0.001$). Moreover, alcohol intake was higher in the ISO rats ($F_{(1,71) \text{ group}} = 12.3, P < 0.001$) which was independent of the

Figure 1

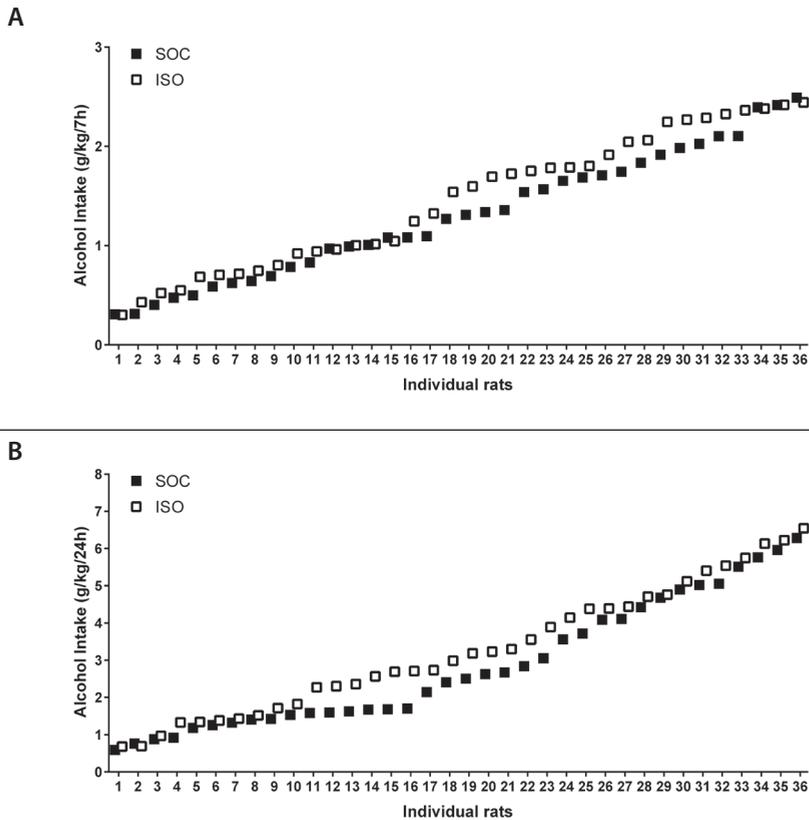


Figure 1. Alcohol consumption for individual isolated rats (ISO) and socially housed control rats (SOC) ($n = 36$). The average levels of alcohol intake (in g/kg) of each individual rat over all 7h (A) and 24h (B) access sessions are shown. There is a leftward shift in the distribution curve for the ISO rats compared with the SOC rats.

subgroup ($F_{(2,71) \text{ group} \times \text{subgroup}} = 1.2, N.S$) (Fig. 3A). The Low, Medium and High alcohol drinking rats also showed differences in their preference for alcohol over water ($F_{(2,71) \text{ subgroup}} = 101.3, P < 0.001$) and early social isolation enhanced the preference for alcohol when compared to the SOC animals ($F_{(1,71) \text{ group}} = 14.6, P < 0.001$). The increase in alcohol preference in the ISO rats was independent of the subgroup ($F_{(2,71) \text{ subgroup} \times \text{group}} = 1.5, N.S.$) (Fig. 3B). The subgroups consumed equal total volumes in the 24 hour sessions ($F_{(2,71) \text{ subgroup}} = 2.6, N.S.$) and there was no effect of early social isolation on total volume consumed ($F_{(1,71) \text{ group}} = 0.1, N.S.$) nor was there an interaction between the two factors on total fluid intake ($F_{(2,71) \text{ subgroup} \times \text{group}} = 0.08, N.S.$) (Fig. 3C).

Figure 2

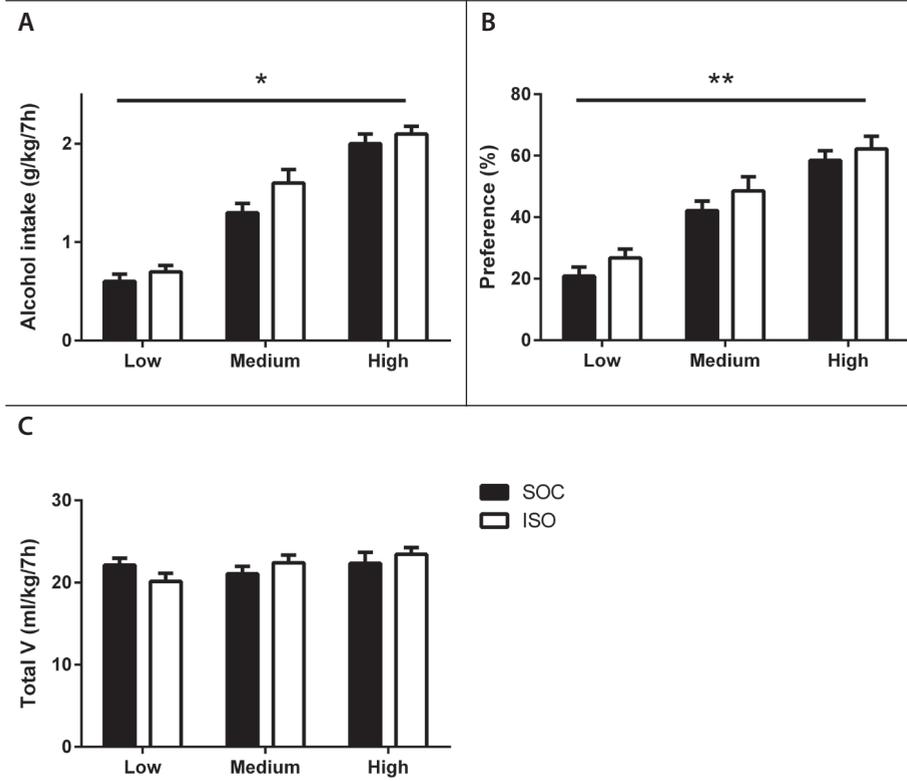


Figure 2. Average alcohol intake (A), alcohol preference (B) and total fluid consumption (C) for all 7h alcohol consumption sessions. The average data for low, medium and high subgroups of socially housed (SOC) and social play-deprived (ISO) rats ($n=12$) are shown. Early social isolation increased alcohol consumption and alcohol preference in 7h sessions without affecting the total fluid intake ($*P < 0.01$, $**P < 0.001$; main effect of early social isolation). Total V, total volume.

Subsequent to the home cage alcohol consumption, the rats were trained to respond for alcohol. Analysis of the FR1 self-administration data revealed no effect of early social isolation ($F_{(1,48) \text{ group}} = 0.32$, N.S.). The reinforcing effects of alcohol were dependent on the subgroup, as apparent from a trend ($F_{(2,48) \text{ subgroup}} = 3.0$, $P = 0.063$), but there was no differential effect of early social isolation in the subgroups of rats ($F_{(2,48) \text{ group} \times \text{subgroup}} = 1.9$, N.S.) (Fig. 4A).

Finally, the rats were tested under PR2 and PR4 schedules of reinforcement to determine whether early social isolation affects the motivation to

Figure 3

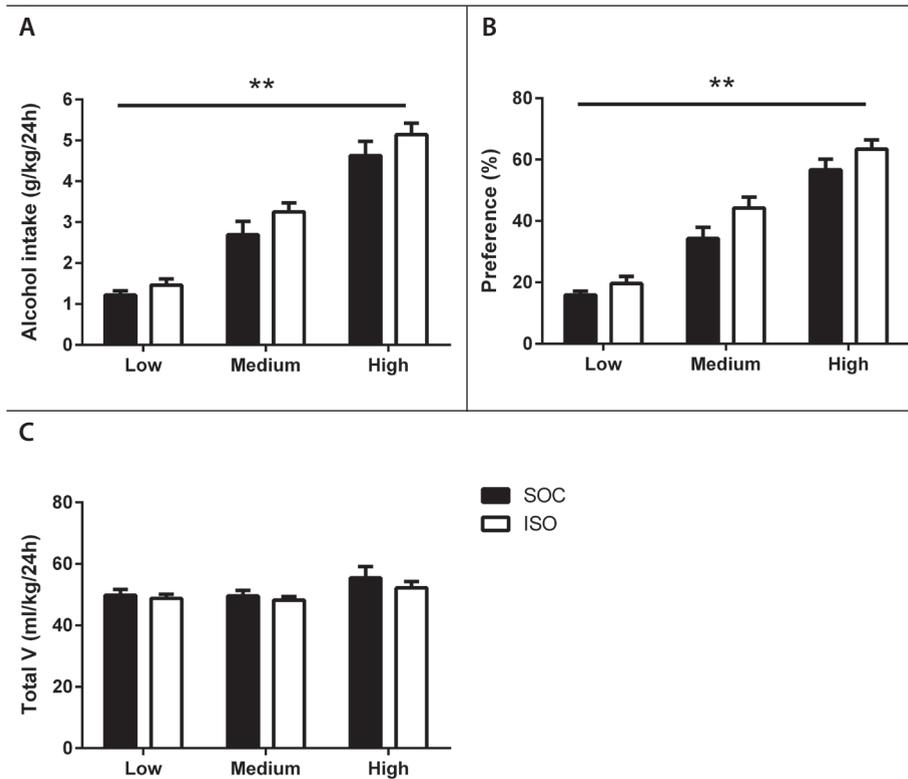


Figure 3. Average alcohol intake (A), alcohol preference (B) and total fluid consumption (C) for all 24h alcohol consumption sessions. The average data for low, medium and high subgroups of socially housed (SOC) and social play-deprived (ISO) rats ($n=12$) are shown. Early social isolation enhanced alcohol consumption and alcohol preference without altering the total fluid intake in 24h sessions (** $P<0.001$; main effect of early social isolation). Total V, total volume.

respond for alcohol (Fig. B-C). As a requirement to progress to PR schedules of reinforcement, each rat had to earn at least 10 rewards for 2-3 sessions before progressing from FR2 to FR5 to FR10 and ultimately to PR2 schedules of reinforcement. Of the SOC rats, 96% met these requirements, as opposed to only 80% of the ISO rats. Analysis of the PR data revealed no effect of early social isolation under both PR schedules ($F_{(1,41) \text{ group}} = 0.69$, N.S. for PR2 and $F_{(1,39) \text{ group}} = 1.3$, N.S. for PR4). In addition, the motivation for alcohol was not different between the Low, Medium and High alcohol drinking rats ($F_{(2,41) \text{ subgroup}} = 1.5$, N.S. for PR2 and $F_{(2,39) \text{ subgroup}} = 0.82$, N.S. for PR4) nor was

Figure 4

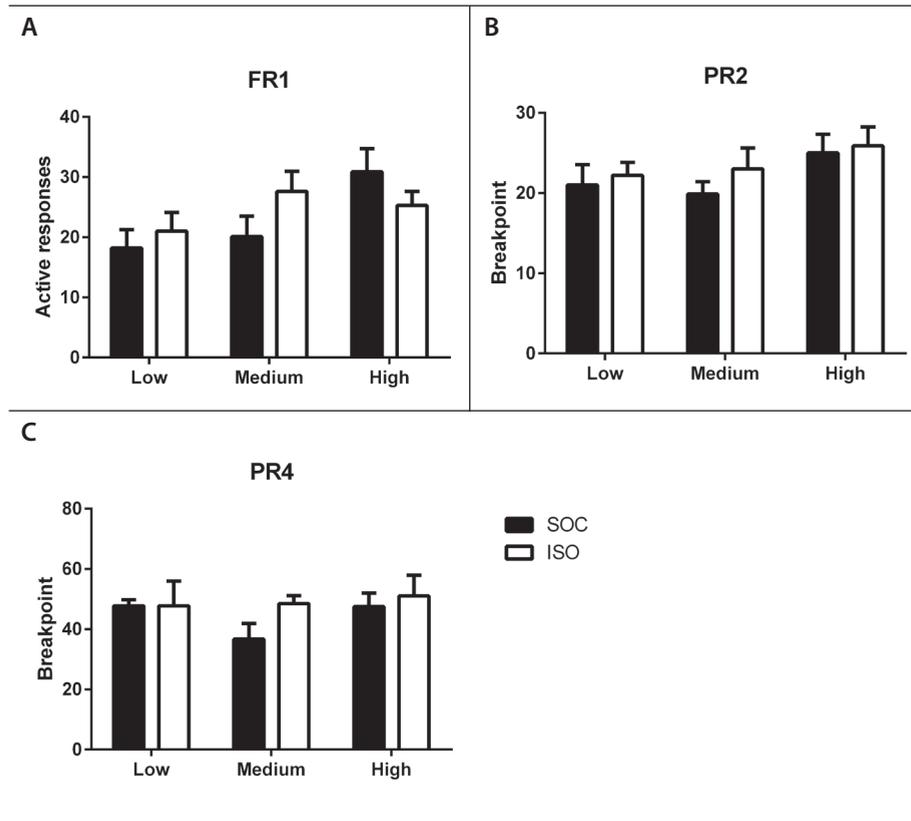


Figure 4. Operant responding for alcohol under different schedules of reinforcement: (A) FR1 ($n = 7-9$), (B) PR2 schedule and (C) PR4 ($n=5-9$). Early social isolation did not affect alcohol reinforcement or the motivation for alcohol self-administration. FR, fixed ratio; ISO, social play-deprived rats; PR, progressive ratio; SOC, socially housed rats.

there a subgroup dependent effect of early social isolation on PR responding ($F_{(2,41) \text{ group} \times \text{subgroup}} = 0.20$, N.S. for PR2 and $F_{(2,39) \text{ group} \times \text{subgroup}} = 0.47$, N.S.).

DISCUSSION

In this study, we show that social isolation during a period in development during which rats display high levels of social play behaviour, that is PND 21-42, augments alcohol consumption in adulthood. The increase in alcohol consumption was restricted to home cage drinking, as responding for alcohol under FR1 or PR schedules of reinforcement was not altered by early social isolation.

Social isolation after weaning has been shown to enhance alcohol consumption in previous studies in rats and mice (Ellison 1981; Schenk et al., 1990; Wolffgramm 1990; Hall et al., 1998; Lodge and Lawrence 2003; Advani et al., 2007; Deehan et al., 2007; McCool and Chappell 2009; Sanna et al., 2011; Chappell et al., 2013; Butler et al., 2014). Importantly, however, in these studies, the animals were reared in isolation from weaning onwards. As a result, it is not possible to discern whether the increase in alcohol consumption results from social isolation during alcohol consumption, or is the consequence of neuroadaptive changes that are induced by social isolation during post-weaning development. In support of the latter possibility, the present findings show that social isolation during a restricted time window (i.e., between PND 21-42) results in increased alcohol consumption in adulthood. This is in line with recent findings of augmented alcohol-induced conditioned place preference after early social isolation (Whitaker et al., 2013). An important element of this study, and ours, is that the animals were re-socialized for at least 5 weeks in between the social isolation and the alcohol consumption or place conditioning tests. Together, these data identify the period between PND21-42, that is characterized by an abundance of social play behaviour (Panksepp 1981), as a sensitive period for social isolation to augment alcohol reward in adulthood. Importantly, the increased sensitivity to alcohol reward (Whitaker et al., 2013; present study) that results from early social isolation extends to other drugs of abuse, as amphetamine-induced conditioned place preference and cocaine self-administration are also enhanced following social isolation during PND21-42 (Whitaker et al., 2013; Baarendse et al., 2014). This suggests that social play experience serves to develop resilience to addictive behaviours in adulthood.

The isolation window between PND 21-42, followed by re-socialization, was chosen to selectively prevent the animals from reaping the benefits of social play experience during post-weaning development, without depriving them completely from social contact during development into adulthood. However, using this approach, it is not possible to specifically attribute the consequences of social isolation to the lack of social play. Previous studies indicate that lack of social play is an important determinant of the consequences of social isolation during early post-weaning development (Einin et al., 1978). Thus, Juarez & Vazquez-Cortes (2003) showed that social isolation during PND 25-35 enhanced alcohol consumption, but not when rats were intermittently exposed to a social partner during the isolation period.

Moreover, Whitaker et al. (2013) reported that the augmented alcohol- and cocaine-induced conditioned place preference after social isolation between PND 21-42 was not observed in animals isolated from PND 21-28 (with the possibility for substantial social play experience after PND 28) or from PND 42-63 (that had social play experience before a period of social isolation of similar length). Taken together, these findings suggest that it is indeed the experience gained during social play behaviour, that serves to properly develop brain mechanisms that are important in reward processes (Whitaker et al., 2013) or cognitive control over behaviour (Baarendse et al., 2013) that protects against addictive behaviour in adulthood.

It is important to mention that the effects of early social isolation were independent of the individual variability in alcohol consumption. That is, they were present in Low, Medium, as well as High alcohol drinking rats. This implies that this social insult, that is, the lack of social play behaviour during post-weaning development, adds up to other factors that determine individual levels of alcohol consumption in adulthood. Interestingly, studies by Ellison (1987) have reported a large degree of individual variation in the propensity to consume alcohol within colonies of rats, which correlated with several aspects of social behaviour, such as grooming, dominance, chasing and aggression (Ellison, 1987), supporting the notion that social behaviour is an important factor contributing to an individual's sensitivity for alcohol consumption. Interestingly, however, the effect of early social isolation on alcohol consumption and alcohol preference was restricted to the situation in which the animals had *ad libitum* access to alcohol in their home cages. The lack of an effect on operant responding for alcohol suggests that early social isolation impacts on the consummatory, perhaps hedonic aspects of alcohol reward, rather than the appetitive and incentive motivational properties of alcohol assessed in operant settings. Alternatively, during the operant sessions, animals could only earn relatively small amounts of alcohol, which may have obscured the effect of early social isolation, that is, socially vulnerable individuals are particularly at risk for enhanced alcohol consumption when large amounts of the substance are available.

In conclusion, our present data show that disruption of early social play interactions during post-weaning development enhances the propensity to consume alcohol in adulthood, identifying early social experience as an important protective factor against excessive alcohol use.

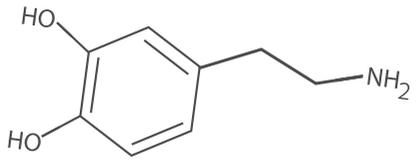
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CHAPTER 7

ADOLESCENT ALCOHOL EXPOSURE AMPLIFIES THE INCENTIVE VALUE OF REWARD-PREDICTIVE CUES THROUGH POTENTIATION OF PHASIC DOPAMINE SIGNALING

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ABSTRACT

Adolescent alcohol use remains a major public health concern due in part to well-established findings implicating the age of onset in alcohol use in the development of alcohol use disorders and persistent decision making deficits in adults. We have previously demonstrated that moderate adolescent alcohol consumption in rats promotes suboptimal decision making and an associated perturbation in mesolimbic dopamine transmission in adulthood. Dopamine-dependent incentive learning processes are an integral component of value-based decision making and a fundamental element to many theoretical accounts of addiction. Thus, we tested the hypothesis that adolescent alcohol use selectively alters incentive learning processes through perturbation of mesolimbic dopamine systems. To assess incentive learning, behavioural and neurochemical measurements were made during the acquisition, maintenance, extinction, and reacquisition of a Pavlovian conditioned approach procedure, in adult rats with a history of adolescent alcohol consumption. We show that moderate adolescent alcohol consumption potentiates stimulus-evoked phasic dopamine transmission, measured *in vivo* by fast-scan cyclic voltammetry, in adulthood and biases individuals toward a dopamine-dependent incentive learning strategy. Moreover, we demonstrate that animals exposed to alcohol in adolescence are more sensitive to an unexpected variation in reward outcomes. This pattern of phasic dopamine signaling and the associated bias in learning may provide a mechanism for the well-documented vulnerability of individuals with early-life alcohol use for alcohol use disorders in adulthood.

INTRODUCTION

Adolescence represents a critical period of maturation in cortical and limbic brain areas involved in reward processing, inhibitory control and decision making (Spear 2000; Chambers et al., 2003; Blakemore and Choudhury 2006; Bava and Tapert 2010). This developmental period is characterized by risky and impulsive behaviours including experimentation with alcohol and other substances of abuse (Casey and Jones 2010). Alcohol is the most commonly used substance by adolescents and a high percentage of consumption during this period occurs in bingeing or uncontrolled use (Witt, 2010). Importantly, an increasing number of studies have revealed that the adolescent brain is particularly vulnerable to alcohol-induced functional changes (Monti et al., 2005; Zeigler et al., 2005; Crews et al., 2007; Philpot et al., 2009; Guerri and Pascual 2010; Squeglia et al., 2012; Schindler et al., 2014). Moreover, adolescent alcohol use increases the likelihood of developing an alcohol use disorder (AUD) in adulthood (Hingson et al., 2006; Dawson et al., 2008; Blomeyer et al., 2013).

We have previously shown that moderate adolescent alcohol consumption in rats promotes suboptimal risk preference and a corresponding increase in striatal dopamine release in response to risky choices in adulthood (Nasrallah et al., 2011). We have further demonstrated that increased risk preference may result from a selective defect in reinforcement learning (Clark et al., 2012) and that this is a specific consequence of alcohol exposure during adolescence, as identical exposure in adults does not produce this effect (Schindler et al., 2014). The mesolimbic dopamine system is implicated in reinforcement learning, goal-directed behaviour, and motivational processes including those for abused substances (Robinson and Berridge 1993; Kelley, 2004; Everitt and Robbins 2005; Schultz, 2007; Salmone and Correa 2012). Alcohol, similar virtually all abused substances, increases dopamine transmission within the ventral striatum (Di Chiara and Imperato 1986; Cheer et al., 2007; Robinson et al., 2009). Importantly, the mesolimbic dopamine system continues to mature during the adolescent period (Chambers et al., 2003), suggesting that adolescent alcohol use may alter its function, resulting in abnormal reward-related learning processes that impact decision making (Zeigler et al., 2005; Goudriaan et al., 2007; Johnson et al., 2008; Philpot et al., 2009; Casey and Jones 2010; Nasrallah et al., 2011; Alaux-Cantin et al., 2013; McClory and Spear 2014; Toalston et al., 2014).

Phasic dopamine transmission is evoked by salient sensory input, rewards, and predictive stimuli that have been paired with rewards during Pavlovian and instrumental conditioning (Roitman et al., 2004; Day et al., 2007; Clark et al., 2013). More recently, it has been shown that dopamine acts selectively in a form of stimulus-reward learning where incentive value is assigned to reward cues (Flagel et al., 2011). Pavlovian conditioning in a wide variety of species has been shown to elicit alternative conditioned responses where some individuals engage with the stimulus itself during cue presentation (sign-trackers) and other individuals engage the site of reward delivery during cue presentation (goal-trackers) (Boakes, 1977; Robinson and Flagel 2009). The sign-tracking response is accompanied by a dynamic pattern of dopamine release in the ventral striatum, is dopamine dependent, and has been interpreted as being indicative of a learning strategy where incentive value is assigned to reward-predictive cues (Flagel et al., 2011; Clark et al., 2012). Importantly, individuals that attribute greater incentive value to reward-predictive cues during Pavlovian conditioning with natural rewards go on to exhibit greater cue-evoked motivational responses to drug-associated cues during cocaine self-administration (Yager and Robinson 2013). Indeed, drug-associated cues exert powerful control over drug-seeking behaviour including the reinstatement of drug self-administration after extinction (Shaham et al., 2003), and individuals that assign greater incentive value to predictive cues (e.g. sign trackers) are more vulnerable to this effect (Saunders and Robinson 2010).

Here, we tested the hypothesis that the behavioural and neurochemical phenotypes promoted by chronic adolescent alcohol use may contribute to the well-documented increased risk for the development of AUD through a potentiation in dopamine-mediated attribution of incentive value to reward-paired cues. We used fast-scan cyclic voltammetry (FSCV) to measure phasic dopamine release in the nucleus accumbens core during Pavlovian conditioned approach behaviour in rats that voluntarily consumed alcohol, or control gelatin, during adolescence. Dopamine release was recorded throughout the acquisition, maintenance, extinction and re-acquisition phases of the Pavlovian conditioned approach procedure. Moreover, animals were exposed to probe trials during the maintenance of Pavlovian conditioned approach behaviour where better-than-expected and worse-than-expected outcomes were isolated to specifically study the effects of adolescent alcohol consumption on reward prediction error signaling. We show that moderate adolescent alcohol

consumption potentiates stimulus-evoked phasic dopamine transmission in adulthood and biases individuals toward a dopamine dependent incentive learning strategy.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River, Hollister, CA) obtained at postnatal day (PND) 27 were housed individually under controlled temperature and humidity conditions on a 12-hour light/dark cycle (lights on at 06:00) with *ad libitum* access to water and chow (Harlan, Kent, WA). One week before the start of behavioural testing, the rats were food-restricted and maintained at 90% of their free-feeding weight. Rats were weighed and handled at least every other day throughout the course of the experiment. An outline detailing the time course of all procedures is presented in Fig. 1A. All experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

Alcohol administration

Rats received 20 days of access to a 10% alcohol (n=15) or control (n=16) gelatin in jars in their home cage from PND 30-49 (Fig. 1B and C). The gelatin was made available 24 h/day in addition to *ad libitum* water and chow, and the jars were replaced with fresh gelatin every day. Alcohol and control gelatin was prepared as previously described (Nasrallah et al., 2009; Clark et al., 2012). Upon completion of the 20-day alcohol and control exposure, jars were removed and the rats were monitored daily for withdrawal symptoms. Systematic measurements of intoxication and withdrawal symptoms were not made in these animals but no overt signs of withdrawal symptoms were observed (e.g. seizures, weight loss and anxious behaviour during handling). In addition, blood ethanol concentration (BEC) measurements were not made as it is challenging to get an accurate measure of peak BEC with voluntary, free access models as we have no control over when the animals consume the gelatin. Therefore, any measure of BEC is likely to be a systematic underestimate because the average BEC will include animals that do not consume alcohol during the sampling period chosen. However, we have previously examined BEC after alcohol gelatin exposure during adolescence (Schindler et al., 2014) as have others (Rowland et al., 2005; McMurray et al., 2014). To assess BEC, we isolated peak intake periods to get an accurate estimate given a specific amount of intake. We observed an average BEC of 35 mg% (the BEC levels

ranged 10 and 80 mg%), and the BEC values were highly correlated with alcohol intake. Daily alcohol intake in the current experiments averaged 11.5 g/kg. This is a substantial amount of alcohol in comparison to other free access models and is consistent with the intake from our BEC studies.

Surgery and electrochemical detection of dopamine

One week after cessation of alcohol access, rats were implanted with bilateral carbon-fiber microelectrodes targeting the nucleus accumbens core (1.3 mm lateral, 1.3 mm rostral, and 6.8 mm ventral of bregma) for *in vivo* detection of phasic dopamine using FSCV as previously described (Clark et al., 2010). Of the 31 rats, 5 were excluded from the voltammetry data analyses; 1 had electrode placements outside the nucleus accumbens core, 3 lost headcaps over the course of the experiment and 1 did not have reliable recordings. Rats were placed in an operant chamber (see below) and connected to a head-mounted voltammetric amplifier. Waveform generation, data acquisition and analysis were carried out on a PC-based system using two PCI multifunction data acquisition cards and software written in LabVIEW (National Instruments). Reward-evoked dopamine release in response to uncued sucrose pellet delivery was used to ensure electrode viability prior to each behavioural session. Dopamine was isolated from the voltammetric signal with chemometric analysis (Heien et al., 2005) using a standard training set based on stimulated dopamine release. Dopamine concentration was estimated based on the average post-implantation electrode sensitivity (Clark et al., 2010). Peak CS- and US-evoked dopamine values were obtained by taking the largest value in the 3 s period after stimulus presentation.

Apparatus

Equipment and procedures for Pavlovian conditioning have been described in detail elsewhere (Flagel et al., 2011). Briefly, the rats were trained and tested in operant conditioning chambers (Med Associates St. Albans, VT), situated in sound-attenuating cubicles. Each chamber was equipped with two retractable levers and a food cup within a recessed magazine situated between the levers. A cue light was present above each lever and a house light was located on the opposite wall. Sucrose pellets (45mg, Bio Serve) were delivered in the food cup via a dispenser. Experimental events and data recording were controlled using MED-PC software for Windows (Med Associates).

Behavioural Procedures

Rats received sucrose pellets in their home cage for two days before training to reduce potential food neophobia. All behavioural sessions were conducted between 10:00 - 19:00 h. The rats were habituated to the operant chamber for one session during which 15 sucrose pellets were randomly delivered over the course of 15 min. The Pavlovian conditioned approach procedure was conducted as previously described (Flagel et al., 2011). Briefly, a trial consisted of the insertion of the left or right lever (counterbalanced between rats) and the illumination of a cue light above the designated lever (conditioned stimulus, CS) for 8 sec, followed by the immediate delivery of 2 sucrose pellets (unconditioned stimulus, US) and the illumination of the light in the recessed magazine. 25 CS-US presentations occurred on a variable inter-trial interval from a range of values (30, 40, 50, 60, 70, 80, and 90 s) in each session. Lever presses and food cup entries during lever presentation were recorded, but had no programmed consequences. After 5 Pavlovian conditioned approach sessions the rats were given two probe sessions consisting of a pseudorandom presentation of different reward sizes (0, 1, 2, 3, or 4 sucrose pellets; 5 trials of each reward size), separated by a standard Pavlovian conditioned approach session with 2 sucrose pellets. The rats then received 5 extinction sessions in which the trial structure remained the same except that CS presentation was not followed by reward delivery. Finally, the rats received a Pavlovian conditioned approach session to assess reacquisition.

Histological verification of recording sites

At the end of experimentation, rats were anesthetized with sodium pentobarbital and recording sites were marked with an electrolytic lesion (300 V) by applying current directly through the recording electrode for 20 sec. Rats were then transcardially perfused with PBS followed with 4% paraformaldehyde. The brains were removed and kept in 4% paraformaldehyde followed by 15% and 30% sucrose solution at 4°C, each for 1-2 days, then rapidly frozen in aluminum foil on dry ice and stored at -20°C. Brains were sliced on a cryostat (50 µm coronal sections, -20°C), and stained with cresyl violet to aid in visualization of anatomical structures. Electrode locations were confirmed to be in the core of the nucleus accumbens (Fig. 1D).

Data analysis

All statistical analyses were conducted using SPSS 20.0 for Windows and GraphPad Prism 6. Behavioural and voltammetry data were binned into

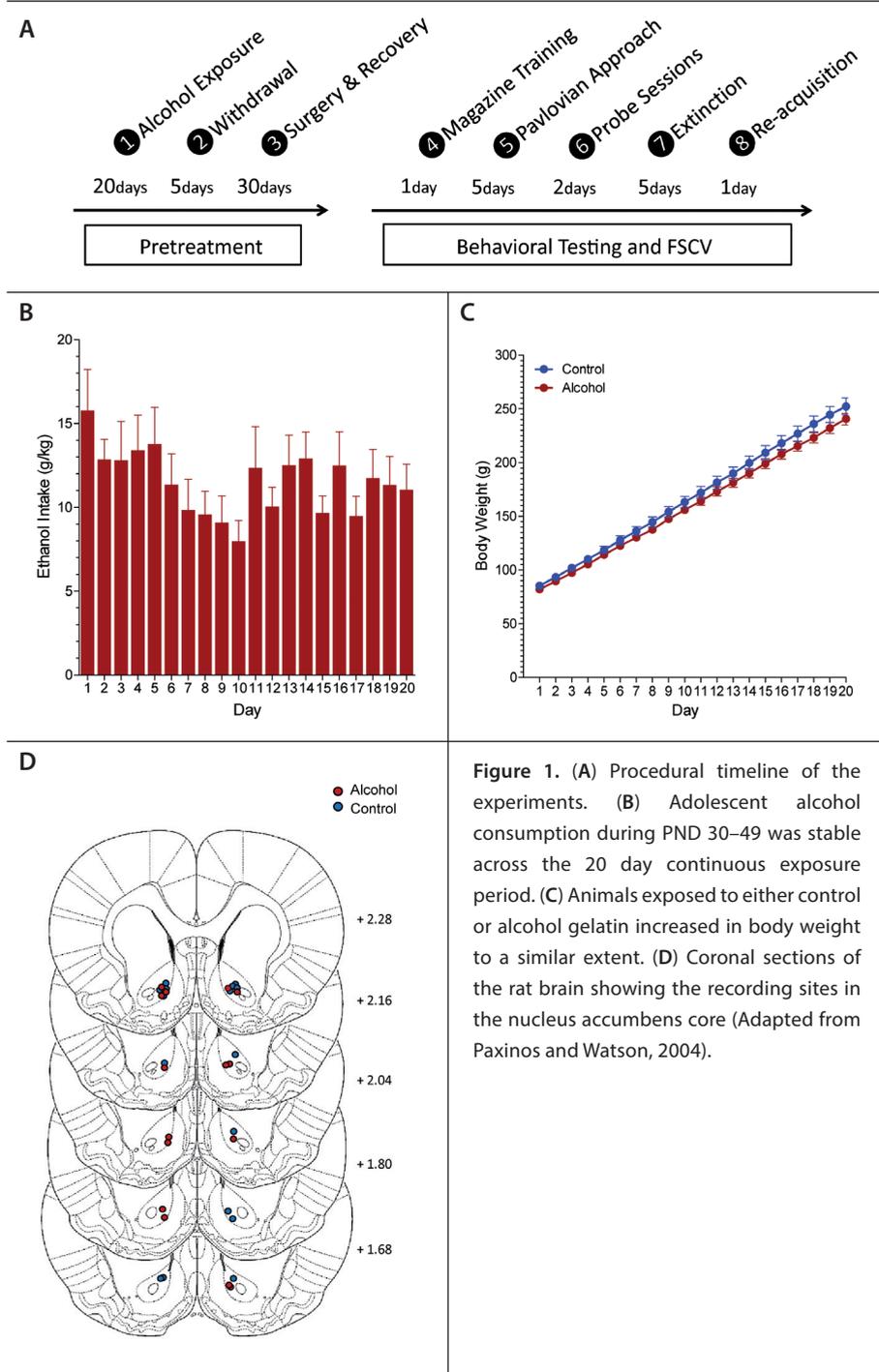
5-trial epochs. A response bias score, i.e. a measure of the relative allocation of behavioural responses, was calculated by subtracting the number of magazine entries from the number of lever presses divided by the sum of both responses: $(\text{lever presses} - \text{magazine entries}) / (\text{lever presses} + \text{magazine entries})$, resulting in a number ranging from -1 (goal tracking response) to +1 (sign-tracking response) (Meyer et al., 2012). Based on previous work, animals with a response bias above +0.70 were defined as animals with a strong sign-tracking bias (Flagel et al., 2008; Meyer et al., 2012) and were used for additional neurochemical analyses. Conditioned responses from all phases of training were analyzed using linear mixed effects models (Verbeke and Molenberghs 2000) in which treatment group (alcohol and control) and trial bin were treated as independent variables. Each parameter and the residuals of the behavioural parameters used in the linear effects model were tested for normality with a Kolmogorov-Smirnov test. For all analyses the covariance structure was explored and modeled appropriately. When significant main effects or interactions were detected, Bonferroni *post hoc* comparisons were made. Statistical analyses of the voltammetry data were performed using one-, two-, and three way repeated-measures ANOVA's with peak dopamine values upon CS and US presentation, reward size or trial bin as within-subject variables and group (alcohol or control) as between-subject variable. In case of significant main effects in the voltammetry data, post hoc analyses were performed using pairwise comparisons with a Bonferroni correction. The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean \pm SEM. Graphs were made using Graphpad Prism 6.

RESULTS

Alcohol intake

Daily adolescent alcohol intake was stable across the 20-day exposure period ($F_{(19,266) \text{ day}} = 1.62$, n.s.) and averaged 11.5 ± 0.98 g/kg, comparable to our previous studies (Fig. 1B; Nasrallah et al., 2011; Schindler et al., 2014). The caloric intake was comparable for alcohol and control exposed animals ($F_{(6,172) \text{ day} \times \text{group}} = 1.69$, n.s.; $F_{(1,29) \text{ group}} = 0.184$, n.s) and both groups increased in bodyweight to the same extent over the course of the 20-day exposure period (Fig. 1C; $F_{(1,35) \text{ day} \times \text{group}} = 1.57$, n.s.; $F_{(1,29) \text{ group}} = 1.48$, n.s.).

Figure 1



Acquisition of Pavlovian conditioned approach behaviour after adolescent alcohol exposure

Pavlovian conditioned approach responses to either the reward predicting lever (sign-tracking) or the food magazine (goal-tracking) during CS presentation developed differentially for the alcohol-exposed and control groups over the course of training (Fig. 2). The response bias developed over trial bins towards a sign-tracking response in both groups ($F_{(24,89) \text{ trial bin}} = 18.81, p < 0.001$); however the alcohol exposed animals showed a significantly stronger sign-tracking bias relative to control animals (Fig. 2A; $F_{(24,89) \text{ trial bin} \times \text{group}} = 2.13, p < 0.01$, $F_{(1,49) \text{ group}} = 4.35, p < 0.05$). Indeed, control animals showed a conditioned response (CR) towards the food magazine or the lever, or both, whereas the distribution of approach behaviour in animals treated with alcohol during adolescence was shifted exclusively towards sign-tracking CRs (Fig. 2B; Levene's Test: $F = 12.47, p < 0.05$). The CR towards the food magazine decreased over trials in alcohol exposed animals, whereas it remained at the same level for control animals (Fig. 2C; $F_{(24,221) \text{ trial bin}} = 5.84, p < 0.001$; $F_{(1,48) \text{ group}} = 6.24, p < 0.05$; $F_{(24,221) \text{ trial bin} \times \text{group}} = 2.37, p < 0.01$). The number of lever contacts upon cue presentation increased in both groups during learning (Fig. 2D; $F_{(24,103) \text{ trial bin}} = 9.15, p < 0.001$). This overall pattern of behaviour and statistical results was unchanged after exclusion of the animals without viable electrodes for FSCV (data not shown).

Stimulus-evoked phasic dopamine signaling during acquisition

Phasic dopamine release was evoked by both CS and US presentation during early acquisition (first 25 trials, session 1) in both groups as previously described (Clark et al., 2013; Day et al., 2007; Flagel et al., 2011). However, phasic dopamine transmission was significantly higher overall in animals with a history of adolescent alcohol exposure during this phase of learning (Fig. 3A-C; $F_{(1,58) \text{ group}} = 5.49, p < 0.05$). Analysis of phasic dopamine release to reward-related stimuli across all trial bins revealed that phasic dopamine release developed differentially in response to CS and US presentation ($F_{(15,426) \text{ stimulus} \times \text{trial bin}} = 10.42, p < 0.001$) independent of treatment ($F_{(15,426) \text{ stimulus} \times \text{trial bin} \times \text{group}} = 1.34, n.s.$).

Previous studies have shown that the pattern of phasic dopamine release evoked by CS and US presentation during learning is linked to the behavioural responses toward reward-related stimuli and the attribution of incentive

Figure 2

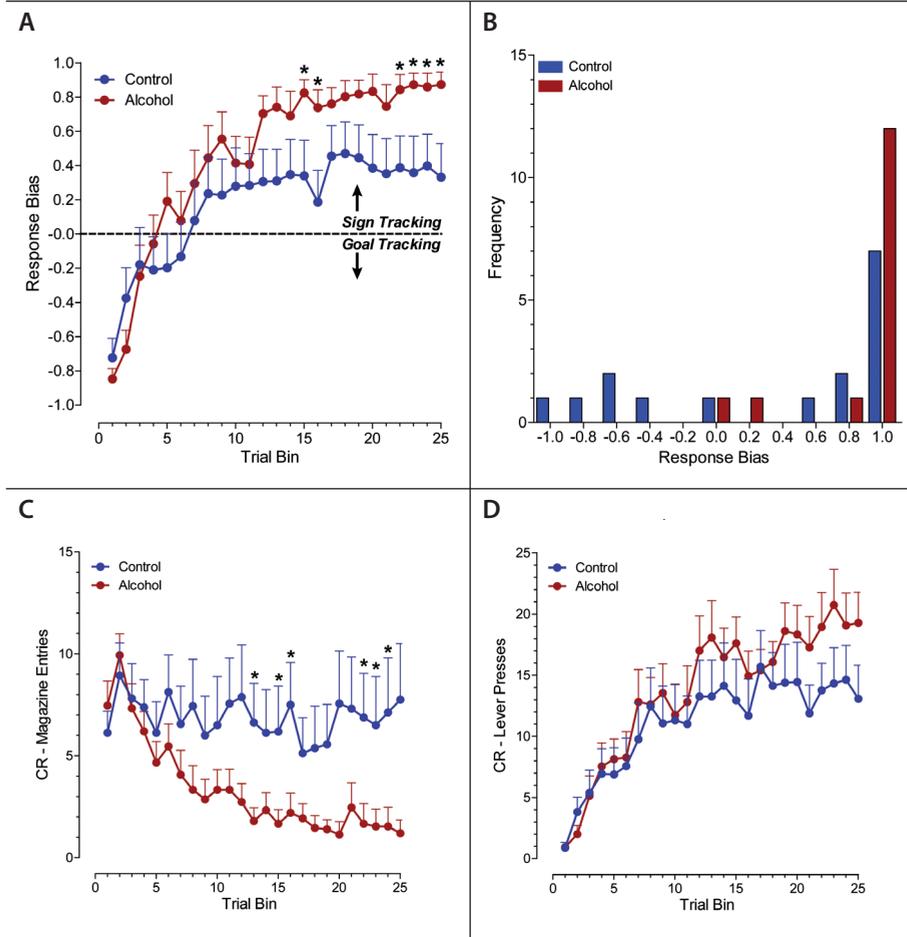


Figure 2. Behavioral responses during the Pavlovian conditioned approach task. (A) Analysis of response bias (lever presses – food cup entries)/(lever presses+food cup entries), a measure of the relative allocation of behavioral responses, revealed that animals exposed to alcohol during adolescence mainly show CRs to the reward-predictive cue. (B) A frequency distribution of response bias scores during the last session of training indicates that animals exposed to alcohol during adolescence shifted the distribution of responses exclusively towards a sign-tracking CR. (C) Over the course of learning, alcohol-exposed animals reduced their CR towards the food cup, whereas control-treated animals continued to approach the food cup. (D) CRs to the reward-predicting lever increased in both groups over training. Data are represented as means+SEM. *Indicates significant difference between groups with *post-hoc* t-tests with a Bonferroni correction ($p < 0.05$).

value to predictive cues (Flagel et al., 2011). Because behavioural responses directed toward either the predictive cue (sign-tracking) or reward location (goal-tracking) may reflect different learning mechanisms (Clark et al., 2012), we performed a similar analysis of phasic dopamine transmission in animals with a strong sign-tracking bias (defined as response bias above +0.70; Fig. 4A) over the course of learning. Importantly, phasic dopamine release was further increased in sign-tracking animals with a history of alcohol exposure in comparison to controls ($F_{(1,23) \text{ group}} = 8.71, p < 0.05$) (Fig. 4B-C), including higher CS-evoked dopamine release throughout learning ($F_{(1,23) \text{ group}} = 6.15, p < 0.05$). Significantly higher CS-evoked dopamine release in animals with a more extreme sign-tracking phenotype after alcohol exposure is consistent with our previous findings where animals selected for extreme phenotypes differed in CS-evoked dopamine release but animals with mixed behavioural responses did not. These data indicate that alcohol exposure further enhances CS-evoked dopamine release in sign-trackers, possibly resulting in a stronger bias toward a dopamine-dependent incentive learning strategy.

Separate analysis of CS- and US-evoked dopamine release in all animals revealed that CS-evoked dopamine release increased during the first trial bins of the Pavlovian conditioning session 2-5 ($F_{(11,326) \text{ trial bin}} = 11.88, p < 0.05$), whereas US-evoked phasic dopamine release steadily decreased during learning ($F_{(10,290) \text{ trial bin}} = 13.26, p < 0.05$). This profile is consistent with the view that phasic dopamine transmission encodes a reward prediction error of the type used as a teaching signal in formal models of reinforcement learning (Schultz et al., 1997). Dopamine transmission after behaviour had reached asymptote (last 25 trials, session 5) was comparable between the treatment groups in response to both the CS and US presentation (Fig. 3D).

Extinction of sign-tracking and goal-tracking responses

During extinction, all animals reduced their conditioned approach behaviour. The response bias score decreased across trials equally in both groups and fluctuated around zero by the end of extinction training (Fig. 5A; $F_{(24,53) \text{ trial bin}} = 27.77, p < 0.001$). Analysis of the food cup directed CR revealed a main effect of extinction training (Fig. 5B; $F_{(24,102) \text{ trial bin}} = 1.67, p < 0.05$) and a main effect of treatment group ($F_{(1,98) \text{ group}} = 6.77, p < 0.05$). The total number of lever contacts upon cue presentation decreased in both groups across trials (Fig. 5C; $F_{(24,47) \text{ trial bin}} = 25.91, p < 0.001$). Consistent with the behavioural data, CS10 evoked dopamine release decreased across the extinction phase

Figure 3

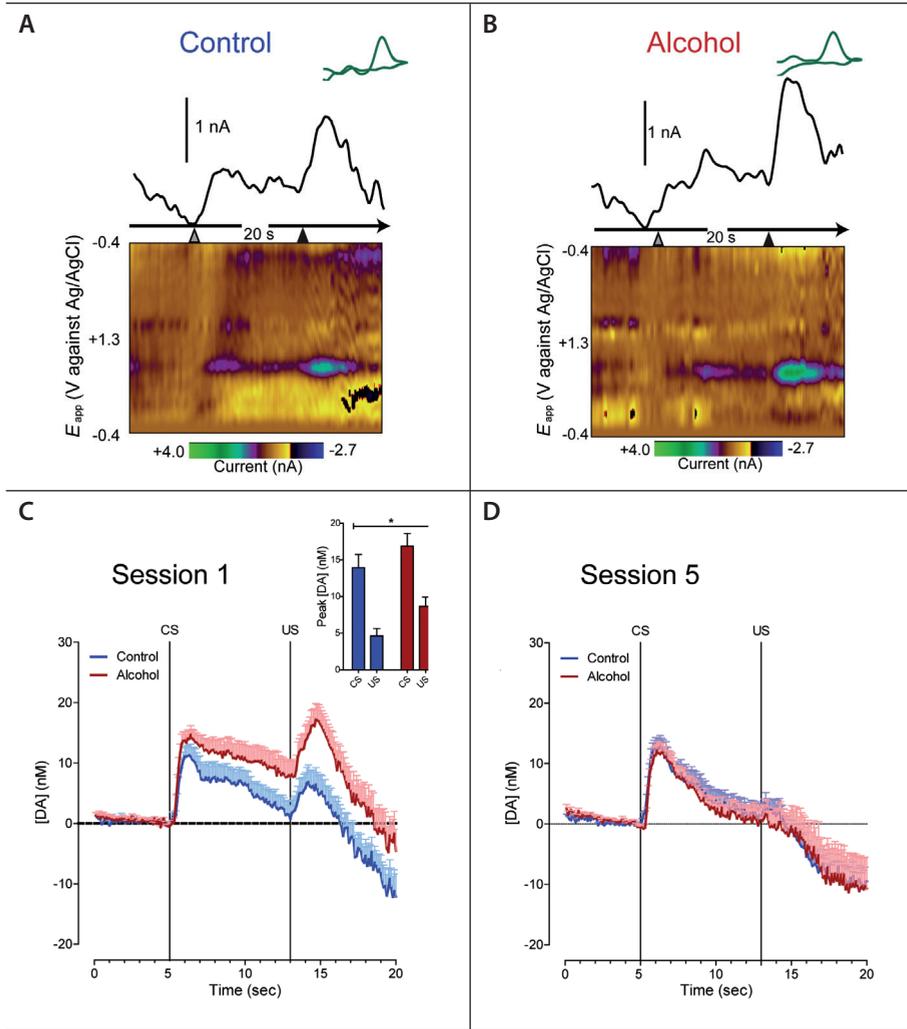


Figure 3. Phasic dopamine signaling during the first and final sessions of Pavlovian conditioned approach behavior. (A, B) Representative traces from the first session and corresponding background-subtracted cyclic voltammograms (inset) depict changes in dopamine oxidative current within the nucleus accumbens core in response to CS presentation (grey arrowhead) after 5 s and US delivery (black arrowhead) after 13 s in control (A) and alcohol-exposed animals (B). The pseudocolor plots depict color-coded observed changes in redox currents as a function of applied potential (y axis) plotted over time (x axis). (C) Average trace of dopamine transmission in a 20-s window around CS and US presentation over the first 25 trials of Pavlovian conditioning. (c: inset) Peak dopamine values for CS and US responses for alcohol- and control-treated animals in the first Pavlovian session. (D) Average trace of dopamine transmission in a 20-s window around CS and US presentation over the final 25 trials of Pavlovian conditioning. Data are represented as means+SEM. *Difference between groups (one-way ANOVA, $p < 0.05$).

Figure 4

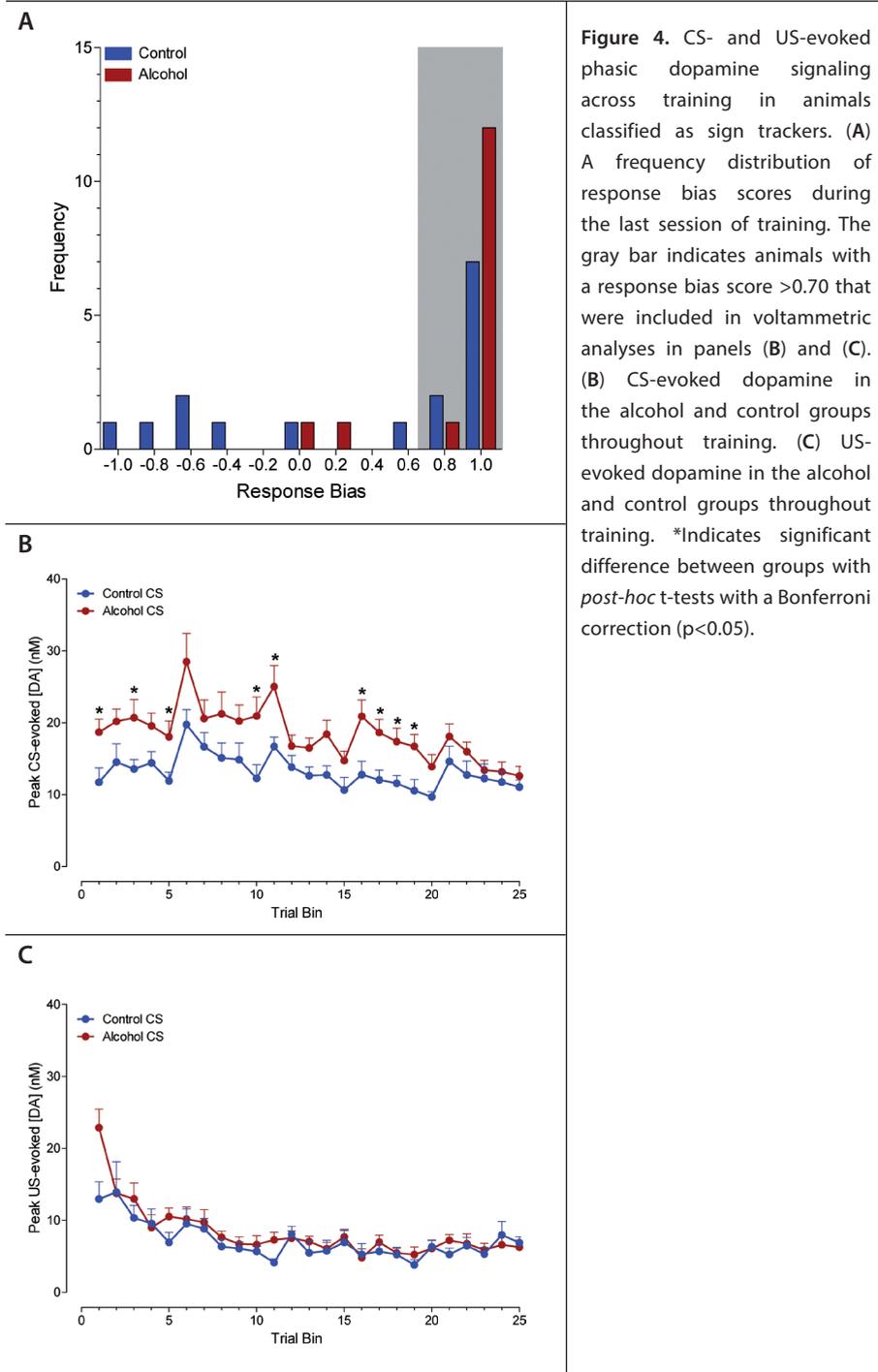
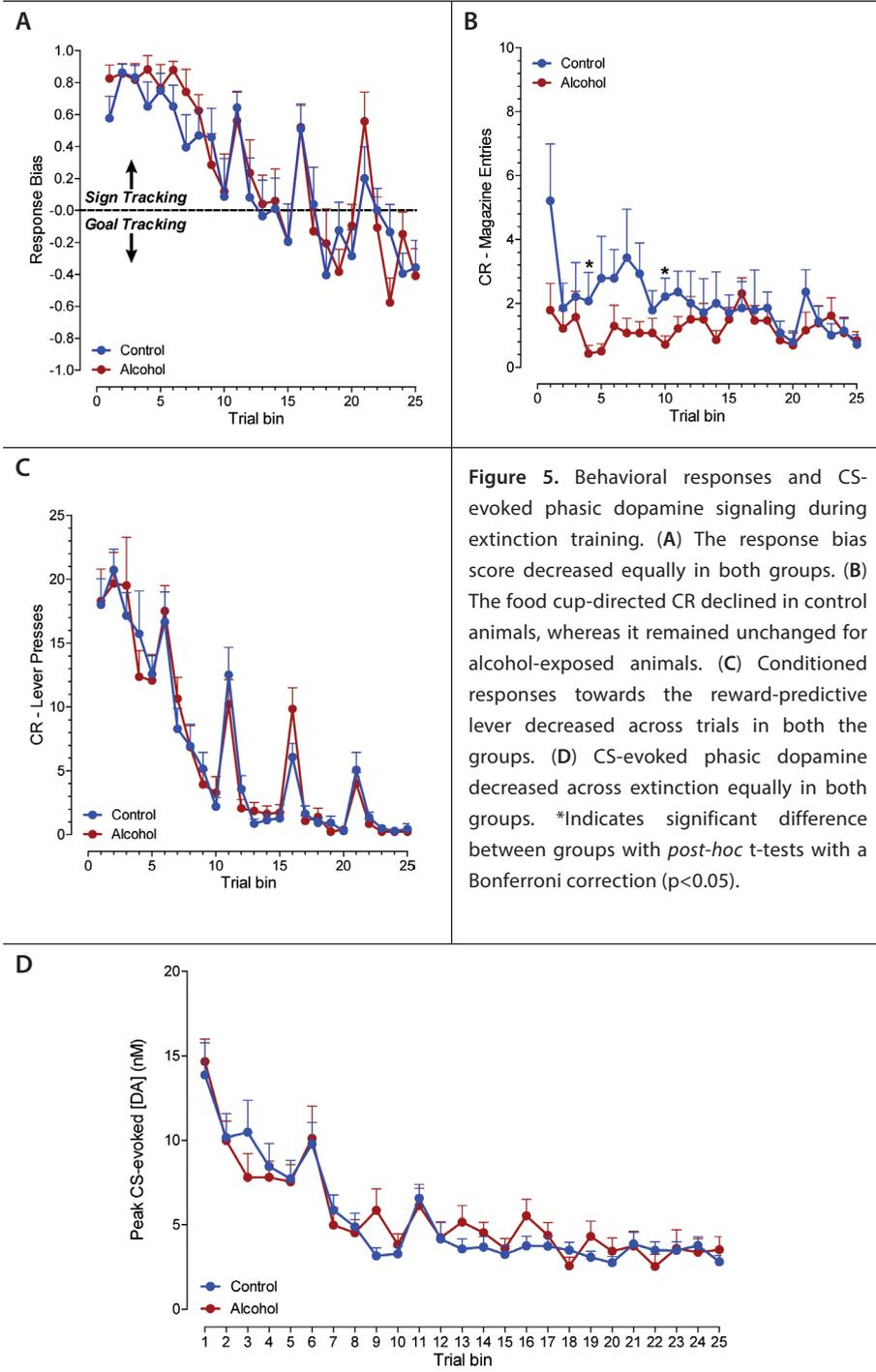


Figure 5



(Fig. 5D; $F_{(9,246) \text{ trial bin}} = 22.17, p < 0.001$) and this was not different between groups ($F_{(9,246) \text{ trial bin} \times \text{group}} = 0.92, \text{ n.s.}$).

Reacquisition of sign-tracking and goal-tracking conditioned responses

After extinction training, the animals were exposed to a final conditioning session in which they were again rewarded with two sucrose pellets upon lever retraction to assess reacquisition of Pavlovian conditioned approach behaviour. In order to analyze the change in behaviour from extinction baseline through reacquisition, we performed a repeated measure analyses over the last 5 trial bins of extinction and the 5 trial bins of reacquisition (Fig. 6A-C). Consistent with acquisition, this analysis revealed that the conditioned response developed differently for the two treatment groups during reacquisition, since alcohol exposed animals showed a greater bias toward a sign-tracking response. This was confirmed by analysis of the response bias, which revealed a main effect of trial bins (Fig. 6A; $F_{(9,225) \text{ trial bin}} = 19.24, p < 0.001$) and a significant interaction between trial bins and group ($F_{(9,225) \text{ trial bin} \times \text{group}} = 2.25, p < 0.05$). Control animals reacquired their approach behaviour toward the food cup during this phase as indicated by a main effect of trial bins ($F_{(9,61) \text{ trial bin}} = 4.79, p < 0.001$) and did so to a greater extent than alcohol treated animals as indicated by a main effect of treatment (Fig. 6B; $F_{(9,61) \text{ group}} = 5.46, p < 0.05$). The total number of lever contacts upon cue presentation increased in both groups (Fig. 6C; $F_{(9,33) \text{ trial bin}} = 19.19, p < 0.001$).

Stimulus-evoked dopamine signaling during reacquisition

CS-evoked (Fig. 6D) and US-evoked (Fig. 6E) phasic dopamine signaling during the re-acquisition session increased above extinction baseline in both groups (CS; $F_{(9,270) \text{ trial bin}} = 22.83, p < 0.001$; US; $F_{(9,270) \text{ trial bin}} = 10.64, p < 0.001$) in parallel with the reacquisition of conditioned behavioural responding. Consistent with the increased response bias score in alcohol-exposed animals, phasic dopamine transmission in alcohol-exposed animals was significantly higher for both stimuli in comparison to controls (CS; $F_{(1,270) \text{ group}} = 4.59, p < 0.05$; US; $F_{(1,270) \text{ group}} = 16.56, p < 0.001$).

Figure 6

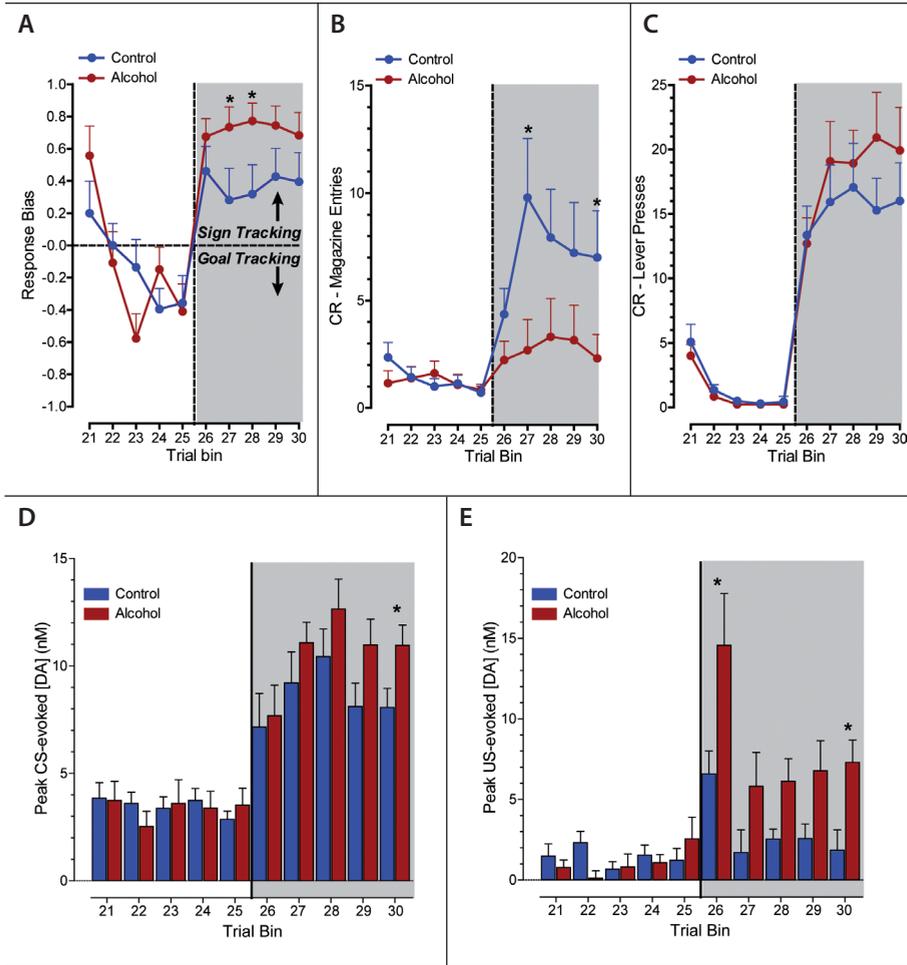


Figure 6. Behavioral (A–C) and dopaminergic responses (D, E) during reacquisition of Pavlovian conditioned approach behavior. (A) Alcohol-exposed animals showed a greater bias toward a sign-tracking response. (B) The CR toward the food cup mainly increased in control animals, whereas (C) both groups increased their CR for the reward-predicting lever. (D, E) CS- and US evoked phasic dopamine signaling increased during reacquisition in both groups. (D) Adolescent alcohol consumption resulted in higher CS-evoked phasic dopamine release in the final trial bin of reacquisition in comparison to controls, as well as a (E) larger US-evoked phasic dopamine release in the first trial bin. Subsequently, in trial bins 2–5, both groups showed a decrease in US-evoked dopamine release, but signaling remained higher in alcohol-exposed animals. *Indicates significant difference between groups with *post-hoc* t-tests with a Bonferroni correction ($p < 0.05$).

Dopamine encoding of positive and negative reward prediction errors

Consistent with phasic dopamine encoding for positive and negative reward prediction errors, phasic dopamine release after unexpected reward presentation and omission was sensitive to variation in reward size (Fig. 7A-D; $F_{(2,54)} = 9.23, p < 0.001$). Moreover, the dopaminergic response to the variation in reward sizes was different upon CS or US presentation ($F_{(2,54) \text{ reward size} \times \text{stimulus}} = 4.15, p < 0.05$), indicating that the US response was affected by the reward size (Fig. 7D; $F_{(2,48)} = 15.07, p < 0.001$), whereas the CS responses remained unaltered (Fig. 7C; $F_{(2,54)} = 0.55, \text{ n.s.}$). Interestingly, alcohol treated animals showed greater overall responsiveness to positive prediction errors ($F_{(2,48) \text{ reward size US} \times \text{group}} = 4.03, p < 0.05$), which is in line with the neurochemical and behavioural data from both acquisition and reacquisition. *Post hoc* analyses indicated that alcohol-exposed animals showed a higher dopamine release upon better-than-expected rewards in comparison to both the neutral and worse-than-expected rewards ($p < 0.004$), whereas the dopamine release upon the better-than-expected reward in the control animals was only higher in comparison to the worse-than-expected reward ($p < 0.01$) (Fig. 7D).

DISCUSSION

To examine a potential mechanism by which adolescent alcohol consumption increases the vulnerability to AUD in adulthood, we investigated phasic dopamine signaling in the nucleus accumbens core during Pavlovian conditioned approach behaviour in adult rats that had voluntarily consumed alcohol during adolescence. We report that moderate alcohol consumption during adolescence increases the assignment of incentive value to reward-predictive cues in adulthood. This perturbation in incentive learning processes was associated with a potentiation of stimulus-evoked phasic dopamine transmission during early acquisition as well as during re-acquisition of Pavlovian conditioned approach behaviour. Importantly, when analysis was restricted to sign-tracking animals, CS-evoked dopamine release was significantly elevated by prior alcohol exposure. Moreover, during probe trials, alcohol-exposed animals showed a heightened sensitivity to outcomes that were better than expected as evidenced by greater phasic dopamine signaling to positive prediction errors. Thus, our results indicate that adolescent alcohol exposure promotes long-lasting alterations in dopamine-dependent incentive learning.

Figure 7

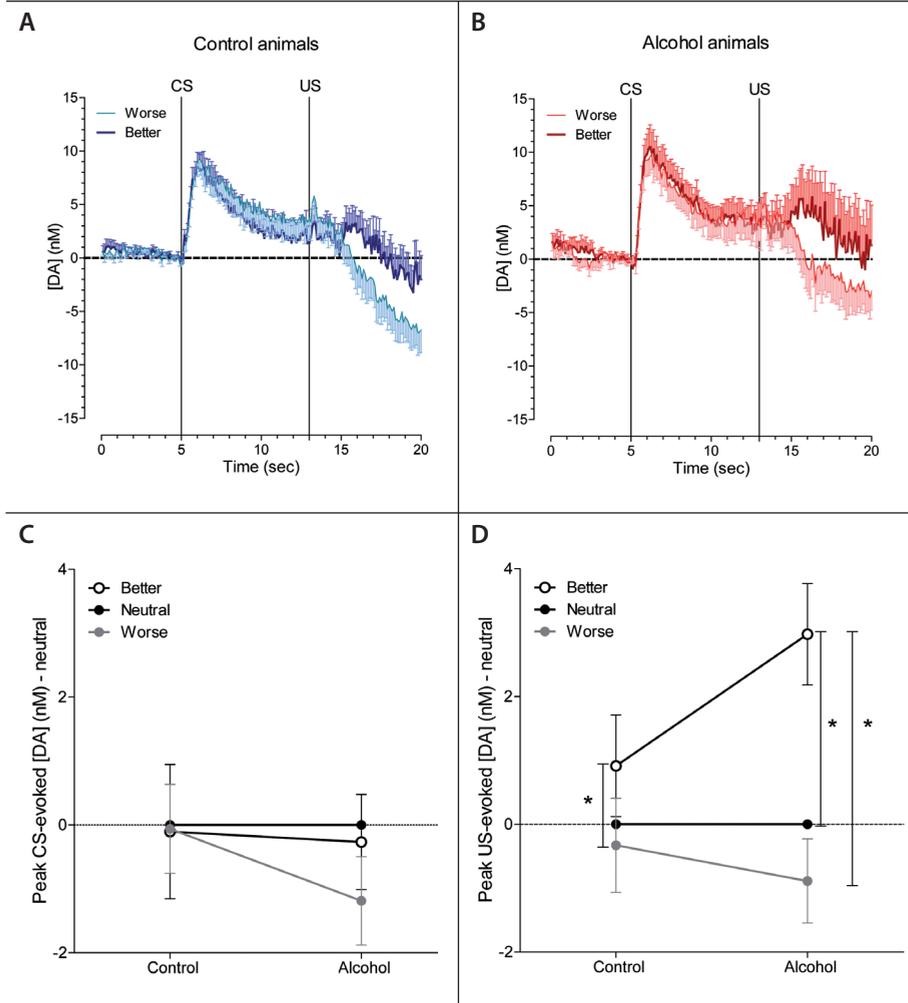


Figure 7. Phasic dopamine signaling in response to worse-than-expected, expected, and better-than-expected reward outcomes in control- and alcohol treated animals. **(A, B)** Average dopamine traces for worse- (reward sizes 0 and 1) and better-than expected (reward sizes 3 and 4) outcomes during the probe sessions where reward size was varied unpredictably. **(C)** CS-evoked dopamine release was not affected by altered reward sizes. **(D)** US-evoked dopamine release was sensitive to varying reward size in both groups but alcohol-treated animals showed greater overall responsiveness to unexpected variation in reward outcomes. *Indicates significant difference in US dopamine release between reward sizes in *post-hoc* within group comparisons with a Bonferroni correction ($p < 0.05$).

Pavlovian conditioning processes are an important contributor to addictive behaviours since substance-associated cues can drive drug craving, drug seeking, and promote relapse following abstinence (Stewart et al., 1984; O'Brien et al., 1998; Shaham et al., 2003; Milton and Everitt 2012). Indeed, individuals that abuse drugs are more likely to exhibit this stimulus-driven affective behaviour (Bickel and Marsch 2001). The sign-tracking phenotype in rats, characterized by the assignment of incentive value to reward-predictive cues, has been previously associated with reduced impulse control and vulnerability to addictive behaviour (Tomie et al., 2008; Flagel et al., 2010; Lovic et al., 2011). Importantly, there is a wide variation in the degree to which individuals engage in sign-tracking behaviour, thus allowing for a comparison between drug-associated behaviours and the degree to which individuals assign incentive value to predictive cues. Multiple reports have shown that Pavlovian cues associated with the delivery of drugs of abuse acquire greater control over motivated behaviour in animals selected for sign-tracking behaviour (Saunders et al., 2013; Yager and Robinson 2013; Yager et al., 2014). This suggests that these animals are more vulnerable to the influence of reward-predicting stimuli, a characteristic which is associated with compulsive and relapsing drug abuse (Stewart et al., 1984; O'Brien et al., 1998; Shaham et al., 2003; Milton and Everitt 2012). Here, we demonstrate that adolescent alcohol exposure shifts the normal distribution of conditioned responses elicited by Pavlovian cues exclusively toward a sign-tracking phenotype. These data are in support of a previous study which reported that alcohol exposure during adolescence, but not during adulthood, increased sign-tracking behaviour in rats (McClory and Spear 2014). Indeed, the sign tracking phenotype has been specifically linked to models of AUD (Tomie and Sharma 2013). Behavioural responses during extinction were similar between groups, replicating our previous findings and supporting the view that adolescent alcohol exposure produces over-fast learning for better-than expected, but not worse-than-expected outcomes (Clark et al., 2012).

The core sub-region of nucleus accumbens is implicated in the acquisition and maintenance of Pavlovian conditioned approach behaviour (Di Ciano et al., 2001; Parkinson et al., 2002) and dopamine transmission in this structure is evoked by rewards and reward-predictive cues (Day et al., 2007; Clark et al., 2013). This pattern of phasic dopamine release is linked to the behavioural responses elicited by reward-related stimuli where sign-tracking animals show this pattern and goal-tracking animals do not (Flagel et al., 2011). Indeed, the

role of dopamine signaling in sign-tracking behaviour has been extensively studied in previous work (Di Ciano et al., 2001; Flagel et al., 2011; Saunders and Robinson 2012; Clark et al., 2013). These studies have shown that systemic and intracranial infusions of the dopamine D1/D2 antagonist flupenthixol into the nucleus accumbens core, reduced sign-tracking behaviour during both the acquisition and performance of Pavlovian conditioned approach behaviour. Moreover, it was shown that dopamine is necessary for the learning of a sign-tracking conditioned response, whereas it is not necessary for learning a goal-tracking conditioned response (Flagel et al., 2011). In the current study, adult animals exposed to alcohol during adolescence showed enhanced phasic dopamine release during early acquisition of Pavlovian conditioned approach behaviour preceding the development of the bias toward a sign-tracking phenotype. Because the behavioural response to either the reward predicting lever (sign-tracking) or the reward location (goal-tracking) may reflect different learning mechanisms (Clark et al., 2012), we also examined phasic dopamine transmission exclusively in sign-tracking animals from both groups over the course of learning. These analyses revealed that alcohol-treated sign-tracking animals had enhanced CS-evoked phasic dopamine release during learning in comparison to control sign-tracking animals. Thus, adolescent alcohol exposure promotes exaggerated sign-tracking responses mirrored by a potentiation in phasic dopamine signaling to incentive cues. Interestingly, after behaviour had reached asymptote (last 25 trials, session 5), we found that phasic dopamine release was comparable between the treatment groups in response to CS presentation, supporting the view that dopamine's involvement is restricted to situations when conditions are changing and differential behaviour is being acquired and established but not after stable responding has been achieved (Di Ciano et al., 2001; Clark et al., 2013). Indeed, significantly potentiated CS- and US-evoked phasic dopamine release in alcohol animals relative to controls during reacquisition mirrored the results from initial acquisition and supports the conclusion that animals exposed to alcohol in adolescence are particularly sensitive to fluctuating conditions and the attribution of updated incentive properties to reward-associated stimuli under those circumstances.

To further examine the hypothesis that alcohol-treated animals may be more responsive under conditions where outcomes are changing and deviating from expectation, all animals were given probe trials where reward size was varied unpredictably after behaviour had reached asymptote. Consistent with previous reports (Ljungberg et al., 1992; Tobler et al., 2005; Hart et al., 2014),

phasic dopamine activity was modulated by reward size and expectation in both groups in a manner consistent with the reporting of a reward prediction error from formal models of reinforcement learning, with increased phasic dopamine signaling after better-than-expected outcomes and decreased phasic dopamine signaling after worse-than-expected outcomes. Interestingly, our data showed that adolescent alcohol consumption promoted a greater sensitivity to the unexpected variation in reward sizes.

One potential limitation of the experiments outlined here is that animals in this study were singly housed during adolescence to permit accurate measures of voluntary alcohol intake, raising the question of whether or not there is an effect of housing condition in adolescence above and beyond that of alcohol exposure or whether the alcohol effects described here are dependent upon housing condition (Anderson et al., 2013). We cannot entirely rule out the possibility that housing conditions contribute to the overall pattern of conditioned responses reported here. However, control animals, housed in the identical conditions to that of the alcohol-exposed animals, show a distribution of conditioned responses that is consistent with previous work that systematically examined the population statistics of these behaviours (Fitzpatrick et al., 2013). This would suggest that singly housing animals in the current work did not shift the overall distribution of response biases and is therefore not the likely explanation for the extreme shift in the alcohol group exclusively to sign-tracking behaviour. Importantly, a previous study in which rats were pair-housed and exposed to intragastric alcohol or control administration during adolescence observed that alcohol-treated animals showed increased lever-pressing behaviour during Pavlovian conditioning consistent with the results outlined here (McClory and Spear 2014), indicating that the effects of alcohol on learning are consistent across housing conditions. Finally, our work demonstrating that adolescent alcohol promotes maladaptive decision making (Nasrallah et al., 2011; Schindler et al., 2014), findings that we have previously linked to learning effects similar to the ones found here (Clark et al., 2012), have been replicated in animals that were group housed during intragastric alcohol administration in adolescence (Boutros et al., 2014). Thus, the observed increase in sign-tracking behaviour reported here is most likely the result of alcohol exposure rather than housing conditions. Indeed, this overall pattern of results suggests that the effects of adolescent alcohol exposure on the attribution of incentive value and decision making are quite robust as they have now been reported under

multiple alcohol delivery paradigms (voluntary and involuntary) and with multiple housing conditions (single, paired, and group). A second potential limitation to the interpretation of the current results is the extent to which the findings are selective to alcohol exposure in adolescence. Previous studies have investigated the effect of nicotine and amphetamine exposure during adolescence on Pavlovian conditioned approach behaviour in adulthood (Doremus-Fitzwater and Spear 2011; Quick et al., 2014). Adolescent nicotine exposure (on PND 31-45) was shown to increase approach to a CS in male animals, while approaches to the CS were reduced in females. In addition, amphetamine sensitization during adolescence also has been shown to increase sign-tracking behaviour. This raises the interesting possibility that an alteration to the assignment of incentive value to reward cues may be a general consequence of substance use in adolescence.

Collectively, these findings support theoretical accounts speculating that an imbalance in learning from positive and negative outcomes may be an important contributor to substance use disorders (Piray et al., 2010; Baker et al., 2011). Specifically, clinical research has demonstrated that the amount of alcohol consumed is reliably correlated with the degree to which individuals have positive over negative alcohol outcome expectancies (Jones et al., 2001). The current findings provide insight into the development of such an imbalance in the weighting of positive and negative experiences and a candidate neural mechanism underlying the neurocognitive and behavioural consequences of adolescent alcohol consumption that may contribute to an enhanced vulnerability for developing AUD in adulthood.

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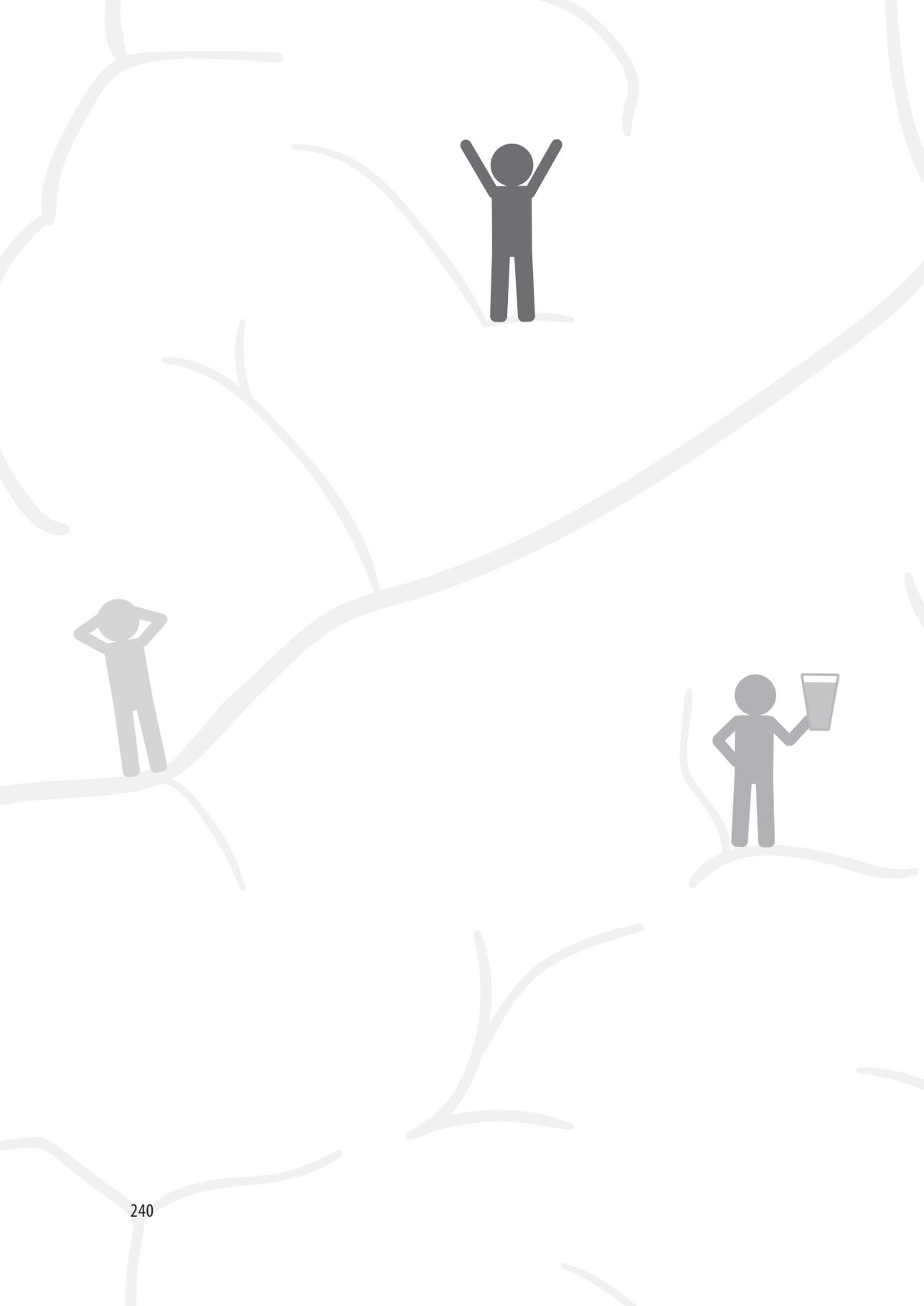
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CHAPTER 8

DOPAMINERGIC NEUROTRANSMISSION IN VENTRAL AND DORSAL STRIATUM DIFFERENTIALLY MODULATES ALCOHOL REINFORCEMENT

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Submitted



ABSTRACT

Dopaminergic neurotransmission in the striatum has been widely implicated in the reinforcing properties of substances of abuse. However, the striatum is functionally heterogeneous, and previous work has mostly focused on psychostimulant drugs. Therefore, we investigated how dopamine within striatal sub-regions modulates alcohol-directed behaviour. To that aim, we examined the role of dopamine in the shell and core of the nucleus accumbens (NAcc) and the dorsolateral striatum (DLS) in responding for alcohol under a fixed ratio 1 (FR1) and a progressive ratio (PR) schedule of reinforcement in rats. Bilateral infusion of the dopamine receptor antagonist alpha-flupenthixol (0 – 15 µg/side) into the NAcc shell dose-dependently reduced responding for alcohol under both schedules, albeit that responding under the PR schedule of reinforcement was decreased by lower doses of flupenthixol. Infusion of flupenthixol into the NAcc core reduced responding for alcohol under both schedules to a comparable extent. By contrast, flupenthixol in the DLS did not affect FR1 responding, but reduced responding under the PR schedule. The flupenthixol-induced decreases in responding were found to be related to earlier termination of responding during the session, whereas the onset and rate of responding remained largely unaffected. These data implicate dopamine in the NAcc shell and DLS in the motivational aspects of obtaining alcohol, whereas NAcc core dopamine plays a more general role in alcohol reinforcement. In conclusion, these findings indicate that dopaminergic neurotransmission acts in concert in sub-regions of the striatum to modulate different aspects of alcohol-directed behaviour.

INTRODUCTION

Alcohol use disorder (AUD) is a chronic relapsing brain disorder characterized by excessive and compulsive alcohol use that affects approximately 76 million people worldwide (WHO 2011; American Psychiatric Association 2013). In order to develop more effective treatments for AUD, the neural mechanisms of this disorder have been intensively investigated during the last decades (Spanagel 2009; Barker and Taylor 2014). In this regard, the dopaminergic innervation of the striatum belongs to the most widely investigated neural systems involved in addictive behaviour, including AUD (Robinson and Berridge 1993; Gonzales et al., 2004; Wise 2004; Everitt and Robbins 2005; Spanagel 2009; Koob and Volkow 2010; Luscher and Malenka 2011; Salamone and Correa 2012). Importantly, it is increasingly understood that there is substantial heterogeneity with regard to the function of dopamine in sub-regions of the striatum in the modulation of reward, motivation and addiction (Zahm 1999; Voorn et al., 2004; Everitt and Robbins 2005; Yin et al., 2008; Balleine and O'Doherty 2010; Floresco 2015).

The involvement of striatal dopamine in alcohol reinforcement has been demonstrated by local infusions of dopamine receptor agonists and antagonists, dopaminergic lesions and the measurement of extracellular dopamine levels during alcohol self-administration (Quarfordt et al., 1991; Weiss et al., 1993; Ikemoto et al., 1997b; Melendez et al., 2002; Doyon et al., 2005; for review see Gonzales et al., 2004). Interestingly, recent findings from neurochemical and electrophysiological studies suggest regional specificity in the effects of alcohol in the striatum (Chen et al., 2011; Adermark et al., 2013; DePoy et al., 2013; Fanelli et al., 2013; Logrip et al., 2015). For example, the NAcc core has been implicated in cue-induced alcohol seeking (Chaudhri et al., 2008; Gremel and Cunningham 2008; Chaudhri et al., 2010) while the NAcc shell is thought to contribute to the primary reinforcing properties of alcohol (Howard et al., 2008; Engleman et al., 2009; Ding et al., 2015) and to context-induced alcohol seeking (Chaudhri et al., 2009; Hauser et al., 2015). Recently, the DLS has been shown to be involved in habitual alcohol seeking (Corbit et al., 2012; Corbit et al., 2014). Together, these studies suggest a differential involvement of ventral and dorsal striatal sub-regions in alcohol-directed behaviour. However, it is unknown how dopamine within these striatal sub-regions modulates alcohol-reinforced behaviour.

In the current study, we therefore used local infusions of the dopamine receptor antagonist alpha-flupenthixol to systematically assess the role of dopamine in different striatal sub-regions in responding for alcohol using a fixed ratio 1 (FR1) and a progressive ratio (PR) schedule of reinforcement. Since the response requirement under an FR1 schedule is minimal, responding under this schedule is thought to reflect consummatory aspects of self-administration, whereas PR schedules, because of their increasing response requirement, tax processes related to the incentive motivational properties of rewards (Katz 1990; Markou et al., 1993; Richardson and Roberts 1996; Arnold and Roberts 1997). Previous studies, using FR schedules of reinforcement, reported reductions in oral alcohol self-administration upon infusion of dopamine receptor antagonists into the ventral striatum (Hodge et al., 1992; Rassnick et al., 1992; Samson et al., 1993; Hodge et al., 1997; Czachowski et al., 2001; Samson and Chappell 2004). Therefore, we hypothesized that dopamine receptor blockade in the NAcc shell and core would reduce responding for alcohol. Considering reported differences in the rewarding effects of alcohol in the NAcc shell and core (Howard et al., 2008; Engleman et al., 2009; Ding et al., 2015), flupenthixol infusion into the NAcc was expected to alter responding for alcohol in a sub-region and reinforcement-schedule dependent manner. With respect to the DLS, recent studies showed that dopaminergic signaling in this brain region might, next to its role in habits, also be involved in the primary reinforcing properties of cocaine (Veeneman et al., 2012; Willuhn et al., 2012; Veeneman et al., 2015). Therefore, dopamine receptor blockade in the DLS may affect alcohol self-administration under both FR and PR schedules of reinforcement.

MATERIALS AND METHODS

Animals

Male Lister Hooded rats (Charles River, Sulzfeld, Germany) weighing 220-250g upon arrival were housed individually under controlled temperature and humidity conditions on a 12 h reversed light-dark cycle (lights off at 7.00 AM) with *ad libitum* access to water and chow. Rats were allowed two weeks of acclimatization to the housing conditions before experiments commenced. They were handled and weighed at least once a week throughout the experiment. All experiments were approved by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op de dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Intermittent alcohol access (IAA) in the home cage

The rats were provided with intermittent access to alcohol (20% v/v) and water in a two-bottle choice setup in the home cage for a period of two months. The rats were exposed to alcohol for three days a week (Monday-Wednesday-Friday) for 7h between 9.00 AM and 16.00 PM (i.e., during the active phase of the animals) in the first month and access was extended to 24h/session in the second month. 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 preference. We observed marked individual differences in alcohol intake and preference between the rats. Therefore, after two months IAA, the rats were ranked based on the animals' average alcohol intake per week and were assigned ranking scores (Spoelder et al., 2015). Rats within the lower and upper 25% of the total ranking score range were designated as low and high alcohol drinking rats, respectively, and were used for other studies. The middle 50% were used in the current study, so that experimental groups with relatively little variability in alcohol reinforcement could be used (Spoelder et al., 2015). These rats were assigned to one of three groups to be implanted with cannulas aimed at NAcc shell, NAcc core or DLS, taking their average alcohol intake into account to ensure similar levels of alcohol intake between groups before operant alcohol self-administration commenced.

Surgery

One week after cessation of IAA, the rats were implanted with bilateral 26-gauge guide cannulas (Plastic One, Roanoke, VA, USA) targeting the NAcc shell (2.0 mm lateral, 1.4 mm rostral, and 6.8 mm ventral at an angle of 5°), the NAcc core (2.0 mm lateral, 1.4 mm rostral, and 5.8 mm ventral at an angle of 5°) or the DLS (3.4 mm lateral, 0.8 mm rostral, and 3.3 mm ventral) with coordinates relative to bregma (Paxinos and Watson 2004). The guide cannula was aimed at 2.0 mm above the target region. Cannulas were fixed to the skull using stainless steel screws and antibiotic cement (Simplex™ P bone cement with tobramycin, Stryker Nederland B.V., The Netherlands). Anaesthesia and analgesia protocols were as previously published (Schaap et al., 2012; Schaap et al., 2013). Briefly, rats were anaesthetized with fentanyl (0.25 mg/kg, IP - Fentanyl Janssen®, Janssen-Cilag B.V., The Netherlands) and dexmedetomidine (0.15 mg/kg, IP - Dexdomitor®, Pfizer Animal Health B.V., The Netherlands) in their home cage. After loss of the pedal reflex the animals were transported to the surgery room and, after endotracheal intubation, anaesthesia was maintained with isoflurane if necessary. Upon completion of the surgery,

anaesthesia was terminated with atipamezole (0.6 mg/kg, IP - Antisedan®, Pfizer Animal Health B.V., The Netherlands), and the animals received buprenorphine (0.05 mg/kg, IP - Buprecare®, AST Farma B.V., The Netherlands) for pain relief. For postoperative analgesia, the rats were treated with buprenorphine (0.05 mg/kg, s.c.) at 12 hour intervals for 3 days after surgery and meloxicam (0.2 mg/kg, s.c. - Metacam, Boehringer Ingelheim B.V., 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.

Alcohol self-administration under FR and PR schedules of reinforcement

The rats were trained and tested, as previously described (Lesscher et al., 2015; Spoelder et al., 2015), in operant conditioning chambers (29.5 cm L, 24 cm W, 25 cm H; Med Associates, Georgia, VT, USA), situated in light- and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8 cm wide retractable levers, placed 11.7 cm apart and 6 cm from the grid floor. A liquid dipper within a recessed magazine was situated between the levers. A cue light was present above each lever (28 V, 100mA) and a house light (28 V, 100mA) was located on the opposite wall. The position of the active and inactive levers was counterbalanced between rats. Pressing the active lever raised the dipper cup containing alcohol (0.1 ml, 20% v/v), illuminated the cue light above the active lever and switched off the house light. Access to alcohol was terminated 10 sec after head entry into the magazine, the cue light was extinguished and after a 5 sec interval a new trial started. Pressing the inactive lever was recorded, but had no programmed consequences. The rats were tested for 3-4 days/week on every other day, and sessions lasted for 30 min. Alcohol consumption during self-administration sessions was calculated by weighing the container with alcohol underneath the liquid dipper before and after each session. To limit fluctuation of the alcohol concentration by evaporation, the alcohol solution was refreshed before each session. Experimental events and data recording were controlled using MED-PC for Windows.

The rats were habituated to the operant chamber for two 30 min sessions during which 15 alcohol rewards were freely available every other minute. After habituation, the rats were trained under a FR1 schedule of reinforcement for 11-15 sessions in which the rats obtained on average 27 ± 0.9 rewards/

session. Microinfusions during FR1 sessions started after all rats acquired a response criterion of at least 10 rewards for 7 consecutive sessions. After completion of the microinfusions for the FR1 schedule of reinforcement, the same rats were trained further and the response requirement was increased to a FR2, FR5 and FR10 schedule, during which each animal had to earn at least 10 rewards per FR schedule before progressing to the PR schedule of reinforcement. The rats required on average 6 ± 0.29 sessions to obtain this criterion. These requirements were set to ensure that the rats made at least 100 presses under the FR10 to obtain reliable response levels during PR sessions. Next, a linear PR schedule of reinforcement was introduced, in which 2 (PR2, i.e. 2, 4, 6, 8, 10, etc.) additional lever presses were required for each subsequent reward. This PR paradigm, rather than the commonly used exponential increase in the response requirement (Richardson and Roberts 1996) was chosen based on the results of previous studies which showed that 1) alcohol non-preferring rats have low breakpoints, 2) the required workload should be increased, however, before the sedative effects of alcohol begin to interfere with operant performance, 3) alcohol is delivered in relatively small sizes (0.1 ml/reinforcement) with a slow absorption rate (Hodos 1961; Ritz et al., 1994; Brown et al., 1998; Rodd et al., 2003). Microinfusions during PR sessions started once responding stabilized, i.e. less than 25% variation in the number of reward deliveries over three consecutive sessions; this required on average 5 ± 0.46 sessions.

Microinfusions

Microinfusions were made, as previously described (Trezza et al., 2011; Veeneman et al., 2012), using 33 gauge injectors (Plastics One, Roanoke, VA, USA) that extended 2.0 mm below the guide cannulas and were connected to a 10 μ L syringe. Using a microinfusion syringe pump (Harvard Apparatus, Holliston, MA, USA), bilateral microinfusions with flupenthixol (0.5 μ L/side) were made over 60 sec and the injectors were left in place for another 60 sec to allow for drug diffusion. Immediately after the microinfusion procedure, rats were placed in the operant chamber where the self-administration session started 5 min later. During two weeks prior to the start of the microinfusions of the FR1 sessions, the rats were habituated to the removal and replacement of the stylets in the cannulas every other day. In addition, each rat was habituated to the infusion procedure in which the rats received one sham control infusion (i.e. injectors were the same length as the guide cannulas, the pump motor was operated but the syringes were not driven) and one actual infusion

with sterile physiological saline (0.9% NaCl). The effects of flupenthixol were examined in a within-subject design in which each rat received all doses of flupenthixol according to a Latin square design. The rats were tested in the operant chambers every other day, and at least one re-baseline session without treatment was scheduled after each drug treatment to verify that response levels remained stable.

Histology

At the termination of the experiment, the rats were sacrificed using an overdose of pentobarbital and ink was infused to aid visual localization of the infusion sites. The brains were removed, flash-frozen in methyl-butane isopentane (-80°C) and subsequently stored at -80°C. Coronal sections were sliced using a cryostat (40 µm), mounted, air-dried, and stained with Cresyl violet. Microinjection sites were verified by light microscopy using a rat brain atlas (Paxinos and Watson 2004). Data from rats with one or both cannulas placed outside of the target area were discarded from the analyses.

Drugs

Cis-(Z)-Flupenthixol-dihydrochloride (Sigma-Aldrich) was dissolved in sterile physiological saline (0.9% NaCl) to concentrations of 0, 3.75, 7.5 and 15 µg in 0.5µl. Doses were based on prior reports (Murray et al., 2012; Veeneman et al., 2012). Alcohol solutions (Klinipath, The Netherlands) were freshly prepared once a week by diluting 99.5% alcohol with tap water to a final concentration of 20% (v/v).

Statistical analysis

Forty-six rats with correct cannula placements were used for statistical analyses for the FR1 sessions (NAcc shell: n=11, NAcc core: n=16, DLS: n=19) (Fig. 1). Three animals with correct cannulae placements (one in the NAcc shell and two in the NAcc core) did not fulfill the response criteria for the PR sessions and were therefore not tested under the PR schedule of reinforcement. The experiments were performed in two batches. Rats of the first batch were treated with three flupenthixol doses (0, 3.75, 7.5 µg/side) (FR1: NAcc shell: n=3, NAcc core: n=9, DLS: n=11; PR: NAcc shell: n=3, NAcc core: n=8, DLS: n=11) and rats of a second batch were treated with four flupenthixol doses (0, 3.75, 7.5, 15 µg/side) (FR1: NAcc shell: n=8, NAcc core: n=7, DLS: n=8; PR: NAcc shell: n=7, NAcc core: n=6, DLS: n=8). To combine both batches in the statistical analyses we made use of linear mixed effects models (Verbeke and Molenberghs 2000). The effect of

Figure 1

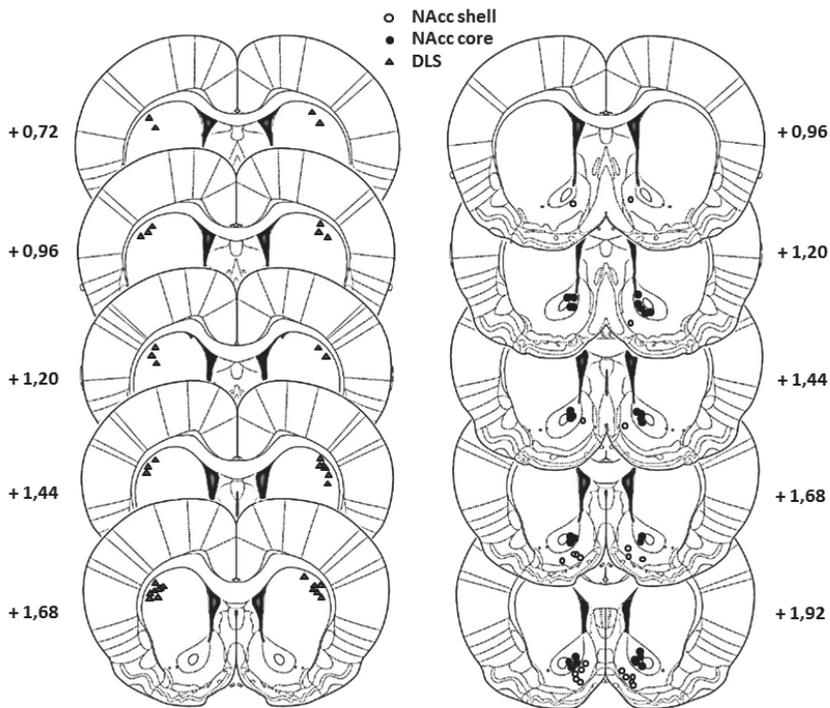


Figure 1. Histological verification of the infusion sites in coronal sections of the rat brain for the NAcc shell (open circles), NAcc core (filled circles), and DLS (grey triangles). Numbers indicate the distances anterior to bregma in millimeters (adapted from Paxinos and Watson, 2004).

flupenthixol on the number of lever presses and the rewards obtained during the FR and PR sessions were analyzed in 4 bins of 7.5 minutes to assess the effects of flupenthixol over time. The session time (i.e. the period of active involvement in the session) was expressed as the duration from the start of the session to the time of the last active lever press. The response rate was calculated by dividing the number of active lever presses by the session time. All parameters were tested for normality with the Kolmogorov-Smirnov test prior to analyses. The latency to the first active lever press and the session time were log transformed prior to statistical analyses, and the inactive lever presses as well as the number of rewards and active lever presses over time bins were square root transformed prior to statistical analyses to obtain a normal distribution of the data. The number of lever presses and rewards, latencies and the response rates were analyzed using linear mixed effects models in which dose and time bin were treated as independent variables. For

all analyses, the covariance structure was explored and modeled appropriately. Alcohol intake and preference in the home cage was analyzed using two-way repeated-measures ANOVAs with session or month as within-subject variables, and group (NAcc shell, NAcc core, DLS) as between-subject variable. Mauchly's test of sphericity was used to test if variances of the differences between treatments were equal. If the assumption of sphericity had been violated, degrees of freedom were corrected using Huynh-Feldt estimates of sphericity to more conservative values; the corrected degrees of freedom are presented rounded to the nearest integer. When significant main effects or interactions were detected, *post hoc* pairwise comparisons with a Bonferroni correction were made. All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, N.Y. USA). The threshold for statistical significance was set at $p < 0.05$. Graphs were made using Graphpad Prism 6. All data are presented as mean \pm SEM.

RESULTS

Home cage alcohol intake and preference

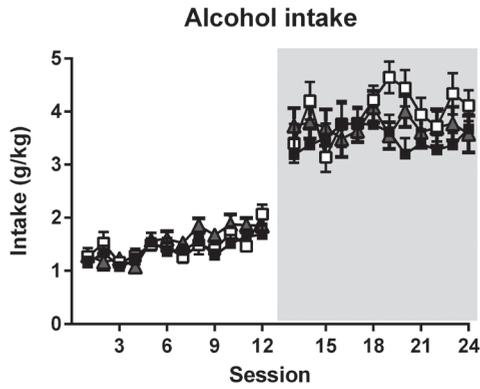
The alcohol intake and preference increased in the first month with 7h alcohol access/day (intake: $F_{(9,377) \text{ session}} = 8.836$, $p < 0.001$; preference: $F_{(11,473) \text{ session}} = 15.135$, $p < 0.001$) (Fig. 2A-B). Increasing the alcohol exposure time in the second month to 24h alcohol access/day further enhanced alcohol intake and preference (intake: $F_{(2,43) \text{ month}} = 301.754$, $p < 0.001$; preference: $F_{(2,43) \text{ month}} = 68.230$, $p < 0.001$). During the second month of voluntary alcohol consumption, alcohol intake remained stable ($F_{(7,312) \text{ session}} = 1.697$, n.s.) (Fig. 2A), whereas the preference for alcohol continued to increase ($F_{(8,350) \text{ session}} = 3.379$, $p < 0.01$) (Fig. 2B). The treatment groups (NAcc shell, NAcc core and DLS) did not differ in their alcohol intake and preference in the first month (intake: $F_{(2,43) \text{ group}} = 1.164$, n.s., $F_{(18,377) \text{ session} \times \text{group}} = 1.115$, n.s.; preference: $F_{(2,43) \text{ group}} = 0.992$, n.s., $F_{(22,473) \text{ session} \times \text{group}} = 1.209$, n.s) or in the second month (intake: $F_{(2,43) \text{ group}} = 0.998$, n.s., $F_{(15,312) \text{ session} \times \text{group}} = 1.389$, n.s; preference: $F_{(2,43) \text{ group}} = 1.327$, n.s., $F_{(16,350) \text{ session} \times \text{group}} = 1.386$, n.s) (Fig. 2A-B).

Effects of flupenthixol infusions on responding for alcohol under the FR1 schedule of reinforcement

During FR1 sessions, the average level of alcohol intake of the rats under vehicle conditions was 0.62 ± 0.05 g/kg; this did not differ between the treatment groups ($F_{(2,43) \text{ group}} = 1.010$, n.s.).

Figure 2

A



B

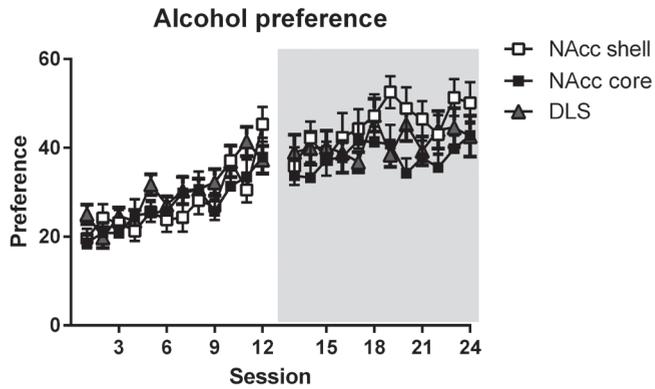


Figure 2. Alcohol intake and preference during intermittent exposure to alcohol (20%, v/v) in the home cage preceding operant alcohol self-administration and microinfusions. (A), Alcohol intake increased over sessions in the first month (7h access/day - white area) but remained stable during sessions in the second month (24h access/day - grey area). (B), Alcohol preference increased over sessions in both months. Alcohol intake and preference did not differ between groups designated for NAcc shell, NAcc core and DLS infusions. Data are shown as mean + SEM per day per infusion group.

Shell

Infusion of flupenthixol into the NAcc shell dose-dependently decreased alcohol self-administration ($F_{(3,30) \text{ dose}} = 4.220, p < 0.05$). *Post hoc* analyses indicated that responding for alcohol was significantly reduced after treatment with 15 μg flupenthixol ($p < 0.02$) (Fig. 3A). Inactive lever presses were not affected by flupenthixol infusions ($F_{(3,40) \text{ dose}} = 2.803, \text{ n.s.}$) (Fig. 3A). The number of rewards obtained declined over the course of the session ($F_{(3,25) \text{ time bin}} = 146.602, p < 0.001$); the number of rewards was significantly reduced by flupenthixol ($F_{(3,43) \text{ dose}} = 5.705, p < 0.005$). *Post hoc* analyses showed that the number of rewards was reduced after infusion of 15 μg flupenthixol ($p < 0.004$) (Fig. 3B). The effect of flupenthixol was dependent on the time bin ($F_{(9,97) \text{ time bin} \times \text{dose}} = 2.120, p < 0.04$); *post hoc* analyses indicated that less rewards were obtained after infusion of 7.5 μg ($p < 0.009$) and 15 μg ($p < 0.025$) flupenthixol in the second time bin (Fig. 3B). The onset of responding was unaffected by flupenthixol infusions (Table 1). While flupenthixol affected session time, no significant *post hoc* differences were apparent after flupenthixol treatment (Table 2). Flupenthixol affected the response rate; *post hoc* analyses indicated a significant reduction after infusion of 7.5 μg flupenthixol (Table 3).

Core

Infusion of flupenthixol into the NAcc core dose-dependently decreased alcohol self-administration ($F_{(3,14) \text{ dose}} = 34.580, p < 0.001$); *post hoc* analyses revealed significant reductions in the number of active responses after infusion of 7.5 μg ($p < 0.04$) and 15 μg flupenthixol ($p < 0.001$) (Fig. 3C). Inactive lever presses were not affected by flupenthixol ($F_{(3,55) \text{ dose}} = 1.018, \text{ n.s.}$) (Fig. 3C). The number of obtained rewards declined over the course of the session ($F_{(3,83) \text{ time bin}} = 35.850, p < 0.001$) and flupenthixol decreased the number of rewards ($F_{(3,173) \text{ dose}} = 10.440, p < 0.001$), independent of time in the session ($F_{(9,178) \text{ time bin} \times \text{dose}} = 1.447, \text{ n.s.}$). *Post hoc* analyses showed that the number of rewards was reduced after infusion of 7.5 μg ($p < 0.001$) and 15 μg flupenthixol ($p < 0.001$) (Fig. 3D). The onset of responding and the response rate were unaffected by flupenthixol infusions (Table 1, 3). Flupenthixol infusions resulted in a trend towards a reduced session time (Table 2).

DLS

Infusion of flupenthixol into the DLS had no effect on the number of active ($F_{(3,47) \text{ dose}} = 1.871, \text{ n.s.}$) and inactive lever presses ($F_{(3,29) \text{ dose}} = 1.548, \text{ n.s.}$) (Fig. 3E). The number of obtained rewards declined over the course of the session

Figure 3

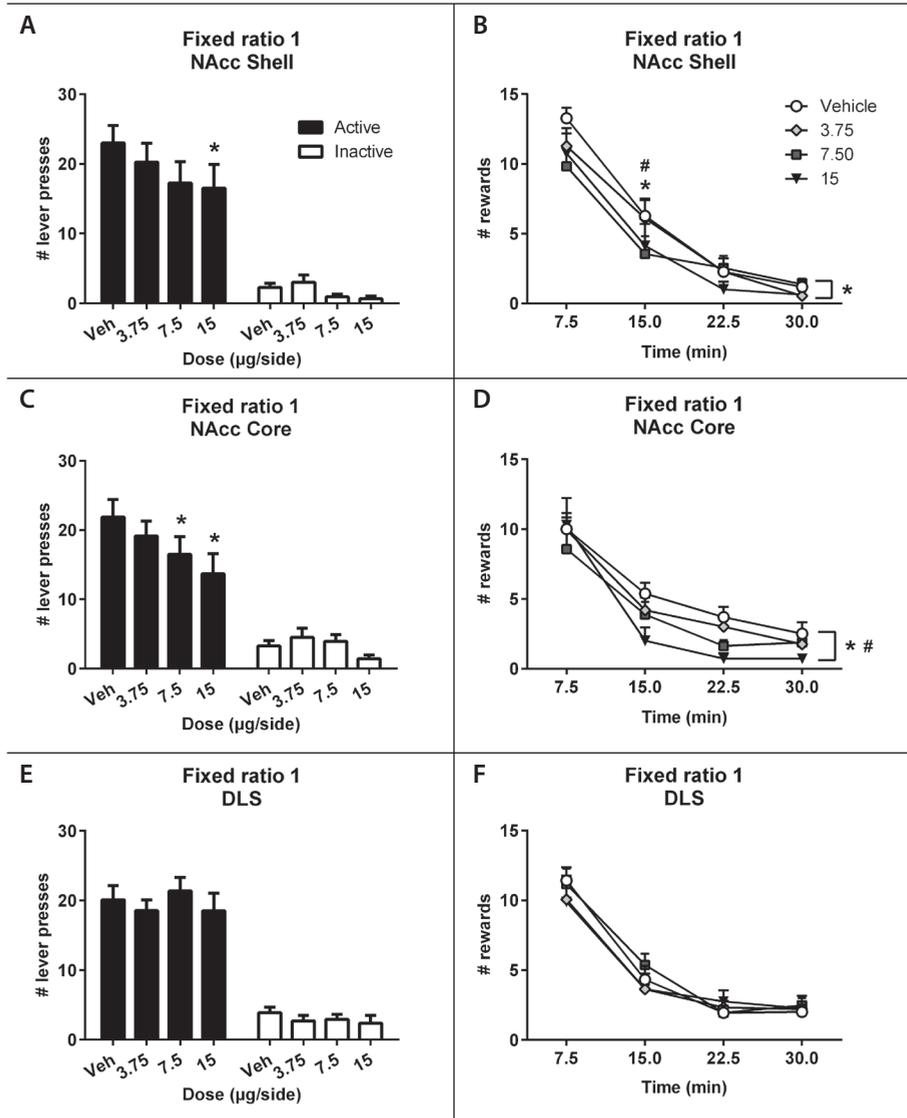


Figure 3. Effects of intracerebral flupenthixol infusions on responding for alcohol under a FR1 schedule of reinforcement. (A, C, E) Total number of active (black bars) and inactive (white bars) lever presses during alcohol self-administration. (B, D, F) Number of rewards obtained over time during alcohol self-administration. Flupenthixol infusions into the NAacc shell and core dose-dependently reduced the number of active lever presses as well as the number of rewards obtained over time during the session, whereas flupenthixol infusions into the DLS were ineffective. Data are presented as mean + SEM. Asterisk in (A, C) and (E): significantly different from vehicle (post-hoc pairwise comparisons with Bonferroni correction). (B, D) and (F): * 15 µg flupenthixol significantly different from vehicle; # 7.5 µg flupenthixol significantly different from vehicle (post hoc pairwise comparisons with Bonferroni correction).

Figure 4

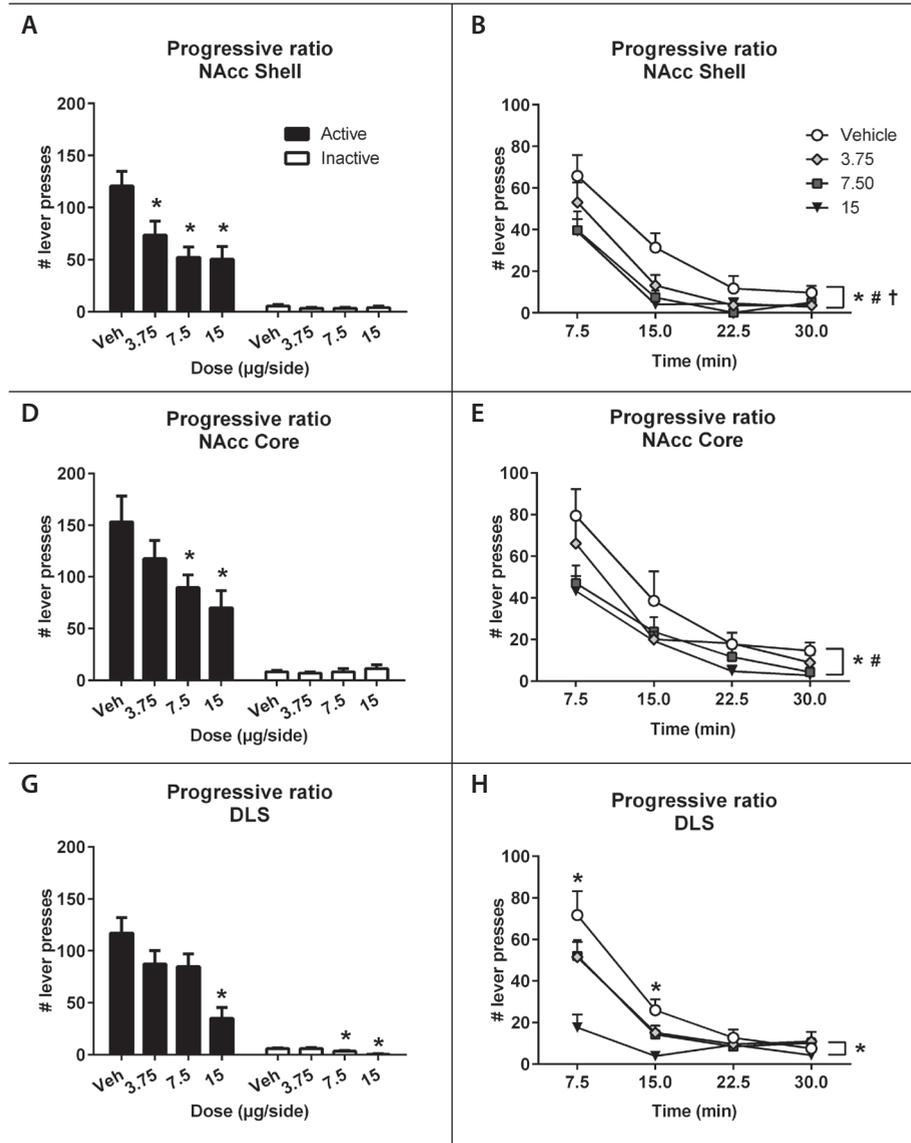


Figure 4

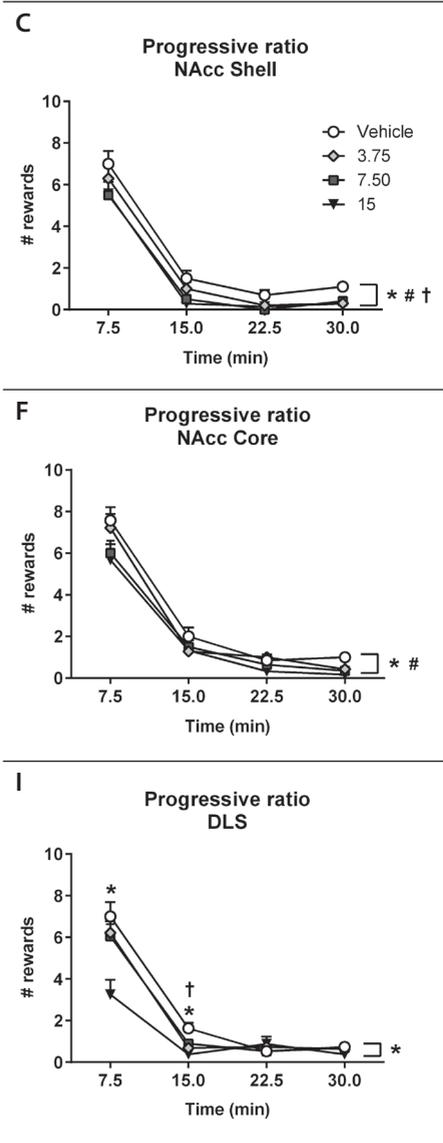


Figure 4. Effects of intracerebral flupenthixol infusions on responding for alcohol under a PR schedule of reinforcement. (A, D, G) Total number of active (black bars) and inactive (white bars) lever presses during alcohol self-administration. (B, E, H) Total number of active lever presses over time during alcohol self-administration. (C, F, I) Number of rewards obtained over time during alcohol self-administration. Flupenthixol infusions into the NAcc shell, NAcc core and DLS dose-dependently reduced responding for alcohol and decreased the number of rewards obtained. Flupenthixol had no effect on the number of inactive lever presses when infused into the NAcc shell and NAcc core, but reduced the number of inactive lever presses when infused into the DLS. Data are presented as mean + SEM. Asterisk in (A, D) and (G): significantly different from vehicle (*post hoc* pairwise comparisons with Bonferroni correction). (B, C, E, F, H) and (I): * 15 µg flupenthixol significantly different from vehicle; # 7.5 µg flupenthixol significantly different from vehicle; † 3.75 µg flupenthixol significantly different from vehicle (*post hoc* pairwise comparisons with Bonferroni correction).

($F_{(3,40) \text{ time bin}} = 37.689$, $p < 0.001$), independent of the flupenthixol dose ($F_{(3,52) \text{ dose}} = 1.303$, n.s.; $F_{(9,84) \text{ time bin} \times \text{dose}} = 1.388$, n.s.) (Fig. 3F). The onset of responding, session time, and the response rate were also unaffected by flupenthixol (Table 1, 2, 3).

Effects of flupenthixol infusions on responding for alcohol under the PR schedule of reinforcement

During PR sessions, the average level of alcohol intake of the rats under vehicle conditions was 0.31 ± 0.02 g/kg; this did not differ between the treatment groups ($F_{(2,40) \text{ group}} = 1.665$, n.s.).

Shell

Infusion of flupenthixol into the NAcc shell dose-dependently decreased responding for alcohol ($F_{(3,27) \text{ dose}} = 10.395$, $p < 0.001$). *Post hoc* analyses indicated that infusion of $3.75 \mu\text{g}$ ($p < 0.002$), $7.5 \mu\text{g}$ ($p < 0.001$) and $15 \mu\text{g}$ flupenthixol ($p < 0.002$) reduced the number of active lever presses (Fig. 4A). Inactive lever presses were not altered by flupenthixol ($F_{(3,26) \text{ dose}} = 1.082$, n.s.) (Fig. 4A). Analyses of the number of active lever presses in time showed that responding declined during the session ($F_{(3,60) \text{ time bin}} = 91.571$, $p < 0.001$). Flupenthixol decreased the number of active lever presses throughout the session ($F_{(3,59) \text{ dose}} = 8.455$, $p < 0.001$; $F_{(9,41) \text{ time bin} \times \text{dose}} = 1.175$, n.s.); *post hoc* analyses indicated that the number of active lever presses was reduced after all flupenthixol doses infused; $3.75 \mu\text{g}$ ($p < 0.015$), $7.5 \mu\text{g}$ ($p < 0.001$) and $15 \mu\text{g}$ ($p < 0.001$) (Fig. 4B). Analyses of the number of obtained rewards in time resembled the results of the active lever presses, indicating a decrease in the number of rewards over the session ($F_{(3,66) \text{ time bin}} = 133.575$, $p < 0.001$), whereby flupenthixol caused an overall reduction in the number of rewards ($F_{(3,105) \text{ dose}} = 12.048$, $p < 0.001$; $F_{(9,108) \text{ time bin} \times \text{dose}} = 0.786$, n.s.). *Post hoc* analyses showed that the number of obtained rewards was reduced after infusion of flupenthixol at all doses tested: $3.75 \mu\text{g}$ ($p < 0.002$), $7.5 \mu\text{g}$ ($p < 0.001$) and $15 \mu\text{g}$ ($p < 0.001$) (Fig. 4C). The onset of responding was unaffected by flupenthixol infusions (Table 1). However, infusion of flupenthixol reduced the session time. *Post hoc* analyses indicated a shorter session time after infusion of $3.75 \mu\text{g}$ flupenthixol, and a trend towards a shorter session time for the $7.5 \mu\text{g}$ and $15 \mu\text{g}$ doses (Table 2). The response rate was not altered by flupenthixol (Table 3).

Table 1

Latency to the first active lever press in the session (sec).

		Dose effect	Vehicle	3.75	7.5	15
FR1	Shell	$F_{3,41 \text{ dose}} = 0.194$, n.s.	9.4 ± 4.4	20.2 ± 9.0	16.5 ± 8.3	9.6 ± 3.5
	Core	$F_{3,40 \text{ dose}} = 1.390$, n.s.	8.4 ± 3.0	15.2 ± 7.6	22.7 ± 7.9	23.1 ± 16.2
	DLS	$F_{3,48 \text{ dose}} = 1.289$, n.s.	12.5 ± 3.9	23.1 ± 12.1	48.8 ± 19.6	16.7 ± 6.6
PR	Shell	$F_{3,24 \text{ dose}} = 0.778$, n.s.	6.4 ± 3.1	8.9 ± 2.7	8.8 ± 5.4	9.8 ± 5.8
	Core	$F_{3,18 \text{ dose}} = 2.973$, n.s.	3.1 ± 0.5	6.9 ± 3.0	3.8 ± 0.8	2.0 ± 0.1
	DLS	$F_{3,30 \text{ dose}} = 1.616$, n.s.	24.0 ± 13.9	22.8 ± 9.9	8.3 ± 2.4	16.4 ± 6.7

Data are presented as mean ± SEM.

Table 2

Session time (min).

		Dose effect	Vehicle	3.75	7.5	15
FR1	Shell	$F_{3,12 \text{ dose}} = 3.497$, $p=0.050$	21.6 ± 1.7	20.6 ± 2.3	22.0 ± 2.4	15.5 ± 3.2
	Core	$F_{3,14 \text{ dose}} = 3.038$, $p=0.065$	26.0 ± 1.0	24.0 ± 1.6	25.8 ± 0.7	19.9 ± 4.1
	DLS	$F_{3,30 \text{ dose}} = 0.781$, n.s.	24.0 ± 1.8	25.3 ± 1.0	25.9 ± 1.1	24.7 ± 1.8
PR	Shell	$F_{3,10 \text{ dose}} = 6.651$, $p<0.02$	26.4 ± 0.7	16.0 ± 2.8*	16.0 ± 3.8§	10.9 ± 3.4§
	Core	$F_{3,12 \text{ dose}} = 17.134$, $p<0.001$	25.4 ± 1.3	22.0 ± 1.4§	19.2 ± 2.0	14.8 ± 3.2*
	DLS	$F_{3,18 \text{ dose}} = 4.976$, $p<0.02$	22.6 ± 1.6	23.4 ± 1.7	21.5 ± 2.1	14.4 ± 3.6*

Data are presented as mean ± SEM. * Significant post hoc analyses with Bonferroni correction ($p<0.05$).§ Trend towards significant *post hoc* analyses with Bonferroni correction ($p<0.082$)

Table 3

Response rate.

		Dose effect	Vehicle	3.75	7.5	15
FR1	Shell	$F_{3,19 \text{ dose}} = 7.982$, $p<0.01$	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.1*	1.2 ± 0.2
	Core	$F_{3,18 \text{ dose}} = 1.171$, n.s.	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.3
	DLS	$F_{3,45 \text{ dose}} = 1.875$, n.s.	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
PR	Shell	$F_{3,14 \text{ dose}} = 1.016$, n.s.	4.3 ± 0.5	6.0 ± 1.3	6.1 ± 1.7	6.6 ± 1.3
	Core	$F_{3,19 \text{ dose}} = 1.478$, n.s.	5.4 ± 0.5	5.3 ± 0.7	4.3 ± 0.6	5.5 ± 1.2
	DLS	$F_{3,11 \text{ dose}} = 2.965$, n.s.	5.4 ± 0.6	3.7 ± 0.5	5.0 ± 1.2	3.1 ± 0.8

Data are presented as mean ± SEM. * Significant *post hoc* analyses with Bonferroni correction ($p=0.050$).

Core

Infusion of flupenthixol into the NAcc core decreased responding for alcohol ($F_{(3,7) \text{ dose}} = 66.662, p < 0.001$) at doses of 7.5 μg ($p < 0.05$) and 15 μg ($p < 0.002$) (Fig. 4D). Inactive lever presses were unaffected by flupenthixol ($F_{(3,9) \text{ dose}} = 2.842, \text{n.s.}$) (Fig. 4D). The number of active lever presses declined over the session ($F_{(3,78) \text{ time bin}} = 25.961, p < 0.001$) and flupenthixol infusions decreased the number of active lever presses throughout the session ($F_{(3,143) \text{ dose}} = 7.734, p < 0.001$; $F_{(9,149) \text{ time bin} \times \text{dose}} = 0.522, \text{n.s.}$). *Post hoc* analyses indicated that the number of active lever presses was reduced after 7.5 μg ($p < 0.002$) and 15 μg flupenthixol ($p < 0.002$) (Fig. 4E). Likewise, the number of rewards obtained decreased over the session ($F_{(3,85) \text{ time bin}} = 88.170, p < 0.001$). Flupenthixol decreased the number of rewards throughout the session ($F_{(3,141) \text{ dose}} = 6.493, p < 0.001$; $F_{(9,145) \text{ time bin} \times \text{dose}} = 0.952, \text{n.s.}$). *Post hoc* analyses showed that the number of obtained rewards was reduced after infusion of 7.5 μg ($p < 0.006$) and 15 μg flupenthixol ($p < 0.002$) (Fig. 4F). The onset of responding was not changed by flupenthixol infusions (Table 1). Infusion of flupenthixol reduced the session time. *Post hoc* analyses indicated a shorter session time after the infusion of 15 μg flupenthixol, and a trend towards significance for the 3.75 μg dose (Table 2). The response rate remained unaffected (Table 3).

DLS

Infusion of flupenthixol into the DLS reduced the number of active ($F_{(3,12) \text{ dose}} = 6.921, p < 0.01$) and inactive lever presses ($F_{(3,47) \text{ dose}} = 9.511, p < 0.001$). *Post hoc* analyses showed that the number of active lever presses was reduced after infusion of 15 μg flupenthixol ($p < 0.02$) and the number of inactive lever presses was reduced after infusion of 7.5 μg ($p < 0.03$) and 15 μg flupenthixol ($p < 0.001$) (Fig. 4G). Analyses of the active lever presses in time showed that responding declined over the course of the session ($F_{(3,241) \text{ time bin}} = 53.240, p < 0.001$) and was decreased by flupenthixol ($F_{(3,245) \text{ dose}} = 4.958, p < 0.01$) at the dose of 15 μg ($p < 0.002$) (Fig. 4H). Moreover, the effect of flupenthixol was dependent on time in the session ($F_{(9,241) \text{ time bin} \times \text{dose}} = 1.919, p = 0.05$); *post hoc* analyses showed a significant reduction in active lever presses after 15 μg flupenthixol during the first ($p < 0.001$) and the second time bin ($p < 0.012$) (Fig. 4H). The number of rewards obtained decreased during the session ($F_{(3,84) \text{ time bin}} = 118.883, p < 0.001$); this was affected by flupenthixol infusions ($F_{(3,200) \text{ dose}} = 4.640, p < 0.01$). *Post hoc* analyses indicated that flupenthixol decreased the number of rewards at a dose of 15 μg ($p < 0.004$) (Fig. 4I). The reduction in the number of rewards evoked by flupenthixol infusions into the DLS was dependent on the time in

the session ($F_{(9,209) \text{ time bin} \times \text{dose}} = 2.974, p < 0.010$). *Post hoc* analyses revealed that the number of rewards was reduced after infusion of 15 μg flupenthixol during the first ($p < 0.002$) and second time bin ($p < 0.005$). In addition, in the second time bin, the dose of 3.75 μg flupenthixol reduced the number of rewards as well ($p < 0.002$) and there was a trend towards a reduction for the 7.5 μg dose ($p = 0.053$) (Fig. 4I). The onset of responding was not altered by flupenthixol infusions (Table 1). The rats ceased responding earlier in the session after infusion of 15 μg flupenthixol (Table 2). The response rate was not affected by flupenthixol (Table 3).

DISCUSSION

In the present study, we found that dopamine receptor blockade in the NAcc shell and core reduced responding for alcohol under both FR1 and PR schedules of reinforcement. Flupenthixol treatment in the NAcc core reduced responding under both schedules at similar doses. In the NAcc shell, however, responding under the PR schedule of reinforcement was more sensitive to the effects of flupenthixol. Infusion of flupenthixol into the DLS decreased responding for alcohol under the PR, but not the FR1 schedule of reinforcement. The flupenthixol-induced reductions in responding were associated with an earlier termination of responding for alcohol. Together, these findings indicate that alcohol reinforcement relies upon coordinated dopamine activity throughout the striatum, whereby different sub-regions play distinct roles in alcohol-directed behaviour.

Flupenthixol infusion into the NAcc shell decreased operant responding for alcohol, which is in agreement with the previously reported role of the NAcc shell in the primary reinforcing properties of food and drug rewards (Ikemoto et al., 1997a; Pecina and Berridge 2000; Di Chiara 2002; Rodd-Henricks et al., 2002; Bassareo et al., 2003; Ikemoto 2003). Alcohol is reliably self-administered into the NAcc shell, but not the NAcc core (Engleman et al., 2009). Moreover, in a recent study, infusion of dopamine receptor antagonists into the NAcc shell, but not the NAcc core, reduced responding for self-infusions of alcohol in the posterior ventral tegmental area (Ding et al., 2015). Importantly, flupenthixol infused into the NAcc shell decreased responding for alcohol under the PR schedule at a fourfold lower dose as compared to the FR1 schedule of reinforcement. Since the increasing response requirement under the PR schedule particularly addresses processes related to incentive motivation, the

current findings suggest that dopamine in the NAcc shell mainly modulates the motivational aspects of responding for alcohol. This finding resonates well with our previous report on cocaine self-administration (Veeneman et al., 2012). Together, these data suggest that NAcc shell dopamine mediates the motivation to obtain substances of abuse from different pharmacological classes.

We observed that the same doses of flupenthixol, infused into the NAcc core, reduced responding for alcohol under both reinforcement schedules, suggesting that NAcc core dopamine plays a more general role in alcohol reinforcement. The NAcc core has been implicated in the control of conditioned cues over behaviour, such that the value of reward-related stimuli is integrated to influence the organization of motor activity (Parkinson et al., 1999; Ito et al., 2004; Day et al., 2007; Flagel et al., 2011; for reviews see Salamone and Correa 2012; Floresco 2015). During alcohol self-administration, the animals are exposed to different alcohol-associated cues (e.g. transportation to the boxes, the context of the boxes, presentation of the levers, the smell of alcohol) as well as response contingent cues (rising of dipper cup and illumination of cue light). These alcohol-associated cues by themselves can induce dopamine release within the NAcc (Weiss et al., 1993; Katner et al., 1996; Gonzales and Weiss 1998; Melendez et al., 2002). Previous studies have shown that the transient rise in dopamine levels in the ventral striatum is associated with the presentation of alcohol-associated cues and the anticipation of alcohol reinforcement, rather than with the concentration of alcohol in the ventral striatum (Weiss et al., 1993; Melendez et al., 2002; Doyon et al., 2003; Doyon et al., 2005). These data suggest that dopamine in the ventral striatum contributes to cue-driven expectations of alcohol reward rather than the positive subjective effects of alcohol itself. Moreover, infusion of dopamine D2 receptor antagonists into the NAcc core has been shown to reduce responding for alcohol, but not its actual consumption (Czachowski et al., 2001; Samson and Chappell 2004). It is therefore likely that blockade of dopaminergic neurotransmission in the NAcc core interfered with the processing of reward-related cues to modulate alcohol self-administration. Importantly, the conditioned cues are largely similar during FR and PR sessions. Because the same doses of flupenthixol in the NAcc core reduced responding for alcohol under both reinforcement schedules, NAcc core dopamine may be especially relevant for processing alcohol-associated cues during alcohol-directed behaviour.

For the DLS, we observed a reduction in responding for alcohol after flupenthixol infusions under the PR schedule, but not the FR1 schedule of reinforcement. These findings suggest that dopamine in the DLS is involved in the motivational aspects of responding for alcohol. The DLS has been implicated in habit formation, which is thought to contribute to compulsive drug seeking after extended exposure to substances of abuse (Yin et al., 2004; Everitt and Robbins 2005; Vanderschuren et al., 2005; Corbit et al., 2012; Barker and Taylor 2014). Indeed, dopaminergic neurotransmission in the DLS has been shown to contribute to habitual responding for alcohol (Corbit et al., 2014), but the role of DLS dopamine in alcohol-reinforced behaviour has not been studied previously. In the current experiments, the effect of flupenthixol was first assessed under the FR1 schedule and subsequently under the PR schedule of reinforcement in the same animals. It is therefore possible that the effects of flupenthixol under the PR schedule are the result of the development of habitual patterns of responding with prolonged operant training and alcohol consumption. However, we think that this explanation is not likely to account for the present findings, because relatively few operant sessions separated the infusions during FR1 and PR sessions in comparison to the alcohol sessions in the home cage and operant chamber prior to infusions during the FR1 schedule. Interestingly, the current findings are, in part, in line with the effects obtained during cocaine self-administration in which responding increased during the FR1 schedule but decreased during the PR schedule of reinforcement upon systemic and intra-DLS dopamine receptor blockade (Ettenberg et al., 1982; Caine and Koob 1994; Bourland and French 1995; Vanderschuren et al., 2005; Veeneman et al., 2012). Taken together, these findings suggest involvement of DLS dopamine in the motivational aspects of drug self-administration.

To gain more insight into the mechanisms by which striatal dopamine modulates responding for alcohol, we determined the effects of flupenthixol on the response onset, the duration of responding (i.e. session time), and the response rate. Treatment with flupenthixol lead to an earlier termination of responding in the session. These effects were especially pronounced for the PR schedule of reinforcement, which emphasizes the involvement of striatal dopamine in the motivation to obtain alcohol, especially when a large effort is required. These findings concur with previous reports on the duration of responding for alcohol after systemic (Pfeffer and Samson 1988; Aberman et al., 1998; Czachowski et al., 2002) and local infusions of dopamine receptor antagonists (Samson et al., 1993; Hodge et al., 1997; Czachowski et al., 2001).

Intra-striatal administration of dopamine receptor antagonists (Fowler 1990; Salamone 1992; Baldo et al., 2002) has previously been reported to reduce motor activity, which could also explain the earlier response termination. However, in the present study, dopamine receptor blockade did not affect the onset of responding, and had no major effects on the response rate. Although we found a decrease in both active and inactive responses upon flupenthixol infusions into the DLS, we did not observe any effects of flupenthixol into the DLS under the FR1 schedule. Moreover, the flupenthixol doses used were in the same range as the doses that increased responding for cocaine upon intra-DLS infusion under a FR schedule of reinforcement (Vanderschuren et al., 2005; Veeneman et al., 2012; Willuhn et al., 2012). Finally, the inactive lever presses remained unaffected after flupenthixol treatment in the NAcc core and shell. Taken together, it is therefore not likely that flupenthixol caused a general decrease in motor function in the current study.

In sum, this study provides novel insight into the differential role of dopamine within sub-regions of the striatal complex in alcohol-directed behaviour. Dopaminergic neurotransmission in the NAcc shell and DLS contributes to the motivational properties of alcohol, while NAcc core dopamine most likely modulates the influence of alcohol-associated cues on alcohol self-administration. Together, these findings show that alcohol reinforcement relies on coordinated dopaminergic activity within the striatum.

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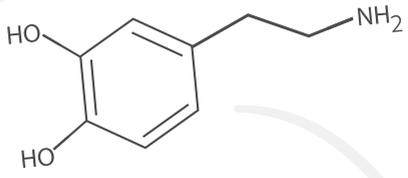
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CHAPTER 9

DOPAMINE RECEPTOR AGONISTS MODULATE VOLUNTARY ALCOHOL INTAKE INDEPENDENTLY OF INDIVIDUAL LEVELS OF ALCOHOL INTAKE

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Submitted



ABSTRACT

Individual susceptibility to alcohol use disorder has been related to functional changes in dopaminergic neurotransmission. The aim of the current work was to assess the effects of selective dopamine D1 and D2 receptor agonists and antagonists on alcohol consumption in rats that display individual levels in alcohol intake. Male rats were exposed to intermittent alcohol access (IAA) to discern groups of low (LD) and high (HD) alcohol drinkers. Subsequently, the effects of the dopamine D1 receptor agonist SKF 82958, the dopamine D1 receptor antagonist SCH 23390, the dopamine D2 receptor agonist sumanirole and the dopamine D2 receptor antagonist L741,626 on alcohol consumption and preference were assessed at 2h, 7h and 24h after alcohol presentation. The dopamine D1 receptor agonist SKF 82958 decreased alcohol intake and alcohol preference throughout the 24h session. The dopamine D2 receptor agonist sumanirole decreased alcohol intake during the first 2h, but increased alcohol intake during the remainder of the 24h session. The effects of SKF 82958 and sumanirole on alcohol intake and alcohol preference were comparable in LD and HD. By contrast, the dopamine D1 receptor antagonist SCH 23390 and the dopamine D2 receptor antagonist L741,626 did not alter alcohol consumption in either group at any time point. These data indicate that stimulation of dopamine D1 receptors reduces alcohol intake, but that endogenous dopamine does not play a primary role in alcohol consumption. Moreover, the difference in alcohol consumption between LD and HD does not involve altered dopamine signaling.

INTRODUCTION

Alcohol use disorder (AUD) is a chronic relapsing brain disorder, which is characterized by a compulsive engagement in alcohol use (American Psychiatric Association 2013). There is substantial heterogeneity in both the etiology and expression of AUD, in which several (e.g. genetic, environmental and personality) factors are thought to contribute to the individual vulnerability for this disorder (Chassin et al., 2002; Anderson 2006; Perry and Carroll 2008; Goudriaan et al., 2011; Enoch 2013). More insight into the mechanisms underlying individual variation in alcohol consumption may provide important knowledge about the development of AUD, which may contribute to improved personalized treatments for AUD.

One prominent hypothesis is that variations in dopaminergic neurotransmission underlie the individual susceptibility to AUD (Noble 2000; Tupala and Tiihonen 2004; Le Foll et al., 2009). The mesolimbic dopamine system has been widely implicated in motivated-, including alcohol-directed behaviour (Berridge 2007; Robbins and Everitt 2007; Spanagel 2009; Volkow et al., 2011; Salamone and Correa 2012; Floresco 2015; Korpi et al., 2015). Acute alcohol administration has been shown to activate dopamine neuronal firing in the ventral tegmental area (VTA) (Gessa et al., 1985; Brodie et al., 1990, 1999), and to increase dopamine release in the ventral striatum upon ingestion (Weiss et al., 1993; Boileau et al., 2003; Doyon et al., 2003). Moreover, acute and repeated alcohol exposure has been shown to alter dopaminergic function at both the pre- and postsynaptic level (Reggiani et al., 1980; Imperato et al., 1987; Imperato and Di Chiara 1988; Nestby et al., 1997, 1999; Gonzales et al., 2004; Sari et al., 2006).

The actions of dopamine are mediated by two principal classes of dopamine receptor subtypes, i.e. the D1-like (D1/D5) and D2-like (D2/D3/D4) dopamine receptors (Le Foll et al., 2009). However, the relative contributions of the different dopamine receptor subtypes to the development and maintenance of AUD remain incompletely understood. In addition, it is unknown whether individual susceptibility to AUD relates to a specific dopamine receptor subtype. Alterations in dopamine D2 receptor function have been the main focus in AUD studies over the last decade (Noble 2000; Connor et al., 2002; Kraschewski et al., 2009). Reduced levels of dopamine D2 receptors in limbic areas have been observed in both AUD patients (Hietala et al., 1994; Volkow et al., 1996, 2002; Tupala et al., 2001, 2003) and in alcohol-preferring rats and

mice (Stefanini et al., 1992; McBride et al., 1993; Zhou et al., 1995; Bice et al., 2008). However, the dopamine D1 receptor has also been implicated in alcohol seeking and consumption. Both dopamine D1 and D2 receptor deficient mice show marked reductions in alcohol-directed behaviour (El-Ghundi et al., 1998; Phillips et al., 1998; Risinger et al., 2000; Thanos et al., 2005). Moreover, the involvement of both dopamine receptor subtypes has been demonstrated in alcohol consumption and reinforcement (Linseman 1990; Silvestre et al., 1996; Files et al., 1998; Cohen et al., 1999; Melendez et al., 2005; Ding et al., 2015).

The aim of this study was to determine the contribution of dopamine D1 and D2 receptors in individual differences in alcohol consumption under intermittent alcohol access (IAA) conditions. IAA results in high levels of alcohol intake that escalate in time, indicating that this paradigm is well suited to investigate biological mechanisms of AUD (Wise 1973; Simms et al., 2008; Hopf et al., 2010; Lesscher et al., 2010; Loi et al., 2010; Hwa et al., 2011; Sabino et al., 2013; Spoelder et al., 2015). We recently observed marked individual differences in alcohol intake in outbred rats using the IAA paradigm, which was related to the motivational properties of alcohol and measures of compulsive alcohol intake (Spoelder et al., 2015). We therefore used the IAA paradigm to determine the effects of dopamine D1 and D2 receptor-selective agonists and antagonists on voluntary alcohol consumption in groups of high and low alcohol drinking rats. We hypothesized that, if variations in dopamine neurotransmission underlie individual vulnerability to AUD, treatment with dopaminergic drugs should have differential effects on alcohol intake in high and low alcohol drinking rats.

MATERIALS AND METHODS

Animals

Male Lister Hooded rats (Charles River, Germany) weighing 320-360 g at the start of the experiment were used. The rats were housed individually under controlled temperature and humidity conditions, a reversed light/dark cycle (lights off 7.00 AM), with *ad libitum* access to water and chow at all times. All rats were weighed and handled at least once per week throughout the experiment. All experiments were approved by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op de dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Intermittent alcohol access (IAA) in the home cage

The rats were provided access to 20% alcohol (v/v) and water in a two-bottle choice IAA setup in the home cage for three days a week (Monday-Wednesday-Friday) using bottles that were fitted with stainless-steel dual ball bearing drinking spouts. Bottle positions were switched between sessions to avoid side bias. Rats were provided with access to alcohol for 7h/day in the first month. Subsequently, access to alcohol was extended to 24h/day in the second month and for the remainder of the experiment. The selection of low and high alcohol drinking rats (LD;HD) was performed as previously described (Spoelder et al., 2015). Briefly, after 2 months of IAA, the rats were ranked based on the animals' average alcohol intake per week and were assigned ranking scores. The weekly ranking scores were summed to calculate a total ranking score per rat. The rats within the lower and upper 25% of the total ranking score range were designated as LD and HD, respectively. The middle 50% were designated as medium alcohol drinking rats and used in other experiments.

Drugs

Alcohol (99.5%, Klinipath, The Netherlands) was freshly diluted with tap water once per week to 20% (v/v). Drug solutions were freshly prepared daily. The dopamine D1 receptor agonist SKF 82958 hydrobromide ((±)-6-Chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide) and the dopamine D2 receptor agonist sumanirole maleate ((R)-5,6-Dihydro-5-(methylamino)-4H-imidazo[4,5,1-ij]quinolin-2(1H)-one maleate) were generously supplied by the NIMH Chemical Synthesis and Drug Supply Program, Maryland, USA. The dopamine D1 receptor antagonist SCH 23390 hydrochloride (R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride) and the dopamine D2 receptor antagonist L741,626 ((±)-3-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]methyl-1H-indole) were purchased from Tocris (UK). SKF 82958, sumanirole and SCH 23390 were dissolved in sterile saline (0.9% NaCl). L741,626 was dissolved in 5% polyethylene glycol (PEG) and 5% Tween 80 in MilliQ water. Saline was used as a vehicle for SKF 82958, sumanirole and SCH 23390; a 5% PEG/Tween solution served as the vehicle for L741,626 treatments. Drug doses were based on previous published reports (Linseman 1990; Silvestre et al., 1996; Cohen et al., 1999; Fernando et al., 2012; Watson et al., 2012).

Drug administration and injection procedures

All drug solutions were administered subcutaneously in a volume of 1 ml/kg body weight, 20 min prior to the drinking session in the home cage. The different drug doses were administered according to a within-subject Latin square design. Alcohol and water bottles were weighed before each session and 2h, 7h and 24h after the start of the session. Because the effects of the drugs were examined under IAA, each treatment session was always followed by at least one alcohol-free day that also served as washout day. Thereafter, there was at least one drug-free re-baseline session between sessions for the same drug and there were at least three re-baseline sessions between different drugs. Two batches of rats were used for this study; the rats in the first batch were treated with the dopamine D2 receptor agonist sumanirole (0; 0.1; 0.3 and 1.0 mg/kg) and the dopamine D2 receptor antagonist L741,626 (0; 0.3; 1.0; 3.0 mg/kg) in a counterbalanced fashion. The rats in the second batch were treated with the dopamine D1 receptor agonist SKF 82958 (0; 0.3; 1.0; 3.0 mg/kg) and the dopamine D1 receptor antagonist SCH 23390 (0; 3; 10; 30 µg/kg). In addition, the effects of the highest dose of sumanirole (0 and 1.0 mg/kg) and L741,626 (0 and 3.0 mg/kg) on alcohol consumption were replicated in this second batch. The order of drugs administered in the second batch was similar for each animal; the rats were first treated with SCH 23390, followed by sumanirole, SKF 82958 and L741,626. All rats received two habituation injections (1.0 ml/kg saline (0.9% NaCl) subcutaneously), prior to alcohol drinking sessions and one week before actual drug testing began.

Data analysis

Alcohol intake and preference during the initial two months IAA was analyzed with two-way repeated-measures ANOVA's with week as the within-subject variable and group (LD;HD) as the between-subject variable. The effects of the pharmacological treatments were analyzed using three-way repeated-measures ANOVA's with time (2h, 7h and 24h) and dose as within-subject variables and group (LD;HD) as the between-subject variable. In case of a significant interaction effect involving the drug dose, follow-up two-way repeated-measures ANOVA's per time-point (2h, 7h and 24h) were conducted with dose as within-subject variable and group (LD;HD) as the between-subject variable. *Post hoc* pairwise comparisons of each drug dose with vehicle were performed with LSD tests. Mauchly's test of sphericity was used to determine if variances of the differences between treatment levels were equal. If the assumption of sphericity were violated, degrees of freedom were

corrected using Huynh-Feldt estimates of sphericity to more conservative values. Corrected degrees of freedom are presented rounded to the nearest integer. All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, N.Y. USA). The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean \pm SEM. Graphs were made using Graphpad Prism 6.

RESULTS

Alcohol consumption during IAA in LD and HD

In agreement with our previous study (Spoelder et al., 2015), when comparing the alcohol intake of the first month (7h/day IAA) to the second month (24h/day IAA), HD increased their alcohol intake to a further extent compared to LD (Batch 1: $F_{(1,30) \text{ month} \times \text{group}} = 96.33$, $p < 0.001$; Batch 2: $F_{(1,10) \text{ month} \times \text{group}} = 29.53$, $p < 0.001$). Statistical analyses confirmed the group differences in alcohol intake and preference over the initial 2 month IAA (Batch 1: Intake: $F_{(1,30) \text{ group}} = 179.78$, $p < 0.001$; Preference: $F_{(1,30) \text{ group}} = 208.34$, $p < 0.001$; Batch 2: Intake: $F_{(1,10) \text{ group}} = 113.31$, $p < 0.001$; Preference: $F_{(1,10) \text{ group}} = 120.55$, $p < 0.001$) (Table 1). Total fluid intake was not different between LD and HD (Batch 1: $F_{(1,30) \text{ group}} = 0.39$, n.s.; Batch 2: $F_{(1,10) \text{ group}} = 3.34$, n.s.) (data not shown).

During treatment with the dopaminergic drugs, HD consumed more alcohol than LD (SKF 82958: $F_{(1,10) \text{ group}} = 4.83$, $p = 0.053$; SCH 23390: $F_{(1,10) \text{ group}} = 16.09$, $p < 0.003$; Sumanriole: Batch 1: $F_{(1,30) \text{ group}} = 27.34$, $p < 0.001$, Batch 2: $F_{(1,10) \text{ group}} = 11.36$, $p < 0.008$; L741,626: Batch 1: $F_{(1,30) \text{ group}} = 38.51$, $p < 0.001$, Batch 2: $F_{(1,10) \text{ group}} = 5.82$, $p < 0.04$). Moreover, group differences in alcohol intake typically became more pronounced as the session progressed (SKF 82958: $F_{(1,12) \text{ time} \times \text{group}} = 4.88$, $p < 0.05$; SCH 23390: $F_{(1,14) \text{ time} \times \text{group}} = 17.62$, $p < 0.001$; Sumanriole: Batch 1: $F_{(1,35) \text{ time} \times \text{group}} = 29.78$, $p < 0.001$, Batch 2: $F_{(1,13) \text{ time} \times \text{group}} = 11.80$, $p < 0.004$; L741,626: Batch 1: $F_{(1,37) \text{ time} \times \text{group}} = 40.19$, $p < 0.001$, Batch 2: $F_{(1,13) \text{ time} \times \text{group}} = 1.68$, n.s.). Preference for alcohol was also greater in HD (SKF 82958: $F_{(1,10) \text{ group}} = 4.74$, $p = 0.055$; SCH 23390: $F_{(1,10) \text{ group}} = 17.11$, $p < 0.003$; Sumanriole: Batch 1: $F_{(1,29) \text{ group}} = 12.21$, $p < 0.003$, Batch 2: $F_{(1,10) \text{ group}} = 4.03$, $p = 0.073$; L741,626: Batch 1: $F_{(1,30) \text{ group}} = 22.36$, $p < 0.001$, Batch 2: $F_{(1,9) \text{ group}} = 4.51$, $p = 0.063$), independent of session time (SKF 82958: $F_{(2,16) \text{ time} \times \text{group}} = 2.78$, n.s.; SCH 23390: $F_{(1,15) \text{ time} \times \text{group}} = 1.71$, n.s.; Sumanriole: Batch 1: $F_{(2,58) \text{ time} \times \text{group}} = 1.14$, n.s., Batch 2: $F_{(1,15) \text{ time} \times \text{group}} = 1.23$, n.s.; L741,626: Batch 1: $F_{(2,51) \text{ time} \times \text{group}} = 2.46$, n.s., Batch 2: $F_{(2,18) \text{ time} \times \text{group}} = 1.19$, n.s) (Fig. 1-3).

Dopamine D1 receptor agonist – SKF 82958

SKF 82958 decreased alcohol intake ($F_{(3,30) \text{ dose}} = 9.58, p < 0.001$), independent of session time ($F_{(6,55) \text{ dose} \times \text{time}} = 1.43, \text{ n.s.}$) or group ($F_{(3,30) \text{ dose} \times \text{group}} = 0.41, \text{ n.s.}$; $F_{(6,55) \text{ time} \times \text{dose} \times \text{group}} = 1.38, \text{ n.s.}$) (Fig. 1A). *Post hoc* analyses showed that alcohol intake was reduced after treatment with 1.0 and 3.0 mg/kg SKF 82958 (Fig. 1A). SKF 82958 decreased the preference for alcohol ($F_{(3,30) \text{ dose}} = 4.04, p < 0.02$), independent of session time ($F_{(5,49) \text{ dose} \times \text{time}} = 1.33, \text{ n.s.}$) or group ($F_{(3,30) \text{ dose} \times \text{group}} = 0.41, \text{ n.s.}$; $F_{(5,49) \text{ time} \times \text{dose} \times \text{group}} = 0.90, \text{ n.s.}$) (Fig. 1B). *Post hoc* analyses showed that the preference for alcohol was decreased after treatment with 1.0 and 3.0 mg/kg SKF 82958 (Fig. 1B).

Because SKF 82958 reduced alcohol intake and preference after 24h of alcohol exposure, we examined if SKF 82958 would affect alcohol consumption in the subsequent re-baseline session, during which the animals received no treatment. Alcohol intake and preference during the re-baseline session were not affected by SKF 82958 treatment in the previous session (alcohol intake: $F_{(3,30) \text{ dose}} = 0.13, \text{ n.s.}$; preference: $F_{(3,30) \text{ dose}} = 0.20, \text{ n.s.}$) (data not shown).

Dopamine D1 receptor antagonist – SCH 23390

SCH 23390 did not affect alcohol intake ($F_{(3,30) \text{ dose}} = 0.27, \text{ n.s.}$) at any of the time points tested ($F_{(4,35) \text{ time} \times \text{dose}} = 0.51, \text{ n.s.}$) independent of group ($F_{(3,30) \text{ dose} \times \text{group}} = 0.20, \text{ n.s.}$; $F_{(4,35) \text{ time} \times \text{dose} \times \text{group}} = 0.14, \text{ n.s.}$) (Fig. 1C). SCH 23390 had no main effect on alcohol preference ($F_{(3,30) \text{ dose}} = 1.68, \text{ n.s.}$), but there was a three-way interaction with group and session time ($F_{(6,60) \text{ time} \times \text{dose} \times \text{group}} = 3.08, p < 0.02$) (Fig. 1D). Subsequent analyses per time point indicated that SCH 23390 influenced the preference for alcohol during the first two hours of the session ($F_{(3,30) \text{ dose} \text{ 2h}} = 2.99, p < 0.05$), independent of group ($F_{(3,30) \text{ dose} \times \text{group} \text{ 2h}} = 2.04, \text{ n.s.}$), without a clear dose-dependent direction. Indeed, *post hoc* analyses did not reveal a significant difference of any of the doses of SCH 23390, when compared to vehicle. Alcohol preference was not affected by SCH 23390 after 7h ($F_{(3,30) \text{ dose} \text{ 7h}} = 1.29, \text{ n.s.}$; $F_{(3,30) \text{ dose} \times \text{group} \text{ 7h}} = 0.69, \text{ n.s.}$) and 24h of alcohol exposure ($F_{(3,30) \text{ dose} \text{ 24h}} = 0.74, \text{ n.s.}$; $F_{(3,30) \text{ dose} \times \text{group} \text{ 24h}} = 0.56, \text{ n.s.}$) (Fig. 1D).

Dopamine D2 receptor agonist – Sumanitrole

Sumanitrole affected the level of alcohol intake, and its effect was dependent on time in the session ($F_{(5,136) \text{ time} \times \text{dose}} = 9.29, p < 0.001$), independent of group ($F_{(5,136) \text{ time} \times \text{dose} \times \text{group}} = 1.55, \text{ n.s.}$) (Fig 2A). Follow-up analyses per time point indicated that sumanitrole decreased alcohol intake during the first two hours

Table 1

Alcohol intake and preference for HD and LD during the initial two months IAA, prior to pharmacological treatment.

		Alcohol intake		Alcohol preference	
		7h/day	24h/day	7h/day	24h/day
Batch 1	HD (n=16)	2.61 ± 0.16	5.46 ± 0.25	46.84 ± 2.47	57.97 ± 2.40
	LD (n=16)	1.00 ± 0.06	1.71 ± 0.12	17.11 ± 1.01	18.30 ± 1.42
Batch 2	HD (n=6)	2.02 ± 0.15	5.25 ± 0.42	59.58 ± 3.87	60.04 ± 3.43
	LD (n=6)	0.49 ± 0.08	1.30 ± 0.26	20.28 ± 4.91	17.30 ± 4.02

Figure 1

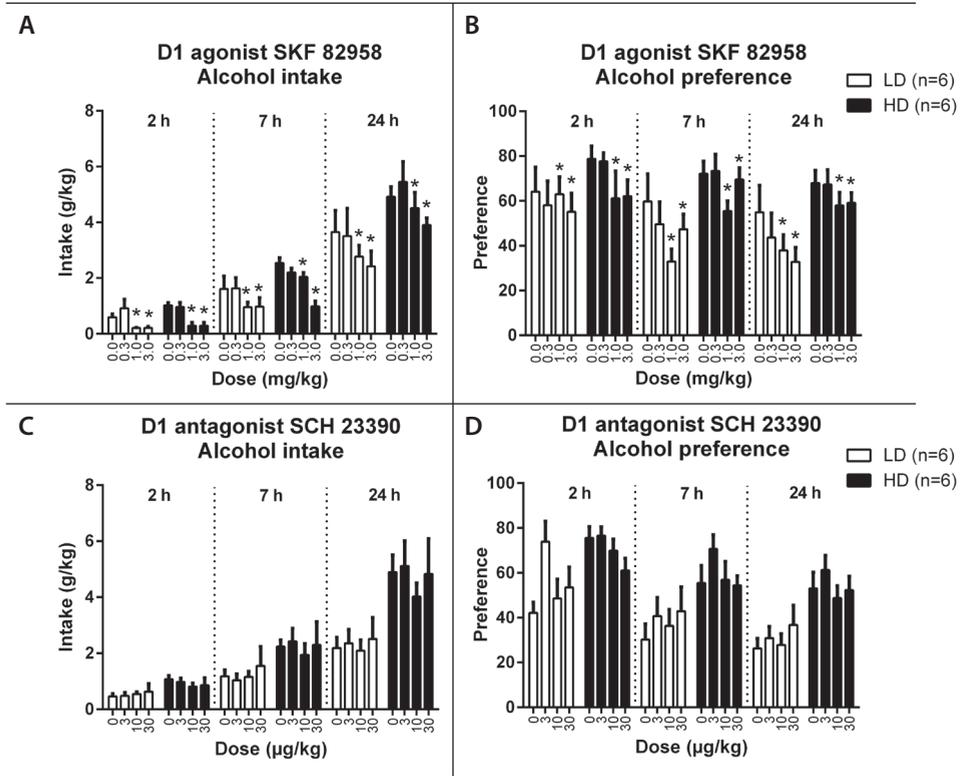


Figure 1. The effects of the dopamine D1 receptor agonist SKF 82958 and the dopamine D1 receptor antagonist SCH 23390 on alcohol intake and preference in HD and LD. SKF 82958 decreased alcohol intake and preference during the entire session to a similar extent in HD and LD (A-B). SCH 23390 did not alter alcohol intake (C). Alcohol preference was affected by SCH 23390 but *post hoc* analyses did not reveal significant differences from vehicle for any of the doses tested (D). Data are presented as the mean + SEM. The effect of SKF 82958 did not interact with the session time. Therefore, the * reflects differences from vehicle in post hoc pairwise comparisons ($p < 0.05$) of the overall analysis.

Figure 2

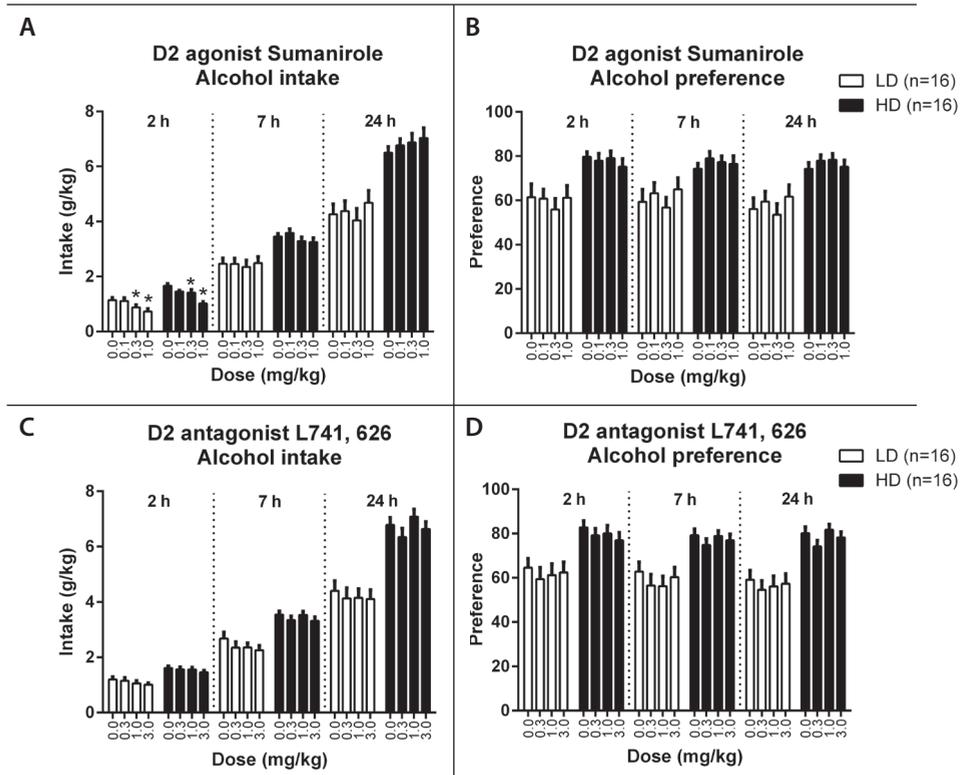


Figure 2. The effects of the dopamine D2 receptor agonist sumanirole and the dopamine D2 receptor antagonist L741,626 on alcohol intake and preference in HD and LD. Sumanriole decreased alcohol intake after 2h of alcohol exposure in both groups, without affecting alcohol intake after 7h or 24h of alcohol exposure (A). Sumanriole had no effect on the preference for alcohol (B). L741,626 did not affect alcohol intake and preference (C-D). Data are presented as the mean + SEM. *Different from vehicle in *post hoc* pairwise comparisons ($p < 0.05$).

of the session ($F_{(3,90) \text{ dose } 2h} = 20.87, p < 0.001$) to a similar extent in LD and HD ($F_{(3,90) \text{ dose } \times \text{ group } 2h} = 1.68, n.s.$). *Post hoc* analyses showed that alcohol intake was reduced after treatment with 0.3 and 1.0 mg/kg sumanirole (Fig 2A). Alcohol intake was no longer affected by sumanirole after 7h of alcohol access ($F_{(3,90) \text{ dose } 7h} = 1.30, n.s.$; $F_{(3,90) \text{ dose } \times \text{ group } 7h} = 0.92, n.s.$). By contrast, analyses of the entire 24h showed a trend towards an increase in alcohol intake ($F_{(3,90) \text{ dose } 24h} = 2.39, p = 0.074$), independent of group ($F_{(3,90) \text{ dose } \times \text{ group } 24h} = 0.95, n.s.$) (Fig 2A). Analysis of the alcohol consumption data between 2h-24h after session onset confirmed that alcohol intake was increased during

Figure 3

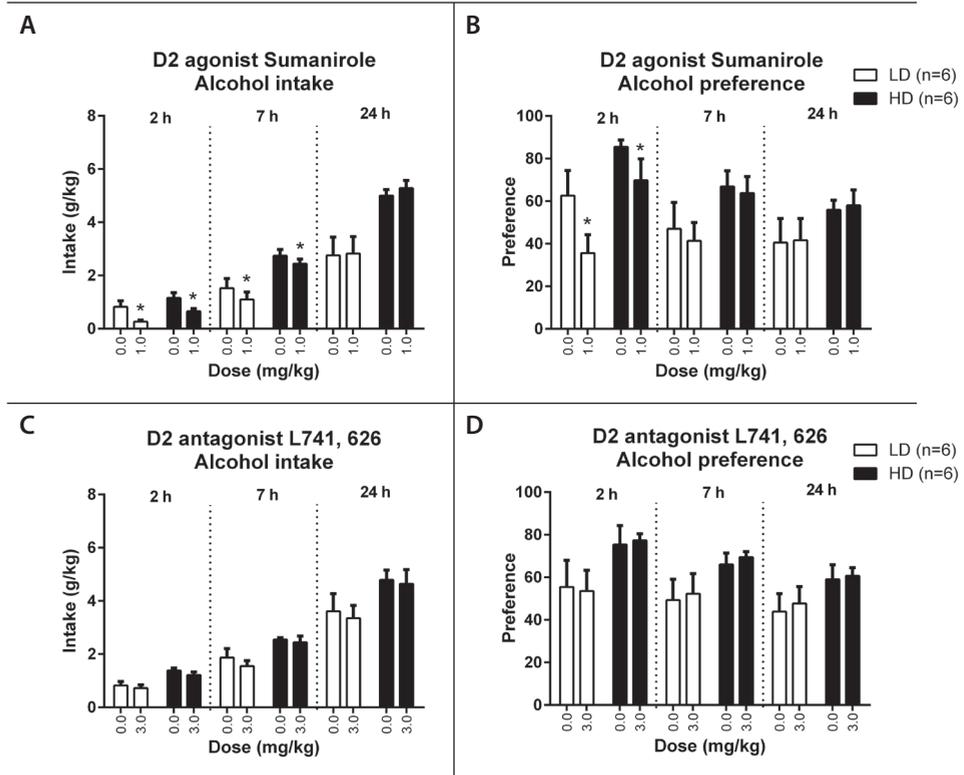


Figure 3. Replication of the effects of the highest dose of the dopamine D2 receptor agonist sumanirole and the dopamine D2 receptor antagonist L741,626 on alcohol intake and preference in HD and LD. Sumanriole decreased alcohol intake in both groups after 2h and 7h of alcohol exposure, but was without effect after 24h of alcohol exposure (A). Sumanriole decreased the preference for alcohol after 2h of alcohol exposure but had no effects after 7h and 24h of alcohol exposure (B). L741,626 did not affect alcohol intake and preference (C-D). Data are presented as the mean + SEM. *Different from vehicle in *post hoc* pairwise comparisons ($p < 0.05$).

the last 22h of the session ($F_{(3,90) \text{ dose } 2\text{h-24h}} = 12.16, p < 0.001$) in both groups ($F_{(3,90) \text{ dose} \times \text{group } 2\text{h-24h}} = 0.99, \text{ n.s.}$) (data not shown).

The effects of sumanirole on alcohol intake were replicated in the second batch of animals (Fig 3A-B), again revealing session time dependent effects ($F_{(2,20) \text{ time} \times \text{dose}} = 6.80, p < 0.007$), independent of group ($F_{(2,20) \text{ time} \times \text{dose} \times \text{group}} = 0.07, \text{ n.s.}$). Subsequent analyses indicated that sumanirole decreased alcohol intake after 2h and 7h ($F_{(1,10) \text{ dose } 2\text{h}} = 13.03, p < 0.006$; $F_{(1,10) \text{ dose } 7\text{h}} = 7.38, p < 0.03$) in both LD and HD ($F_{(1,10) \text{ dose} \times \text{group } 2\text{h}} = 0.05, \text{ n.s.}$; $F_{(1,10) \text{ dose} \times \text{group } 7\text{h}} = 0.21, \text{ n.s.}$), without

affecting alcohol intake over the full 24h of the session ($F_{(1,10) \text{ dose } 24h} = 1.09$, n.s.; $F_{(1,10) \text{ dose} \times \text{group } 24h} = 0.41$, n.s.) (Fig 3A-B). Interestingly, alcohol intake increased between 2-24h of exposure to alcohol ($F_{(1,10) \text{ dose } 2-24h} = 10.96$, $p < 0.009$) in both groups ($F_{(1,10) \text{ dose} \times \text{group } 2-24h} = 0.13$, n.s.), similar to the results from the initial experiment (data not shown).

Sumanriole did not affect alcohol preference in the first batch ($F_{(3,87) \text{ dose}} = 0.88$, n.s.; $F_{(4,119) \text{ time} \times \text{dose}} = 1.81$, n.s., $F_{(4,119) \text{ time} \times \text{dose} \times \text{group}} = 0.10$, n.s.) (Fig. 2B), but did alter alcohol preference in the second batch ($F_{(1,10) \text{ dose}} = 5.75$, $p < 0.04$), independent of group ($F_{(1,10) \text{ dose} \times \text{group}} = 0.53$, n.s.). The effect of sumanirole on alcohol preference in the second batch was dependent on the time in the session ($F_{(1,15) \text{ time} \times \text{dose}} = 9.53$, $p < 0.005$), but was independent of group ($F_{(1,15) \text{ time} \times \text{dose} \times \text{group}} = 0.51$, n.s.). Subsequent analyses in the second batch revealed that sumanirole decreased preference for alcohol after 2h ($F_{(1,10) \text{ dose } 2h} = 11.52$, $p < 0.008$) but had no effects after 7h ($F_{(1,10) \text{ dose } 7h} = 1.21$, n.s.) and 24h of alcohol exposure ($F_{(1,10) \text{ dose } 24h} = 0.30$, n.s.), independent of group (2h: $F_{(1,10) \text{ dose} \times \text{group } 2h} = 0.79$, n.s.; 7h: $F_{(1,10) \text{ dose} \times \text{group } 7h} = 0.10$, n.s.; 24h: $F_{(1,10) \text{ dose} \times \text{group } 24h} = 0.03$, n.s.) (Fig. 3B).

Dopamine D2 receptor antagonist – L741,626

There was a trend for an effect of L741,626 on alcohol intake ($F_{(3,90) \text{ dose}} = 2.63$, $p = 0.055$), independent of the time in the session ($F_{(4,124) \text{ time} \times \text{dose}} = 1.85$, n.s.) or the group ($F_{(4,124) \text{ time} \times \text{dose} \times \text{group}} = 1.04$, n.s.) (Fig. 2C). L741,626 did not affect alcohol intake in the second batch ($F_{(1,10) \text{ dose}} = 1.38$, n.s.; $F_{(1,15) \text{ time} \times \text{dose}} = 0.05$, n.s.; $F_{(1,15) \text{ time} \times \text{dose} \times \text{group}} = 0.13$, n.s.) (Fig. 3C). L741,626 did not influence the rats' preference for alcohol in the first ($F_{(3,90) \text{ dose}} = 1.58$, n.s.; $F_{(5,141) \text{ time} \times \text{dose}} = 0.56$, n.s., $F_{(5,141) \text{ time} \times \text{dose} \times \text{group}} = 0.52$, n.s.) (Fig. 2D) or the second batch ($F_{(1,9) \text{ dose}} = 0.69$, n.s.; $F_{(2,18) \text{ time} \times \text{dose}} = 0.25$, n.s.; $F_{(2,18) \text{ time} \times \text{dose} \times \text{group}} = 0.11$, n.s.) (Fig. 3D).

DISCUSSION

In the present study, treatment with the dopamine D1 receptor agonist SKF 82958, reduced alcohol intake and preference. Treatment with the dopamine D2 receptor agonist sumanirole, induced a transient reduction, followed by an increase in alcohol intake. By contrast, the dopamine D1 and D2 receptor antagonists, SCH 23390 and L741,626, did not alter alcohol consumption. Interestingly, the effects of the dopamine D1 and D2 receptor agonists were similar in LD and HD, suggesting that individual variation in alcohol consumption does not involve altered dopamine signaling.

The reductions in voluntary alcohol consumption upon treatment with dopamine D1 and D2 receptor agonists are in agreement with previous studies (Linseman 1990; Dyr et al., 1993; George et al., 1995; Silvestre et al., 1996), despite differences in experimental procedures (e.g. continuous alcohol access, sweetened vs unsweetened alcohol, different alcohol concentrations, food restriction procedures, inclusion criteria, species and strain). Interestingly, the current study, as well as previous reports show that dopamine D1 receptor agonists are more powerful in reducing alcohol intake than dopamine D2 receptor agonists (Linseman 1990; Ng and George 1994; Silvestre et al., 1996; El-Ghundi et al., 1998). After dopamine D1 receptor stimulation using SKF 82958, alcohol intake and preference was reduced throughout the session. In contrast, the selective dopamine D2 receptor agonist sumanirole mainly reduced alcohol intake during the first phase of the alcohol consumption session, and concurrently reduced preference for alcohol during the first 2h of the session. Importantly, upon the initial decrement in alcohol intake after sumanirole administration, an increase in alcohol intake was apparent for the remainder of the session. The initial decrease in alcohol intake, followed by a subsequent rise in alcohol intake after treatment with sumanirole suggests a rebound effect after the initial suppression of alcohol intake. Importantly, however, a similar increment in alcohol intake did not occur upon SKF 82958 treatment, indicating that an initial decrease in alcohol intake is not necessarily followed by a rebound increase in alcohol intake. Together, these data indicate that dopamine D1 and D2 receptors play different roles in the modulation of alcohol drinking, whereby dopamine D1 receptor stimulation evokes a clear-cut reduction in alcohol intake and preference.

The dopamine D1 and D2 receptor antagonists SCH 23390 and L741,626 did not alter alcohol intake and preference. These findings are in agreement with the lack of effect of dopamine D1 and D2 receptor antagonists on voluntary alcohol consumption reported previously (Brown et al., 1982; Goodwin et al., 1996; Silvestre et al., 1996). However, decreases in voluntary alcohol consumption upon treatment with either dopamine D1 and D2 receptor antagonists have been reported as well by several studies (Pfeffer and Samson 1986; Dyr et al., 1993; Panocka et al., 1995; El-Ghundi et al., 1998; Bulwa et al., 2011; Sabino et al., 2013), while only one study reported an increase in alcohol consumption (Dyr et al., 1993). Importantly, the doses that reduced alcohol consumption often also decreased water intake, reflecting either a non-specific suppression of fluid intake or a more general impairment in motor

activity (Linseman 1990; Hubbell et al., 1991; Dyr et al., 1993). In any event, the lack of an effect of dopamine receptor antagonists on alcohol consumption suggests that endogenous dopamine does not play a primary role in alcohol consumption, at least not under IAA conditions.

Comparable dopamine receptor drug treatments have been performed in the context of operant alcohol self-administration, which are important to consider because it has been suggested that dopamine is especially involved in tasks requiring effort (Salamone and Correa 2012). Indeed, it has been observed that the dopamine D2 receptor antagonist reduced responding for alcohol, but not its actual consumption (Czachowski et al., 2001, 2002; Samson and Chappell 2004). Interestingly, both dopamine D1 and D2 receptor agonists and antagonists have been shown to reduce operant responding for alcohol (Pfeffer and Samson 1988; Rassnick et al., 1993; Files et al., 1998; Cohen et al., 1999; Czachowski et al., 2002; Samson and Chappell 2004), while increasing responding for water (Weiss et al., 1990). An explanation for the reduction in operant self-administration of alcohol upon treatment with both dopamine receptor agonists and antagonists may be that dopamine receptor agonists substitute for the reinforcing effects of alcohol (Hodge et al., 1993; Samson and Chappell 1999), whereas dopamine receptor antagonist, may attenuate the reinforcing properties of alcohol (Imperato et al., 1987; Imperato and Di Chiara 1988; See et al., 1991; Santiago et al., 1993), all resulting in reduced alcohol consumption levels. Taken together with the consumption studies, these findings suggest that both dopamine D1 and D2 receptors are important for the regulation of alcohol intake, but especially so when an effort is required to obtain alcohol.

Individual susceptibility to AUD has been related to dopamine receptor deficiency and an altered dopaminergic response to alcohol. Previous preclinical studies showed that alcohol preferring rodents have reduced levels of dopamine in the terminal regions of the mesolimbic dopamine system (Murphy et al., 1987; Gongwer et al., 1989; McBride et al., 1990; George et al., 1995), which led to the hypothesis that their response to dopamine D1 or D2 receptor stimulation or inhibition might be altered. Interestingly, both humans at risk for AUD and rats bred or selected for high alcohol intake, respond to alcohol exposure with greater increases in extracellular dopamine levels (Weiss et al., 1993; Katner and Weiss 2001; Doyon et al., 2005; Bustamante et al., 2008; Setiawan et al., 2014). Moreover, although treatment with the dopamine D2 receptor antagonist in

AUD patients and social drinkers has been shown to reduce alcohol craving and to increase control over alcohol intake (Borg 1983; Modell et al., 1993; Peters and Faulds 1994; Enggasser and de Wit 2001; Martinotti et al., 2010), dopaminergic treatments do not affect all individuals (Modell et al., 1993; Enggasser and de Wit 2001; Walter et al., 2001; Tupala and Tiihonen 2004, 2005; Barrett et al., 2008). To assess the potential involvement of dopamine signaling in individual differences in alcohol intake, we classified outbred rats as LD and HD based on their alcohol consumption. As reported previously (Spoelder et al., 2015), we observed large differences in alcohol intake and preference between groups which were most pronounced during the 24h measurement. We observed that the dopamine D1 and D2 receptor agonists SKF 82958 and sumanirole reduced alcohol intake to a similar extent in LD and HD. These findings are consistent with previous reports, that showed that treatment with dopamine D1 and D2 receptor agonists and antagonists in alcohol preferring rodents led to similar results on voluntary alcohol consumption as observed in outbred cohorts (Weiss et al., 1990; Dyr et al., 1993; George et al., 1995; Panocka et al., 1995; Goodwin et al., 1996; Sabino et al., 2013). Together, the current and previous findings suggest that individual differences in voluntary alcohol intake are not related to altered dopaminergic signaling.

To conclude, both the dopamine D1 and D2 receptor agonists affect voluntary alcohol consumption, although the reduction in alcohol intake and alcohol preference was most pronounced after activation of dopamine D1 receptors. The dopamine receptor antagonists did not alter alcohol intake and alcohol preference, suggesting that endogenous dopamine is not essential for alcohol consumption under IAA conditions. Moreover, the comparable effects of dopamine D1 and D2 receptor agonists in LD and HD, suggest that the individual level of alcohol intake is not related to differences in dopamine signaling. Taken together, these data increase our knowledge on the modulatory role of dopamine in alcohol intake. Stimulation of dopamine D1 receptors may aid in the treatment of AUD.

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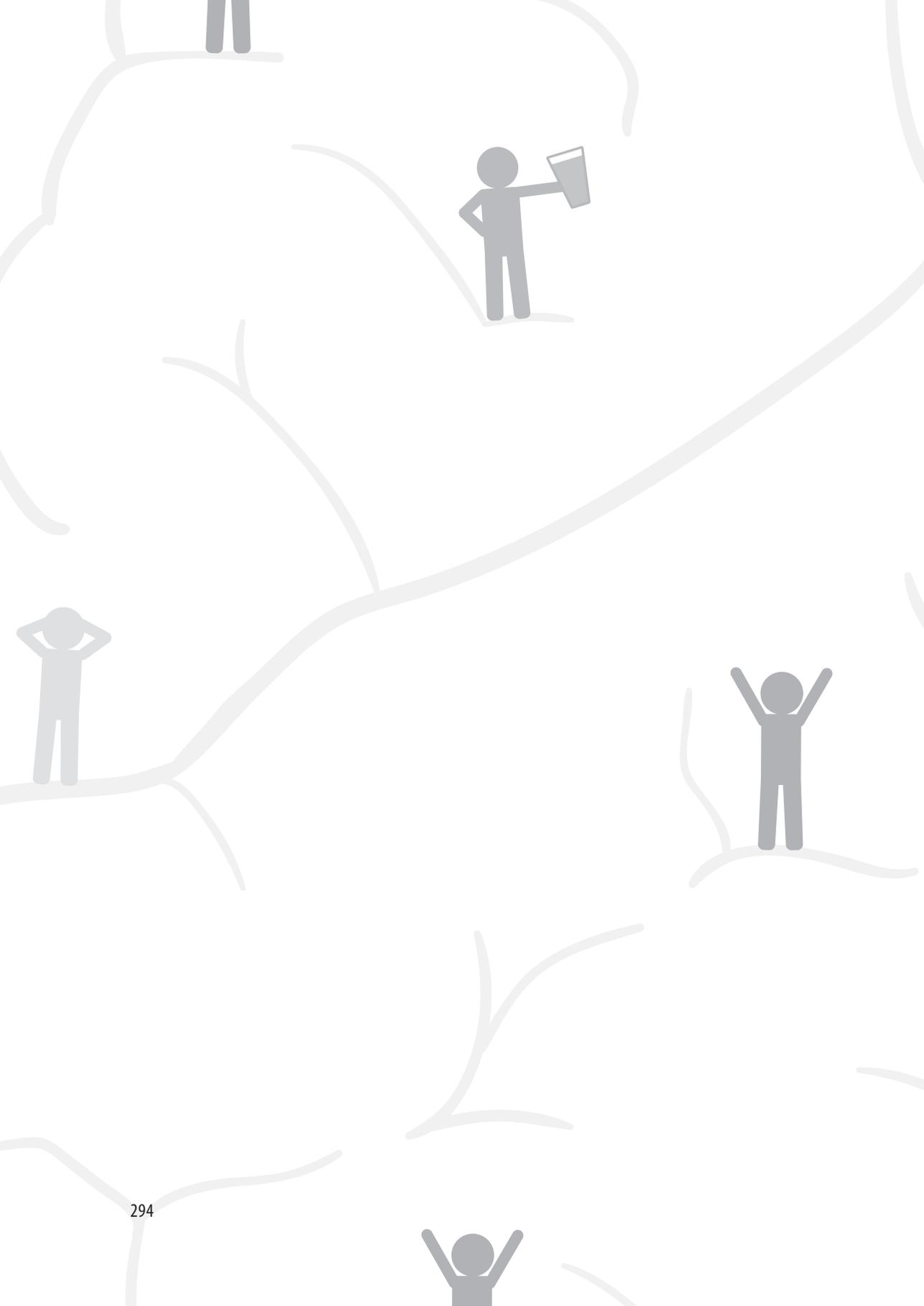
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CHAPTER 10

GENERAL DISCUSSION

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SUMMARY OF THE FINDINGS

Alcohol use disorder (AUD) is a chronic and relapsing brain disorder which is the result of a complex interaction between personality characteristics, the environment of an individual and alcohol-induced neurobiological changes. There is a substantial individual variability in the risk to develop AUD. Till today it is still unclear why some individuals are more vulnerable to develop AUD than others. In the present thesis, we addressed this topic using an animal model of individual variation in voluntary alcohol consumption. We related individual differences in alcohol consumption to alcohol reinforcement, loss of control over alcohol use, impaired social behaviour during adolescence, incentive learning processes, decision making and impulsive behaviour. In addition, we investigated the role of the mesolimbic dopamine system in alcohol consumption, alcohol reinforcement and incentive learning processes upon adolescent alcohol exposure. The results in this thesis contribute to the understanding of the underlying behavioural and neurochemical factors associated with AUD. Therefore, the present findings may aid the development of improved, personalized treatments for AUD.

Chronic excessive alcohol use and loss of control over alcohol use are key characteristics of AUD. In order to understand the individual vulnerability for AUD, models that capture both the individual variability in alcohol consumption and the transition from casual to compulsive alcohol use are therefore essential. In **Chapters 2 and 3**, we investigated the relationship between individual differences in voluntary alcohol intake with the progression of excessive alcohol use, the motivation to obtain alcohol and the persistence of alcohol seeking despite negative consequences. In **Chapter 2**, using male Lister hooded rats, we found marked individual differences in alcohol consumption and preference under both intermittent (IAA) and continuous alcohol access (CAA) conditions, whereby subgroups of high and low alcohol drinking rats (HD; LD) could be discerned. Under IAA conditions, individual differences between rats were more pronounced. Moreover, HD showed a profound increase in alcohol intake over time and even more so upon extension of the alcohol access duration. In addition, we observed that HD were more motivated to obtain alcohol. Finally, HD showed continued intake of alcohol despite adulteration of the alcohol solution with quinine, tolerating higher quinine concentrations than LD. In **Chapter 3**, we used the conditioned suppression model to investigate loss of control over alcohol

seeking by confronting the rats with a conditioned aversive stimulus during alcohol seeking. Conditioned suppression of alcohol seeking was reduced after an extended period of IAA. Moreover, HD showed reduced conditioned suppression of alcohol seeking compared to LD. These findings show that the development of loss of control over alcohol seeking, a key characteristic of AUD in humans, is dependent on both the extent of alcohol exposure and on the individual's propensity to consume alcohol.

Suboptimal decision making and exaggerated impulsivity are thought to have a bidirectional relationship with AUD. In **Chapters 4 and 5**, we aimed to gain more insight into this relationship. In **Chapter 4**, we found that repeated alcohol treatment during acquisition of the rodent gambling task (rGT) increased risky choice behaviour in a rGT version that entailed long punishment delays. Moreover, repeated alcohol treatment during task acquisition reduced the ability to adjust choice behaviour on the basis of feedback. Interestingly, alcohol pre-treated rats showed a behavioural disinhibition upon subsequent alcohol challenges. In **Chapter 5**, we report that HD showed more efficient choice behaviour in the rat gambling task (rGT) and the delayed reward task (DRT) than LD. At the same time, HD showed an increase in cue-driven behaviour which was reflected by greater motor impulsivity and enhanced sign-tracking behaviour. Importantly, no differences in incentive learning processes between LD and HD were apparent prior to alcohol consumption. In addition, acute alcohol challenges affected choice behaviour in the rGT and DRT equally in both subgroups. Taken together, these findings suggest that, while involuntary repeated alcohol treatment impaired decision making, voluntary high alcohol drinking individuals show more optimal decision making.

Adolescence is thought to represent a period of increased risk for AUD. It has been reported that disruptions in early social behaviour are associated with an increased risk for AUD (Bardo et al., 2013; Varlinskaya et al., 2015). In **Chapter 6**, we observed that early social isolation, effectively depriving rats from social play, increases alcohol consumption in adulthood independent of the individual levels of alcohol consumption. However, operant responding for alcohol was not altered. This suggests that socially vulnerable individuals are particularly at risk for enhanced alcohol consumption under free consummatory conditions, but do not show altered appetitive or incentive motivation to obtain alcohol. In **Chapter 7**, we observed that adolescent

alcohol exposure biases individuals toward a sign-tracking conditioned response during Pavlovian conditioning and potentiates stimulus-evoked phasic dopamine transmission. In addition, adolescent alcohol-exposed rats showed larger differences in phasic dopamine transmission to unexpected variation in reward outcomes, compared to control subjects. In **Chapters 8 and 9**, we further investigated the role of the dopaminergic system in relation to alcohol reinforcement and consumption. The results in **Chapter 8** suggest that dopamine in the NAcc shell and DLS play a major role in the motivational aspects of obtaining alcohol, whereas NAcc core dopamine plays a more general role in alcohol reinforcement. Together, these findings highlight the functional heterogeneity of striatal dopamine in the context of alcohol-directed behaviour. Finally, in **Chapter 9**, we report that treatment with a dopamine D1 receptor agonist decreased alcohol intake whereas a dopamine D2 receptor agonist initially decreased alcohol intake and subsequently increased alcohol intake, independent of the individual level of alcohol consumption. Dopamine D1 and D2 receptor antagonist did not alter alcohol consumption. These findings suggest that the difference in alcohol consumption between LD and HD does not involve altered dopamine signaling.

WHY DO HIGH DRINKERS CONSUME MORE ALCOHOL?

One of the main findings of this thesis is that male Lister hooded rats show marked individual variability in voluntary alcohol intake that predicts the development of measures of loss of control over alcohol seeking, a key characteristic of human AUD (**Chapters 2 and 3**). As such, this approach provides an important tool to assess the mechanisms that determine the development of AUD. We have found several factors that may explain the differences in alcohol consumption between LD and HD. For example, in **Chapter 5**, we showed that LD and HD differ in reward sensitivity and cue-driven behaviour. More specifically, after alcohol consumption, HD show improved cognitive performance in decision making tasks. Moreover, HD display more approach behaviour towards a reward-predicting cue after alcohol consumption, but not prior to alcohol consumption. Therefore, these effects are most likely induced by alcohol, rather than reflecting a pre-existing trait effect in HD. Indeed, in **Chapter 7** we showed that alcohol consumption during adolescence resulted in enhanced approach behaviour towards a reward-predicting cue, i.e. a sign-tracking conditioned response. Together,

these data suggest that high alcohol consumption may lead to enhanced reward sensitivity and cue-driven behaviour but that these may not be a pre-existing trait that predicts the development of AUD-like behaviour.

In **Chapters 4 and 5**, we observed that acute alcohol exposure caused subtle changes in decision making and impulsive choice, but that repeated alcohol administrations or a period of voluntary alcohol intake, did substantially affect decision making and impulsive choice. It has been shown that individuals with an impulsive phenotype are more likely to initiate substance use (Galvan et al., 2007; Verdejo-Garcia et al., 2008; de Wit 2009; Fernie et al., 2013). Moreover, these substances of abuse can enhance impulsivity and extended substance use may trigger compulsive characteristics of substance use, implying an indirect relationship between impulsivity and compulsive substance use (Perry and Carroll 2008). Interestingly, we observed that HD were *less* impulsive in the DRT. Moreover, in the rGT, HD showed increased an increased preference for the optimal option, thereby increasing their total gain in the task. Likewise, by preferring the large delayed reward, HD managed to obtain a larger gain in the DRT as well. Therefore, our data suggest that loss of control over alcohol use in rats that consume high amounts of alcohol is not related to enhanced choice impulsivity. Rather, HD might be more sensitive to obtain rewards in general, which explains their higher motivation to obtain alcohol as well as a higher motivation to maximize the gain in cognitive tasks. Importantly, we did not observe group differences in the preference for sweet solutions or in aversion for quinine adulterated water (**Chapter 2**). This is in contrast to previous reports, showing that more concentrated sweet solutions are preferred in AUD patients as well as in alcohol-preferring rats and mice (Sinclair et al., 1992; Kampov-Polevoy et al., 1997, 1999). Interestingly, a previous study showed that alcohol-preferring rats have a tendency to consume sucrose and saccharin solutions far beyond the limits of their normal fluid intake in comparison to their non-alcohol-preferring counterparts (Overstreet et al., 1997). In subsequent studies, it will be of particular interest to assess whether HD show also a higher motivation to obtain sucrose and a loss of control over sucrose intake.

Previous studies have suggested a strong relationship between conditioned sign-tracking behaviour and substance abuse liability (Tomie and Sharma 2013; Yager and Robinson 2013; Yager et al., 2014). It is well known that alcohol-associated cues contribute to alcohol seeking and consumption (Krank

1989; Katner et al., 1999; Liu and Weiss 2002; Nie and Janak 2003; Palfai 2006; Christiansen et al., 2012) and that they produce sign-tracking behaviour (Krank 2003). Moreover, it has been shown that alcohol by itself augments the sign-tracking conditioned response (Tomie et al., 1998). In addition, over time, the alcohol-predicting cues can develop into conditioned reinforcers, indicating that the cues themselves become rewarding (Saunders and Robinson 2013). In **Chapters 2 and 3**, we observed that HD show a greater motivation to lever press to obtain alcohol compared to LD and that HD are more resistant to conditioned suppression of alcohol seeking. If HD are more attracted by the reward predictive lever, as the Pavlovian conditioned approach data suggest (**Chapter 5**), this likely also affects the number of lever presses they make to obtain alcohol. Thus, enhanced responding for alcohol may be due to enhanced conditioned responses directed at the reward predicting lever (Rescorla and Solomon 1967). Although a sign-tracking phenotype may not be directly related to loss of control over alcohol use, sign-tracking may result in a higher alcohol consumption (Christiansen et al., 2012), which may consequently lead to loss of control over alcohol use. Moreover, it is conceivable that HD are resistant to conditioned suppression because they are more attracted by the reward predicting lever, despite the presentation of an aversive tone (**Chapter 3**). To investigate whether a reward-predicting stimulus alters responding for alcohol differently in LD versus HD, it is of interest to compare LD and HD in a Pavlovian to instrumental transfer (PIT) task (Balleine 1994). PIT can be used to determine whether the alcohol-predicting lever or the presentation of another stimulus, not associated with alcohol, increases responding for alcohol (Kruse et al., 1983; Colwill and Rescorla 1988; Corbit et al., 2001; Corbit and Balleine 2005). It has, for example, been reported that detoxified AUD patients perform instrumental actions for access to alcohol at a higher rate when presented with alcohol-associated cues (Ludwig et al., 1974). Interestingly, the alcohol-paired stimulus was found to have a general excitatory effect on reward-seeking behaviour, affecting both alcohol-directed and sucrose-directed responding equally (Glasner et al., 2005; Corbit and Janak 2007). Another study found comparable results and showed that heavy alcohol drinkers performed a strong automatic response tendency towards alcohol-related cues and other appetitive stimuli, but not upon general positive or negative stimuli (Houben and Wiers 2009). Together, these results suggest that the increase in response tendencies upon the presentation of an appetitive stimulus, does not depend upon the relationship with alcohol specifically, but to reward processes in general. Therefore, the consumption of

high levels of alcohol and the development of AUD may be related to enhanced responsiveness to appetitive stimuli in general.

In **Chapter 7**, we investigated the effect of adolescent alcohol exposure on sign-tracking behaviour in the Pavlovian conditioned approach task during adulthood. During the alcohol (or control) exposure period, the rats were housed individually. We observed that the alcohol-exposed rats showed more sign-tracking behaviour compared to rats in the control condition. Because we observed that social isolation during adolescence increased alcohol consumption (**Chapter 6**), it is an interesting question whether or not housing conditions have impacted our findings above and beyond the effect of alcohol on Pavlovian conditioned approach behaviour. For the studies in **Chapter 7**, the rats arrived at an age of 27 days and were individually housed for the entire experiment. For the experiments described in **Chapter 6**, the rats arrived at an age of 14 days, together with a nursing mother, were socially isolated during 21-43 days of age, were thereafter housed in pairs for 4 weeks and subsequently individually housed for the remainder of the experiment. Although the housing conditions were different in **Chapters 6 and 7**, the rats were exposed to a period of social isolation during adolescence. Interestingly, the effects of intra-gastric alcohol administration during adolescence on Pavlovian conditioned approach behaviour in adulthood were investigated in rats that were pair-housed in a previous study. In line with our findings, this study showed that adolescent alcohol exposure enhanced sign-tracking behaviour (McClory and Spear 2014). Moreover, Pavlovian conditioned approach behaviour was not different between adolescent rats that were pair- or individually housed but were not exposed to alcohol (Anderson et al., 2013). Therefore, it is likely that the increase in sign-tracking behaviour is the result of alcohol exposure rather than the housing conditions. Together, these results suggest that social isolation induces more alcohol consumption in adulthood, while adolescent alcohol exposure enhances sign-tracking behaviour which may in turn also affect alcohol consumption (see previous section).

Loss of control over use is a key characteristic of substance use disorder in humans, which has been captured in different animal models (Wolffgramm and Heyne 1991; Ahmed and Koob 1998; Wolffgramm et al., 2000; Deroche-Gamonet et al., 2004; Vanderschuren and Everitt 2004; Cooper et al., 2007; Pelloux et al., 2007; Hopf et al., 2010; Lesscher et al., 2010). Chronic and excessive alcohol use may provoke uncontrolled alcohol use. However, not all individuals develop

AUD and prolonged exposure to alcohol may not be sufficient to induce loss of control over alcohol use. We addressed this question and investigated whether (1) individual variation in alcohol intake predicts AUD-like behaviour and (2) prolonged alcohol use increases the propensity to develop loss of control over alcohol use. In **Chapter 2**, we showed that HD continued to consume alcohol despite quinine adulteration, where they tolerated higher quinine concentrations than their LD counterparts. This was determined after lengthy exposure to alcohol, i.e. 6-7 months IAA. These findings were comparable to a previous study which showed that individual rats which displayed continued intake of bitter-tasting alcohol solutions, had, in retrospect, consumed more alcohol compared to rats that showed flexible, quinine-sensitive alcohol intake after a period of 7-12 months CAA (Turyabahika-Thyen and Wolffgramm 2006). In **Chapter 3**, we observed that HD continued to seek alcohol despite the presentation of a tone which was previously associated with a mild electric footshock, while the LD showed conditioned suppression of alcohol seeking. Hence, with this method, we showed that only 2 months of IAA is sufficient to induce loss of control over alcohol seeking in individuals that are vulnerable to develop AUD-like behaviour. On a group level, other research groups report quinine- and footshock-resistant motivation after 3-4 months of alcohol consumption but not after 1.5 months of IAA (Wolffgramm and Heyne 1991; Hopf et al., 2010). Indeed, in **Chapter 3**, we show in a group of medium alcohol drinkers, that prolonged alcohol consumption reduced conditioned suppression of alcohol seeking, suggesting that a certain cumulative level of alcohol exposure is sufficient to induce quinine-resistant alcohol intake. Together, we conclude from these findings that individuals that consume high levels of alcohol are at increased risk for AUD, but that prolonged alcohol consumption may also put less vulnerable individuals at risk for AUD.

The studies in this thesis devoted to the assessment of the behavioural characterization of LD and HD showed consistent individual differences in alcohol intake and preference in male Lister hooded rats. We decided to select LD and HD based on each cohort separately by selecting the extremes of each cohort. During our studies we noted subtle differences in alcohol intake between cohorts (**Chapters 2 and 9**). What caused the differences between cohorts is speculative at the moment, but it may be due to differences in the offspring at the breeder, experimental design or seasonal differences. Considering the variation between cohorts of rats, we think that using the absolute levels of alcohol intake as a criterion to classify animals as LD or HD

would not be the best strategy. In that case, the distribution of LD and HD may be skewed in certain cohorts, compromising the statistical power of individual experiments. Rather, we chose to compare the behavioural characteristics between the most extreme animals within each cohort. The threshold to define the extremes was different across the studies described in this thesis. For example, in **Chapters 3 and 6**, the LD and HD were subdivided into two experimental groups (e.g. socially isolated group or control group) resulting in a lower number of rats in the group, and therefore the extreme 33% of the cohort were selected as LD and HD. In **Chapter 5**, we selected the 12.5% extremes of the cohorts as LD and HD, and observed that these subgroups differed in their decision making pattern and Pavlovian conditioned approach behaviour. However, comparisons between less extreme subgroups, e.g. 25%, reflected less robust group differences in this study. These findings suggest that different behavioural and environmental factors contribute to the alcohol consumption and AUD phenotype of the rats, but to a different degree. Apparently, decision making patterns contribute only to the most extreme variations in alcohol consumption while alcohol consumption patterns predict loss of control over alcohol seeking across a wider range of alcohol intake levels.

THE IMPORTANCE OF DOPAMINE IN AUD-RELATED BEHAVIOURS

The importance of dopamine in substance use disorders, including AUD, suggests that dopaminergic compounds may have potential in the treatment of AUD. It has been suggested that treatment with dopamine receptor antagonists may attenuate the rewarding value of alcohol, or alternatively, increase endogenous dopamine synthesis and release (Imperato et al., 1987; See et al., 1991; Santiago et al., 1993), thereby reducing the motivation to drink alcohol. Indeed, treatment with a dopamine D2 receptor antagonist has been shown to reduce craving for alcohol and to have positive effects on the control over alcohol intake in AUD patients (Borg 1983; Modell et al., 1993; Peters and Faulds 1994; Martinotti et al., 2010). However, contrasting findings of the dopamine D1/D2 receptor antagonist, flupenthixol, have also been reported, i.e. increased relapse rates in AUD patients (Wiesbeck et al., 2001). In **Chapter 9**, we investigated the effect of dopamine D1 and D2 receptor agonists and antagonists on voluntary alcohol and water consumption in the home cage in LD and HD. We observed that treatment with agonists,

but not antagonists, reduced alcohol consumption in both subgroups to a similar extent. The lack of an effect of dopamine D1 and D2 receptor antagonists on alcohol intake or preference suggests that endogenous dopamine is not directly required for voluntary alcohol consumption. In contrast, dopamine receptor antagonists primarily show a reduction in responding for alcohol (Pfeffer and Samson 1988; Files et al., 1998; Czachowski et al., 2002; Samson and Chappell 2004), as well as a reduction in the initiation and maintenance of alcohol seeking behaviour (Liu and Weiss 2002). These findings suggest that dopamine is important for the regulation of alcohol intake when an appetitive, operant response is required, but is less important during voluntary alcohol consumption. Indeed, in **Chapter 8**, we showed modulation of alcohol reinforcement upon treatment with the dopamine D1/D2 receptor antagonist, flupenthixol, which depended on the striatal sub-region as well as the reinforcement schedule. Since the response requirement under an fixed-ratio 1 schedule is minimal, responding under this schedule is thought to reflect consummatory aspects of self-administration, whereas progressive ratio schedules of reinforcement, because of their increasing response requirement, tax processes related to the incentive motivational properties of rewards (Katz 1990; Markou et al., 1993; Richardson and Roberts 1996; Arnold and Roberts 1997). We observed that dopamine in the NAcc shell and the DLS are especially important for the motivational aspects of alcohol reinforcement, whereas the NAcc core dopamine plays a more general role in alcohol reinforcement.

The observation that LD and HD showed a similar response to systemic treatment with dopamine receptor agonists and antagonists during intermittent alcohol access suggests that the subgroups do not differ in dopaminergic signaling. Considering the sub-region dependent effects of flupenthixol, however, it remains to be determined whether LD and HD differ in dopamine signaling within the striatal circuitry. Preliminary Western blot analyses, however, revealed no differences in dopamine D2 receptor expression in the NAcc core, shell and DLS between LD and HD (unpublished observations). Nevertheless, the possibility remains that LD and HD respond differently to systemic or intracerebral dopamine receptor agonists and antagonists in operant alcohol self-administration tests.

In **Chapter 5**, we observed that HD show an enhanced sign-tracking conditioned response, which is known to be paralleled with a phasic dopaminergic response in the NAcc core (Di Ciano et al., 2001; Flagel et

al., 2011; Saunders and Robinson 2012; Clark et al., 2013). Interestingly, it was shown that dopamine is necessary for the learning of a sign-tracking conditioned response, whereas it is not necessary for learning a goal-tracking conditioned response (Flagel et al., 2011). Moreover, alcohol consumption has been shown to alter phasic dopamine release (Grace 2000). It would therefore be interesting to compare phasic dopamine release in the NAcc core between LD and HD upon the presentation of reward- (preferably alcohol-) predictive cues. Indeed, we have shown in **Chapter 7** that rats exposed to alcohol during adolescence are characterized by augmented dopamine release during acquisition of Pavlovian conditioned approach behaviour. A similar pattern in behaviour and phasic dopamine release was observed upon re-exposure to the reward after several extinction sessions. Moreover, the alcohol-exposed rats showed a stronger dopaminergic response upon unexpected changes in reward size. The pattern of phasic dopamine signaling and the associated bias in learning observed after adolescent alcohol exposure provides a potential mechanism for the well-documented vulnerability of individuals with early-life alcohol use for the development of AUD in adulthood (Hingson et al., 2006; Dawson et al., 2008; Blomeyer et al., 2013). Taken together, dopamine may not be required for alcohol consumption, but it does contribute to alcohol reinforcement and Pavlovian conditioned approach behaviour. However, we have no evidence thus far that dopamine signaling underlies the individual variation in alcohol consumption observed in Lister Hooded rats.

TRANSLATIONAL AND CLINICAL IMPLICATIONS

The great advantages of using animal models are that 1) very specific aspects of a certain disease can be studied in isolation under controlled genetic and environmental influences, 2) the causality of associations between certain factors and the disease can be studied, and 3) the underlying neurobiological mechanisms can be investigated. However, a drawback may be that complex interactions that are inherent to the clinical cases of AUD cannot be modelled in animals, thereby compromising their translational value. Hence, a careful coordination between animal and human studies is essential to facilitate the clarification of the complex processes underlying AUD needed to generate clinical advances.

Translational implications

In the current thesis, we show individual differences in voluntary alcohol consumption which are related to a higher motivation to obtain alcohol and a loss of control over alcohol seeking. The escalation in alcohol consumption is an important characteristic of human AUD; it is one of the earliest signs in the development of substance use disorders (Uhart and Wand 2009; Koob and Volkow 2010). We, and others, have observed that IAA on a group level results in an escalation of alcohol intake across the first month of alcohol access (Simms et al., 2008; Loi et al., 2010; Hwa et al., 2011). Moreover, rats with IAA develop quinine resistance while rats with CAA do not (Hopf et al., 2010). In **Chapter 2** we observed that HD continued to consume alcohol despite an aversive taste. Importantly, it has been reported that AUD patients drink non-beverage alcohol despite the bad taste (Soo Hoo et al., 2003; Leon et al., 2007). Together, both the individual differences and the development of loss of control during IAA implicate translational value of these models to the human condition.

Several theories regarding the explanation of substance use disorder attribute a significant role of substance-associated stimuli in supporting compulsive substance use, craving and relapse (O'Brien et al., 1998; Carter and Tiffany 1999; Everitt and Robbins 2005). We observed enhanced sign-tracking conditioned approach behaviour towards reward-predicting stimuli after alcohol exposure, as well as in HD. Although speculative, these sign-tracking conditioned responses may relate to the observed 'attentional bias' for alcohol-related stimuli in AUD patients. Attentional bias has been described as the strength of automatic appetitive impulsive processes (Wiers et al., 2006, 2007, 2010; Thush et al., 2008). It remains to be further determined in future studies whether these concepts describe similar behaviour.

In order to correctly interpret and translate the results described in this thesis regarding the cognitive tasks and the Pavlovian conditioned approach task to the human situation, we would like to stress four important differences between human and animal models. First, during tasks that require attention and effort, such as decision making tasks, humans are generally rewarded with money whereas animals are often rewarded with palatable food. The most important difference between these two types of reward is that money is an abstract secondary reinforcer, whereas food is a primary reinforcer. Moreover, primary rewards are consumed immediately and satisfies the subject after each

trial of the task, whereas with the use of a secondary reward, the reward usually accumulates during the task and is given upon the completion of the task. Second, in order to increase the motivation of animals to perform a cognitive task for a reasonable amount of time that results in many food rewards, animals are generally food-restricted. Unfortunately, hunger or thirst affect the incentive value of rewards and reward-predicting cues and can therefore modulate the motivational value of the learned reward cues (Berridge 2001; Cardinal et al., 2002; Anderson et al., 2013). Moreover, rewarding a hungry animal with food, directly contributes to its homeostatic state and elicits a strong hedonic response upon receiving the food. Therefore, the use of food-restriction, but also differences in food-restriction protocols between labs may complicate the interpretation of the results. Importantly, it is unknown how the incentive value for food in food-restricted animals relates to the monetary rewards in humans. When we pre-fed the rats with sucrose prior to being tested on the rGT, i.e. devalued the sucrose reward, the rats performed the task sub-optimally (Fig. 1, unpublished observations). However, investigation of the effects of different food deprivation levels did not impact decision making in another rodent gambling task (Rivalan et al., 2009). Third, the way punishments is modelled in cognitive tasks differs between human and animal studies. For human decision making tasks, punishment is mainly provided by reducing the monetary budget, while in animals punishment is provided by delays, reward omissions, unpalatable tastes, air-puffs and shocks (de Visser et al., 2011). Importantly, in animal models, a punishment generally does not result in actual loss of reward, but merely loss of reward opportunity. Fourth, animals are typically trained for several weeks until they have established a stable choice pattern. In human studies, however, cognitive tasks are usually performed in one single session, both under uncertain, i.e. when the task contingencies are not fully known, and more certain conditions, i.e. when the choice contingencies become known to the participant. Hence, investigating the effects of alcohol in animals can either be performed by multiple treatments during task acquisition or after a stable choice pattern has been achieved. Hence, the repeated versus single alcohol administrations results in different effects on choice behaviour, which is exactly what we observed in **Chapter 4**. Moreover, it is likely that different types of memory processes are employed within a single session compared to a between-sessions paradigm.

The findings in the present thesis may extend well beyond the scope of human addiction. The fact that rats are willing to work to receive a reward

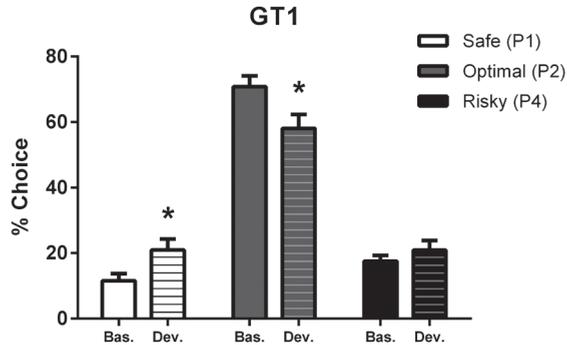
emphasizes the key role of the reward system in animals, which serves to meet fundamental behavioural needs across the animal kingdom. Reward sensitivity may reflect the internal state of an animal and therefore may also be considered an important indicator of an individual's emotional state or welfare status. In that respect, it is important to consider that high levels of anxiety predict the escalation of alcohol use (Hayton et al., 2012). However, we did not find consistent anxiety-like phenotypes in HD (unpublished observations).

Clinical implications

The compulsive drive to consume alcohol, defined by the persistence of alcohol use despite adverse consequences, represents a major challenge when attempting to treat AUD clinically (Tiffany and Conklin 2000; Sanchis-Segura and Spanagel 2006; Spanagel 2009; Koob and Volkow 2010). The substantial number of factors that contribute to the development of AUD results in a large clinical heterogeneity in terms of symptom dimensions and severity, complicating the development of treatment strategies. Therefore, it does not seem likely that a single strategy will be sufficient as a treatment for AUD. It is more likely that a combination of several strategies will potentially lead to a true cure rather than merely suppressing symptoms (Anton et al., 2006). Hence, an individualized approach, targeting specific aspects of the behaviour of a specific patient, will likely be most successful (Miller 2008). The results described in this thesis indicate that a subgroup of rats, the HD, showed escalated alcohol consumption over time as well as loss of control over alcohol seeking and consumption. In addition, HD showed a higher reward sensitivity and an increased motor impulsivity and sign-tracking conditioned response. However, we found no evidence for altered dopaminergic signaling in HD, while previous human studies with AUD patients showed clear alterations in the dopaminergic system (Volkow et al., 2011). This discrepancy may lie in the amount of alcohol consumed by HD in comparison to AUD patients. The question whether the alcohol intake of the HD subgroup is representative for the amount of alcohol consumed in AUD patients is very hard to answer, not least since alcohol metabolism differs markedly between rats and humans. Nevertheless, HD showed certain behavioural characteristics which reflect compulsive aspects of alcohol seeking. Therefore, these findings suggest that improved control over behaviour may reduce alcohol use. Moreover, it may be effective if AUD patients are trained not to focus on rewards or reward-predicting cues (Wiers et al., 2007).

Figure 1

A



B

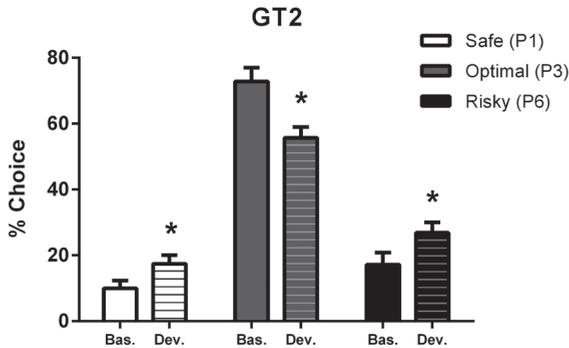


Figure 1. Effect of sucrose pre-feeding on choice behaviour in the rGT. Bas. = baseline; Dev. = devalued (i.e. sucrose pre-fed). * $p < 0.05$, Student's t-test.

It has been reported that only 20-30% of treated AUD patients respond to the current pharmacotherapies (Spanagel 2009). Pharmacotherapies to treat AUD are currently limited in number and efficacy (Heilig and Egli 2006; Johnson 2008; O'Brien 2008; Spanagel and Kiefer 2008; Koob et al., 2009; Spanagel 2009; Pierce et al., 2012; van den Brink 2012). Acamprosate (calcium acetyl homotaurinate), naltrexone (an opiate receptor antagonist) and disulfiram (better known as Antabuse) are the three pharmacotherapies which are currently approved clinically to treat the symptoms of craving for alcohol (Bouza et al., 2004; Johnson 2008). The therapeutic effects of acamprosate appear to be related to the glutamatergic system, although homotaurine itself is a GABA receptor agonist (Boismare et al., 1984; Dahchour and De Witte 2000;

De Witte et al., 2005; Heilig and Egli 2006; Mann et al., 2008). Disulfiram blocks aldehyde dehydrogenase resulting in the accumulation of acetaldehyde after alcohol consumption which produces several aversive symptoms like nausea and headache. Hence, disulfiram acts more as a punishment upon alcohol ingestion and the drug has been questioned for its safety and effectiveness (Heilig and Egli 2006). In animal studies it has been shown that naltrexone and acamprosate reduce alcohol self-administration (Boismare et al., 1984; Ulm et al., 1995; Czachowski and Delory 2009; Spanagel 2009; Sabino et al., 2013). The fact that these animal models show comparable effects supports their predictive validity, and therefore the discovery of a new putative compound provides a good rationale for further translational studies and randomized controlled trials. In future studies, it might therefore be an interesting question to study if the effects of naltrexone and acamprosate have different effects on voluntary alcohol consumption and alcohol seeking behaviour in HD compared to LD. The identification of several new very promising compounds discovered by animal studies has been reviewed elsewhere (Spanagel 2009). Some of them are currently being developed by pharmaceutical companies or have already passed Phase 1 of clinical testing.

Over the last decades, many studies have focused on the mesolimbic dopamine system in relation to substance use disorders. However, dopaminergic compounds are not clinically used to treat AUD. In **Chapter 9** we observed comparable findings of dopamine receptor agonists in LD and HD, suggesting that the modulation of alcohol consumption by dopamine receptor agonists is independent of individual alcohol consumption levels. On the other hand, tasks that require an operational response do show differences in the effects of dopamine receptor antagonists on alcohol seeking between rats which received a different degree of alcohol exposure (Liu and Weiss 2002). In a recent meta-analysis, it was observed that patients with AUD or heavy alcohol users show a striatal hyper-activation when reward-predicting cues are present and a hypo-activation when reward-predicting cues are absent (Leyton and Vezina 2013). Because dopamine is a major modulator of signal transduction in the striatum, these findings may indicate that dopaminergic signaling in AUD patients is especially important under the influence of reward-predicting cues. Interestingly, the effectiveness of dopamine D2 receptor antagonists in reducing the attentional bias for substance-related cues has been reported for other substances of abuse (Franken et al., 2004), while dopamine receptor agonists increase attentional bias (Ersche et al.,

2010). Moreover, partial dopamine receptor agonists have been suggested as a putative pharmacotherapy in AUD (Tupala and Tiihonen 2004) because these compounds could help to restore the suboptimal levels of dopaminergic activity by reducing both the euphoria subsequent to the release of dopamine by alcohol and by reducing craving.

The recent advances in the neurobiological and psychological mechanisms underlying AUD has led to several new concepts of behavioural treatments (Everitt and Robbins 2015). First, because of the comorbidity of impulsive control disorders with AUD, it may be that the approved medications to treat these disorders are also effective in the treatment of AUD (Everitt 2014, Broos et al., 2015). Second, the influence of conditioned alcohol-related stimuli may be reduced via behavioural treatment in which repeated pairings of the alcohol-related stimuli is not followed by the consumption of alcohol; hence extinguishing the association. Moreover, according to the ‘Sign-Tracking Model’ suggested by Tomie and others, the negative association will lower the likelihood that the sign-tracking conditioned response directed at alcohol-related stimuli will be elicited, improving the control over alcohol consumption (Tomie and Sharma 2013). This so-called ‘cue exposure treatment’ has shown to be context-independent for patients with an AUD, i.e. the extinction learning performed in the context of the laboratory continued to be effective even when the patient is confronted with the alcohol-related stimuli in its own context (Monti et al., 1999; Stasiewicz et al., 2007; MacKillop and Lisman 2008; Tomie and Sharma 2013). Indeed, interventions aimed to restore the balance between impulsive and reflective processes by attentional re-training (Wiers et al., 2006) or through approach-bias re-training have shown to be effective (Wiers et al., 2010, 2011, 2013; Eberl et al., 2013). A third approach relates to memory reconsolidation. During this process, the presentation of an alcohol-related stimulus would reactivate a certain memory and causes the memory to become labile and therefore may be disrupted or erased (Lewis 1979; Nader et al., 2000; Milton and Everitt 2010). Promising results from studies in healthy volunteers and patients suffering from post-traumatic stress disorder suggest that the oral administration of the beta-adrenergic receptor antagonist propranolol before or (directly) after the memory reactivation reduced the behavioural expression of the fear memory (Brunet et al., 2008; Kindt et al., 2009). In a study using heroin addicts, the retrieval of drug-associated memories 10 minutes before extinction training attenuated cue-induced heroin craving 1, 30 and even 180 days later, indicating that a memory

retrieval-extinction procedure might be a promising non-pharmacological treatment option (Xue et al., 2012). Hence, these studies indicated the feasibility of the reconsolidation disruption approach, and should certainly be further investigated. Finally, the existing therapies to treat AUD are mainly directed at reducing reward or craving (van den Brink 2012), but are not aimed at restoring control over alcohol seeking. We report individual differences in alcohol consumption, that predict the development of loss of control over alcohol seeking, which is a key characteristic of AUD. This has important implications for future studies, directed at unravelling the neurobiological mechanisms that underlie the development of AUD. Indeed, recent studies have started to show the contribution of the prefrontal cortex and the dorsal striatum to aversion resistance that characterizes substance use disorders (Jonkman et al., 2012; Chen et al., 2013; Seif et al., 2013, 2015; Limpens et al., 2014).

CONCLUDING REMARKS

In the present thesis, we report individual differences in AUD-related behaviours. We observed that HD show a loss of control in their alcohol use and continue to seek and consume alcohol despite adverse consequences. These profound individual differences in alcohol intake are related to alcohol reinforcement, motivation, Pavlovian conditioned approach behaviour, decision making and social stimuli during early development. This model therefore provides a promising tool to unravel the neurobehavioural underpinnings of individual vulnerability for AUD. Eventually, these new insights may help to develop improved treatment strategies to regain control over alcohol use and reduce the impact of this devastating disorder.

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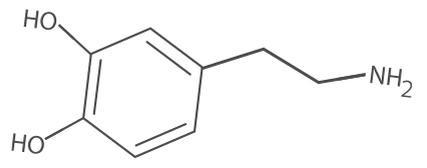
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ADDENDUM

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DUTCH SUMMARY

RATIONALE

Alcoholverslaving is een groot medisch en maatschappelijk probleem. Alcoholverslaving ontstaat door een complexe interactie tussen persoonlijkheidskenmerken, ontwikkelings- en omgevingsfactoren en neurobiologische veranderingen in de hersenen als gevolg van alcoholgebruik. Alhoewel wereldwijd veel mensen regelmatig alcohol nuttigen, ontwikkelt slechts een klein percentage (3-5%) een alcoholverslaving. Het is nog steeds niet duidelijk waarom de ene persoon wel een alcoholverslaving ontwikkelt en de andere persoon niet. In dit proefschrift hebben we bij ratten onderzocht of er verband bestaat tussen een hoge mate van alcoholinname en 1) de motivatie om alcohol te verkrijgen, 2) het verlies van controle over alcoholgebruik, 3) sociaal spelgedrag, 4) de gevoeligheid voor stimuli die geassocieerd zijn met beloning, 5) beslissingsvermogen en 6) impulsiviteit. Daarnaast hebben we gekeken naar de rol van het mesolimbische dopaminesysteem in de hersenen 1) op gedragsveranderingen na adolescent alcoholgebruik, 2) op de motivatie om alcohol te verkrijgen en 3) op alcoholconsumptie. De resultaten van dit proefschrift leveren nieuwe inzichten in de gedragskenmerken en neurobiologische factoren die bijdragen aan het ontstaan van alcoholverslaving. Deze bevindingen kunnen worden toegepast in de ontwikkeling van betere en meer persoonsgerichte behandelingen.

ALCOHOLVERSLAVING EN CONTROLEVERLIES

Alcoholverslaving is een chronische recidiverende hersenziekte die wordt gekenmerkt door veelvuldig alcoholgebruik, ondanks dat men zich bewust is van de negatieve consequenties van het alcoholgebruik. Wereldwijd hebben circa 76 miljoen mensen een alcoholverslaving. In Nederland zijn tussen 2007 en 2009 478.000 mensen gediagnosticeerd met een alcoholverslaving. Omdat alcoholverslaving geassocieerd is met medische problemen, verminderde productiviteit op het werk, criminaliteit, ongelukken en risicovol gedrag, brengt alcoholverslaving grote economische en psychologische schade toe aan de samenleving.

Alcoholverslaving is een complexe ziekte die vaak voor komt in combinatie met andere psychiatrische stoornissen zoals depressie, angst, schizofrenie, bipolaire stoornissen, ADHD en slapeloosheid. Chronisch en/of excessief alcoholgebruik vergroot de kans om een alcoholverslaving te ontwikkelen,

maar zijn niet de enige factoren die het ontstaan van alcoholverslaving voorspelt. Eerdere studies hebben aangetoond dat 40-60% van het risico op alcoholverslaving wordt veroorzaakt door genetische factoren. Daarnaast spelen persoonlijkheidskenmerken en veranderingen in de hersenen als gevolg van alcoholgebruik ook een rol bij het ontstaan van alcoholverslaving. In dit proefschrift staat onderzoek beschreven waarin we hebben gekeken naar een aantal neurobiologische en gedragsfactoren die geassocieerd zijn met alcoholverslaving.

Diermodellen hebben de afgelopen decennia een belangrijke bijdrage geleverd aan het begrijpen van de gedrags- en hersenprocessen die betrokken zijn bij alcoholverslaving. In dit proefschrift hebben we gebruik gemaakt van groepen ratten die veel individuele variatie in gedrag vertonen. In **hoofdstuk 2, 3, 5, 6 en 9** hebben we een groep ratten 2 maanden lang de beschikking gegeven over een 20% alcoholoplossing. Ze kregen deze alcohol om de dag aangeboden in hun thuishok. We zagen grote verschillen tussen de ratten m.b.t. de hoeveelheid alcohol die ze dronken. Na 2 maanden vrijwillig alcohol te hebben gedronken, hebben we de ratten onderverdeeld in drie groepen die weinig, matig of veel alcohol dronken (LD, MD en HD). Het viel ons op dat HD, de ratten die veel alcohol drinken, ook na verloop van tijd meer gingen drinken. De motivatie om alcohol te verkrijgen kan bij dieren worden bepaald door te meten hoe vaak ze bereid zijn om op een pedaaltje te drukken voor alcohol, als ze voor elke volgende portie alcohol vaker op het pedaaltje moeten drukken. We zagen dat HD ook een hogere motivatie hebben voor alcohol dan LD (zie **hoofdstuk 2**). In **hoofdstuk 2 en 3** van dit proefschrift hebben we ook bepaald of LD en HD verschilden in hun mate van controle over alcoholgebruik. De meerderheid van de diagnostische criteria voor alcoholverslaving (zoals in de DSM5, een handboek dat veel gebruikt wordt om mentale aandoeningen te beschrijven) duiden op verlies van controle over alcoholgebruik, terwijl de huidige behandelingen vooral zijn gericht op het verminderen van beloning en van 'trek' in alcohol. Om controleverlies nader te onderzoeken, hebben we in **hoofdstuk 2 en 3** onderzocht of de ratten doorgaan met het gebruiken van of zoeken naar alcohol, als dit negatieve consequenties heeft. Dit hebben we gedaan door de alcohol bitter te maken door er kinine (dat heel bitter smaakt) aan toe te voegen. In een ander experiment hebben we dit gedaan door een waarschuwingssignaal te laten horen aan de dieren. Dit waarschuwingssignaal was een pieptoon, die ze eerder in verband hebben gebracht met milde elektrische schokken, vergelijkbaar met schrikdraad. HD dronken alcohol met

hogere concentraties kinine (**hoofdstuk 2**) en waren minder gevoelig voor het waarschuwingssignaal dan LD (**hoofdstuk 3**). Bovendien zagen we in MD, de 'matige drinkers', dat ze na langdurige alcoholconsumptie ook minder gevoelig werden voor het waarschuwingssignaal. Deze resultaten geven aan dat controleverlies over alcoholgebruik afhankelijk is van zowel hoe lang de dieren alcohol hadden gedronken als van hun individuele gevoeligheid voor alcoholgebruik. Deze waarnemingen zijn heel nuttig voor vervolgonderzoek, zodat we kunnen onderzoeken welke processen in de hersenen betrokken zijn bij alcoholverslaving.

DE ROL VAN IMPULSIVITEIT, BESLISSINGS- VERMOGEN EN DE GEVOELIGHEID VOOR STIMULI DIE GEASSOCIEERD ZIJN MET BELONING

Eerdere studies hebben aangetoond dat patiënten met een alcoholverslaving impulsiever zijn, risicovoller gedrag vertonen en minder goede beslissingen nemen. Echter, in gezonde proefpersonen of dieren veroorzaakt alcohol geen eenduidige effecten op impulsief gedragen beslissingsvermogen. Dit suggereert dat blootstelling aan alcohol op zichzelf wellicht niet verantwoordelijk is voor verhoogde impulsiviteit en een verminderd beslissingsvermogen. Tegelijkertijd vertonen patiënten met een alcoholverslaving of dieren die herhaaldelijk alcohol hebben gekregen, na hernieuwde toediening van alcohol wel meer impulsief gedrag, wat aangeeft dat alcohol wellicht een andere werking heeft in individuen die langdurig aan alcohol zijn blootgesteld. Omdat deze gedragingen zowel de oorzaak als het gevolg van een alcoholverslaving kunnen zijn, is het moeilijk om te achterhalen wat de causaliteit is. We kunnen hier alleen een antwoord op krijgen door prospectief onderzoek te doen. In dit proefschrift hebben we gekeken naar 3 gedragskenmerken die zowel een risicofactor zijn voor alcoholverslaving, dan wel een gevolg kunnen zijn van alcoholmisbruik, te weten 1) impulsiviteit, 2) beslissingsvermogen en 3) de gevoeligheid voor stimuli die geassocieerd zijn met beloning.

Impulsief gedrag kan worden omschreven als '*handelingen die slecht doordacht zijn, onnodig risicovol, ongepast, te vroeg uitgevoerd worden en vaak ongewenste gevolgen hebben*'. Impulsief gedrag is een heteroog fenomeen; het wordt vaak onderverdeeld in *impulsief handelen* en *impulsief kiezen*. Een voorbeeld van een *impulsieve handeling* is voor je beurt spreken, terwijl een *impulsieve keuze* bijvoorbeeld de voorkeur is voor een kleine directe beloning ten opzichte van een grotere, maar uitgestelde beloning.

In **hoofdstuk 4 en 5** hebben we gekeken naar het effect van alcohol op impulsiviteit en beslissingsvermogen. Er zijn verschillende tests ontwikkeld om impulsiviteit en beslissingsvermogen te meten bij mensen en dieren. De 'Iowa Gambling Task' (IGT) is een test voor beslissingsvermogen die in de psychiatrie veel gebruikt wordt. Met een variant van deze taak, de rat Gambling Task (rGT) kan ook beslissingsvermogen bij dieren worden gemeten. In dit proefschrift hebben we het beslissingsvermogen getest door de ratten in een rGT uit 3 opties te laten kiezen: een veilige, een optimale en een risicovolle optie. De opties verschillen van elkaar doordat bijvoorbeeld de veilige optie een grote kans biedt op een kleine beloning (in dit geval een kleine hoeveelheid suikerkorrels) en een kleine kans op een klein verlies (in dit geval een korte wachtperiode, waarbinnen het dier geen suiker kan verdienen), terwijl de risicovolle optie een kleine kans biedt op een grote beloning (een grotere hoeveelheid suikerkorrels) en een grote kans op een groot verlies (een lange wachtperiode). Een *impulsieve keuze* kan bestudeerd worden met de 'Delayed Reward Task' (DRT), die in zowel humaan als dierexperimenteel onderzoek wordt toegepast. In deze taak kan het dier kiezen tussen een kleine beloning die direct wordt gegeven, of een grote beloning waarop het dier moet wachten. In het begin van de test is de wachtperiode voor zowel de kleine als de grote beloning even groot, en kiezen de ratten meestal voor de grote beloning. Naarmate de test vordert wordt de wachtperiode voor de grote beloning steeds langer. Wanneer het dier al bij een redelijk korte wachttijd kiest voor de kleine beloning, duidt dit op impulsief keuzegedrag, namelijk het onvermogen om te wachten op een grote beloning.

In **hoofdstuk 4** hebben we de effecten van acute en herhaaldelijke alcoholtoediening op het beslissingsvermogen onderzocht in twee versies van de rGT. Het belangrijkste verschil tussen deze twee versies is de wachttijd van de risicovolle keuze, die in versie A lang was en kort in versie B. Daardoor konden we meten wat het effect is van alcohol op de gevoeligheid voor straf. De resultaten uit dit hoofdstuk wijzen uit dat, nadat het dier de consequenties van iedere keuze heeft geleerd, een acute behandeling met alcohol weinig effect heeft op het beslissingsvermogen. Wanneer we echter de dieren herhaaldelijk alcohol gaven tijdens het aanleren van de taak, zagen we dat in versie A, waarbij de risicovolle keuze vaak resulteerde in een lange wachttijd, de dieren die behandeld waren met alcohol meer risicovolle keuzes maakten. Daarnaast zagen we dat behandeling met alcohol ervoor zorgde dat de dieren minder goed hun gedrag aanpasten op basis van de feedback die ze kregen

na de keuzes die ze hadden gemaakt. Deze resultaten wijzen erop dat alcohol ertoe leidt dat het individu minder gevoelig wordt voor feedback en straf, en daardoor minder goed in staat is om beslissingen te nemen die uiteindelijk het meest voordelig zijn. Ook zagen we dat de dieren die voorheen herhaaldelijk alcohol hadden gekregen, ontremd gedrag vertoonden na acute toediening van alcohol, zo reageerden ze vaker al voordat ze überhaupt een keuze konden maken in de taak. Deze laatstgenoemde bevinding geeft aan dat een acute toediening van alcohol een andere uitwerking heeft op dieren die al eerder zijn blootgesteld aan alcohol.

In **hoofdstuk 5** zagen we dat de dieren die veel alcohol drinken (HD), efficiënter keuzegedrag lieten zien in zowel de rGT als de DRT en zodoende meer suiker verdienden. In de DRT waren HD meer bereid om te wachten op de grote beloning en lieten minder impulsief keuzegedrag zien dan LD. HD lieten tegelijkertijd wel meer impulsieve handelingen zien, ze reageerden vaak al voordat ze een keus konden maken. Na acute behandeling met alcohol lieten zowel LD als HD meer impulsief keuzegedrag zien, maar alcohol had geen effect op impulsief handelen. Samenvattend geven de bevindingen uit **hoofdstuk 5** aan dat HD keuzegedrag laten zien dat resulteert in meer beloning, wat suggereert dat deze dieren meer gefocust zijn op beloningen.

Tijdens consumptie van alcohol raken de effecten van alcohol geassocieerd met bijbehorende 'cues', zoals glazen, flessen, de geur van alcohol of het zien van een alcoholisch drankje. Het is aangetoond dat deze alcohol-geassocieerde cues de 'trek' in alcohol kunnen vergroten, hetgeen bijvoorbeeld kan leiden tot terugval in alcoholmisbruik na een periode van abstinentie. Opmerkelijk genoeg blijken er grote individuele verschillen te zijn in de waarde die mensen en dieren hechten aan stimuli die geassocieerd zijn met beloning. Ratten reageren allemaal op een stimulus die geassocieerd is met een beloning, maar de manier waarop ze dat doen verschilt. Sommige dieren zoeken contact met de stimulus zelf, zogenoemde 'sign-trackers', terwijl andere dieren naar de plaats toegaan waar de beloning wordt verstrekt; deze dieren noemen we 'goal-trackers'. Ratten die sign-tracking gedrag vertonen blijken impulsiever te zijn en hebben een grotere neiging om zichzelf amfetamine, cocaïne, nicotine, morfine en alcohol toe te dienen. Bij mensen is aangetoond dat overmatig alcoholgebruik samenhangt met een meer uitgesproken benadering naar stimuli die geassocieerd zijn met alcohol. Daarnaast blijkt de gevoeligheid voor stimuli die geassocieerd zijn met beloning een hoge mate

van alcoholconsumptie bij mensen te voorspellen. De precieze causaliteit van deze associatie en de onderliggende neurobiologische mechanismen zijn onbekend. In **hoofdstuk 5 en 7** van dit proefschrift hebben wij daarom de relatie tussen alcoholgebruik en sign-tracking onderzocht. In **hoofdstuk 5** zagen we dat sign-tracking niet voorspellend is voor de hoeveelheid alcoholconsumptie. Echter, na een periode van alcoholgebruik, zagen we een hogere mate van sign-tracking. Daarnaast zagen we dat HD meer sign-tracking lieten zien dan LD. Samenvattend geven deze resultaten uit **hoofdstuk 5** aan dat alcoholconsumptie resulteert in meer benaderingsgedrag naar stimuli die geassocieerd zijn met beloning. Dit kan bijdragen aan de ontwikkeling van alcoholverslaving doordat de individu gevoeliger wordt voor stimuli in de omgeving die samenhangen met alcohol. De resultaten van **hoofdstuk 7** worden hieronder besproken.

DE ADOLESCENTIE ALS EEN PERIODE VAN KWETSBAARHEID VOOR VERSLAVING

Adolescentie is de periode tussen puberteit en volwassenheid. Tijdens deze fase in de ontwikkeling laten mensen vaak risicovol en impulsief gedrag zien, zoals het experimenteren met genotmiddelen, voornamelijk alcohol. Eerder onderzoek heeft aangetoond dat het gebruik van alcohol tijdens de adolescentie de kans op alcoholverslaving vergroot. Men denkt dat dit komt doordat de hersenen van adolescenten gevoeliger zijn voor de functionele verstoringen die kunnen ontstaan als gevolg van alcoholgebruik. Onderzoek met ratten heeft aangetoond dat ratten die alcohol drinken tijdens de adolescentie, op volwassen leeftijd meer risicovol keuzegedrag laten zien in vergelijking met controle dieren of ratten die alcohol hadden genuttigd op volwassen leeftijd. In **hoofdstuk 7** hebben we onderzocht wat de consequentie is van adolescent alcoholgebruik op sign-tracking. Wij zagen dat adolescent alcoholgebruik resulteerde in een toename in sign-tracking; de dieren zochten dus meer contact met de stimulus die geassocieerd was met beloning i.p.v. contact met de beloning zelf. Deze gedragsverandering zou kunnen verklaren waarom adolescent alcoholgebruik het risico op een alcoholverslaving op volwassen leeftijd verhoogt.

Sociale ervaringen tijdens de ontwikkeling, zoals spelgedrag, zijn van groot belang voor de ontwikkeling van hersenen en gedrag. Ratten vertonen veel spelgedrag gedurende de vroege ontwikkeling. Onderzoek bij ratten heeft aangetoond dat spelgedrag belonend is en dat spelgedrag gereguleerd wordt

door hersengebieden die ook betrokken zijn bij de belonende effecten van verslavende middelen. Verstoringen in het spelgedrag kunnen leiden tot gedragsveranderingen en afwijkingen in de hersenen. In **hoofdstuk 6** hebben we onderzocht wat de consequenties zijn van een periode van sociale isolatie tijdens de ontwikkeling (het afnemen van de mogelijkheid om te kunnen spelen) op alcoholinname in de volwassenheid. Onze resultaten laten zien dat ratten die op jonge leeftijd niet de mogelijkheid hadden om te spelen, op latere leeftijd meer alcohol drinken, al vertonen ze geen hogere motivatie om de alcohol te verkrijgen.

HET MESOLIMBISCHE DOPAMINESYSTEEM

Om alcoholverslaving beter te begrijpen, is het belangrijk om onderzoek te doen naar wat er in de hersenen gebeurt bij verslaving. Voorgaand onderzoek heeft uitgewezen dat vrijwel alle genotmiddelen een effect hebben op het zogenaamde mesolimbische dopaminesysteem, dat een belangrijk deel uitmaakt van het beloningssysteem in de hersenen. Alhoewel verschillende genotmiddelen een ander primair werkingsmechanisme hebben, leiden ze allemaal, dus ook alcohol, tot een verhoogde afgifte van de signaalstof dopamine. Signaalstoffen zoals dopamine kunnen informatie doorgeven aan andere hersencellen door te binden aan de op hersencellen gelegen ontvangende eiwitten (zogenaamde receptoren). In voorgaand onderzoek heeft men aangetoond dat dopamine een belangrijke rol speelt bij verschillende vormen van gedrag dat gericht is op beloning. Zo blijkt dopamine belangrijk te zijn voor het leren van de associaties tussen stimuli en beloningen die ik eerder besproken heb en ook aan de waarde die toegekend wordt aan zulke stimuli. Dopamine speelt verder ook een rol bij het verwerken van negatieve stimuli, maar het is tot op heden onduidelijk of dezelfde of juist verschillende dopaminerge cellen verantwoordelijk zijn voor de effecten van positieve en negatieve stimuli.

Dopamine wordt geproduceerd door cellen in de middenhersenen, die projecteren naar verschillende hersengebieden in de voorhersenen, waaronder het striatum en delen van de hersenschors. Het striatum is een heterogeen hersengebied. Omdat is gebleken dat bepaalde delen van het striatum verschillende functies hebben en in verbinding staan met verschillende andere hersengebieden, is het striatum onderverdeeld in een aantal onderdelen. Daarnaast wordt er gedacht dat de verschillende delen van het striatum met elkaar in verband staan, waarbij informatie van het ene

deel wordt doorgespeeld naar het volgende deel. Het onderste ('ventrale') deel van het striatum wordt de nucleus accumbens genoemd; dit kan worden onderverdeeld in de nucleus accumbens shell (rand) en nucleus accumbens core (kern). Eerdere studies hebben aangetoond dat de nucleus accumbens shell een belangrijke rol speelt bij het toekennen van waarde aan beloningen. De nucleus accumbens core is belangrijk voor het aanleren van associaties tussen stimuli en beloningen. Vanwege de verbindingen van de nucleus accumbens met andere hersengebieden wordt deze beschouwd als een belangrijk schakelpunt dat emotionele en cognitieve informatie integreert en op grond van die informatie gedrag aanstuurt. Het bovenste ('dorsale') deel van het striatum wordt onderverdeeld in een dorsolateraal (aan de buitenkant gelegen) en een dorsomediaal (aan de binnenkant gelegen) deel. Het dorsolaterale striatum is belangrijk voor het aanleren van gewoontes en mogelijk ook bij compulsief gedrag, terwijl het dorsomediale striatum betrokken is bij doelgericht gedrag.

In **hoofdstuk 7, 8 en 9** hebben we de rol van het mesolimbische dopamine-systeem bij verschillende gedragingen die te maken hebben met alcoholgebruik onderzocht. In **hoofdstuk 7** hebben we onderzocht of alcoholgebruik tijdens de adolescentie leidt tot een verandering in dopamine-afgifte in een belangrijk deel van het mesolimbische dopaminesysteem: nucleus accumbens core. Daartoe hebben we een elektrochemische onderzoekstechniek gebruikt, zogenaamde 'Fast-Scan Cyclic Voltammetry' (FSCV), waarmee we dopamine-afgifte kunnen meten in een levend proefdier door middel van geïmplanteerde elektrodes. We hebben dopamine-afgifte gemeten tijdens het aanleren van de associatie tussen een stimulus en een beloning. We zagen dat de ratten die alcohol hadden gekregen tijdens adolescentie, meer dopamine-afgifte lieten zien bij zowel de presentatie van de stimulus, als bij het krijgen van de beloning zelf. Vervolgens hebben we de ratten onverwachts een grotere of kleinere beloning gegeven dan ze verwachtten. De ratten die alcohol hadden gekregen tijdens adolescentie lieten hierbij duidelijke verschillen in dopamine-afgifte zien, afhankelijk van de grootte van de beloning, terwijl het verschil in dopamine-afgifte bij controledieren een stuk kleiner was. Deze resultaten suggereren dat blootstelling aan alcohol tijdens de adolescentie leidt tot een aanpassing in het mesolimbische dopaminesysteem zodat de ratten gevoeliger worden voor de grootte van een beloning. Bovendien lieten de met alcohol behandelde ratten meer sign-tracking zien, wat suggereert dat dit gedrag samenhangt met een grotere afgifte van dopamine in de nucleus accumbens core.

In **hoofdstuk 8** hebben we gekeken naar de rol van dopamine in verschillende hersengebieden bij alcohol-zelftoediening. Er is de afgelopen jaren veel onderzoek gedaan naar de rol van de verschillende delen van het striatum bij cocaïneverslaving, maar het is onduidelijk wat hun rol is van bij alcoholverslaving. Daarom hebben we in **hoofdstuk 8** gekeken naar de rol van dopamine in de verschillende delen van het striatum bij zelftoediening van alcohol. Hiervoor hebben we ratten eerst getraind om in een zogenaamde Skinnerbox op een pedaaltje te drukken voor alcohol. Vervolgens hebben we buisjes geïmplantéerd in de delen van het striatum en een stof geïnjecteerd die de werking van dopamine remt door de dopaminereceptoren te blokkeren. We zagen dat dopamine in de nucleus accumbens shell en het dorsolaterale striatum vooral belangrijk zijn voor de motivatie om alcohol te verkrijgen. De nucleus accumbens core lijkt een meer algemene rol te spelen bij de belonende effecten van alcohol, mogelijk doordat de nucleus accumbens core belangrijk is voor het verwerken van stimuli die geassocieerd zijn met de effecten van alcohol.

Omdat het mesolimbische dopaminesysteem zo belangrijk is voor de effecten van genotmiddelen, is het mogelijk dat medicijnen die aangrijpen op dit systeem, nuttig zijn voor de behandeling van verslaving. Er zijn echter geen eenduidige resultaten gevonden voor het effect van deze medicijnen bij patiënten met een verslaving. Er zijn 5 verschillende dopaminereceptoren bekend, die worden onderverdeeld in 2 subtypen: de dopamine D1-type en de dopamine D2-type receptoren. Beide subtypes van dopaminereceptoren zijn in verband gebracht met alcoholgebruik en alcoholverslaving. Zo heeft men geconstateerd dat zowel patiënten met een alcoholverslaving als dieren met een sterke voorkeur voor alcohol minder dopaminereceptoren hebben in het beloningssysteem. In **hoofdstuk 9** hebben we onderzocht wat de rol is van het activeren en remmen van dopamine D1 of D2 receptoren tijdens alcoholconsumptie en of deze rol verschillend is voor LD en HD. De resultaten gaven aan dat het activeren van het dopaminesysteem resulteert in minder alcoholinname, terwijl de remming van het dopaminesysteem geen effecten had. Deze effecten waren niet verschillend tussen LD en HD. Deze resultaten geven daarom aan dat dopamine waarschijnlijk niet betrokken is bij het consumeren van alcohol. Echter, de resultaten in **hoofdstuk 8** wijzen uit dat dopamine in het striatum wel een rol speelt bij de motivatie voor alcohol. Samen geven deze resultaten aan dat dopamine een bepalende rol speelt bij hoeveel moeite men wil doen om aan alcohol te komen, maar niet zozeer bij het nuttigen en genieten van de alcohol zelf.

CONCLUSIES EN KLINISCHE TOEPASBAARHEID

De resultaten in dit proefschrift hebben ons inzicht in de neurobiologische en psychologische mechanismen die een rol spelen bij alcoholverslaving vergroot. Deze inzichten kunnen mogelijk worden gebruikt in een aantal gedragstherapieën. Zo hebben we in dit proefschrift aangetoond dat alcohol impulsief handelen stimuleert. Het zou daarom kunnen dat bij bepaalde patiënten, medicatie die impulsiviteit vermindert (bijvoorbeeld geneesmiddelen die worden gebruikt voor ADHD), effectief is bij de behandeling van alcoholverslaving. Daarnaast hebben we gezien dat blootstelling aan alcohol tijdens de adolescentie of overmatige consumptie van alcohol tijdens de volwassenheid, sign-tracking versterkt (gedrag dat gericht is op meer benaderingsgedrag naar stimuli die een beloning voorspellen). Dit kan ertoe leiden dat iemand sneller getriggerd wordt door deze stimuli om weer alcohol te gebruiken. Voor de behandeling van alcoholverslaving kan het daarom nuttig zijn om patiënten te trainen deze stimuli te negeren om zo meer controle te krijgen over de effecten van de omgeving op het gedrag.

Gezien het feit dat verschillende factoren een rol spelen bij alcoholverslaving, is het niet aannemelijk dat er een behandeling bestaat die goed aanslaat bij iedere patiënt. Er zijn enkele medicijnen beschikbaar waarmee alcoholverslaving wordt behandeld, maar deze zijn effectief bij slechts 20-30% van de patiënten. Deze medicijnen verlagen ook bij proefdieren de alcoholconsumptie en de motivatie voor alcohol. Behandeling met dopamine receptor antagonisten heeft tot dusver niet tot een effectieve behandeling van verslaving geleid. In dit proefschrift zagen we dat de effecten van dopamine agonisten en antagonisten op alcoholconsumptie onafhankelijk zijn van de individuele gevoeligheid voor alcoholmisbruik. Omdat dopamine een belangrijke rol speelt bij het verwerken van beloningscues in de omgeving, zou medicatie gericht op het dopaminesysteem wellicht vooral effectief zijn bij patiënten die sterk beïnvloed worden door stimuli in de omgeving.

De resultaten in dit proefschrift suggereren dat een hoge mate van alcoholconsumptie leidt tot controleverlies over de inname van alcohol, een hogere beloningsgevoeligheid en meer naderingsgedrag naar stimuli die beloningen voorspellen. Dit verlies van controle lijkt echter niet noodzakelijkerwijs samen te hangen met impulsief gedrag. Daarnaast blijkt dopamine niet zozeer belangrijk voor de consumptie van alcohol, maar wel bij de motivatie voor alcohol en bij het aanleren van associaties tussen stimuli

en beloning. Verlies van controle is een belangrijk gedragskenmerk van alcoholverslaving. Deze gedragingen zien we duidelijk terug bij een deel van onze dieren, in overeenstemming met de hoge mate van individuele variatie in de gevoeligheid voor alcoholverslaving bij mensen. De in dit proefschrift gebruikte opzet kan daarom worden beschouwd als een betrouwbaar model om alcoholverslaving te onderzoeken. In vervolgonderzoek kan deze opzet bijvoorbeeld worden gebruikt om inzicht te krijgen in welke processen in de hersenen hierbij een rol spelen. Herstel van controle over alcoholgebruik zou kunnen bijdragen aan een efficiëntere behandeling van alcoholverslaving.

CURRICULUM VITAE



Marcia Spoelder was born on April 7th 1987 in Heeg. In 2005 she obtained her Athenaeum degree from secondary school 'RSG Magister Alvinus' in Sneek and started a Bachelor in Human Movement Sciences at the University of Groningen. Because of her growing interest in the brain and its psychopathologies, she worked as a research assistant conducting neuropsychological assessments, interviews and interventions in patients with a dementia under supervision of Prof. Dr. Erik Scherder and was a teaching assistant in neuro-anatomy courses. Marcia performed her Bachelor internship at the University Medical Centre Groningen where she studied movement characteristics of patients with osteoarthritis of the hip and compared the improvement in movement upon surgery Type A versus B. Thereafter, she started the Research Master Cognitive Neuropsychology at the VU University Amsterdam. Here, she continued to participate as a research assistant in dementia research and spend her free hours in the anatomy dissecting room. She performed her Master internship in Cambridge, UK, at the Department of Experimental Psychology under the supervision of Dr. Jeff Dalley where she investigated the underlying brain substrates of impulsive behaviour. Marcia obtained her Master's degree *cum laude* in 2010. Subsequently, she started working on her PhD project regarding the individual susceptibility to alcohol use disorder under supervision of Prof. Dr. Louk Vanderschuren and Dr. Heidi Lesscher at the University of Utrecht.

In addition to her PhD, Marcia assisted the PhD student platform of the PhD program Clinical and Experimental Neuroscience of the Graduate School of Life Sciences Utrecht and assisted many students during their internships. Moreover, she is assisting the Drug Information and Monitoring System of the Trimbos Institute and is a volunteer in the Dutch organisation 'Unity' at addiction care center 'Victas' to educate people regarding the risks of the use of recreational drugs and thereby prevent drug-harm. In 2014, Marcia initiated a collaboration with Dr. Jeremy Clark at the Department of Psychiatry and Behavioural Sciences at the University of Washington in Seattle. Between March – September 2014, Marcia performed experiments to study the consequences of adolescent alcohol consumption on phasic dopamine release during stimulus-reward learning. The results obtained at the University of Utrecht and the University of Washington are presented in this thesis. In 2015, Marcia finished her PhD thesis and was appointed as a postdoctoral scientist at the University of Utrecht under the supervision of Prof. Dr. Louk Vanderschuren and Dr. Corette Wierenga (Department of Cell Biology, Faculty of Science) and performed behavioural and neurochemical studies to investigate cognitive flexibility. In 2016, Marcia will be active as a member in the Animal Ethics Committee of the University of Utrecht.

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SUBMITTED MANUSCRIPTS

Marcia Spoelder, Peter Hesseling, Matthew Styles, Annemarie Baars, José G. Lozeman-van 't Klooster, Heidi M.B. Lesscher, Louk J.M.J. Vanderschuren. Dopaminergic neurotransmission in ventral and dorsal striatum differentially modulates alcohol reinforcement.

Marcia Spoelder, Jacques P. Flores Dourojeanni, Catherina G. De Git, Annemarie Baars, Heidi M.B. Lesscher, Louk J.M.J. Vanderschuren. Individual differences in voluntary alcohol intake in rats: relationship with impulsivity, decision making and Pavlovian conditioned approach behaviour.

Marcia Spoelder, Annemarie Baars, Marthe D. Rotte, Louk J.M.J. Vanderschuren, Heidi M.B. Lesscher. Dopamine receptor agonists modulate voluntary alcohol consumption independently of the individual level of alcohol intake.

Marcia Spoelder, Annemarie Baars, Sylvana Pol, Boris Janssen, Louk J.M.J. Vanderschuren, Heidi M.B. Lesscher. Loss of control over alcohol seeking in rats depends on individual vulnerability and duration of alcohol consumption.

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