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RESEARCH PAPER

Cue-conditioned alcohol seeking in rats following abstinence: involvement of metabotropic glutamate 5 receptors

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Background and purpose: The current study was designed to: (i) examine whether functional interactions occur between receptors known to regulate alcohol self-administration; and (ii) characterize relapse to alcohol seeking following abstinence.
Experimental approach: The selective cannabinoid CB_1 receptor antagonist SR141716A (0.03–1.0 mg·kg^{-1} i.p.) resulted in a dose-dependent reduction in ethanol self-administration in ethanol-preferring Indiana-preferring rats. SR141716A was then co-administered with either the selective glutamate metabotropic glutamate 5 ($mGlu_5$) receptor antagonist 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) or the selective adenosine A_{2A} receptor antagonist SCH58261.
Key results: When administered at individually sub-threshold doses, a combination of SR141716A (0.1 mg·kg^{-1}) and SCH58261 (0.5 mg·kg^{-1} i.p.) produced a reduction (28%) in ethanol self-administration. Combinations of threshold doses of SR141716A (0.3 mg·kg^{-1}) and SCH58261 (2.0 mg·kg^{-1}, i.p.) caused an essentially additive reduction (68%) in alcohol self-administration. A combination of individually sub-threshold doses of CB_1 and $mGlu_5$ receptor antagonists did not affect alcohol self-administration; however, combined threshold doses of SR141716A (0.3 mg·kg^{-1}) and MTEP (1.0 mg·kg^{-1} i.p.) did reduce ethanol self-administration markedly (80%). Cue-conditioned alcohol seeking was attenuated by pretreatment with MTEP (1.0 mg·kg^{-1}) co-administered with SR141716A (0.3 mg·kg^{-1} i.p.). In contrast, SCH58261 (2.0 mg·kg^{-1}) co-administered with SR141716A (0.3 mg·kg^{-1} i.p.) did not reduce cue-conditioned alcohol seeking.
Conclusions and implications: Adenosine A_{2A} and cannabinoid CB_1 receptors regulated alcohol self-administration additively, but combined low-dose antagonism of these receptors did not prevent cue-conditioned alcohol seeking after abstinence. In contrast, combined low-dose antagonism of $mGlu_5$ and CB_1 receptors did prevent relapse-like alcohol seeking after abstinence, suggesting a prominent role for $mGlu_5$ receptors in this paradigm.
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Keywords: alcohol; relapse; glutamate $mGlu_5$ receptor; cannabinoid CB_1 receptor; adenosine A_{2A} receptor

Abbreviations: AGS3, activator of G protein signalling 3; CS+, conditioned stimulus; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; DMSO, dimethyl sulphoxide; ERK, extracellular signal-regulated kinase; iP, Indiana-preferring rat; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; MTEP, 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine; NAcc, nucleus accumbens; NMDA, *N*-methyl-D-aspartic acid; PKA, protein kinase A; PKC, protein kinase C; S+, unconditioned stimulus; SCH58261, 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine; sP, Sardinian alcohol-preferring rat; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; SR147778, 5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl-*N*-(1-piperidinyl)-1H-pyrazole-3-carboxamide; THC, tetrahydrocannabinol

Introduction

Ethanol is the second most commonly abused psychotropic drug after caffeine (Nevo and Hamon, 1995). The consequence of alcohol abuse is far more serious, however, resulting in significant social and economic costs. The World Health Organization estimates that 2 billion people consume alcohol, and of those 76.3 million have diagnosable alcohol use disorders. In 1998, the estimated cost of alcohol abuse to the United States was $184.6 billion (World Health Organization, 2004). Due to the polymodal nature of alcohol addiction, relapse rates remain high under treatment (Graham

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et al., 2002; Heidbreder, 2005), and further investigation into the pathophysiology of the disease is required. Early theories on the mechanism of action of ethanol suggested that membrane lipids were a target, but more recent research refutes this simple view, with enzymes, receptors and various ion channels shown to be targets for ethanol (see Vengeliene *et al.*, 2008).

The metabotropic glutamate 5 ($mGlu_5$) receptor (nomenclature follows Alexander *et al.*, 2008) is a member of the seven-transmembrane, G protein-coupled receptor family. High levels of mRNA encoding the $mGlu_5$ receptor and associated protein are found within the olfactory tubercle and bulb, nucleus accumbens, caudate–putamen, lateral septum, hippocampus and cortex (Romano *et al.*, 1995; Sahara *et al.*, 2001). The $mGlu_5$ receptor is positively coupled to adenylate cyclase through G_s/G_{olf} proteins, and has been associated with phosphoinositide hydrolysis and activation of phospholipase C (Conn and Pin, 1997; Hermans and Challiss, 2001).

The $mGlu_5$ receptor appears to be involved in the reinforcing properties of a number of drugs of abuse. Concerning ethanol, the $mGlu_5$ receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) decreased consumption in mice (Olive *et al.*, 2005) and rats (McMillen *et al.*, 2005), and operant self-administration in mice (Hodge *et al.*, 2006) and rats (Schroeder *et al.*, 2005). MPEP also prevented the reinstatement of ethanol-seeking behaviour induced by olfactory cues (Backstrom *et al.*, 2004) or repeated deprivations (Schroeder *et al.*, 2005) in rats. Administration of 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP), a $mGlu_5$ receptor antagonist with greater selectivity and bioavailability than MPEP (Anderson *et al.*, 2002; Cosford *et al.*, 2003), decreased ethanol self-administration in two strains of alcohol-preferring rats (Cowen *et al.*, 2005b) and reduced both consummatory and appetitive responding for ethanol in C57/BL6J mice (Cowen *et al.*, 2007). $mGlu_5$ receptor-deficient mice consume less ethanol than their wild-type littermates, in a two-bottle free-choice paradigm, and are more susceptible to the hypnotic effects of ethanol (Bird *et al.*, 2008).

The adenosine A_{2A} receptor belongs to a family of four G protein-coupled adenosine receptors that are widely distributed throughout the body, with particularly strong expression in the basal ganglia (Fredholm *et al.*, 2000; Yaar *et al.*, 2005). Adenosine A_{2A} receptors are localized pre-synaptically on glutamatergic afferents from the prefrontal cortex, as well as post-synaptically on enkephalin/dopamine D_2 receptor expressing GABAergic medium spiny neurones, where they appear to modulate cortico-limbic-striatal glutamatergic neurotransmission (Schiffmann *et al.*, 2007). The A_{2A} receptor is positively coupled to adenylate cyclase via G_s or G_{olf} proteins, and, upon activation, elevates cAMP and intracellular calcium levels (Ongini and Fredholm, 1996; Kull *et al.*, 1999; Fredholm *et al.*, 2000; Yaar *et al.*, 2005).

Worldwide, the most commonly used psychoactive drug is caffeine, a non-selective adenosine receptor antagonist. The adenosine A_{2A} receptor in particular appears to be involved in the reinforcing properties of ethanol and other drugs of abuse (Brown and Short, 2008; Castane *et al.*, 2008; Brown *et al.*, 2009). Adenosine A_{2A} receptor-deficient mice are observed to be less sensitive to the acute intoxicating effects of ethanol (Naassila *et al.*, 2002), and exhibit blunted ethanol withdrawal effects, a phenotype replicated in wild-type mice following treatment with A_{2A} receptor antagonists (El Yacoubi *et al.*, 2001). A_{2A} receptor antagonism also attenuates operant self-administration of ethanol in rats (Arolfo *et al.*, 2004; Thorsell *et al.*, 2007; Adams *et al.*, 2008).

Cannabinoid receptors are also seven-transmembrane-spanning, G protein-coupled receptors. There are two known subtypes, one of which, the CB_1 receptor, is the most abundantly expressed G protein-coupled receptor within the CNS (Solinas *et al.*, 2008). The CB_1 receptor is negatively coupled through G_i/G_o proteins to adenylate cyclase, and positively coupled to mitogen-activated protein kinases (Solinas *et al.*, 2008). The CB_1 receptor antagonist rimonabant (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; SR141716A) inhibits phasic dopamine release in the nucleus accumbens evoked by nicotine, ethanol and cocaine administration in freely moving Sprague-Dawley rats (Cheer *et al.*, 2007) without altering basal dopamine release (Cheer *et al.*, 2004). Considering the effect of many drugs of abuse upon phasic dopamine release, it is logical that the CB_1 receptor is more widely implicated in the reinforcing properties of many drugs of abuse (Cheer *et al.*, 2007).

CB_1 receptor knockout mice display reduced consumption of ethanol in a two-bottle free-choice paradigm (Lallemand and de Witte, 2005; Thanos *et al.*, 2005), a reduction in place preference for ethanol in a conditioned place preference paradigm (Thanos *et al.*, 2005), and impaired neuroadaptation of NMDA and $GABA_A$ receptors following chronic ethanol exposure (Warnault *et al.*, 2007). CB_1 receptor knockout mice also show reduced cocaine self-administration, reduced drug-lever pairing discrimination and a lower progressive ratio breakpoint, results which mirror those obtained when the CB_1 antagonist SR141716A is given to wild-type mice (Soria *et al.*, 2005). Interestingly, however, when CB_1 receptor-deficient mice are administered cocaine, they show similar dopamine release in the nucleus accumbens as their wild-type littermates (Soria *et al.*, 2005), a finding perhaps suggestive of developmental adaptations within the nucleus accumbens.

Another CB_1 receptor antagonist, SR147778, attenuates ethanol acquisition, drinking and the deprivation effect in Sardinian alcohol-preferring (sP) rats in a two-bottle free-choice paradigm (Gessa *et al.*, 2005). sP Rats exhibit greater CB_1 receptor mRNA expression than Wistar rats within the mesocorticolimbic system (Cippitelli *et al.*, 2005), suggesting that the differences observed may correlate with alcohol drinking behaviours. SR147778 significantly reduces ethanol preference and intake in a two-bottle free-choice paradigm (Lallemand and De Witte, 2006), and also self-administration under operant conditions (Economidou *et al.*, 2007) in Wistar rats. Furthermore, SR141716A significantly decreases the motivation to self-administer ethanol, and blocks cue-induced reinstatement in both Wistar (Cippitelli *et al.*, 2005; Economidou *et al.*, 2006) and sP rats (Cippitelli *et al.*, 2005), but does not affect stress-induced reinstatement in Wistar rats (Economidou *et al.*, 2007).

We have previously reported that the selective adenosine A_{2A} receptor antagonist SCH58261 and the selective glutamate $mGlu_5$ receptor antagonist MTEP interact to produce an apparently synergistic reduction in ethanol

self-administration, and in combination completely block cue-induced reinstatement in alcohol-preferring Indiana-preferring rat (iP) rats (Adams *et al.*, 2008). The logic for investigating $mGlu_5$ and A_{2A} receptor interactions arose from the co-localization of the A_{2A} and $mGlu_5$ receptors within the mesocorticolimbic pathway, a circuit intimately involved in natural and drug-induced reward. The existence of heteromeric receptor complexes (Ferre *et al.*, 2002), signal transduction commonalities (Agnati *et al.*, 2003) and other functional evidence (Nishi *et al.*, 2003; Kachroo *et al.*, 2005; Rodrigues *et al.*, 2005) also highlight the need to explore interactions between these receptors in relation to complex behavioural patterns.

Likewise, interactions between the cannabinoid CB_1 receptor and other receptors have been reported. For example, μ-opioid and CB_1 receptors have been found to interact synergistically via a common signal transduction pathway in cultured primary striatal and accumbal neurones, and this phenomenon was regulated by the adenosine A_{2A} receptor (Yao *et al.*, 2006). Reductions in tetrahydrocannabinol-induced rewarding or aversive effects have been found in mice lacking the A_{2A} receptor, which display a normal distribution of the CB_1 receptor (Soria *et al.*, 2004).

Given the ability of the CB_1 receptor to regulate alcohol self-administration, the similarities in the signal transduction pathways of CB_1, $mGlu_5$ and A_{2A} receptors, and the co-localization of the receptors within the mesocorticolimbic pathway, here we seek to extend previous work and examine if interactions exist between A_{2A}, $mGlu_5$ and CB_1 receptors in an operant, ethanol self-administration paradigm, utilizing alcohol-preferring (iP) rats. The choice of cue-conditioned alcohol seeking following abstinence, rather than typical extinction–reinstatement paradigms, is based on the premise that many human alcohol/drug users do not undergo the equivalent of extinction training via rehabilitation programmes. Indeed, it is common for humans with alcohol use disorders to relapse following a period of abstinence (40–60% within months, 70–80% by 1 year; Dawson *et al.* (2007). Consequently, we have established a model to address this often overlooked issue.

Methods

Animals

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Inbred alcohol-preferring (iP) rats were obtained from the breeding colony at the Howard Florey Institute (University of Melbourne). Parental stock had previously been obtained from Professor TK Li (while at Indiana University, Indianapolis, IN, USA). The animals were pair housed with *ad libitum* access to standard rat chow and water, with a 12 h light/dark cycle; lights on 0700 h.

Operant alcohol self-administration

Alcohol-preferring iP rats (n = 30) were trained to self-administer ethanol (10% v/v; Merck Pty Ltd, Victoria, Australia) under operant conditions using a fixed ratio of 3 (FR3) during 20 min sessions as previously described (Cowen *et al.*, 2005a,b; Lawrence *et al.*, 2006; Liang *et al.*, 2006). Operant chambers supplied by Med Associates (St Albans, VT, USA) were employed. Each chamber was housed individually in sound attenuation cubicles, featuring a fan to provide airflow and mask external noise. The chambers were connected to a computer running Med-PC IV software (Med Associates) to record the activity. Availability of ethanol was conditioned by the presence of an olfactory cue (S+; 2 drops of vanilla essence, placed on the bedding of the operant chamber directly under the active lever), plus a 1 s light stimulus (CS+) occurred when FR3 was obtained. Ultimately, the rats were responding for a 10% ethanol solution under a fixed ratio requirement of 3 (FR3) with the presentation of alcohol and water randomized to minimize side preference. For each session, total ethanol and water responses were recorded with each delivery consisting of 100 μL of either water or ethanol solution, and the difference of fluid in the ethanol reservoir between the beginning and end of the session was also recorded to ensure the correct calibration of the delivery system. Following acquisition of lever-pressing behaviour and stable alcohol self-administration (<10% variation across sessions, 10% ethanol v/v), the rats were given the adenosine A_{2A} receptor antagonist SCH58261 (5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine, Sigma, St Louis, MO, USA; 1 and 2 $mg \cdot kg^{-1}$ i.p. and co-administered as detailed) suspended in methyl cellulose 30 min prior to the beginning of operant sessions. The $mGlu_5$ receptor antagonist MTEP (Ascent Scientific, N. Somerset, UK; 0.25 and 1 $mg \cdot kg^{-1}$ i.p.) was dissolved in 1% dimethyl sulphoxide (DMSO) as previously described (Cowen *et al.*, 2005b; 2007; Adams *et al.*, 2008) and administered 20 min prior to the operant session. The CB_1 receptor antagonist SR141716A (Sanofi Synthelabo Recherche, Montpelier, France; 0.03, 0.1, 0.3 and 1.0 $mg \cdot kg^{-1}$ i.p. and co-administered as detailed) was dissolved in Tween 80/saline, and administered 20 min prior to the operant session. Administration of MTEP with either SCH58261 or SR141716A involved two injections both at 0.5 $mL \cdot kg^{-1}$ at the time-points mentioned; vehicle controls were delivered in the same way. Drug administration weeks were structured so that Mondays and Fridays were no injection days; vehicle was injected either Tuesday or Wednesday (i.p.) followed by drug the following day (i.p.).

Cue-conditioned alcohol seeking

Following standard operant training as detailed earlier, iP rats (n = 30) were returned to their home cages for a period of 4 weeks. Alcohol seeking was then triggered by replacing S+ (i.e. the olfactory cue) under the 'active' lever and also reprogramming the software such that the stimulus light (CS+) was activated (for 1 s) after every FR3 response, although there was no delivery of ethanol into the receptacle. Prior to the session, the rats were treated with either vehicle or a combination of SCH58261 (0.5 $mg \cdot kg^{-1}$ i.p. 30 min prior) and SR141716A (0.1 $mg \cdot kg^{-1}$ i.p. 20 min prior) or MTEP (0.25 $mg \cdot kg^{-1}$ i.p. 20 min prior) and SR141716A (n = 10 per treatment).

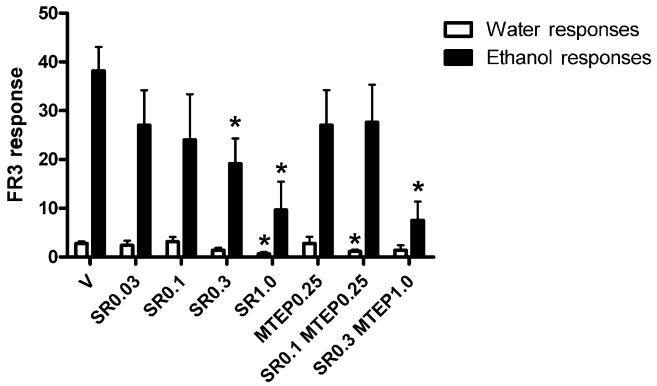

Figure 1 The effect of SR141716A and MTEP on operant ethanol self-administration in iP rats ($n = 10$). Ethanol self-administration was significantly reduced by SR141716A administration at 1.0 mg·kg^{-1} ($P = 0.003$) and 0.3 mg·kg^{-1} ($P = 0.006$) i.p. When co-administered at a low dose, SR141716A and MTEP had no effect on ethanol self-administration at 0.1 and 0.25 mg·kg^{-1}, respectively, but did significantly reduce ethanol self-administration when co-administered at 0.3 and 1 mg·kg^{-1} i.p. ($P = 0.001$). Water responses were significantly reduced by SR141716A at 1 mg·kg^{-1} ($P = 0.009$) and SR141716A, and MTEP co-administered at 0.1 and 0.25 mg·kg^{-1} i.p. ($P = 0.011$). V, Vehicle; SR0.1 or 0.3, SR141716A at 0.1 or 0.3 mg·kg^{-1} i.p.; MTEP 0.25 or 1.0, MTEP at 0.25 or 1.0 mg·kg^{-1} i.p. *Significantly different to vehicle.

Statistics

Statistical analysis was performed with SigmaStat (version 3; SPSS Inc., Chicago, IL, USA). The data are presented as mean ± SEM. A significance level of $P = 0.05$ was used. In general, session totals and time-courses were analysed using a repeated-measures two-way analysis of variance (ANOVA) with Student–Newman–Keuls *post hoc* tests. For every dose of drug, there is a corresponding vehicle injection, thus the factors for the session totals were treatment versus drug/vehicle; for the time-course analysis, the factors were drug/vehicle versus time-point. The effect of 0.5 mg·kg^{-1} i.p. SCH58261 was also examined using a paired *t*-test. Vehicle injections on each day were not significantly different for ethanol or water lever presses (as examined via a paired *t*-test), and were therefore pooled for graphical representation. The resultant data were analysed using a one-way ANOVA with a Student–Newman–Keuls *post hoc* test.

Results

Effect of SR141716A on operant responding for ethanol

We have previously published dose–response curves demonstrating the effect of MTEP and SCH58261 upon ethanol self-administration in iP rats (Cowen *et al.*, 2005b; Adams *et al.*, 2008). Therefore, we first conducted a dose–response curve for the selective CB_1 antagonist SR141716A. A cohort of 10 iP rats responding stably and preferentially for 10% v/v ethanol (38.7 ± 11.3 responses representing 0.6 ± 0.1 g·kg^{-1} ethanol per 20 min session) compared with water (3.8 ± 1.0 responses) were treated with either vehicle or SR141716A (Figure 1). A significant effect of treatment occurred [treatment: $F_{(6,42)} = 1.095$, $P = 0.381$; drug vs. vehicle $F_{(1,7)} = 7.858$, $P = 0.026$] indicating that SR141716A significantly reduced ethanol self-administration at 1.0 mg·kg^{-1} i.p. (–75%, $P = 0.003$) and 0.3 mg·kg^{-1} i.p. (–50%, $P = 0.006$). SR141716A at 1.0 mg·kg^{-1} i.p. also significantly attenuated water responding ($P = 0.009$), but 0.3 mg·kg^{-1} was without effect on water responding. No significant effects on self-administration of ethanol or water were noted when SR141716A was administered at 0.1 or 0.03 mg·kg^{-1} i.p.

Effect of MTEP and SR141716A on operant responding for ethanol

From previously published experiments, the highest sub-threshold dose of MTEP in iP rats was 0.25 mg·kg^{-1} i.p. (Adams *et al.*, 2008). This was repeated in the current cohort of iP rats to confirm no significant reduction in ethanol self-administration (Figure 1, $P > 0.05$). When co-administered at individually sub-threshold doses, SR141716A (0.1 mg·kg^{-1}) and MTEP (0.25 mg·kg^{-1} i.p.) produced no reduction in ethanol self-administration; however, a small, but significant, reduction in water responding was observed ($P = 0.011$). When co-administered at individually threshold doses, SR141716A (0.3 mg·kg^{-1}) and MTEP (1.0 mg·kg^{-1} i.p.) in combination produced a significant reduction in ethanol self-administration (–80%, $P = 0.001$). It should be noted that MTEP administered alone (1.0 mg·kg^{-1} i.p.) in a previous study with similar protocols and the same strain of rat significantly reduced ethanol (~ –55%), but not water self-administration (Cowen *et al.*, 2005b).

Effect of SCH58261 and SR141716A on operant responding for ethanol

From previously published experiments, the highest sub-threshold dose of SCH58261 in iP rats was 0.5 mg·kg^{-1} i.p. (Adams *et al.*, 2008). This was repeated in a cohort of iP rats with no significant reduction in ethanol self-administration (Figure 2). SR141716A (0.1 mg·kg^{-1} i.p.) was also administered to this cohort to reverify that this dose was sub-threshold for alcohol self-administration (Figure 2).

When co-administered at the highest sub-threshold doses, SR141716A (0.1 mg·kg^{-1}) and SCH58261 (0.5 mg·kg^{-1} i.p.) produced a significant reduction in ethanol self-administration (–28%, $P = 0.030$, Figure 2). When co-administered at threshold doses, SR141716A (0.3 mg·kg^{-1}) and SCH58261 (2.0 mg·kg^{-1} i.p.) together produced a significant reduction in ethanol self-administration (–68%, $P = 0.005$). We have previously shown that SCH58261 (2.0 mg·kg^{-1} i.p.) significantly reduces ethanol self-administration (–47%) in this rat strain using this paradigm (Adams *et al.*, 2008).

Effect of SR141716A/SCH58261 and SR141716A/MTEP on cue-conditioned alcohol seeking

Following a period of withdrawal (4 weeks), the rats were tested for cue-conditioned alcohol seeking under extinction conditions (cues present, but no delivery of ethanol subsequent to lever pressing). Figure 3 shows that under these conditions, iP rats show robust alcohol seeking in this paradigm. MTEP (1.0 mg·kg^{-1}) co-administered with SR141716A

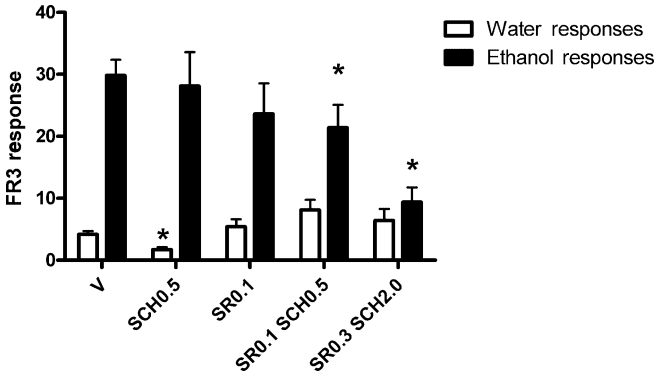

Figure 2 The effect of SCH58261 and SR141716A on operant ethanol self-administration in iP rats (n = 10). Ethanol self-administration was not significantly altered by SCH58261 at 0.5 mg·kg^{-1} i.p., although water responding was reduced (P = 0.035). SR141716A at 0.1 mg·kg^{-1} i.p. did not alter responding. When co-administered, SR141716A and SCH58261 significantly reduced responding for ethanol without affecting responding for water at 0.1 and 0.5 mg·kg^{-1} (P = 0.030), and 0.3 and 2 mg·kg^{-1} (P = 0.005) respectively. V, Vehicle; SCH0.5 or 2, SCH58261 at 0.5 or 2.0 mg·kg^{-1} i.p.; SR0.1 or 0.3, SR141716A at 0.1 or 0.3 mg·kg^{-1} i.p. *Significantly different to vehicle.

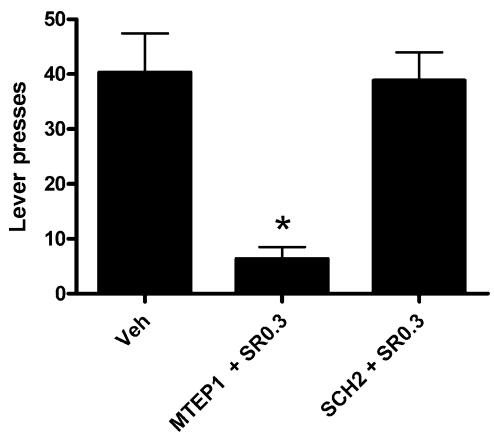

Figure 3 The effect of SCH58261 and MTEP, co-administered with SR141716A, on cue-conditioned alcohol seeking in iP rats (n = 10 per treatment). MTEP (1.0 mg·kg^{-1}) and SR141716A (0.3 mg·kg^{-1}) significantly reduced cue-conditioned alcohol seeking compared with vehicle (P < 0.001). SCH58261 (2.0 mg·kg^{-1}) and SR141716A (0.3 mg·kg^{-1}) co-administered i.p. had no effect on cue-conditioned alcohol seeking. Veh, Vehicle; SCH2.0, SCH58261 at 2.0 mg·kg^{-1} i.p.; SR0.3, SR141716A at 0.3 mg·kg^{-1} i.p.; MTEP1.0, MTEP at 1.0 mg·kg^{-1} i.p. *Significantly different to vehicle (P < 0.001).

(0.3 mg·kg^{-1} i.p.) significantly reduced alcohol seeking (P < 0.001) compared to vehicle-treated iP rats. In contrast, SCH58261 (2 mg·kg^{-1}) co-administered with SR141716A (0.3 mg·kg^{-1} i.p.) did not reduce active lever pressing during relapse-like behaviour (P = 1.0).

Effects of combinations of SCH58261, MTEP and SR141716A on operant ethanol self-administration in iP rats

Figure 4A shows the normalized, synergistic effect of combining individually sub-threshold doses of SCH58261 and MTEP on ethanol self-administration in iP rats (primary data published in Adams *et al.*, 2008). From the present data, it is clear that combining either individually sub-threshold doses of

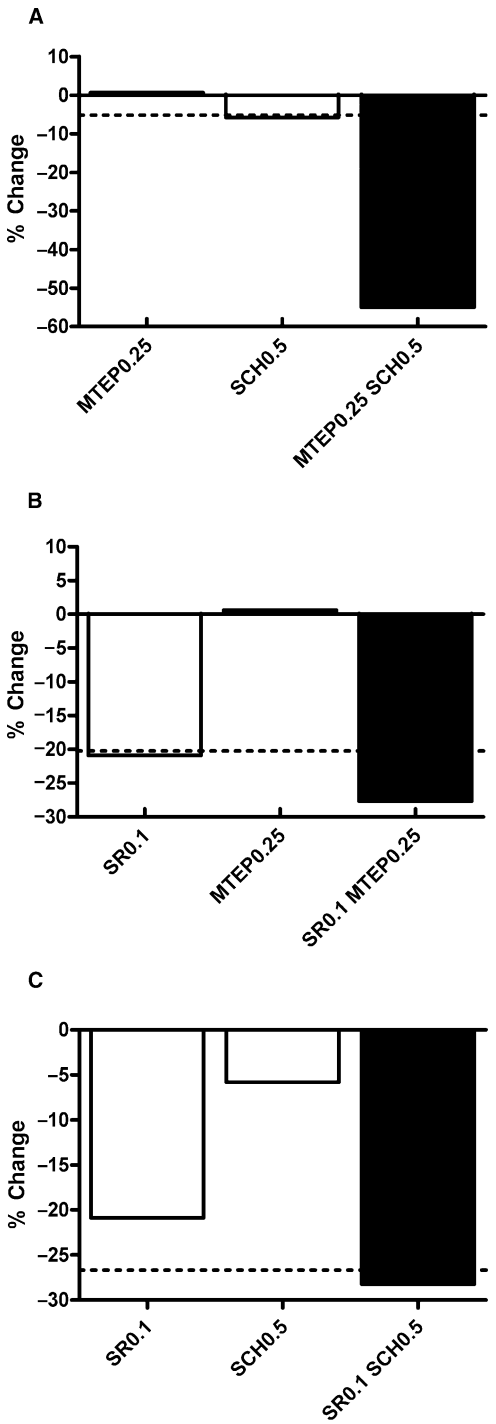

Figure 4 Summary figures describing the effects of SCH58261, MTEP and SR141716A on operant ethanol self-administration in iP rats. Open bars represent the percentage change in deliveries of 10% v/v ethanol on an FR3 schedule. The dotted, horizontal line represents the theoretical additive effect of each combination. The solid bar represents the actual percentage reduction in ethanol self-administration achieved with co-administration. (A) The combination of SCH58261 (0.5 mg·kg^{-1}) and MTEP (0.25 mg·kg^{-1}) injected i.p. produces a synergistic reduction in ethanol self-administration in iP rats. Primary data for Figure 4a have been reported previously (Adams *et al.*, 2008). (B) The combination of SR141716A (0.1 mg·kg^{-1}) and MTEP (0.25 mg·kg^{-1} both i.p.) and (C) the combination of SR141716A (0.1 mg·kg^{-1}) and SCH58261 (0.5 mg·kg^{-1} i.p.) produced essentially additive effects on ethanol self-administration. SCH: SCH58261, SR: SR141716A.

SR141716A (0.1 mg·kg^{-1} i.p.) and MTEP (0.25 mg·kg^{-1} i.p.; Figure 4B), or individually sub-threshold doses of SR141716A (0.1 mg·kg^{-1} i.p.) and SCH58261 (0.5 mg·kg^{-1} i.p.; Figure 4C), produces effects on alcohol self-administration that are essentially additive.

Discussion

Here, we report that combinations of antagonists that target glutamate $mGlu_5$, adenosine A_{2A} and cannabinoid CB_1 receptors can regulate alcohol self-administration and/or seeking following a period of abstinence. We have previously noted that co-administration of low doses of A_{2A} and $mGlu_5$ receptor antagonists results in a dramatic reduction of alcohol self-administration and blocks cue-induced reinstatement following extinction (Adams *et al.*, 2008). We now provide more evidence for the specificity of this $A_{2A}/mGlu_5$ interaction, because essentially additive effects were observed for combinations of A_{2A}/CB_1 or $mGlu_5/CB_1$ receptor antagonists in relation to self-administration of alcohol. Moreover, combined low-dose CB_1/A_{2A} receptor antagonists had no effect on alcohol seeking following a period of abstinence. In contrast, while an $mGlu_5/CB_1$ receptor antagonist combination again suggested a simple additive effect in terms of reducing alcohol self-administration, this combination was able to prevent alcohol seeking following abstinence. Consequently, this study demonstrates that in iP rats, following a period of withdrawal the re-exposure to cues previously associated with the availability of alcohol precipitates a relapse-like response that apparently involves $mGlu_5$-mediated signalling.

At sub-threshold doses, the effect of SR141716A and MTEP in combination produced essentially an additive effect (Figure 4B), suggesting that CB_1 and $mGlu_5$ receptors may not functionally interact in a co-operative or facilitatory manner within this paradigm to reduce operant ethanol self-administration. Although sub-threshold combinations of SR141716A co-administered with SCH58261 i.p. reduced ethanol self-administration, the effect was no greater than additive (Figure 4C), suggesting that CB_1 and adenosine A_{2A} receptors probably work independently within this paradigm.

Striatal cannabinoid CB_1 receptors are localized pre-synaptically on GABAergic terminals of interneurones, collaterals from GABAergic efferent neurones and also on glutamatergic but not dopaminergic terminals (Kofalvi *et al.*, 2005). If CB_1 receptors are found post-synaptically, their population is sparse (for review, see Ferre *et al.*, 2009). Adenosine A_{2A} receptors are localized within the striatum post-synaptically on dopamine D_2 receptor/enkephalin expressing GABAergic medium spiny neurones receiving glutamatergic input, and a smaller population pre-synaptically (Hettinger *et al.*, 2001; Rosin *et al.*, 2003). In the striatum, CB_1 and A_{2A} receptors are co-localized on glutamatergic nerve terminals and in the dendritic spines of GABAergic/enkephalinergic neurons (Shindou *et al.*, 2008). As a heteromer in cell culture, cannabinoid CB_1 receptor function is co-dependent on adenosine A_{2A} receptor function, with activation of the heteromer resulting in G_i protein signalling (Carriba *et al.*, 2007).

$mGlu_5$ receptors are mainly located within the post-synaptic density of glutamatergic synapses (Pin *et al.*, 2003). The anatomical co-localization of the A_{2A} and $mGlu_5$ receptors, and their existence as a heteromer in striatal extracts (Ferre *et al.*, 2002) suggest opportunities for interaction. Receptor heteromer interactions are proposed to occur via various mechanisms. These include direct, protein–protein interactions and intra-membrane lipid and scaffolding protein interactions resulting in allosteric modulation of receptors and subsequent alterations in ligand affinity, and phosphorylation and signal transduction interactions (Franco *et al.*, 2003). One interesting finding from Yao *et al.* (2008) reveals a substantial cross-talk between PKA ($mGlu_5$ and dopamine D_2 receptors) and PKC (cannabinoid CB_1 and adenosine A_{2A} receptors) signalling occurs whereby PKC activation leads to potentiation of G_s receptor signalling. Additionally, the activator of G protein signalling 3 (AGS3) levels within the nucleus accumbens core are significantly elevated following abstinence, knockdown of which normalizes heightened alcohol-seeking responses in rats (Bowers *et al.*, 2008). Further work exploring these interactions within signalling transduction pathways would seem essential.

There is evidence to support the existence of adenosine $A_{2A}/mGlu_5$/dopamine D_2 receptor mosaics. Indeed, A_{2A} or $mGlu_5$ receptor agonists reduce the affinity of dopamine D_2 binding sites, with concurrent stimulation resulting in synergistic interactions for c-*fos* expression, ERK phosphorylation and DARPP-32 (Ferre *et al.*, 2002; Nishi *et al.*, 2003). Functional interactions also occur between A_{2A} and $mGlu_5$ receptor antagonists, which synergistically increase locomotion in reserpinized mice (Coccurello *et al.*, 2004; Kachroo *et al.*, 2005).

The CB_1 receptor, generally located across the synapse from the $A_{2A}/mGlu_5$ receptor complex, has no such opportunity for 'direct' interaction as a mosaic, although the cannabinoid system has been demonstrated to signal via retrograde messaging (Matyas *et al.*, 2006), and so the possibility exists for A_{2A} and/or $mGlu_5$ receptors to modulate the synthesis and/or release of endocannabinoids from medium spiny neurones. Excited cortico-striatal glutamatergic inputs induce retrograde endocannabinoid signalling, which is involved in dopamine D_2 receptor-mediated long-term synaptic plasticity in this region (Giuffrida *et al.*, 1999; Centonze *et al.*, 2004). Ethanol self-administration dose-dependently increases dialysate levels of 2-arachidonoylglycerol within the nucleus accumbens shell of rats (Caille *et al.*, 2007). This plasticity subsequently results in the reduced probability of glutamate release (Choi and Lovinger, 1997), and as previously alluded to, adenosine A_{2A} or $mGlu_5$ receptor antagonists effectively function as positive allosteric modulators of dopamine D_2 receptors within the receptor mosaic. Further work to extend the current experiment into a chronic setting is required to examine striatal plasticity within the context of alcohol studies.

The association of instrumental actions (lever pressing) followed by reward is mediated within the dorsal striatum, as lesions to, or dopamine antagonists infused into this area, abolish this association (Faure *et al.*, 2005; Vanderschuren *et al.*, 2005). Drug-primed reinstatement is associated with glutamatergic inputs into the basal ganglia (Kalivas and McFarland, 2003), and reversible inactivation of the anterior cingulate prevents cue-, foot shock stress- and cocaine-primed reinstatement in rats (McFarland and Kalivas, 2001; See, 2002). In fact, glutamatergic innervation of the accumbens

core via the anterior cingulate is critical for cue-induced reinstatement (Di Ciano and Everitt, 2001). As $mGlu_5$ receptors (Homayoun *et al.*, 2004) can modulate glutamatergic transmission onto medium spiny neurones, it is possible that striatal $mGlu_5$ receptors may play a role in relapse to alcohol seeking following abstinence.

Threshold doses of SCH58261 (2.0 $mg \cdot kg^{-1}$) and SR141716A (0.3 $mg \cdot kg^{-1}$) did not block relapse-like alcohol seeking, although SR141716A (0.3 $mg \cdot kg^{-1}$) and MTEP (1.0 $mg \cdot kg^{-1}$ i.p.) did (Figure 3), suggesting that attenuation of relapse in the current paradigm is mediated primarily by the $mGlu_5$ receptor. Importantly, MPEP alone (3.0 $mg \cdot kg^{-1}$ i.p.) reduces cue-induced reinstatement of ethanol-seeking (Backstrom *et al.*, 2004), and MTEP alone (1.0 $mg \cdot kg^{-1}$ i.p.) reduces operant ethanol self-administration (Cowen *et al.*, 2005b). MPEP blocks nicotine-induced drug-seeking behaviour and reinstatement in Wistar rats (Tessari *et al.*, 2004; Bespalov *et al.*, 2005), and reduces incentive motivational properties of nicotine, cocaine and food (Paterson and Markou, 2005). While SR141716A can prevent reinstatement of alcohol seeking, this typically occurs at doses of 1 $mg \cdot kg^{-1}$ or greater, depending on rat strain (Cippitelli *et al.*, 2005). In the present study, we used SR141716A at a dose of 0.3 $mg \cdot kg^{-1}$ during drug combination trials, which in combination with MTEP (1.0 $mg \cdot kg^{-1}$) did prevent relapse, although the same dose of SR141716A in combination with SCH58261 (2.0 $mg \cdot kg^{-1}$) had no impact on alcohol seeking. Higher doses of SR141716A were confounded by altered water responding, and therefore not pursued further. Therefore, while cannabinoid CB_1 receptors and adenosine A_{2A} receptors can regulate drug seeking, it is likely that under the conditions employed in the present study, the $mGlu_5$ receptor plays a more relevant role in alcohol seeking following abstinence. It would also appear that $mGlu_5$ receptors do not synergistically interact with CB_1 receptors in this context, perhaps due to the subcellular localization of the receptor types.

One explanation as to why CB_1–$mGlu_5$ receptor antagonists attenuate cue-conditioned alcohol seeking while CB_1–A_{2A} antagonists do not is the $mGlu_5$–NMDA receptor heterodimer, found in medium spiny neurons on the post-synaptic membrane of striatal glutamate terminals (Fuxe *et al.*, 2007). The attenuation of function of this receptor complex would reduce glutamatergic function from the prefrontal cortex, a structure known to modulate reinstatement (Weitlauf and Woodward, 2008), but less involved in self-administration.

There is debate as to the validity of relapse and reinstatement models in animal research. Issues of volition, for example, are difficult to model. A valid model of relapse is significant considering up to 85% of abstinent alcoholics displaying no withdrawal symptoms relapse (Boothby and Doering, 2005). Extinction–reinstatement and cue-conditioned relapse appear to be mediated via differing neural circuitry (Fuchs *et al.*, 2006). Extinction training, perhaps the rodent correlate of human rehabilitation clinic attendance, has questionable construct validity as the vast majority of drug users do not seek help in achieving abstinence (Cunningham, 1999), but rather become 'spontaneously abstinent'. Thus, forced abstinence may be a more accurate model of the typical human experience. Abstinence also induces incubation of craving, suggested to be an important component of the persisting susceptibility for relapse in humans (Grimm *et al.*, 2001). Unlike many studies using a brief extinction protocol, the current study used a 1 month abstinence period as human relapse is an enduring phenomenon (Epstein *et al.*, 2006).

We found evidence for an apparently additive effect between antagonists of cannabinoid CB_1 and either adenosine A_{2A} or glutamate $mGlu_5$ receptors in relation to alcohol self-administration. Combination treatment approaches may potentially reduce doses of individual drugs, and thus minimize off-target effects. We also demonstrate that relapse to alcohol seeking can be precipitated following a period of abstinence, and this appears to be mediated in part by $mGlu_5$ receptors.

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