

Giuliano *et al.*

Title:

The novel μ -opioid receptor antagonist GSK1521498 decreases both alcohol seeking and drinking: evidence from a new preclinical model of alcohol seeking

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Running Title:

Cue-maintained alcohol seeking and μ -opioid receptors

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Abstract:

Distinct environmental and conditioned stimuli influencing ethanol-associated appetitive and consummatory behaviors may jointly contribute to alcohol addiction. To develop an effective translational animal model that illuminates this interaction, daily seeking responses, maintained by alcohol-associated conditioned stimuli (CSs), need to be dissociated from alcohol drinking behavior. For this, we established a procedure whereby alcohol seeking maintained by alcohol-associated CS is followed by a period during which rats have the opportunity to drink alcohol. This cue-controlled alcohol seeking procedure was used to compare the effects of naltrexone and GSK1521498, a novel selective μ -opioid receptor antagonist, on both voluntary alcohol-intake and alcohol-seeking behaviors. Re-derived alcohol-preferring, alcohol-nonpreferring and high alcohol-drinking replicate 1 lines of rats (Indiana University, USA) first received 18 sessions of 24-h home-cage access to 10% alcohol and water under a 2-bottle choice procedure. They were trained subsequently to respond instrumentally for access to 15% alcohol under a second-order schedule of reinforcement, in which a prolonged period of alcohol-seeking behavior was maintained by contingent presentations of an alcohol-associated CS acting as a conditioned reinforcer. This seeking period was terminated by 20 min of free alcohol drinking access that achieved significant blood alcohol concentrations. The influence of pre-treatment with either naltrexone (0.1-1-3 mg/kg) or GSK1521498 (0.1-1-3 mg/kg) prior to instrumental sessions was measured on both seeking and drinking behaviors, as well as on drinking in the 2-bottle choice procedure. Naltrexone and GSK1521498 dose-dependently reduced both cue-controlled alcohol-seeking and alcohol-intake in the instrumental context as well as alcohol intake in the choice procedure. However, GSK1521498 showed significantly greater effectiveness than naltrexone, supporting its potential use for promoting abstinence and preventing relapse in alcohol addiction.

Key words:

Addiction, Alcohol seeking, Alcohol-preferring rats, Conditioned stimulus, Naltrexone, μ -opioid receptor antagonist.

Main Text:

Introduction

Alcohol is the most commonly used addictive substance worldwide, and alcohol use disorders place a major socioeconomic and public health burden on modern societies. Some 17.6 million people are estimated to abuse alcohol in US (National Council on Alcoholism and Drug Dependence, Inc), while 1.6 million people are addicted to alcohol in the UK, where alcohol consumption has increased by 9% over the last 3 decades (Alcohol Concern, UK). Despite a major need, there are however few effective pharmacological treatments for alcohol addiction, especially those that promote abstinence and prevent relapse.

Preclinical research on alcohol addiction and associated treatment development strategies have focused almost exclusively on the factors determining the propensity to maintain high levels of alcohol intake, reflecting the importance of intoxication and neuroadaptive changes during protracted withdrawal as the driving force to addiction (Koob, 2013). Commonly used strains of laboratory rats do not readily drink high quantities of alcohol unless sweet taste (e.g. sucrose) fading techniques are used to induce it (e.g. (Samson, 1986; Czachowski and Samson, 2002), or animals are exposed intermittently to alcohol for a prolonged period of time under 2- or 3-bottle choice procedures (Wise, 1973; Simms *et al*, 2008; Carnicella *et al*, 2014), or are first made dependent by exposing them for long periods to alcohol in vapour chambers to induce drinking in withdrawal (Koob, 2013).

A complementary approach is to study rats that have, through selective breeding strategies, a spontaneous propensity to drink and prefer alcohol. Thus, alcohol-preferring (P) and high alcohol-drinking (HAD) lines have been established on the basis of their preference for a 10% alcohol solution over water and their consumption of more than 5 g of

alcohol/kg body weight/day. Both P and HAD lines have been shown to drink ethanol under free-choice conditions (McBride *et al.*, 2014), to be highly motivated for ethanol compared to alcohol-nonpreferring (rNP) rats and to readily acquire instrumental responding for ethanol (Czachowski and Samson, 2002).

However, while alcohol drinking to intoxication is the major behavioral characteristic of those addicted to alcohol, such individuals also crave alcoholic beverages and spend time actively seeking alcohol, as well as drinking compulsively. Effective treatments for alcoholism may therefore not only be those reducing volumes drunk, such as the recently approved nalmefene (Gual *et al.*, 2014), but may also be those that can reduce the craving and alcohol seeking that leads to drinking.

Alcohol-associated conditioned stimuli are known to elicit craving in alcohol-dependent individuals and cue-reactivity procedures have been used to identify relapse prevention treatments (Niaura *et al.*, 1988). The invigorating impact of drug-associated CSs on seeking behavior has been operationalized in models of CS-induced relapse (Ciccocioppo *et al.*, 2002; Gipson *et al.*, 2013; Lee *et al.*, 2006; Marchant *et al.*, 2013) as well as cue-controlled cocaine or heroin seeking behavior (Arroyo *et al.*, 1998; Economidou *et al.*, 2011; Giuliano *et al.*, 2013) and CS-dependent seeking of high incentive foods that is associated with obesity and binge-eating (Giuliano *et al.*, 2012). Although outbred rat strains will respond instrumentally for alcohol (Augier *et al.*, 2014), and alcohol-associated CSs can elicit approach (Tomie and Sharma, 2014) and serve as conditioned reinforcers (Smith *et al.*, 1977; Panlilio *et al.*, 2004; Milton *et al.*, 2012; Rodd *et al.*, 2004), attempts to establish vigorous CS-dependent alcohol seeking that mediate delays to the opportunity to drink have proven difficult, probably because alcohol is an apparently weak reinforcer for rats with little or no propensity to drink.

Here we established a procedure in which P (and NP), and HAD rats seek alcohol for prolonged periods of time during which the response-contingent presentation of an alcohol-associated CS would bridge delays before animals eventually earned the opportunity freely to drink alcohol for 20 minutes (to 'drink at the bar').

Having established this novel CS-controlled alcohol seeking and drinking procedure, we then investigated the effects of a novel, putative treatment for alcohol dependence, GSK1521498. We further compared its effects with those of a clinically approved treatment for alcoholism, naltrexone, that has previously been shown to reduce ethanol drinking (Davidson and Amit, 1997; Froehlich *et al.*, 1990; Henderson-Redmond and Czachowski, 2014) and cue-induced reinstatement (Ciccocioppo *et al.*, 2002) in animal models, as well as to decrease cue-induced craving in human alcohol abusers (Rohsenow *et al.*, 2000). Both compounds target the μ -opioid receptors (MORs), but GSK1521498 is a more selective antagonist at MOR than naltrexone, and unlike naltrexone, has no partial agonist activity (Nathan *et al.*, 2012; Kelly *et al.* 2014) and instead, in some in vitro assays, minor inverse agonist activity (Ignar *et al.* 2011; Kelly *et al.* 2014). The effects of both treatments on CS-controlled alcohol-seeking behavior, alcohol intake during the free-access period earned by rats through making seeking-responses, as well as in a home environment 2-bottle choice procedure, were investigated.

Materials and Methods

(i) Animals

Male rP (~30 days old, n=63), rNP (n=20) and rHAD1 rats (n=20) were obtained from Indiana University Medical Center (Indiana, USA). See **SI**.

Rats from the 1st and the 2nd cohorts were divided into 2 sub-groups: some were exposed to alcohol in a 2-bottle choice procedure (EtOH-experienced) whereas others were screened first for anxiety-like behavior and left in their home cages for the same period of time (EtOH-naïve). All rats then began instrumental training at the same time. rP and rNP rats from the 2nd cohort were first tested on the Elevated Plus Maze (EPM), before undergoing instrumental training and pharmacological treatments under the second-order schedule of reinforcement for 15% EtOH to assess effects on alcohol seeking behavior. The EPM tested the anxiety-like phenotype previously, but variably, shown in P rats (Stewart *et al.*, 1993). Finally, rHAD1 rats from the 2nd cohort and rP rats from the 3rd cohort underwent pharmacological manipulations on the 2-bottle choice procedure (**Table 1a**).

(ii) Alcohol consumption – Two-bottle choice procedure

Animals were presented with either continuous or intermittent concurrent access (depending on the cohort of animals, see **Table 1a**) to 10% EtOH (v/v) and water in their home-cage for a total of 18 sessions. Voluntary consumption was assessed every 24h. See **SI**.

(iii) Elevated Plus-Maze (EPM)

EtOH-naïve animals were assessed for anxiety on the EPM as previously described (Dilleen *et al.*, 2012). See **SI**.

(iv) Alcohol consumption – Instrumental conditioning

(iv.a) Apparatus

Behavioral training was conducted in operant chambers previously described (Giuliano *et al.*, 2013). See **SI**.

(iv.b) Alcohol seeking: Second-order schedule of reinforcement for 15% EtOH

Rats were water-deprived for 22 h/day during the first 5 days of training, which consisted of the following 4 phases.

- i. Rats were initially trained to associate a light stimulus presentation with access to drinking EtOH (5 daily sessions): 1 min-CS was illuminated during 1 min free access to a bottle containing 15% EtOH (v/v) in the operant chamber (Pavlovian conditioning) under a Random Time 60-sec procedure. The rats received in average 38 CS-alcohol pairings in each 45 min-session.
- ii. Rats were then trained to press a lever under a fixed-ratio 1 schedule (FR1) to have free access to 15% EtOH: each active lever press resulted in 20 sec CS illumination above the active lever, retraction of both levers, free access to a 15% EtOH bottle and 20 sec turning off of the house-light. After these 20 sec, the house-light was again illuminated, the alcohol-associated CS was turned off, and the two levers again inserted into the chamber. Rats were limited to a maximum of 45 rewards/2 h-session. Following acquisition of EtOH self-administration (7 daily sessions), a fixed-interval schedule (FI) of reinforcement was introduced.
- iii. The FI increased daily from 1 min to 2-4-6-8-10 min, before stabilizing at FI15 min for 3 consecutive sessions (total 9 daily sessions). The end of each FI was associated with a progressively longer drinking period of CS-paired free access to 15% EtOH: from 1 min under FI1 to 20 min under FI15.
- iv. Subsequently, a second-order schedule of reinforcement was introduced, in which every 10th active lever press during one FI15 resulted in a 1sec-CS presentation [FI15(FR10:S)]. At the end of the FI15 rats earned 20 min of CS-paired free access to 15% EtOH in the operant chamber (minimum 25 daily sessions). Representative values of blood alcohol

concentrations (BACs) following the 20 min-free access to 15% EtOH were measured. See

SI.

After alcohol seeking behaviour had been acquired, dependence of seeking responses on contingent presentations of the alcohol-associated CS was assessed by omitting the CS in some sessions. Since completion of each FR10 in these CS-omission sessions had no consequence (in terms of CS presentation), these were effectively simple FI15 min sessions, as earlier in training. The CS omission sessions were followed by additional sessions in which the 1sec-CS-presentations were again contingent on completion of each FR10, i.e. the FI15(FR10:S) second-order schedule was reinstated.

(v) Drugs

EtOH solutions were prepared by mixing 99.8% EtOH (Sigma, UK) in tap water to obtain 10% EtOH (v/v; 2-bottle choice procedure) or 15% EtOH (v/v; instrumental conditioning).

Naltrexone (Sigma, UK) and GSK1521498 (GlaxoSmithKline, UK) were prepared freshly on each test day following the protocol previously described (Giuliano *et al.*, 2012, 2013). See **SI.**

Statistical analysis

Data were analyzed using repeated measures ANOVA (SPSS 21) with time, schedule, or dose as within-subject factors. Two-tailed values of $p \leq 0.05$ were considered statistically significant. Significant main effects and interactions were analysed further using the Dunnett's *post-hoc* test where appropriate. See **SI.**

Results

Alcohol consumption – Two-bottle choice procedure

When given free choice between 10% EtOH and water, rP and rHAD1, but not rNP, rats displayed a high propensity to drink alcohol and readily developed a marked preference for alcohol. Average intakes from the last 3 of the 18 total sessions in animals from all the cohorts used are presented in **Table 1b**. Alcohol intakes and preferences over 18 sessions in animals from the 1st and the 2nd cohorts are presented in **Figure 1a-1b** and **Figure 2a-2b**, respectively.

rP rats are more anxious than rNP rats on the Elevated Plus-Maze

EtOH-naïve rP rats showed more anxiety-like behavior (i.e. more time in the open arms) on the EPM relative to EtOH-naïve rNP rats, (**Figure 2c-2d**). See **SI**.

Cue-controlled alcohol-seeking behavior under a second-order schedule of 15% EtOH reinforcement

1st cohort: rP rats were divided into 2 sub-groups before being trained in the cue-controlled alcohol seeking task: (i) rP EtOH-experienced rats, exposed to 10% EtOH under the 2-bottle choice procedure; (ii) rP EtOH-naïve rats, screened first for anxiety-like behavior and left in the home-cage for the same period of time on *ad libitum* food and water. All the animals then underwent instrumental seeking training (**Table 1a**).

rP EtOH-experienced rats trained instrumentally to seek 15% EtOH under FI15 made 42 ± 2.87 lever presses in the 15 min alcohol-free period (**Figure 1c**), whereas EtOH-naïve rats which displayed 29 ± 2.38 alcohol-seeking responses in the same 15 min alcohol-free period (**Figure 1d**). When the brief 1 sec-CS presentation contingent on lever pressing was introduced (i.e. a second-order schedule [FI15(FR10:S)]), the alcohol seeking lever presses during the 15 min alcohol-free period increased in EtOH-experienced and EtOH-naïve rats to 126 ± 13.65 (**Figure 1c**) and to 104 ± 17.91 (**Figure 1d**), respectively, during the last three of 12

sessions of training ($p < 0.001$ vs FI15). A further 8 sessions, in which the CS was omitted (in EtOH-experienced subjects only) resulted in a marked decrease in seeking responses during the 15 min alcohol-unaffected interval to 65 ± 4.37 lever presses during the seventh and eighth sessions ($p < 0.01$ vs [FI15(FR10:S)]) (**Figure 1c**). This demonstrates that the vigorous alcohol seeking behavior of EtOH experienced rats depended on contingent presentations of the alcohol CS. This control over alcohol seeking by alcohol CSs was further confirmed by the re-establishment of high levels of seeking responses (105 ± 13.06 lever presses in 15 min ($p < 0.05$ vs FI15 and $p = ns$ vs [FI15(FR10:S)]) on subsequent reintroduction of contingent presentations of the CS in 8 additional sessions ([FI15(FR10:S)]) (overall schedule effect [$F(3,39) = 27.48$; $p < 0.001$]) (**Figure 1c**).

At the end of the alcohol-unaffected seeking interval, under either FI15 or [FI15(FR10:S)], rP rats had earned 20 min-free-drinking access to 15% EtOH in the operant chamber. EtOH-experienced and EtOH-naïve rats consumed 1.62 ± 0.16 g/kg (**Figure 1e**) and 1.09 ± 0.13 g/kg (**Figure 1f**) of alcohol, respectively under the FI15 schedule of reinforcement (i.e. when no CS was presented). Their intake in the 20 minutes following seeking when CSs were presented was 1.54 ± 0.15 g/kg (**Figure 1e**) and 0.99 ± 0.09 g/kg (**Figure 1f**), respectively (EtOH exposure: [$F(1,32) = 20.35$; $p < 0.001$]; schedule \times time \times EtOH exposure [$F(2,64) = 4.34$; $p < 0.05$]; no effect of schedule).

2nd cohort: rP and rNP (but not rHAD1) rats were divided into two subgroups based on their EtOH-experience (**Table 1a**).

rP rats: After concurrent access to 10% EtOH and water for 18 consecutive days in the home-cage (**Figure 2a-2b**), rP EtOH-experienced and rP EtOH-naïve rats trained instrumentally to seek 15% EtOH under FI15 made respectively 44 ± 6.51 (**Figure 2e**) and 41 ± 5.78 (**Figure 2f**) lever presses in 15 min. When the brief 1 sec-CS presentation contingent

on lever pressing was introduced, EtOH-experienced and EtOH-naïve rats increased their lever presses to 96 ± 13.25 (**Figure 2e**) and to 98 ± 13.67 (**Figure 2f**), respectively, in 15 min after 12 sessions of training (scheduleXtime interaction [$F(2,36)=11.91$; $p < 0.001$; no effect of EtOH exposure]. Alcohol intake in the 20 min free-access period under either FI15 or [FI15(FR10:S)] was respectively 0.60 ± 0.06 g/kg and 0.70 ± 0.06 g/kg in EtOH-experienced rats (**Figure 2g**), and it was respectively 0.59 ± 0.07 g/kg and 0.63 ± 0.09 g/kg in EtOH-naïve rats (**Figure 2h**) (no effect of schedule or time).

rNP rats: In marked contrast to rP rats, rNP EtOH-experienced and rNP EtOH-naïve rats made respectively only 9 ± 1.97 (**Figure 2e**) and 14 ± 2.75 (**Figure 2f**) active lever presses in 15 min under FI15. They only increased their responding to respectively 16 ± 4.45 (**Figure 2e**) and 28 ± 7.62 (**Figure 2f**) when the alcohol-associated CS was introduced response-contingently (schedule effect [$F(1,8)=3.35$; ns]). Alcohol intake was very low under both schedules, reaching 0.18 ± 0.05 g/kg under FI15 and 0.17 ± 0.06 under [FI15(FR10:S)] in rNP EtOH-experienced (**Figure 2g**), and 0.21 ± 0.05 g/kg under FI15 and 0.15 ± 0.05 under [FI15(FR10:S)] in rNP EtOH-naïve rats (**Figure 2h**).

rHAD1 rats: Under FI15, rHAD1 rats made 19 ± 3.63 active lever presses (**Figure 2e**) and drank 0.76 ± 0.09 g/kg of EtOH (**Figure 2g**) in the following 20 min drinking period. Once the response-contingent alcohol CS was introduced ([FI15(FR10:S)]), active lever presses increased to 47 ± 6.05 (**Figure 2e**), with an alcohol intake of 0.74 ± 0.07 g/kg in the following 20 min (**Figure 2g**). Their seeking performance was significantly lower than rP rats, despite similar EtOH intake during the 20 min of earned free access (main effect of line [$F(1,18)=10.59$; $p < 0.01$]; timeXscheduleXline interaction [$F(2,36)=5.60$; $p < 0.01$]; no differences in EtOH intake [$F(1,18)=1.26$; ns]).

During the 20 min period of free-access to 15% EtOH in the operant chamber after CS-controlled seeking, the rP and rHAD1 rats consumed rather large amounts of alcohol, reaching BACs in the range 15-79mg% and 11-85mg%, respectively, which were correlated with the amount of alcohol drunk during 20 minutes ($R=0.52$, $p<0.05$ and $R=0.82$, $p<0.01$ for rP and rHAD1 rats, respectively).

Pharmacological manipulations

GSK1521498 decreased both cue-controlled alcohol seeking and alcohol drinking

rP rats: GSK1521498 dose-dependently decreased seeking behavior (rP EtOH-experienced [$F(3,27)=20.98$; $p<0.001$, partial effect size=0.70] (**Figure 3a**); rP-naïve [$F(3,27)=27.60$; $p<0.001$, partial effect size=0.75]) (**Figure 3b**), and alcohol intake during the 20 min free-drinking period (rP EtOH-experienced [$F(3,27)=33.72$; $p<0.001$, partial effect size=0.79] (**Figure 3c**); rP-naïve [$F(3,27)=29.65$; $p<0.001$, partial effect size=0.77]) (**Figure 3d**).

rNP rats: GSK1521498 affected neither seeking behavior [$F(3,24)=1.91$; ns, partial effect size=0.19] (**Figure 3a**) nor alcohol intake [$F(3,24)=2.32$; ns, partial effect size=0.22] in rNP EtOH-experienced rats (**Figure 3c**), but reduced the seeking behavior [$F(3,27)=7.70$; $p<0.01$, partial effect size=0.46] (**Figure 3b**) and alcohol intake [$F(3,27)=9.28$; $p<0.01$, partial effect size=0.51] in rNP-naïve rats (**Figure 3d**).

rHAD1 rats: GSK1521498 reduced both seeking behavior [$F(3,24)=17.21$; $p<0.001$, partial effect size=0.68] (**Figure 3a**) and alcohol intake [$F(3,24)=13.85$; $p<0.001$, partial effect size=0.63] (**Figure 3c**).

Naltrexone decreased both cue-controlled alcohol seeking and alcohol drinking

rP rats: Naltrexone reduced alcohol-seeking behavior (rP EtOH-experienced [F(3,27)=9.02; $p < 0.01$, partial effect size=0.50] (**Figure 3e**); rP EtOH-naïve [F(3,27)=15.81; $p < 0.001$, partial effect size=0.64] (**Figure 3f**), and the 15% EtOH intake during the 20 min drinking period (rP EtOH-experienced [F(3,27)=29.63; $p < 0.001$, partial effect size=0.77] (**Figure 3g**); rP EtOH-naïve [F(3,27)=45.69; $p < 0.001$, partial effect size=0.83] (**Figure 3h**).

rNP rats: Naltrexone reduced the low level of seeking-behavior of rNP rats (rNP EtOH-experienced [F(3,24)=3.95; $p = 0.05$, partial effect size=0.33] (**Figure 3e**); rNP-naïve [F(3,27)=5.28; $p < 0.05$, partial effect size=0.37] (**Figure 3f**), and the low level of alcohol intake (rNP EtOH-experienced [F(3,24)=5.08; $p < 0.05$, partial effect size=0.39] (**Figure 3g**); rNP-naïve [F(3,27)=8.50; $p < 0.01$, partial effect size=0.48] (**Figure 3h**), but *post-hoc* pairwise comparisons revealed no effect of dose in rNP EtOH-experienced rats.

rHAD1 rats: Naltrexone reduced both EtOH seeking [F(3,24)=5.67; $p < 0.05$, partial effect size=0.79] (**Figure 3e**) and alcohol intake [F(3,24)=18.26; $p < 0.001$, partial effect size=0.69] (**Figure 3g**).

GSK1521498 decreased the consumption of EtOH in the 2-bottle choice procedure

rP and rHAD1 rats: GSK1521498 was tested on EtOH consumption in the intermittent access two-bottle choice procedure at 30 min-1h-2h-4h-6h-12h-24h after the beginning of the access period (30 min after GSK1521498 treatment). GSK1521498 dose-dependently reduced 10% EtOH intake during the 24h alcohol vs water access (rP: [F(18,396)=32.61; $p < 0.001$, partial effect size for dose=0.77, partial effect size for doseXtime=0.60] (**Figure 4a-**

4b); rHAD1: [F(18,342)=42.02; $p < 0.001$, partial effect size for dose=0.77, partial effect size for doseXtime=0.69] (**Figure 4e-4f**). See **SI**.

Naltrexone decreased the consumption of EtOH in the 2-bottle choice procedure

rP and rHAD1 rats: The effects of naltrexone on EtOH consumption were investigated at 30 min-1h-2h-4h-6h-12h-24h after the beginning of the access period (10 min after naltrexone SC injection). Naltrexone dose-dependently decreased 10% EtOH intake during the 24h alcohol vs water access (rP: treatmentXtime [F(18,396)=3.88; $p < 0.001$, partial effect size for doseXtime=0.63] (**Figure 4c-4d**); rHAD1: treatment [F(3,57)=14.90; $p < 0.001$, partial effect size=0.44], time [F(6,114)=550.61; $p < 0.001$, partial effect size=0.97], treatmentXtime [F(18,342)=1.45; ns] (**Figure 4g-4h**). See **SI**.

Discussion

Alcohol-preferring, but not -nonpreferring rats, were shown to acquire high levels of CS-controlled alcohol-seeking behavior for 15% EtOH and to achieve blood alcohol concentrations up to 80 mg% in the immediately following 20 min earned drinking periods. Selective antagonism at the MOR by the novel compound GSK1521498 markedly reduced both cue-controlled alcohol seeking and alcohol drinking, effects that were both greater and longer lasting than those of naltrexone.

Cue-controlled alcohol-seeking behavioral procedure

Alcohol-related cues, including sight and smell of alcohol drinks, acquire conditioned incentive properties (Barker and Taylor, 2014; Field and Duka, 2002), can provoke intense

craving in individuals that abuse or have lost control over alcohol (Ludwig, 1986; De Bruijn *et al*, 2004), and this may be a predictor of relapse after abstinence (Schneekloth *et al*, 2012).

The first aim of this study, then, was to develop a procedure to quantify CS-controlled alcohol seeking over protracted periods of time in rats selectively bred for the propensity to drink alcohol. This was achieved by training rats daily to seek alcohol such that instrumental responding was under the control of alcohol-associated CSs that mediated delays to drinking and alcohol reinforcement by acting as conditioned reinforcers. This novel procedure therefore differs from extinction-reinstatement tasks by measuring the daily propensity for CS-elicited and maintained alcohol seeking and drinking, which was only seen in rats predisposed to drink, rather than the reinstatement of instrumental responding after extinction which provides a low response baseline from which to measure CS effects on reinstatement (generally assumed to be a measure of relapse), but without the opportunity to drink (Marchant *et al*, 2013).

Even though a CS-alcohol Pavlovian association can be established in rats not genetically predisposed voluntarily to drink alcohol and this alcohol-associated CS can elicit approach (Tomie and Sharma, 2014), support Pavlovian-to-instrumental transfer (Milton *et al*, 2012), and act as a conditioned reinforcer (Samson *et al*, 2001; Milton *et al*, 2012), the present data show that an alcohol-associated CS cannot sustain prolonged and vigorous periods of alcohol seeking behavior in animals not predisposed to drink alcohol (i.e. in the NP selected line).

The P, NP and HAD rats used here have been bred over many generations as a rodent model to investigate the genetic basis of excessive alcohol consumption by humans (Czachowski and Samson, 2002; McBride *et al*, 2014). These lines have been both behaviorally and neurobiologically characterized, and show phenotypic differences in their

responses to ethanol that may be of relevance to the neurogenetic mechanisms underlying ethanol preference or non-preference (McBride *et al.*, 2014). For example, P rats have a lesser aversive response to ethanol in a conditioned taste aversion procedure (Froehlich *et al.*, 1988) and may show more anxiety-like behavior in an elevated plus maze compared to NP rats (Stewart *et al.*, 1993), the latter observation being confirmed in the rP cohorts studied here, although this high anxiety phenotype is not always observed (Viglinskaya *et al.*, 1995).

In the present study we established an alcohol-seeking procedure in alcohol-preferring rats by adapting the second-order schedule of reinforcement used previously to study not only cocaine and heroin seeking (Arroyo *et al.*, 1998; Schindler *et al.*, 1988; Everitt and Robbins, 2000; Giuliano *et al.*, 2013), but also the seeking of high incentive foods (Giuliano *et al.*, 2012) or sexual rewards (Everitt, 1990), in which seeking was measured prior to unrestrained access to, and consummatory interaction, with these rewards. Similarly here, rats were trained to make instrumental seeking responses over 15 min in order to have access to alcohol for an unrestricted 20 min drinking period in each daily session. The rats were never food deprived, nor did they undergo either sucrose fading or prior induction of dependence. Alcohol seeking was markedly dependent on response-contingent presentations of the alcohol-associated CS, since it decreased following CS omission and increased to pre CS-omission levels following its reintroduction. This effect of CS omission is key evidence that seeking behavior depends brief presentations of the CS that probably has conditioned reinforcing properties (Goldberg *et al.*, 1981; Everitt and Robbins, 2000). It is well-established that response-contingent, brief presentations of a CS paired with a drug reward results in significantly greater control over second-order responding than presentation of CSs explicitly not paired with drug, further supporting the conditioned

reinforcement account (Goldberg *et al.*, 1979). A related approach – i.e. a period of instrumental responding followed by free alcohol drinking - has been used previously (Samson *et al.*, 1999, 2001; Czachowski and Samson, 2002; Henderson-Redmond and Czachowski, 2014), in which seeking behavior measured over repeated extinction sessions (Czachowski and Samson, 1999) was suggested to be elicited by the Pavlovian context in which the rats had been trained (Conklin *et al.*, 2008). By contrast, in the present experiments rats were always trained in the same alcohol-associated context, but the addition of response-contingent CS presentations resulted in more vigorous and sustained seeking responses that were maintained during repeated test sessions and always terminated by the attainment of an alcohol free-drinking period (i.e. the animals were not under extinction conditions), thereby also making a clear distinction between appetitive and consummatory behavior (Czachowski and Samson, 2002; Kaminski *et al.*, 2008; Samson *et al.*, 2001).

Two cohorts of rP and rNP were trained to respond for 15% EtOH under cue-controlled seeking procedure either after having been allowed previously to drink 10% EtOH in a 2-bottle choice procedure, which confirmed the alcohol-preferring phenotype, or without such experience and therefore being EtOH-naïve at the time of instrumental training. Their seeking performance was very similar regardless of their alcohol drinking history.

We also studied a cohort of rHAD1 rats following confirmation of their high alcohol-drinking phenotype in the 2-bottle choice procedure with 10% EtOH. They showed on initial exposure a higher level of ethanol intake than the rP rats, confirming previous findings (Czachowski and Samson, 2002), however, both lines subsequently showed similar levels of ethanol consumption (~1 g/kg/20 min) during the 20 min drinking period following CS-seeking behavior. However, rP rats that tended to drink less ethanol than rHAD1 rats in our

studies, nevertheless responded more for the opportunity to drink. This observation is consistent with previous findings that P rats emit more appetitive lever press responding under ratio schedules than HAD rats (Czachowski and Samson, 2002). P rats have also been reported to show more activity in locomotor chambers and greater responsivity to a novel odour compared with HAD rats (Nowak *et al.*, 2000), perhaps indicating that P rats display greater exploratory behavior or novelty seeking.

Effects of manipulating the opioid system on alcohol seeking and drinking

Having established this novel cue-controlled alcohol seeking and drinking procedure in alcohol-preferring rats, we investigated the effects on both appetitive and consummatory behavior of manipulating MOR activity. A large body of evidence indicates that alcohol, as well as other rewarding stimuli, may enhance the activity of the endogenous opioid system (Herz, 1997) and MOR levels (Krishnan-Sarin *et al.*, 1998). Moreover, MOR antagonists can decrease drinking in individuals that abuse alcohol (Volpicelli JR *et al.*, 1992; Drobles *et al.*, 2003) and alcohol craving in the presence of alcohol-associated CSs (Monti *et al.*, 1999).

Initially we investigated the effects of naltrexone on alcohol drinking in the 2-bottle choice procedure, as well as on CS-dependent alcohol seeking and on ethanol intake in the free drinking sessions earned by making seeking responses. As expected (Froehlich *et al.*, 1990), pre-treatment with naltrexone dose-dependently reduced voluntary alcohol intake both in the 2-bottle choice procedure and in the instrumental context, but it also decreased alcohol-seeking responses (Czachowski and Delory, 2009; Henderson-Redmond and Czachowski, 2014). However, although used to treat alcoholism (Volpicelli JR *et al.*, 1992), naltrexone is not universally viewed as an effective medication (Rösner *et al.*, 2010) and the recent introduction of nalmefene has been to reduce the volume of alcohol consumed, rather than to maintain abstinence.

We have previously shown significantly greater effects over naltrexone of the novel MOR antagonist GSK1521498, developed for over-eating and substance dependence disorders (Nathan *et al.*, 2012), in reducing highly palatable food-, cocaine- and heroin-seeking behavior in rats (Giuliano *et al.*, 2012, 2013), but has no effect on locomotor activity (**Figure S1**). Treatment with GSK1521498 dose-dependently reduced alcohol-seeking behavior and voluntary alcohol consumption in rP and rHAD1 rats, more effectively than naltrexone and with a longer duration of effect significantly, reflecting its longer half-life. This apparently greater effectiveness of GSK1521498 is also convergent with prior results and is possibly due to the higher selectivity for the MOR (14-20 fold greater over delta and kappa opioid receptors, compared to 4-10 fold selectivity reported for naltrexone) or the 100-fold selectivity for MOR over nociceptin/orphanin receptors (Ignar *et al.*, 2011; Kelly *et al.*, 2014). Moreover, its more complete antagonist profile, and its lesser inverse agonist activity under some assay conditions, compared to the incomplete antagonism and partial agonist activity of naltrexone, also possibly contribute to the different behavioral profile of GSK1521498 (Ignar *et al.*, 2011; Kelly *et al.*, 2014).

Clinical implications

In humans, GSK1521498 has been shown generally to be well tolerated compared with placebo, with no detectable deleterious effects on anxiety, mood or other aspects of hedonic function, or on liver or other blood safety parameters. Importantly, its co-administration with ethanol did not affect its tolerability (Ziauddeen *et al.*, 2013b).

That GSK1521498 treatment reduces the tendency to seek cocaine, heroin, high incentive chocolate and, from the present results, also alcohol in rats with a propensity to drink, suggests effects on a common conditioned incentive motivational system, in addition to its effects to reduce the hedonic impact of the taste of chocolate and alcohol and hence reduce

ingestion. In humans, GSK1521498 also reduced attentional bias to food-related stimuli in obese binge-eaters, for whom the food-cues had higher motivational value (Ziauddeen *et al*, 2013a). However, GSK1521498 did not reduce the attentional bias to alcohol-related cues in healthy social drinkers, for whom the motivational properties of alcohol-cues may have been low (Ziauddeen *et al*, 2013b). Together these observations suggest an important specificity in the behavioral effects of GSK1521498 in individuals having lost control over specific drugs or ingestive rewards. In addition, the present data suggest that administering GSK1521498 in an appropriate therapeutic context should not only decrease the propensity of alcohol dependent patients, including those wanting to maintain abstinence, to seek, or crave alcoholic drinks, especially when elicited by alcohol cues such as those in advertising signs. It may also reduce the volume of alcohol consumed by patients seeking harm reduction treatments, as well as patients relapsing from abstinence. Thus the novel behavioral procedure we have developed for evaluating alcohol seeking and consumption in rats can generate therapeutic predictions that may be important in guiding the clinical development of GSK1521498 as a new treatment for alcohol dependence.

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With this paper we would like to commemorate Daina Economidou who died in December 2012 after heroic fight against lung cancer. The best way to remember her is to

share with the scientific community her dedication and enthusiastic contribution to science through the data collected until a few months before her death, including the data in Figure 1.

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Figure Legends

Figure 1: Voluntary alcohol intake, instrumental conditioning and alcohol seeking characterisation in rP rats (1st cohort)

Rats from the 1st cohort were divided into 2 sub-groups: some were exposed to alcohol in a 2-bottle choice procedure (EtOH-experienced, **on the left**) whereas others stayed in their home-cage for the same period of time (EtOH-naïve, **on the right**). (a) 10% Alcohol intake (g/kg) and (b) percentage of alcohol preference over the total fluid intake (%) were assessed in rP (n=20) rats every 24h using the 2-bottle choice procedure, for 18 continuous days.

All the animals (EtOH-experienced and EtOH-naïve) were then instrumentally trained to seek 15% EtOH. Lever presses (mean \pm SEM) under FI15 (white bars) and under second-order schedule of reinforcement (diagonal bars) are shown in **c** and **d**. Further 8 sessions, in which the CS was omitted (grey bars), were delivered to EtOH-experienced rats to assess the role of the CS as a conditioned reinforcer. Additional sessions under second-order schedule of reinforcement were then delivered to re-establish CS-maintained seeking behavior (**c**). The acquisition of ethanol-seeking behavior in rats with no previous history of ethanol exposure was similar to that of EtOH-experienced rats (scheduleXtimeXEtOH experience interaction [F(2,64)=1.31; ns]; EtOH experience effect [F(1,32)=1.97; ns]). Alcohol intake (g/kg) during the 20 minute drinking period earned by responding during the FI15 schedule (white bars) and the second-order schedule of reinforcement (diagonal bars) are shown in **e** and **f**. EtOH intake in rP EtOH-naïve rats was overall less than in rP EtOH-experienced rats (scheduleXtimeXEtOH exposure interaction [F(2,26)=4.34; p<0.05]; EtOH experience effect [F(1,32)=20.35; p<0.001]).

Figure 2: Voluntary alcohol intake, instrumental conditioning and alcohol seeking characterisation in rP, rNP and rHAD1 rats (2nd cohort)

Rats from the 2nd cohort were divided into 2 sub-groups: some were exposed to alcohol in a 2-bottle choice procedure (EtOH-experienced, **on the left**) whereas others were screened for anxiety-like profile and stayed in their home-cage for the same period of time (EtOH-naïve, **on the right**). (a) 10% Alcohol intake (g/kg) and (b) percentage of alcohol preference over the total fluid intake (%) were assessed every 24h using the 2-bottle choice procedure, for 18 continuous days, in rP (n=20), rNP (n=10) and rHAD1 (n=18) EtOH-

experienced rats. In the meantime, EtOH-naïve rats (n=10 rP rats; n=10 rNP rats) were assessed for anxiety-like behavior on the EPM. The % of time spent in the open arms (% time OA/(OA+CA), mean \pm SEM) and % of open arm entries (% entries OA/(OA+CA), mean \pm SEM) are shown in **c** and **d**, respectively. All the animals (EtOH-experienced and EtOH-naïve) were then instrumentally trained to seek 15% EtOH. Lever presses (mean \pm SEM) under FI15 (white bars) and under second-order schedule of reinforcement (diagonal bars) are shown in **e** and in **f** in EtOH-experienced and EtOH-naïve rats, respectively. Alcohol intake (g/kg) during the 20 minute drinking period earned by responding during the FI15 schedule (white bars) and the second-order schedule of reinforcement (diagonal bars) are shown in **g** and in **h** in EtOH-experienced and EtOH-naïve rats, respectively.

EtOH-naïve rP rats were no different to EtOH-experienced rats in their acquisition of alcohol seeking (scheduleXtimeXEtOH experience interaction [F(2,36)=0.41; ns]; EtOH experience effect [F(1,18)=0.03; ns]).

Figure 3: GSK1521498 and naltrexone decreased EtOH seeking and voluntary consumption

Effects of GSK1521498 (1st and 2nd rows) and effects of naltrexone (3rd and 4th rows) on alcohol seeking and taking under a second-order schedule of reinforcement in EtOH-experienced rats (n=10 rP, n=9 rNP, n=9 rHAD1), **on the left**, and in EtOH-naïve rats (n=10 rP, n=9 rNP), **on the right**, are shown. Number of active lever presses (**a**, **b**, **e**, **f**) and alcohol intake (g/kg) during the 20 minute drinking period earned by responding for alcohol-associated CS presentation (**c**, **d**, **g**, **h**) are presented.

Both GSK1521498 and naltrexone significantly reduced seeking behavior and alcohol drinking in rP rats regardless of their alcohol-experience (naltrexone: doseXalcohol experience [F(3,57)=0.27; ns]; GSK1521498: doseXalcohol experience [F(3,54)=0.69; ns]).

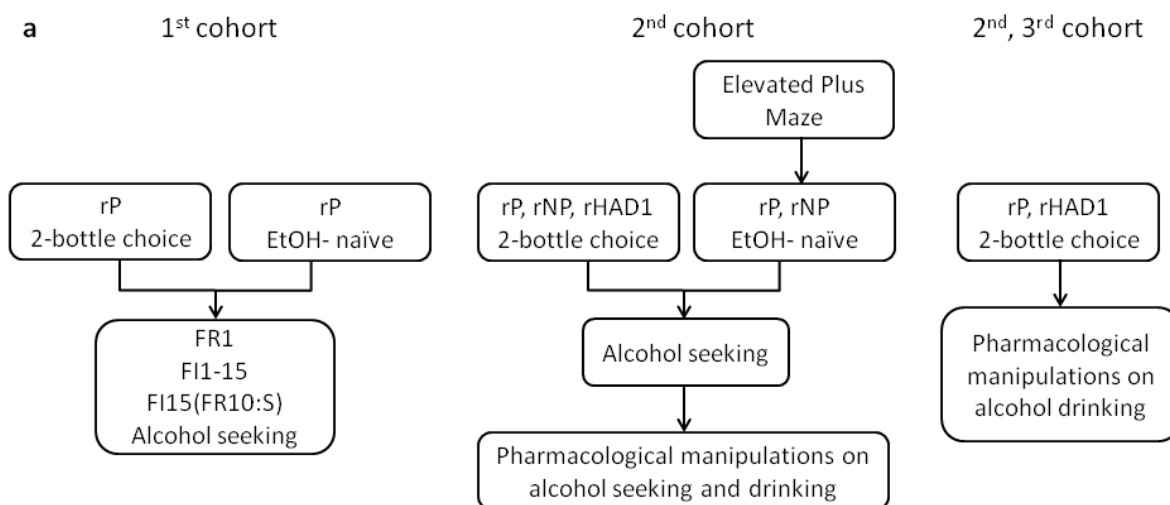
The data are expressed as mean \pm SEM. *p<0.05, **p<0.01, ***p<0.001 compared with vehicle treated animals.

Figure 4: GSK1521498 and naltrexone decreased EtOH intake in the 2-bottle choice procedure

On the top: Effects of GSK1521498 (1st row, **a** and **b**) and naltrexone (2nd row, **c** and **d**) on alcohol (**a**, **c**) and water (**b**, **d**) intake in the 2-bottle choice procedure in rP rats (n=23).

On the bottom: Effects of GSK1521498 (3rd row, **e** and **f**) and naltrexone (4th row, **g** and **h**) on alcohol (**e, g**) and water (**f, h**) intake in the 2-bottle choice procedure in rHAD1 rats (n=23). Alcohol and water intake were assessed at different time points: 30 min, 1h, 2h, 4h, 6h, 12h and 24h after the beginning of the access period and 30 min, or 10 min, after GSK1521498 or naltrexone treatment, respectively. The data are expressed as mean \pm SEM. *p<0.05, **p<0.01, ***p<0.001 compared with vehicle treated animals at each time point, following repeated measures ANOVA per each time point.

Tables:



b

| | | Alcohol intake (g/kg/24h) | Alcohol preference (%) |
|--------------------------------|-------------------------------|---------------------------|------------------------|
| Continuous 10%EtOH access | 1 st cohort: rP | 5.08 ± 0.32 | 82.27 ± 3.10 |
| | 2 nd cohort: rP | 3.52 ± 0.29 | 68.02 ± 4.96 |
| | 2 nd cohort: rNP | 0.01 ± 0.01 | 0.09 ± 0.09 |
| | 2 nd cohort: rHAD1 | 6.58 ± 0.65 | 78.50 ± 5.31 |
| Intermittent 10%EtOH access | 2 nd cohort: rHAD1 | 5.12 ± 0.20 | 95.15 ± 1.58 |
| | 3 rd cohort: rP | 5.78 ± 0.25 | 95.69 ± 1.12 |

Table 1:

a) Schematic representation of the experimental design

Rats from 3 different cohorts were used. Those from the 1st and the 2nd cohorts were divided into 2 sub-groups: some were exposed to alcohol in a 2-bottle choice procedure (EtOH-experienced) whereas others stayed in their home-cage for the same period of time (EtOH-naïve). All the rats started the instrumental training at the same time. rP and rNP rats from the 2nd cohort were tested on the EPM, before undergoing the instrumental training and pharmacological manipulations on the seeking-behavior under the second-order schedule of reinforcement for 15% EtOH. Finally, rHAD1 rats from the 2nd cohort and rP rats from the 3rd cohort underwent pharmacological manipulations on the 2-bottle choice procedure.

b) Summary of alcohol intakes under 2-bottle choice procedure

Average alcohol intakes (g/kg) and alcohol preferences over the total fluid intake/24h (%) from the last 3 of the 18 sessions of 2-bottle choice procedure in the several cohorts of animals tested are shown: the 1st cohort consisted of rP rats, the 2nd cohort consisted of rP, rNP and rHAD1 and the 3rd cohort consisted of additional rP rats. The data are expressed as mean \pm SEM.