Loss of control over alcohol seeking in rats depends on individual vulnerability and duration of alcohol consumption experience

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Alcohol use disorder (AUD) is characterized by excessive alcohol use and persistent alcohol seeking despite knowledge of its negative consequences. Importantly, AUD typically 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 alcohol exposure. The present study assessed the contribution of prolonged exposure to alcohol and individual levels of alcohol intake to the development of loss of control over alcohol seeking in a conditioned suppression model. To investigate the impact of prolonged alcohol exposure, conditioned suppression of alcohol seeking was assessed after 2 and 4 months of intermittent alcohol access (IAA) in a subgroup of rats drinking moderate amounts of alcohol. We observed that suppression of alcohol seeking was reduced after 4 months compared with 2 months of IAA. The influence of individual levels of alcohol intake on loss of control over alcohol seeking was subsequently determined by assessing conditioned suppression in subgroups of low and high alcohol drinking rats. Unlike the low alcohol drinking rats, the high alcohol drinking rats showed aversion-resistant alcohol seeking after 2 months of IAA, although both groups showed comparable levels of conditioned freezing. 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. ***Behavioural Pharmacology*** **28:334–344**

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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 individuals, that is, ~76 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, a number of preclinical models of loss of control over substance use have been developed, whereby rodents show continued substance use or seeking despite adverse consequences (Lesscher and Vanderschuren, 2012; Vanderschuren and Ahmed, 2013; Hopf and Lesscher, 2014; Vanderschuren *et al.*, 2017). For example, it has been shown that rats show continued cocaine seeking despite the presentation of footshocks or footshock-associated stimuli (Deroche-Gamonet *et al.*, 2004; Vanderschuren and Everitt, 2004; Pelloux *et al.*, 2007, 2015; Belin *et al.*, 2008, 2009, 2011; Jonkman *et al.*, 2012a, 2012b; Chen *et al.*, 2013). In the case of alcohol, resistance to quinine adulteration has been shown in alcohol self-administering mice and rats (Wolffgramm, 1991; Wolffgramm and Heyne, 1995; Wolffgramm *et al.*, 2000; Hopf *et al.*, 2010; Lesscher *et al.*, 2010; Vendruscolo *et al.*, 2012; Leao *et al.*, 2015; Seif *et al.*, 2015; Spoelder *et al.*, 2015; Warnault *et al.*, 2016). However, except for one study in rats (Seif *et al.*, 2013) and one in mice (Radke *et al.*, 2015), insensitivity of alcohol seeking to footshocks or associated stimuli has not been shown.

Considering that only a minority of the individuals who use alcohol develop AUD, it is important to understand the factors that determine the transition from recreational and controlled to compulsive and uncontrolled alcohol use. Previous studies have shown 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;

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Lesscher *et al.*, 2010; Jonkman *et al.*, 2012a; Chen *et al.*, 2013). For example, it has been shown that rats show reduced suppression of cocaine seeking upon presentation of footshock-associated cues after prolonged cocaine self-administration (SA) (Vanderschuren and Everitt, 2004; Limpens *et al.*, 2014b). Hopf *et al.* (2010) have shown that rats become resistant to quinine modulation and footshock-modulation of alcohol SA after 3–4 months of alcohol consumption (Seif *et al.*, 2013, 2015). Importantly, however, loss of control does not inevitably occur in all animals that take alcohol or cocaine (Deroche-Gamonet *et al.*, 2004; Pelloux *et al.*, 2007; 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 toward the development of substance use disorders as well. Indeed, we have recently shown that individual differences in alcohol consumption in Lister hooded 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, 2014b) in Lister Hooded rats. Optimal parameters to induce conditioned suppression of alcohol seeking were first determined in a selected group of medium alcohol drinking rats. Next, we retested conditioned suppression of alcohol seeking after prolonged 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 rats that consume high levels of alcohol are more prone to lose control over alcohol seeking compared with low alcohol drinking rats.

Methods

Subjects

Adult male Lister hooded rats (Charles River, Sulzfeld, Germany) were housed individually under controlled conditions, with a reversed 12 h light/dark cycle (lights off 07.00 h) and free access to water and chow. Rats were acclimatized to the facility for 2 weeks upon arrival 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).

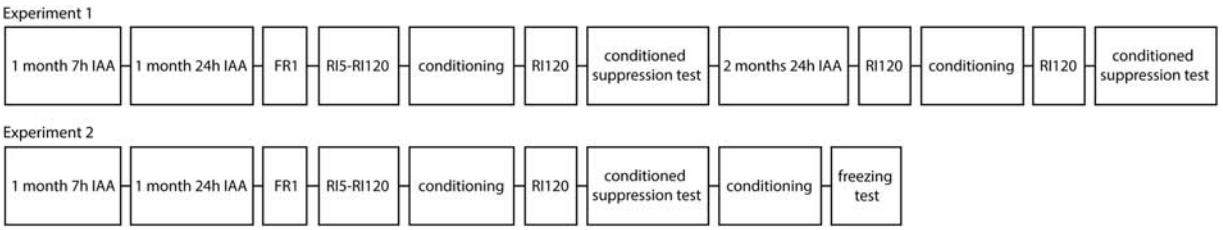
Voluntary home-cage alcohol consumption

The rats were allowed access to 20% (v/v) alcohol (Klinipath, Duiven, the Netherlands) and water in a two-bottle choice setup in the home-cage, with intermittent alcohol access (IAA) for 3 days a week (Monday–Wednesday–Friday). In the first month, alcohol was presented for 7 h/day (09.00–16.00 h) and access to alcohol was extended to 24 h/day thereafter (Spoelder *et al.*, 2015). Alcohol intake and preference were calculated per rat per session and averaged per week. Rats that consistently consumed low or high amounts of alcohol were selected on the basis of the sum of weekly ranking scores for average alcohol intake per week (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. The low alcohol drinking rats (LD) and the high alcohol drinking rats (HD) from this cohort were used for other studies. For experiment 2, which was designed to assess the relationship between individual levels of alcohol consumption and loss of control over alcohol seeking, 16 LD and 16 HD were selected from 48 rats. These animals originated from two batches. We chose to use a tertile split for this experiment, among other reasons to ensure a large enough sample size upon assigning the animals to CS− and CS+ subgroups. Use of two separate batches was necessary for logistical reasons to ensure that there were enough animals in each group to achieve sufficient statistical power. Ranking was performed separately per batch. Figure 1 shows an overview of the sequence of procedures for both experiments.

Alcohol self-administration

After 2 months of voluntary home-cage alcohol consumption, the rats were trained and tested 3 days/week (Monday–Wednesday–Friday) to lever press for alcohol in operant conditioning chambers as described previously (Spoelder *et al.*, 2015). The position of the active and inactive levers was counterbalanced between rats. Upon meeting the response requirement, the dipper cup containing alcohol (0.1 ml, 20% v/v) was raised, the cue light was illuminated above the active lever and the house light was switched off. Ten seconds after a head entry into the magazine, access to alcohol was terminated, the cue light was turned off, and 5 s later a new trial was started. Pressing the inactive lever was recorded, but had no programmed consequences. The rats were initially trained under a fixed ratio 1 schedule of reinforcement for three sessions. Thereafter, the rats were trained under a random interval (RI) schedule of reinforcement, whereby the first active lever press initiated a RI during which lever pressing was recorded, but was without consequences. After the RI had elapsed, the first active lever press resulted in the delivery of alcohol. The rats were tested in 30 min RI sessions with increasing RI durations (3 × RI: 5 s, 3 × RI: 15 s, 2–3 × RI: 30 s, and 2–3 × RI: 60 s). Finally, the rats were trained in five 60-min RI 120 s sessions. Stable responding was defined as less than 25% variation in active responses during the RI in the first 15 min of the last three RI 120 s sessions. Experimental events and data recording were controlled using MED-PC software; Med Associates, St. Albans, Vermont, USA.

Fig. 1



Sequential outline of the procedures for experiments 1 and 2. FR, fixed ratio; IAA, intermittent alcohol access; RI, random interval.

Conditioned suppression of alcohol seeking

Once responding on the RI 120 s schedule stabilized, the conditioned suppression test was performed according to previously described procedures (Vanderschuren and Everitt, 2004; Limpens *et al.*, 2014a, 2014b). The rats were assigned to groups that either underwent fear conditioning, with conditioned stimulus (CS) – footshock pairings (CS+), or underwent control conditioning (CS−). The rats' mean seeking responses per minute during the first 15 min of the last three RI 120 s sessions were used to assign the rats to CS− and CS+ groups to ensure equal mean seeking rates for CS+ and CS− groups before conditioning. In experiment 1, different shock intensities were used to determine the optimal shock intensity for conditioned suppression of alcohol seeking. Thirty-two MD rats (described above) were assigned to one of four CS groups to receive either no footshocks ($n=10$: CS−) or one of three shock intensities ($n=7$: 0.35 mA, CS+; $n=8$: 0.40 mA, CS+; $n=7$: 0.45 mA, CS+). In experiment 2, the 16 LD and 16 HD rats were either fear-conditioned with 0.40 mA footshocks (CS+; LD: $n=8$; HD: $n=8$) or underwent control conditioning (CS−; LD: $n=8$; HD: $n=8$). Acquisition of the CS-shock association was established in conditioning chambers that were physically different from operant SA chambers in that the conditioning chamber had a curved wall with five nose poke holes and, in contrast to the SA chambers, no levers or stimulus lights. To facilitate CS-shock, rather than context-shock, associations, the rats were habituated to the conditioning chambers in three 30-min sessions before conditioning. 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 a 10-min intertrial-interval) was constantly presented. During the 10-min tone presentations, 10 unpredictable, scrambled footshocks (1 s duration) were delivered, resulting in a total of 20 shocks for each CS+ rat. The conditioning session was completed after a final 5-min period without tone presentation. Rats in the CS− control group were subjected to the same procedure, but without footshocks.

After conditioning, the rats received two additional RI 120 s training sessions. Subsequently, conditioned suppression of alcohol-seeking behavior was assessed in the SA chambers. The house light was illuminated and 2 min after the start of the session, the levers were extended for the remaining 12 min of the test. Alcohol seeking during the conditioned suppression test was examined in extinction, that is, responding on the levers was recorded, but had no programmed consequences. To avoid altered responding because of the lack of (smell of) alcohol in the SA chamber, the cup containing 20% alcohol (v/v) was present underneath the liquid dipper, but the rats did not have access to the solution. Two-minute intervals in which the tone CS was presented (CS-ON interval) were alternated with 2-min intervals where the tone CS was absent (CS-OFF interval). Active lever presses and latency to the first lever press were recorded and compared for CS− and CS+ subgroups as a measure for control over alcohol seeking.

After the conditioned suppression test, the MD in experiment 1 received 24 h IAA for another 2 months and were subsequently retrained under fixed ratio 1 (1×), RI 30 s (1×), RI 60 s (2×), and RI 120 s schedules of reinforcement (5×). The rats were again habituated to the conditioning chamber for 3 days and were subsequently reconditioned using the same CS-conditioned footshock intensity or control procedure that they were exposed to before. Thereafter, they received two RI 120 s baseline sessions and were retested for conditioned suppression of alcohol seeking.

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. One week after the conditioned suppression test, the rats were subjected to fear conditioning (CS+) or control conditioning (CS−); they were assigned to the same group as before. Fear conditioning procedures were similar, as described in the previous section. After 24 h, the rats were placed in the conditioning chamber for 2 min without the CS+ tone and subsequently 2 min with the CS+ tone. The frequency and duration of freezing

behavior, defined as the absence of any movement other than breathing (Blanchard and Blanchard, 1969; Bouton and Bolles, 1980; LeDoux *et al.*, 1984), was scored from DVD-taped behavior using Observer software (Noldus, Wageningen, the Netherlands) by an observer blinded to treatment.

Statistical analysis

The alcohol consumption data were analyzed by one-way or two-way repeated measures analysis of variance (ANOVA) with time as the within-subjects factor and subgroup (LD or HD) as the between-subjects factor (experiment 2). The conditioned suppression data for experiment 1 were analyzed by four-way repeated-measures ANOVA with CS group (CS−, 0.35, 0.40 or 0.45 mA) as a between-subjects variable and IAA access duration (2 or 4 months), interval (CS-ON and CS-OFF minute periods), and tone (no-tone vs. tone) as within-subjects variables. The conditioned suppression data for experiment 2 were analyzed by four-way repeated-measures ANOVA with CS group (CS− and CS+) and group LD and HD as between-subjects factors and interval (CS-ON and CS-OFF minute periods) and tone (no-tone vs. tone) as within-subjects variables. The conditioned fear data were analyzed by three-way repeated-measures ANOVA with CS group (CS− and CS+) and group LD and HD as between-subjects factors and interval (tone-OFF vs. tone-ON) as a within-subjects factor. 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, *d.f.* were corrected to more conservative values using Huynh–Feldt estimates of sphericity; corrected *d.f.* are presented rounded to the nearest integer. When appropriate, post-hoc analyses were carried out using Student's *t*-tests or pairwise comparisons. Each parameter was tested for normality using a Kolmogorov–Smirnov test. In case the behavioral parameters were not normally distributed, data were square root transformed (active responses in the conditioned suppression test and conditioned freezing) or log transformed (latency data) before statistical analyses, which resulted in normal distribution of the data. Statistical analyses were carried out using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, New York, USA). The threshold for statistical significance was set at P less than 0.05. All data are presented as 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 7 h in the first month to 24 h in the second month of IAA ($P < 0.001$), increased further after the first conditioned suppression test ($P < 0.01$), and remained stable during the last 2 months of IAA (Fig. 2a). A near-significant trend toward 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 to increase after the first conditioned suppression test from the second to the third month ($P < 0.001$), but remained unchanged thereafter (Fig. 2b). Importantly, the CS groups did not differ in alcohol intake or alcohol preference at any of the time points tested [intake: $F_{(3,28)\ \text{CS group}} = 0.38$, NS; $F_{(6,58)\ \text{month} \times \text{CS group}} = 0.79$, NS; preference: $F_{(3,28)\ \text{CS group}} = 0.53$, NS; $F_{(6,58)\ \text{month} \times \text{CS group}} = 0.90$, NS; data not shown].

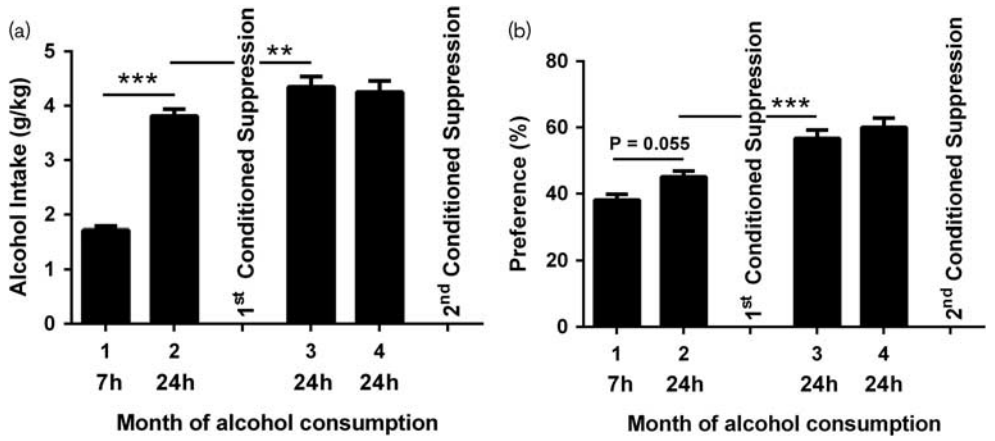
Analysis of responding during the first 15 min of the last three RI 120 sessions, corresponding to the duration of the conditioned suppression test, before fear conditioning after 2 months and after 4 months of alcohol consumption, showed that the CS groups (CS−: 0.35, 0.40 and 0.45 mA) responded equally during baseline sessions before fear conditioning [$F_{(3,28)\ \text{CS group}} = 0.13$, NS], independent of the duration of access to alcohol [$F_{(3,28)\ \text{time} \times \text{CS group}} = 2.6$, NS]. The same analysis showed that overall, responding before the second conditioned suppression test (i.e. after 4 months of alcohol consumption) was lower compared with responding before the first test (i.e. after 2 months of alcohol consumption) [$F_{(1,28)\ \text{time}} = 9.5$, $P < 0.01$; data not shown].

Conditioned suppression of alcohol seeking – active responses

Analysis of the number of active responses during the conditioned suppression test showed a significant effect of tone (ON/OFF) [$F_{(1,28)\ \text{tone}} = 5.5$, $P < 0.05$], which was different between the CS groups [$F_{(3,28)\ \text{tone} \times \text{CS group}} = 9.3$, $P < 0.001$] and dependent on the interval [$F_{(6,56)\ \text{tone} \times \text{time} \times \text{CS group}} = 5.9$, $P < 0.001$]. Moreover, responding during the suppression test was different for rats with a history of limited versus extended alcohol exposure [$F_{(2,56)\ \text{time} \times \text{IAA access}} = 6.8$, $P < 0.01$].

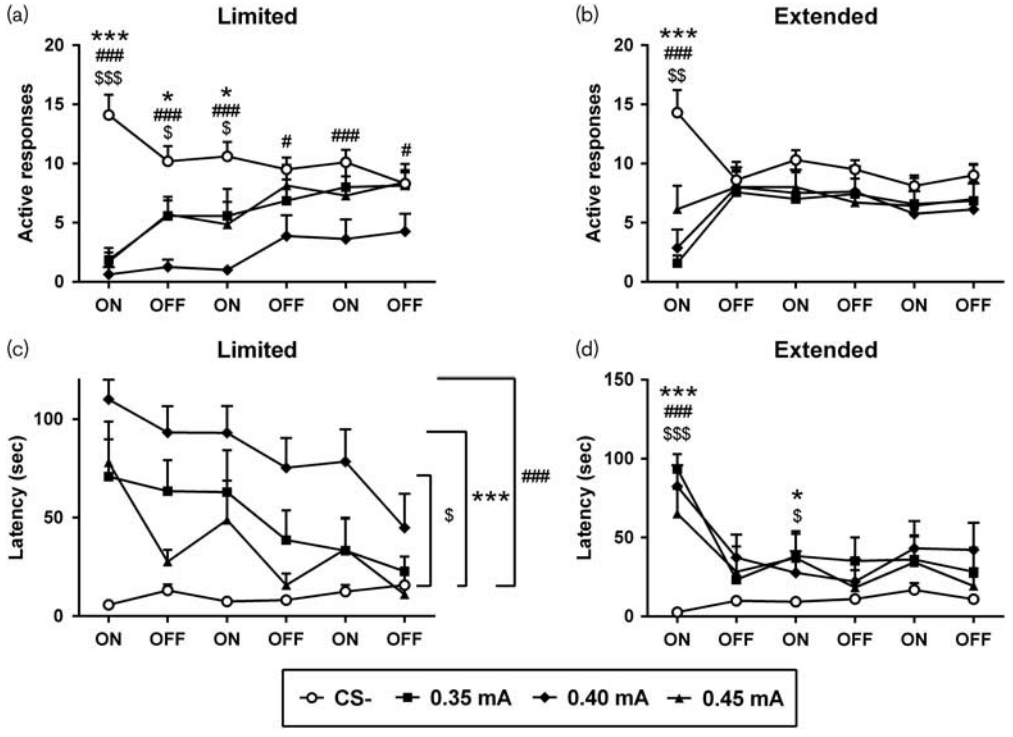
Separate analysis of the data for the rats with limited alcohol exposure showed differential effects of the tone presentation for the CS groups [$F_{(3,28)\ \text{tone} \times \text{CS group}} = 6.0$, $P < 0.01$] and an overall effect of fear conditioning on alcohol seeking [$F_{(3,28)\ \text{CS group}} = 11.6$, $P < 0.001$] (Fig. 3a). Post-hoc pairwise comparisons per interval confirmed significant conditioned suppression of alcohol seeking in all CS+ groups during the first tone presentation ($P < 0.001$), the first tone-OFF interval ($P < 0.05$ for 0.35 and 0.45 mA and $P < 0.001$ for 0.4 mA), and the second tone-ON interval ($P < 0.05$ for 0.35 and 0.45 mA and $P < 0.001$ for 0.4 mA). Conditioned suppression was only persistent throughout the rest of the session in the 0.40 mA CS group (ON3, $P < 0.001$; OFF2 and OFF3, $P < 0.05$).

Fig. 2



Alcohol consumption in the home cage during the 2 months preceding each conditioned suppression test. (a) Alcohol intake increased upon extension of the access duration from 7 to 24 h, 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 7 to 24 h and continued to increase over time. **, ***Significant differences between months of alcohol consumption (post-hoc pairwise comparisons). Data are presented as mean + SEM.

Fig. 3



Conditioned suppression of alcohol seeking after limited (2 months; a, c) and prolonged (4 months; b, d) alcohol consumption. (a–b) Number of active responses during consecutive CS-ON and CS-OFF intervals in rats conditioned with different footshock intensities (0.35, 0.40 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, 0.40 and 0.45 mA) after limited (c) and extended (d) alcohol consumption. Data are presented as mean + SEM active responses or latencies, binned per 2 min intervals. *, ***Significant difference between the 0.35 mA group and the CS− group (post-hoc pairwise comparisons, $P < 0.05$ and < 0.001, respectively). #, ###Significant difference between the 0.40 mA group and the CS− group (post-hoc pairwise comparisons, $P < 0.05$ and < 0.001, respectively). $, $$$Significant difference between the 0.45 mA group and the CS− group (post-hoc pairwise comparisons, $P < 0.05$ and < 0.001, respectively). CS, conditioned stimulus.

After extended alcohol exposure, there was no overall difference between the CS groups, but there was a significant difference in the effect of tone presentation between the CS groups [$F_{(3,28)\ \text{tone}\times\text{CS group}} = 4.4$, $P < 0.05$], which also depended on the interval [$F_{(6,56)\ \text{tone}\times\text{time}\times\text{CS group}} = 6.8$, $P < 0.001$] (Fig. 3b). Post-hoc pairwise comparisons per interval showed suppression of alcohol seeking only during the first CS-ON interval ($P < 0.001$ for the 0.35 and 0.40 mA group; $P < 0.01$ 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 showed a significant effect of tone (ON/OFF) [$F_{(1,28)\ \text{tone}} = 19.0$, $P < 0.001$], which was different between the CS groups [$F_{(3,28)\ \text{CS group}} = 9.9$, $P < 0.001$] and dependent on the interval [$F_{(6,56)\ \text{tone}\times\text{time}\times\text{CS group}} = 3.3$, $P < 0.01$]. Moreover, the effect of tone presentation on the latency to respond during the suppression test was different for rats with a history of limited versus extended alcohol exposure [$F_{(2,56)\ \text{tone}\times\text{time}\times\text{IAA access}} = 7.4$, $P < 0.01$; $F_{(6,56)\ \text{tone}\times\text{time}\times\text{IAA access}\times\text{CS group}} = 3,0$, $P < 0.05$].

Separate analysis of the latency to respond for rats with limited alcohol exposure showed a significant difference between the conditioning groups [$F_{(3,28)\ \text{CS group}} = 20.4$, $P < 0.001$], independent of tone presentation [$F_{(3,28)\ \text{tone}\times\text{CS group}} = 2.6$, NS] or session interval [$F_{(6,56)\ \text{interval}\times\text{CS group}} = 2.0$, NS] (Fig. 3c). Post-hoc pairwise comparisons showed an overall increase in the active response latency in all CS+ groups relative to the CS− group (0.35 mA: $P < 0.001$; 0.40 mA: $P < 0.001$; 0.45 mA: $P < 0.05$).

The latency to the first active response was increased in the conditioned groups during the conditioned suppression test after extended alcohol exposure as evident from an overall effect of the CS group [$F_{(3,28)\ \text{CS group}} = 4.5$, $P < 0.05$], which was dependent on the tone presentation and the session interval [$F_{(3,28)\ \text{tone}\times\text{CS group}} = 10.0$, $P < 0.001$; $F_{(6.56)\ \text{interval}\times\text{CS group}} = 3.6$, $P < 0.01$; $F_{(6,56)\ \text{tone}\times\text{interval}\times\text{CS group}} = 6.0$, $P < 0.001$] (Fig. 3d). Post-hoc pairwise comparisons per interval showed that the active response latency was consistently enhanced during the first CS-ON interval ($P < 0.001$ for all intensities tested) and to a lesser extent also during the second CS-ON interval (0.35 and 0.45 mA, $P < 0.05$).

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 [$F_{(1,24)\ \text{group}} = 139.3$, $P < 0.001$; $F_{(1,24)\ \text{group}} = 127.6$, $P < 0.001$, respectively], which was independent of batch [$F_{(1,24)\ \text{group}\times\text{batch}} = 2.14$, NS; $F_{(1,24)\ \text{group}\times\text{batch}} = 1.54$, NS, respectively] (Fig. 4). Consistent with our previous studies (Spoelder *et al.*, 2015), the augmented alcohol intake when access to alcohol was increased from 7 h/day in the first month to 24 h/day in the second month was more pronounced in HD compared with LD [$F_{(1,24)\ \text{month}\times\text{group}} = 67.9$, $P < 0.001$], which was also comparable for both batches [$F_{(1,24)\ \text{month}\times\text{group}\times\text{batch}} = 0.24$, NS]. Moreover, the preference for alcohol increased with extended access time in HD, but not in LD [$F_{(1,24)\ \text{month}\times\text{group}} = 9.3$, $P < 0.01$], independent of the batch [$F_{(1,24)\ \text{month}\times\text{group}\times\text{batch}} = 1.30$, NS]. There were no differences in alcohol intake and preference between the CS− and the CS+ groups [intake: $F_{(1,24)\ \text{month}\times\text{group}\times\text{CS group}} = 0.51$, NS, $F_{(1,24)\ \text{group}\times\text{CS group}} = 1.9$, NS; preference: $F_{(1,24)\ \text{month}\times\text{group}\times\text{CS group}} = 1.02$, NS, $F_{(1,24)\ \text{group}\times\text{CS group}} = 1.99$, NS]. Figure 4c shows the individual levels of alcohol intake for the selected LD and HD rats.

Analysis of the RI 120 s sessions showed that the HD made more active responses during the first 15 min of the last three RI 120 s sessions before fear conditioning than LD (38.8 ± 3.5 vs. 26.1 ± 2.9; $F_{(1,32)\ \text{group}} = 7.5$, $P < 0.05$). Importantly, there were no differences between CS− and CS+ groups [$F_{(1,32)\ \text{CS group}} = 0.0$, NS; $F_{(1,32)\ \text{group}\times\text{CS group}} = 0.45$, NS] in baseline responding under the RI 120 s schedule of reinforcement (data not shown).

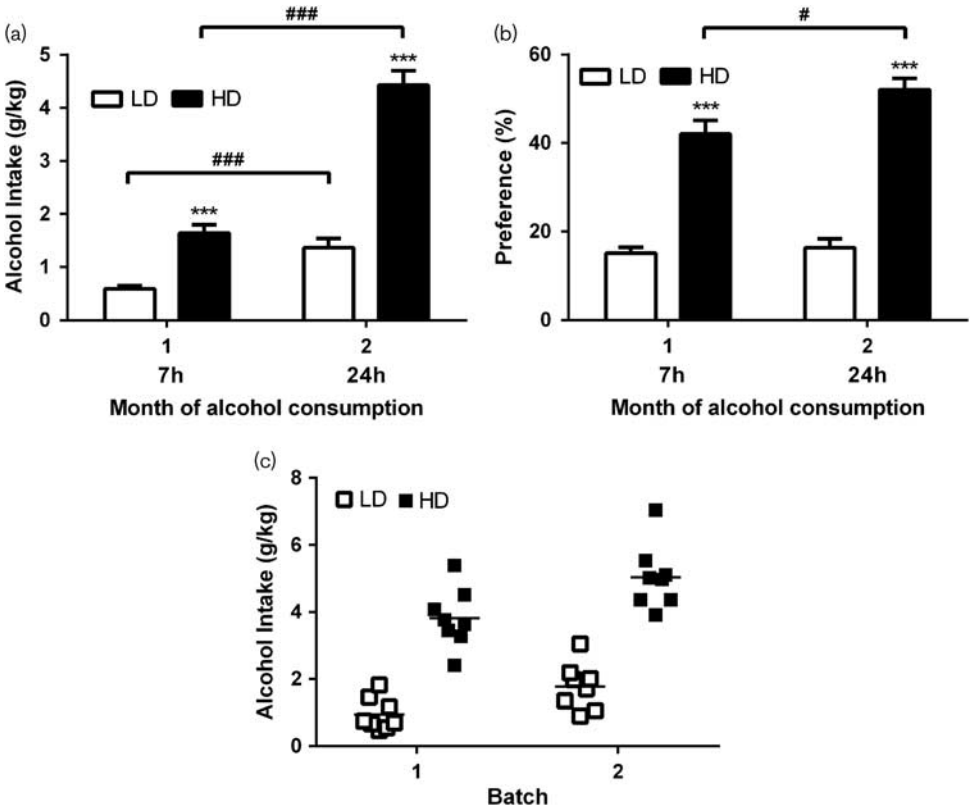
Conditioned suppression of alcohol seeking – active responses

Analysis of the number of active responses during the conditioned suppression test showed a significant difference between the CS groups [$F_{(1,28)\ \text{CS group}} = 5.1$, $P < 0.05$], that depended on presentation of the tone [$F_{(1,28)\ \text{tone}\times\text{CS group}} = 5.8$, $P < 0.05$]. Moreover, there was a significant tone × alcohol drinking group (LD, HD) interaction [$F_{(1,28)\ \text{tone}\times\text{group}} = 5.8$. $P < 0.05$]. Separate analysis of the active responses during the conditioned suppression test for the LD (Fig. 5a) showed a significant effect of fear conditioning on alcohol seeking [$F_{(1,14)\ \text{CS group}} = 7.4$, $P < 0.05$], which was dependent on the interval [$F_{(2,28)\ \text{interval}\times\text{CS group}} = 4.2$, $P < 0.05$]. Post-hoc pairwise comparisons per interval confirmed significant conditioned suppression of alcohol seeking during the first ON and first OFF interval ($P < 0.01$ and < 0.05, respectively) and a near-significant reduction in responding in the CS+ group during the second and third tone-ON intervals ($P = 0.066$ and 0.055, respectively). By contrast, the number of active lever presses was not changed by the CS presentation in HD [$F_{(1,14)\ \text{tone}} = 2.2$, NS; $F_{(1,14)\ \text{tone}\times\text{CS group}} = 3.2$, NS; $F_{(1,14)\ \text{CS group}} = 0.52$] (Fig. 5b).

Conditioned suppression of alcohol seeking – latency to first active response

Analysis of the latency to the first active lever press showed a significant effect of tone presentation

Fig. 4



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 with LD upon the extension of the alcohol access duration from 7 h/day in the first month to 24 h/day in the second month. (b) Alcohol preference was higher in HD and increased in the second month in HD only. (c) Scatter plots showing the individual levels of alcohol intake within the LD and HD groups. Data are presented as mean + SEM.***Significant difference between LD and HD (post-hoc student's *t*-test, $P < 0.001$). #, ###Significant difference between the first month (7 h sessions) and second month (24 h sessions) of alcohol consumption (post-hoc pairwise comparisons, $P < 0.05$ and < 0.001, respectively). HD, high alcohol drinking rats; LD, low alcohol drinking rats.

$[F_{(1,28)\ \text{tone}} = 7.3,\ P < 0.05]$ and an overall increase in the latency to respond for CS+ rats compared with CS− animals $[F_{(1,28)\ \text{CS group}} = 22.8,\ P < 0.001]$. However, there was no effect of group (LD, HD) $[F_{(1,28)\ \text{tone} \times \text{group}} = 0.94$, NS; $F_{(1,28)\ \text{tone} \times \text{group} \times \text{CS group}} = 0.026$, NS; $F_{(1,28)\ \text{group} \times \text{CS group}} = 22.8,\ P < 0.001]$ (Fig. 5c and d).

Conditioned freezing

Analysis of the freezing behavior of the rats during 2 min before (no-tone) and during CS (tone) presentation showed that the CS+ rats spent significantly more time freezing compared with the CS− controls $[F_{(1,28)\ \text{CS group}} = 73.7,\ P < 0.001]$. Moreover, conditioned freezing was augmented upon presentation of the tone $[F_{(1,28)\ \text{tone} \times \text{CS group}} = 18.1,\ P < 0.001]$, but it was not dependent on the alcohol drinking group $[F_{(1,28)\ \text{group} \times \text{CS group}} = 0.29;\ F_{(1,28)\ \text{tone} \times \text{group} \times \text{CS group}} = 0.35$, NS] (Fig. 6).

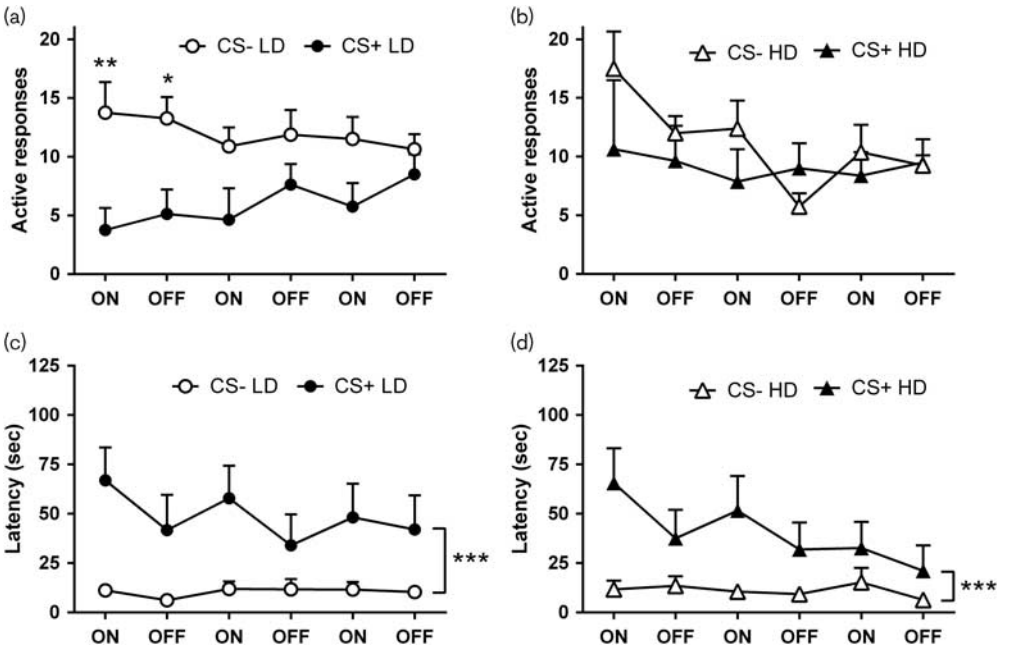
Discussion

In the present study, we investigated the role of prolonged alcohol consumption and individual differences in alcohol drinking in the development of loss of control over alcohol seeking. To this end, we determined 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 selected groups of rats showing high and low levels of alcohol consumption. Consistent with our hypothesis, we observed aversion-resistant alcohol seeking (i) in rats with a protracted alcohol drinking history and (ii) 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 who display high levels of alcohol consumption are therefore at increased risk for AUD, but individuals who 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

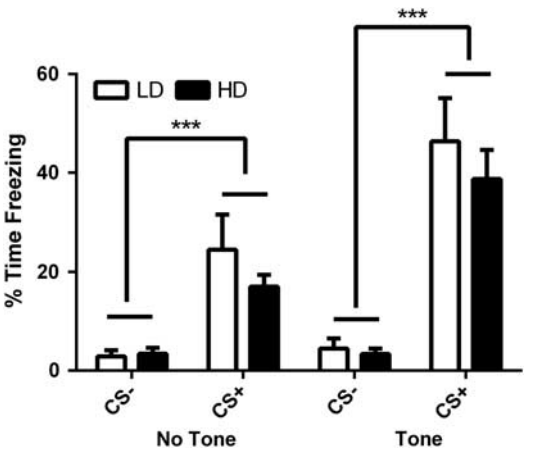
To optimize the assessment of conditioned suppression, we first determined the effects of different footshock

Fig. 5



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 the reduced number of active responses made by the CS+ compared with the CS− group (a). In contrast, the HD show no significant conditioned suppression, that is, 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 compared with their respective CS− controls. Data are presented as mean + SEM.*, **, ***Significant difference between CS− and CS+ groups (post-hoc pairwise comparisons, $P < 0.05$, < 0.01 and < 0.001, respectively). CS, conditioned stimulus; HD, high alcohol drinking rats; LD, low alcohol drinking rats.

Fig. 6



Freezing behavior in LD and HD during the 2 min before (no-tone) and during 2-min presentation of the footshock-associated CS+ (tone) in the conditioning chamber. The LD and HD CS+ groups showed significant context-induced and CS-induced freezing compared with their respective CS−control groups. The fear conditioning test was performed after completion of the conditioned suppression test and a reconditioning session (Fig. 1). Data are presented as mean + SEM. ***Significant difference between CS+ and CS− groups (post-hoc pairwise comparisons, $P < 0.001$). CS, conditioned stimulus; HD, high alcohol drinking rats; LD, low alcohol drinking rats.

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, 0.40 and 0.45 mA) resulted in 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 to be the 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; Piazza and

Deroche-Gamonet, 2013; Vanderschuren and Ahmed, 2013). Indeed, extended cocaine SA 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.*, 2012a) and conditioned suppression of cocaine seeking (Vanderschuren and Everitt, 2004; Limpens *et al.*, 2014a, 2014b). Punishment-induced abstinence of alcohol SA has been described using similar footshock intensities after 3–4 weeks of IAA alcohol consumption (Marchant *et al.*, 2013, 2016). Hopf *et al.* (2010) have shown 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 (Seif *et al.*, 2013). We now 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 they are resistant to presentation of aversive stimuli during alcohol seeking 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 s 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 s 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 (Fig. 5a and 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 behaviorally different expressions of addictive behavior.

A limitation of the present study is that the differences in responding between tone-ON and tone-OFF intervals were less pronounced compared with our previous findings with conditioned suppression of cocaine or sucrose SA (Vanderschuren and Everitt, 2004; Limpens *et al.*, 2014b). This is most likely because of spill-over of the response-suppressant effect of the CS from the CS-ON period to the subsequent CS-OFF period. Although reduced responding for alcohol by the CS+ animals was dependent on the presentation of the tone, suggesting that suppression of responding was stimulus bound, we cannot rule out that the consequences of alcohol exposure reflect general resistance to aversion, rather than conditioned suppression. Another 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 toward 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 with 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 adversity of alcohol seeking after 4 months, compared with 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 substances of abuse, including alcohol, for the transition to full-blown substance use disorder.

Individual differences in alcohol consumption and loss of control

Our recent studies have shown a high degree of individual variability in alcohol consumption in outbred Lister hooded rats (Lesscher *et al.*, 2015; Spoelder *et al.*, 2015), whereby groups of HD and LD can be discerned on the basis of their voluntary alcohol consumption under IAA conditions. In line with our previous findings (Spoelder *et al.*, 2015), the HD make more active responses for alcohol during operant responding, in this case under the RI120 schedule of reinforcement. However, these findings do not transfer to the conditioned suppression test (CS− groups), perhaps because responding was not reinforced during the conditioned suppression test. Importantly, the present findings indicate 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 reduced control over alcohol use. The LD and HD responded equally to both the fear conditioning context and the footshock-associated tone (Vanderschuren and Everitt, 2004), thus ruling out the possibility that the relative resistance to aversive manipulation of alcohol seeking observed in the HD was merely the result of impaired fear conditioning. 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 an unpleasant tactile stimulus (Hopf and Lesscher, 2014). The adverse consequences of human alcohol ingestion often do not directly coincide with actual alcohol consumption. As a result, the relevance of taste aversion resistance for human AUD, where the bad taste of a quinine-adulterated alcohol solution accompanies each drinking bout, has been questioned, although AUD patients are known to ingest non-beverage, taste-aversive alcohol solutions (Soo Hoo *et al.*, 2003; Leon *et al.*, 2007). By contrast, the warning signal in

conditioned suppression, that is, the footshock-associated tone, represents anticipation of adverse consequences that are 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 show 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 characterize AUD.

There is considerable 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 (This 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 just a high degree of alcohol consumption, shown by a subgroup of animals, but also the cumulative degree of exposure to alcohol is an important determinant for the development of AUD. The HD showed aversion-resistant alcohol seeking already after 2 months of alcohol consumption, whereas LD and MD showed considerable suppression of alcohol seeking after 2 months of IAA. However, MD do develop characteristics of addictive behavior after having consumed alcohol for a total duration of 4 months. Together, 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 individual level of alcohol consumption and the duration of alcohol exposure.

Conclusion

Our findings suggest 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. 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; Pierce *et al.*, 2012; van den Brink, 2012). Moreover, the available treatments are directed toward reducing reward or relapse (van den Brink, 2012), but not at restoring control over behavior. The individual variation in alcohol consumption, predicting the degree of aversion resistance of alcohol seeking observed in our studies, provides an important tool to assess the neurobiological mechanisms that determine loss of control over alcohol use, which may contribute toward the development of innovative treatments for AUD and other forms of addiction.

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Conflicts of interest

There are no conflicts of interest.

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