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Role of $GABA_A$ α5-containing receptors in ethanol reward: The effects of targeted gene deletion, and a selective inverse agonist

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Abstract

$GABA_A$ receptors containing α5 subunits have been suggested to mediate the rewarding effects of ethanol. We tested this hypothesis in mice with deletion of α5 subunits. α5 knockout mice did not differ from wildtypes in operant responding for 10% ethanol/10% sucrose, but responded less for 10% sucrose. The benzodiazepine (BZ) site inverse agonist, Ro 15-4513, has higher affinity for $GABA_A$ receptors containing 5 subunits and dose-dependently (0–27 mg/kg, i.p.) reduced lever pressing for ethanol/sucrose in wildtype mice, but had less effect in knockout mice; lever pressing for sucrose was unaffected. These data suggest that α5 subunits are not essential for ethanol reward, but the reduction of operant responding for ethanol by Ro 15-4513 is mediated by α5-containing $GABA_A$ receptors. In measures of ethanol consumption, α5 knockout mice did not differ from wildtypes at low ethanol concentrations (2–8%), but consumed less ethanol at higher concentrations; these differences were not attributable to increased behavioural disruption of the knockout by ethanol, since no differences were seen in sensitivity to ethanol's sedative or ataxic effects. Ro 15-4513's ability to reduce ethanol consumption was unaffected, suggesting that this effect is not mediated by the α5 subtype. Secondly, we tested the ability of a novel α5-efficacy-selective benzodiazepine receptor ligand, α5IA-II, that possesses greater inverse agonist activity at α5- than at α1-, á2- or α3-containing $GABA_A$ receptors, to influence operant responding. α5IA-II (0.03–3 mg/kg) dose-dependently decreased lever pressing for 10% ethanol, the minimally effective dose of 1 mg/kg, corresponding to over 90% receptor occupancy, but did not affect lever pressing for 4% sucrose. Although inverse agonists acting at α5-containing receptors reduce ethanol self-administration, α5 subunits may not be essential to signaling ethanol reward.

Keywords: Ethanol abuse; Reward; Knockout; Inverse agonist

1. Introduction

Ethanol achieves its effects on the central nervous system through diverse mechanisms, including interaction with several ligand-gated ion channels. A major contribution is played by $GABA_A$ receptors, at which ethanol acts to facilitate chloride flux, resulting in enhanced neuronal inhibition. Compounds acting as inverse agonists at the benzodiazepine (BZ) binding site of $GABA_A$ receptors (i.e. they reduce GABA-stimulated chloride flux, thereby reducing the membrane potential) may antagonize behaviorally sedative effects of ethanol (Bonetti et al., 1988; Dar, 1992; Suzdak et al., 1986; Wood et al., 1989), and it has also been suggested that such compounds may also reduce ethanol's euphoric and rewarding properties (June et al., 1994).

$GABA_A$ receptors are a family of oligomeric proteins that show considerable heterogeneity in their regional distribution and function. It has been suggested that receptors containing the α5 subunit isoform may be particularly important in mediating the rewarding properties of ethanol. This conclusion is predicated on three lines of pharmacological evidence. First, the imidazobenzodiazepine, RY 023 which has 67-fold higher affinity for and is an inverse agonist at recombinant $GABA_A$ receptors made up of β2, γ2 plus α5 subunits compared to the

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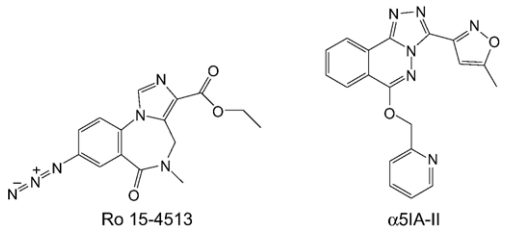
Table 1
Chemical structures of Ro 15-4513 and α5IA-II, and their affinity for human recombinant $GABA_A$ receptors made up from α subunits combined with β3 and γ2 subunits



Binding affinity (Ki nM) at human recombinant $GABA_A$ receptors containing β3, and γ2 subunits plus named α subunit

	α1	α2	α3	α4	α5	α6
Ro 15-4513 [a]	4.8	7.3	2.4	5.1	0.13	6.5
α5IA-II [b]	1.4	2.7	1.4	244	0.8	4410

[a] Data from Hadingham et al. (1995) and Smith et al. (2001).
[b] Data from Street et al. (2004).

analogous receptors containing either an α1, α2 or α3 subunit (Liu et al., 1996, 1995), suppressed operant responding for ethanol reinforcers in the alcohol-preferring rat, when administered into dorsal hippocampal sites (June et al., 2001) rich in α5-containing receptors (Fritschy and Mohler, 1995; Pirker et al., 2000; Wisden et al., 1992). Higher doses of RY023 were required to reduce responding for saccharin, consistent with this latter effect being mediated by other $GABA_A$ receptor subtypes (June et al., 2001). Second, RY 024, which has an α5 binding selectivity (76-fold) comparable to RY 023 but has greater α5 inverse agonism (Liu et al., 1996, 1995), decreases ethanol self-administration in rats when given systemically (McKay et al., 2004). In keeping with a role for α5-containing receptors in ethanol reward, chronic alcohol administration increases expression of α5 mRNA in hippocampus (Charlton et al., 1997; Devaud et al., 1995). Thirdly, the prototypic imidazobenzodiazepine inverse agonist, Ro 15-4513, which has 10–20-fold higher affinity for α5-compared to α1-, α2-, α3-, α4- or α6-containing receptors (Hadingham et al., 1993; Lingford-Hughes et al., 2002; Liu et al., 1996; Smith et al., 2001), has long been known to antagonize the rewarding effects of ethanol in operant self-administration tests (Bao et al., 1992; Glowa et al., 1988; Rassnick et al., 1993; Samson et al., 1989, 1987). The ability of Ro 15-4513 to disrupt responding for ethanol might thus be related to its action at α5-containing receptors.

Nevertheless, the conclusions that can be reached with the hitherto available pharmacological tools are limited. Liu et al. (1996) suggest that both RY 023 and RY 024 can exert actions, presumably inverse agonism, at other $GABA_A$ subtypes, although it should be noted that to date only efficacy data for the α5 subtype has been presented (Liu et al., 1995). Similarly, the fact that Ro 15-4513 is proconvulsant (Lister and Nutt, 1988) and possesses inverse agonism at the α1, α2 and α3 subtypes (Benson et al., 1998; Hadingham et al., 1995; Kelly et al., 2002; Wafford et al., 1993) raises the possibility that the ability of Ro 15-4513 to disrupt ethanol reinforcement may represent actions at other receptor subtypes. An alternative approach to investigating the role of α5-containing subunits in ethanol's effects is to exploit the availability of mice in which the gene encoding subunits have been deleted (Collinson et al., 2002). If α5-containing receptors play an important role in mediating either the reinforcing effects of ethanol, or the ability of drugs like Ro 15-4513 to interfere with such reinforcing effects, then α5 knockout mice should show lower rates of responding for ethanol reward, and should be less sensitive than corresponding wildtype control mice to the disruptive effects of Ro 15-4513. To ensure that the α5 knockout mice were not generally different from wiltypes in the effects of ethanol, the pharmacological effects of ethanol on motor performance and operant performance were compared between the knockout and wildtype mice.

In addition, and as a complement to the knockout mice studies, we have used a novel compound, α5IA-II, which preferentially modulates the functions of α5-containing $GABA_A$ receptors (21). This differs from the α5 binding-selective compounds RY 023, RY 024 and Ro 15-4513 in that α5IA-II

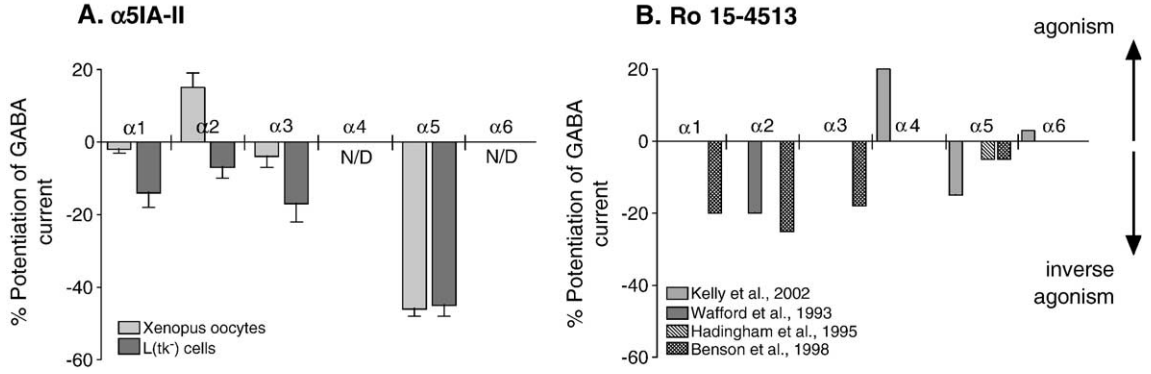


Fig. 1. The efficacy profiles of α5IA-II and Ro 15-4513 in $GABA_A$ receptors containing β, γ2 and α1-6 subunits. A. Efficacy of α5IA-II was measured in either *Xenopus* oocytes or mouse fibroblast L(tk⁻) cells expressing human recombinant $GABA_A$ receptors. In both systems, α5IA-II decreased the GABA EC_{20}-induced currents to a much greater extent (i.e. had greater inverse agonism) at the α5 relative to α1, α2 or α3 subtypes (data from (Street et al., 2004)). N/D=not determined. B. Ro 15-4513 has partial inverse agonism at the α1, α2, α3 and α5 subtypes and despite having high binding affinity for the α4 and α6 receptors has only negligible weak agonist efficacy at these subtypes.

possesses subtype selective efficacy. In other words, whilst α5IA-II binds with comparable affinity to the α1, α2, α3 and α5 $GABA_A$ subtypes, it has much greater inverse agonist efficacy at the α5 compared to α1, α2 or α3 subtypes and consequently it's in vivo effects will be exerted primarily via α5-containing $GABA_A$ receptors (see Table 1 and Fig. 1 for summary).

2. Materials and methods

2.1. Experiments with α5-knockout mice

2.1.1. Self-administration experiment

2.1.1.1. Animals. Wildtype and $\alpha5^{-/-}$ mice (Collinson et al., 2002) were bred from wildtype and homozygous $\alpha5^{-/-}$ mice in a mixed 50% C57BL6 and 50% 129SvEv genetic background, constructed and selectively bred by Dr Thomas Rosahl, Merck Sharp and Dohme Neuroscience Centre, Terlings Park Essex, UK. Female animals of the F3 generation only were used for this study. On arrival at the University of Sussex, the animals were housed in pairs and maintained on a 12-h light/dark cycle with lights off at 19:00; temperature was kept within the range of 19–21 °C and humidity was 50±10%. Except where specified, the mice had ad libitum access to laboratory chow (Bekay Feeds, Hull, UK) and water within the home cage. All experiments were carried out under the authority of the UK Animal (Scientific Procedures) Act, 1986.

Body weight at the start of the experiment was approximately 35 g (31.5 g during the operant experiment, due to food restriction).

2.1.1.2. Self-administration of ethanol. Eight knockout, and 8 wildtype mice were food restricted to reduce body weights to 90% of the free-feeding weight, and trained to press a lever for 10% sucrose in one 15 h session, which included the dark phase, in mouse operant chambers (model ENV-307; Med Associates, Georgia, VT, USA) constructed of clear Perspex (18×18×15 cm), and contained in sound and light attenuating cubicles. Each operant chamber possessed a single house light located on the wall opposite the levers. The front wall was fitted with a liquid dipper (model ENV-202A), located between 2 ultrasensitive mouse levers (model ENV-310A). Following initial training, in daily 60 min sessions, the response requirement to obtain a reinforcer (10% sucrose solution) was increased over consecutive days (range 2–7) from FR1 to FR2 to FR4. Training continued at FR4 until animals received at least 30 reinforcers per session on 2 consecutive days. Over the following 15 sessions the sucrose concentration was decreased and the ethanol concentration was increased as follows; 10% sucrose+3% ethanol; 10% sucrose+5% ethanol, 7% sucrose +5% ethanol, 10% sucrose+10% ethanol, 5% sucrose+10% ethanol. Food restriction was stopped as soon as the mice were pressing for 10% sucrose/10% ethanol solution. Seven animals (4 wildtype and 3 KO) were trained with 5% ethanol alone for 4 sessions, but since responding was not maintained, further training was carried out with 5% sucrose+5% ethanol solution as reinforcer. Each solution was available as the reinforcer for 3 days. Finally, since these genotypes did not perform for ethanol alone, we adopted the 10% ethanol+10% sucrose mixture for further experiments.

A second group of 8 wildtype and 8 KO mice was trained in a similar fashion as far as the 10% sucrose step, and maintained on this reinforcer for the remainder of the experiment. When animals had reached stable levels of responding for their respective reinforcers, the effects of Ro 15 4513 were tested. The mice were habituated to the injection procedure over 3 days; they were then administered, in a Latin-Square design, 4 doses of Ro 15 4513 (0, 3, 9, 27 mg/kg, i.p.) 20 min before being placed in the operant chambers for 60 min. Drug testing took place once weekly and animals were retrained drug-free on the intervening 6 days.

In order to compare the sedative effects of ethanol in the knockout and wildtype mice, animals trained to perform for sucrose reinforcers were given one week's training drug free, before being administered 20% ethanol (0.5 g/kg, i.p.) 10 min before being placed in the operant chambers. This procedure was repeated for 5 consecutive days to study the development of tolerance to the sedative effects of ethanol. Since no evidence of tolerance was observed, all mice were administered 2 g/kg ethanol for 5 days in their home cages before again being administered 0.5 g/kg 10 min before placing in the operant chamber.

2.1.1.3. Statistical analysis. Two way ANOVA, with the between subject factor genotype (2 levels) and within subject factor drug dose (4 levels), was used to evaluate potential genotype and drug effects, and any interactions, for the variables "number of lever presses" and "number of reinforcers", using SPSS statistical software.

2.1.2. Home cage drinking: 2-bottle choice

2.1.2.1. Animals. 24 female adult mice (12 knockouts and 12 wildtype) initially weighing 22–50 g were tested in the two-bottle choice paradigm. All mice were housed individually with ad libitum food and water at all times (except from the last week when they were placed on a 16-h water-restriction schedule). The experiment was conducted during the light phase at the same time each day for each mouse.

2.1.2.2. Water and ethanol consumption. Following 4 weeks habituation to their home cages, mice were given access to two drinking tubes containing ethanol and water, respectively (two-bottle ethanol/water preference test). Food was freely available. Initially, both tubes were continuously available (24-h a day) and contained tap water. They were refilled daily and their location (i.e. left or right side of the cage) alternated daily to reduce position preferences. After 7-day habituation, for the next 7 days the mice were given free access to one tube of water and one filled with a 2% v/v ethanol solution. The quantities of water and ethanol consumed were measured by weighing the tubes every 24-h. Over a period of weeks, ethanol in increasing (2/4/6/8/10/16%) concentrations was presented together with

water. Each condition was tested for 7 days. The animals were weighed daily before the drinking bottles were placed into cages. The data presented represents intakes on the seventh day at each concentration.

In the next step, the fluid access was restricted to 2-h per day (for 7 days). After two days to customize the mice to the procedure, the mice were weighed, received i.p. injection of saline (10 ml/kg), and replaced in their home cage. Twenty minutes later, both bottles were placed in cages simultaneously. Weights of ethanol and water consumed were measured at the end of each 2-h session. On the third day of the week the animals within each genotype were randomly allocated to two groups, one group of mice received vehicle (saline with Tween 80) and the other group received Ro 15-4513 (9 mg/kg) suspended in saline with 0.1% Tween 80. Both Ro 15-4513 and vehicle were administered i.p., 20 min before session. On day 4, the mice received saline, as on days 1 and 2, and on day 5 again treated with Ro 15-4513 (9 mg/kg) or vehicle, counterbalanced to the first drug treatment day. The next two days (Day 6 and day 7) the mice went through the same procedure as on the day 1 and 2 and their fluid intakes were measured. Intakes were expressed both as ethanol intake (g ethanol/kg body weight) and as preference (proportion of fluid taken as ethanol solution).

2.1.2.3. Statistical analysis. The amounts of ethanol solution consumed were analyzed, using a 2-way repeated measures ANOVA, with two factors, genotype, and ethanol concentration as a repeated measure. To test the effects of Ro 15-4513, a 2-way ANOVA with the factors genotype and Ro 15-4513 dose as repeated measure was used.

2.1.3. Acute effects of ethanol

2.1.3.1. Rotarod. On Day 1, 9 knockout and 10 wildtype mice were trained in three-minute sessions trials to remain on a rotating rod (Rotarod; Ugo Basile, Comerio, Italy), revolving at 16 revolutions per min. On the next day, the mice were re-tested, all of them maintaining their position on the rotating rod for 3 min. Animals were then dosed i.p. with 2.5 g/kg ethanol (20% v/v) and tested on the rotarod every 20 min until the animal completed 2 consecutive 3 min trials without falling.

2.1.3.2. Loss of righting reflex. One week later 22 mice were dosed with 3g/kg ethanol i.p. (20% v/v). At intervals of one minute, the mice were laid gently on their backs, and their ability to recover an upright posture within 15 s noted. The time of loss of righting reflex and duration of loss were recorded. On recovery of righting reflex animals were immediately sacrificed. A central blood sample was collected and brains removed and placed on dry ice. Blood and brain alcohol values were estimated using an Analox Instruments (London, UK) ethanol analyser.

2.1.3.3. Statistics.

Rotarod. The time spent on the rotarod during each 3-min probe test was compared between genotypes across the time course of the experiment using 2-way ANOVA with the factors genotype and time (as a within subjects factor).

Loss of righting reflex. The time required between alcohol administration and loss of righting reflex, and the time taken to regain it were compared between the genotypes using Student's *t*-tests.

2.1.4. Effects of a selective α5 inverse agonist on self-administration

2.1.4.1. α5IA-II. α5IA-II was obtained from the Medicinal Chemistry department at Merck Sharp and Dohme (Harlow, UK) and was synthesized as described previously (Street et al., 2004).

2.1.4.2. Receptor occupancy. Doses of α5IA-II (0.1–1mg/kg) dissolved in 5% cremophor were administered i.p. to mice of a mixed C57BL × sv129 strain. Twenty-seven minutes later, the mice (5–6 per dose) were administered 5 μL/g of a 10 μCi/mL stock solution of [^{3}H]-flumazenil (equivalent to 1.5 μCi /30g mouse) into the tail vein, and 3-min later were killed by stunning and decapitation. Brains were rapidly excised, weighed and homogenised in 10 volumes of ice-cold buffer (10 mM phosphate buffer/100 mM KCl, pH 7.4). Two hundred microliter aliquots were filtered over Whatman GF/B filters which were rapidly washed with 10 mL ice-cold buffer. The filters were used for conventional scintillation counting. Non-specific in vivo binding of [^{3}H]flumazenil was estimated by measuring radioactivity in mice pretreated 30 min earlier with 5 mg/kg bretazenil, administered i.p.

2.1.4.3. Animals. The subjects used were male mice bred in-house at the University of Sussex from a C57Bl × 129sv-derived line. Housing conditions were as in Experiment 1.

2.1.4.4. Operant food shaping and sucrose fading. Operant training was carried out essentially as in experiment 1, except that, following initial training, animals were randomly assigned to one of two groups for which the reinforcers differed. The ethanol reinforcement group was trained by sucrose fading to lever press for a 10% ethanol solution containing no sucrose, while the sucrose reinforcement group received a 4% sucrose solution. The concentration of the sucrose reinforcer was progressively decreased from 10% to 4% in an unsuccessful attempt to achieve a similar rate of lever pressing as that maintained by the ethanol reinforcer. Although 4% was the lowest concentration of sucrose that maintained stable responding, it supported higher response rates than 10% ethanol.

When animals had reached stable levels of responding for their respective reinforcers, the effect of the α5 inverse agonist, α5IA-II, on responding was tested over a range of doses. The mice were habituated to the injection procedure and then administered i.p. with 6 doses of α5IA-II (0, 0.03, 0.1, 0.3, 1.0, 3.0 mg/kg, in a pseudo Latin-Square design), 10 min before being placed in the operant chambers for 60 min. The animals were trained using this programme 5 days each week, with drug testing taking place once a week. Computer recordings were made of number of active lever presses, inactive lever presses, head entries into the magazine, and number of reinforcers delivered. Calculations were then made to reveal the number of reinforcers retrieved and the

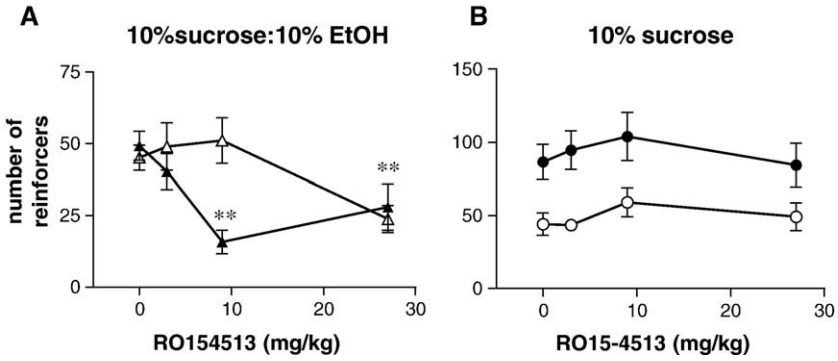


Fig. 2. The numbers of reinforcers obtained by wildtype (closed symbols) and α5 knockout mice (open symbols) performing an operant lever press response to obtain 0.01 ml aliquots of either a 10% ethanol/10% sucrose solution (Panel A), or a 10% sucrose solution (Panel B), available on a fixed ratio 4 (FR4) schedule. ** Effect of Ro 15-4513 significantly different from vehicle, $p<0.01$.

percentage of reinforcers taken of those that were delivered; reinforcers retrieved were taken as head entries into the magazine during the time the dipper was in the up position.

Statistics. Data on numbers of lever presses on active and inactive levers were initially analysed using a 3-way mixed ANOVA with the reinforcer (ethanol vs. sucrose) as a between subject factor, and lever (active vs. inactive), and drug dose, as within subject factors. Data on reinforcers delivered, on lever presses as a percentage of baseline, and on reinforcers retrieved were subjected to 2-way ANOVA, and following significant interaction effects data for each reinforcer was analysed separately with one-way ANOVAs.

3. Results

3.1. Experiment with α5-knockouts

3.1.1. Self-administration

Both genotypes learned to lever press on an FR4 schedule to obtain 0.01 ml of 10% sucrose/10% ethanol mixture, obtaining approximately 50 reinforcers in a 60 min session. There were no differences between genotypes at any stage of training (data not shown). Fig. 2A shows that Ro 15-4513 administration reduced the number of reinforcers delivered during the operant session, in a dose-related fashion ($F_{3,36}=6.50$; $p=0.001$). A significant dose by genotype interaction ($F_{3,36}=5.55$; $p<0.01$) indicated that the genotypes differed in their response to drug. Inspection of Fig. 1 suggests that this was due to Ro 15-4513 being more potent in the wildtype mice. Post hoc comparisons indicated that the effects of the 9 mg/kg dose differed between the genotypes ($F_{1,12}=19.85$; $p<0.001$), but there were no differences at other doses.

In contrast to ethanol/sucrose reinforcers, the genotypes differed in their responding for sucrose reinforcers. Fig. 2B shows that α5 knockouts obtained significantly fewer reinforcers than wildtype mice, giving rise to a significant main effect of genotype ($F_{1,15}=11.75$; $p=0.01$). Ro 15-4513 had no effects on responding for sucrose in either genotype ($F_{2,30}=2.75$; $p=0.08$).

In the groups responding for sucrose, ethanol (0.5 g/kg, i.p.) significantly reduced the number of reinforcers obtained (Fig. 3A; $F_{1,14}=44.36$; $p<0.0001$), but there were no differences between genotypes ($F_{1,14}=0.26$; ns). The depressant effect of ethanol increased over the 5 days of administration (Fig. 3A; $F_{5,70}=3.44$; $p<0.01$), but there was no effect of genotype

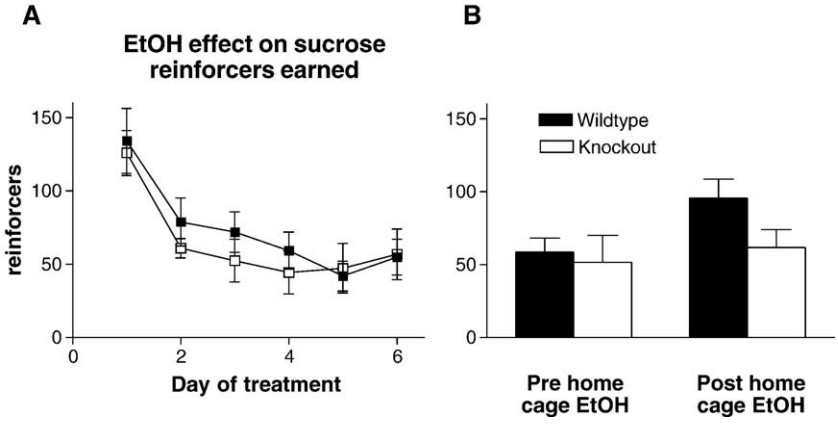


Fig. 3. A. Effect of treatment with 0.5 g/kg ethanol, i.p., on the number of sucrose reinforcers obtained. Filled symbols represent wildtype mice, and open symbols the α5 knockouts. Day 1, the animals received injection of water, and on successive days 0.5 g/kg ethanol. The depressant effect of ethanol showed no tendency to decrease over 5 days administration. B. When the mice were given a higher dose of ethanol (2 g/kg) daily for 5 days, in their home cages, the ability of ethanol to reduce responding for sucrose was decreased, suggesting tolerance development, but there were no differences between the genotypes.

($F_{1,14}$=0.23; ns). However, Fig. 3B shows that when the mice were given a higher dose of ethanol (2 g/kg) daily for 5 days, in their home cages, the ability of ethanol to reduce responding for sucrose was decreased (Main effect of day, $F_{1,14}$=9.88; p<0.01), suggesting tolerance development, but there were no significant differences between the genotypes.

3.1.2. Two-bottle choice

Fig. 4A and B show the amounts of water and ethanol solution taken in 24 h, when the ethanol concentration was systematically increased. The volume of diluted ethanol consumed declined monotonically with increasing concentration (Fig. 4A; $F_{5,110}$= 8.9; p<0.0001), and water intake increased (Fig. 4B; $F_{5,110}$=5.7; p<0.0001), so that daily fluid consumption remained steady. Fig. 4D shows that the proportion of fluid taken as ethanol also decreased with concentration ($F_{5,110}$=7.9; p<0.0001). There were no differences between genotypes in this pattern for any parameter ($F_{5,110}$<0.13; ns). When ethanol consumption was expressed as g/kg ethanol consumed per day (see Fig. 4C), consumption increased with increasing ethanol concentration ($F_{5,110}$=8.8; p<0.0001). Although there were no differences between the genotypes ($F_{1,22}$=0.8; ns), there was a tendency towards an interaction between concentration and genotype ($F_{5,110}$=2.1; p=0.075) that inspection of Fig. 4C suggests was attributable to reduced consumption by the α5 knockout mice at higher ethanol concentrations.

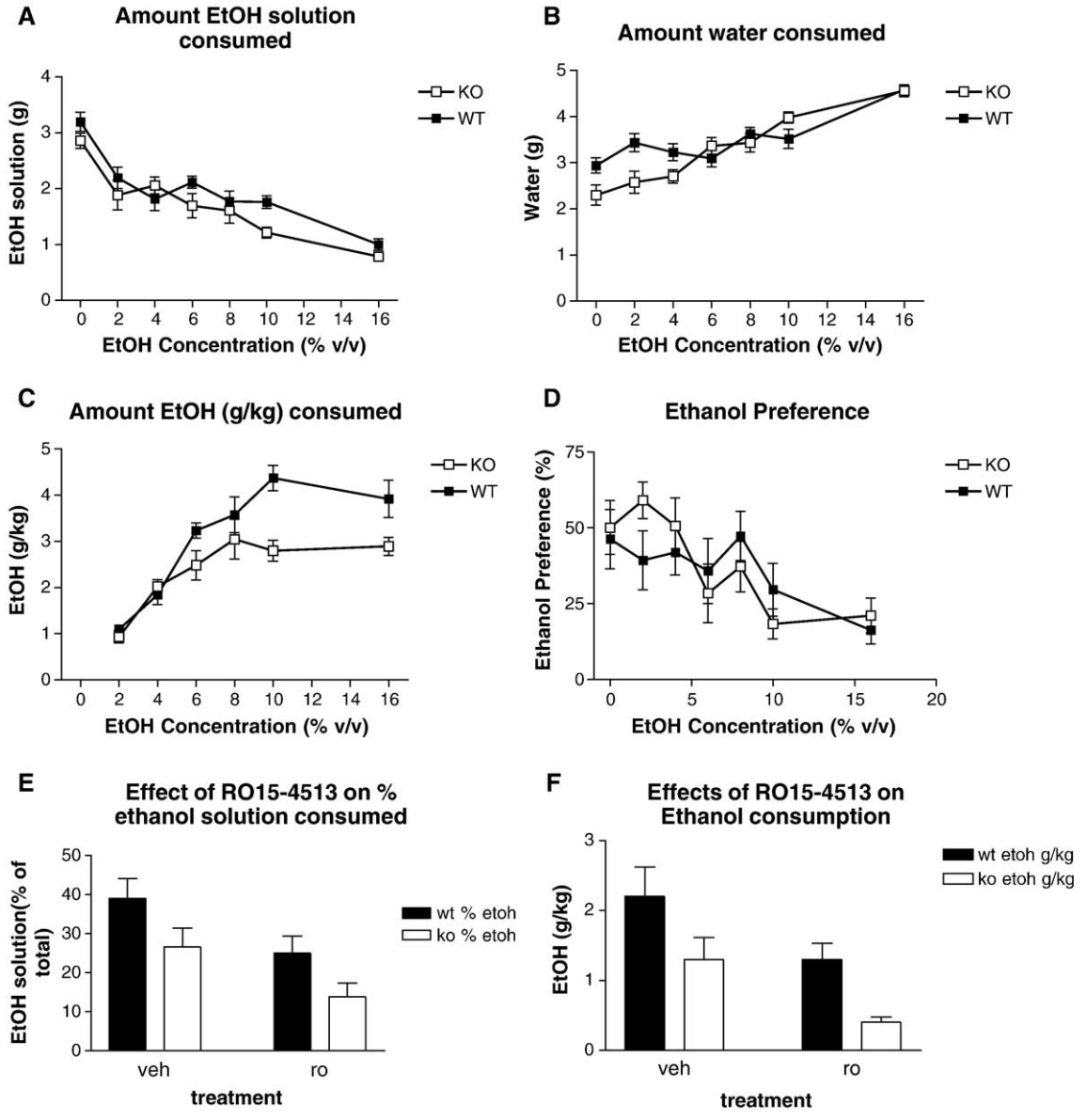


Fig. 4. Effects of α5 deletion on ethanol and water consumption in two-bottle choice tests. Open symbols represent knockout mice, and filled symbols, wildtype mice. A. The effect of increasing the concentration of ethanol on the volume consumed. B. Effect of varying the ethanol concentration on water consumption. C. Ethanol consumption expressed as g ethanol consumed per kg body weight. D. Effect of ethanol concentration on preference for the ethanol solution, expressed as ethanol consumption as a percentage of total volume consumed. E. Effect of 9 mg/kg Ro 15-4513 on consumption of 8% ethanol in a two-bottle choice experiment. 2-way ANOVA revealed significant effects of genotype, and of Ro 15-4513 treatment, but no interaction. F. Effect of Ro 15-4513 on consumption of 8% ethanol, when ethanol intake is expressed per kg body weight. 2-way ANOVA revealed significant effects of genotype, and of Ro 15-4513 treatment, but no interaction.

In order to investigate the effects of Ro 15-4513 in this paradigm, a single concentration (8%) of ethanol was chosen because it was the highest concentration of ethanol that did not give rise to markedly different effects in knockout and wildtype mice when presented alone for 2 h, following 16 h fluid deprivation. A single dose (9 mg/kg) Ro 15-4513 or vehicle was administered i.p 20 min before allowing access to the two bottles; the same procedure was followed two days later, when vehicle/Ro 15-4513 was given counterbalanced to the previous treatment day. The mice consumed more water than ethanol ($F_{1,22}=13.61$; $p=0.001$), with a trend for the knockout mice to consume more water and less ethanol than the wildtypes (genotype × fluid type interaction; $F_{1,22}=3.0$; $p=0.1$). While Ro 15-4513 did not significantly reduce the volume of fluid intake overall (main effect of Ro 15-4513; $F_{1,22}=0.78$; ns), there was a significant effect of the drug and the genotype on ethanol consumed when expressed in g/kg (Fig. 4F; main effect of Ro 15-4513 $F_{1,22}=12.39$; $p<0.01$) and this measure again indicated that the knockouts showed a reduced ethanol consumption (main effect of genotype $F_{1,22}=7.21$; $p=0.01$). Fig. 4E shows EtOH preference (EtOH solution consumed as % of total fluid). Ro 15-4513 decreased preference (main effect of Ro 15-4513 $F_{1,22}=12.88$; $p<0.010$), and a main effect of genotype ($F_{1,22}=4.70$; $p<0.05$) again indicated reduced ethanol preference by the knockout. Thus, Ro 15-4513 impaired ethanol drinking more than it reduced water consumption, and the knockout also showed reduced ethanol consumption and preference. There was no significant interaction between drug and genotype, or three-way interaction, suggesting that the genotypes did not differ in their response to Ro 15-4513 in this measure.

3.1.3. Acute effects of ethanol

3.1.3.1. Rotarod. Following i.p. administration of 2.5 g/kg ethanol, rotarod performance was impaired within 20 min of administration, but had recovered at 4 h after ethanol, leading to a main effect of time ($F_{5,13}=27.9$; $p<0.001$; data not shown). There were no differences between genotypes ($F_{1,17}=0.4$; ns), and no evidence for differences in the rate of recovery from ethanol (genotype × time interaction: $F_{1,17}=0.8$; ns).

3.1.3.2. Loss of righting reflex. Administration of 3 g/kg ethanol led to a loss of the righting reflex within 3–4 min, the animals recovering within approximately 50 min (data not shown). There were no differences between the genotypes in either the time to loss of righting reflex, nor time to recovery. At the time of recovery of the reflex, blood alcohol levels of wildtype and KO mice were 401.5±16.6 and 390.4±17.9 mg/dL (ns) while brain levels were 383.5±12.4 and 389.8±11.8 mg/dL, respectively (ns).

3.1.4. Effects of a selective α5 inverse agonist on self-administration

3.1.4.1. Pharmacokinetics. Fig. 5 shows that 30 min following i.p administration, α5IA-II dose-dependently inhibited the binding of [^{3}H]-flumazenil binding to mouse brain membranes,

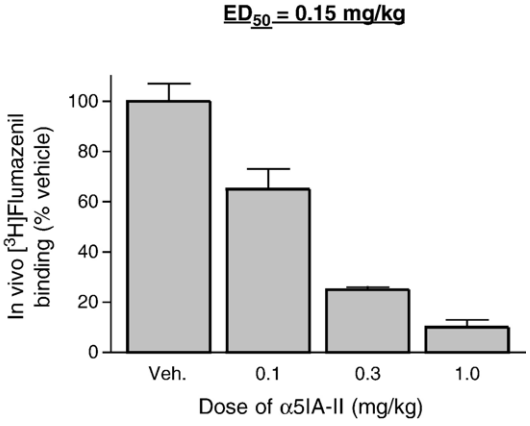


Fig. 5. Inhibition of the in vivo binding of [^{3}H]-flumazenil to mouse brain membranes by α5IA-II (pre-treatment time = 30 min., vehicle = 5% Cremophor). The extent to which the binding is inhibited represents the occupancy of mouse brain BZ binding sites by α5IA-II. Thus, at 1.0 mg/kg the extent of in vivo [^{3}H]-flumazenil binding was 10% of vehicle treated animals and represents an occupancy of BZ binding sites by α5IA-II of 90%. Values shown are mean ± SEM (n = 5–6/group).

with an estimated ED_{50} of 0.15 mg/kg. The highest dose tested, 1 mg/kg, gave rise to 90% receptor occupancy at this time. Since α5IA-II has equivalent affinity for the BZ binding site of $GABA_A$ receptors containing either an α1, α2, α3 or α5 subunit (Fig. 1), any given dose of α5IA-II will occupy these different receptor populations to the same extent. Thus this dose of α5IA-II is likely to have occupied over 90% of α5-containing receptors.

3.1.4.2. Self-administration. Despite using low concentrations of sucrose, the mice performed at higher rates of lever pressing to obtain sucrose than ethanol. α5IA-II administration reduced the number of lever presses on the active lever, in a dose related fashion (main effect of drug dose; $F_{5,7}=9.64$; $p<0.01$), leading to fewer reinforcers being delivered (Fig. 6C; $F_{5,7}=8.44$; $p<0.01$). Comparison of Fig. 6A and B suggests that α5IA-II was more effective in reducing responding for ethanol than sucrose, and this was confirmed statistically, a significant dose by reinforcer interaction ($F_{5,7}=12.06$; $p<0.01$) indicating that the drug effect on lever presses depended on the reinforcer. Subsequent 1-way ANOVA conducted separately for each reinforcer revealed a significant effect of dose when ethanol was the reinforcer ($F_{5,35}=2.73$; $p<0.05$), but no significant effect of dose when sucrose was the reinforcer.

Whether the mice consumed reinforcers when they were delivered cannot be known in this apparatus; however, an attempt to derive this information was made by observing entries into the reinforcer-delivery magazine within the time period that the dipper was available. When reinforcers retrieved was expressed as a percentage of reinforcers delivered (Fig. 6D), 2-way ANOVA revealed a main effect of dose ($F_{5,7}=10.45$; $p<0.01$) and of reinforcer ($F_{1,11}=11.19$; $p<0.01$) and an interaction ($F_{5,7}=8.72$; $p<0.01$). Inspection of Fig. 6D suggests that there were no effects of drug on the number of sucrose reinforcers retrieved (one-way ANOVA $F_{5,42}=1.42$;

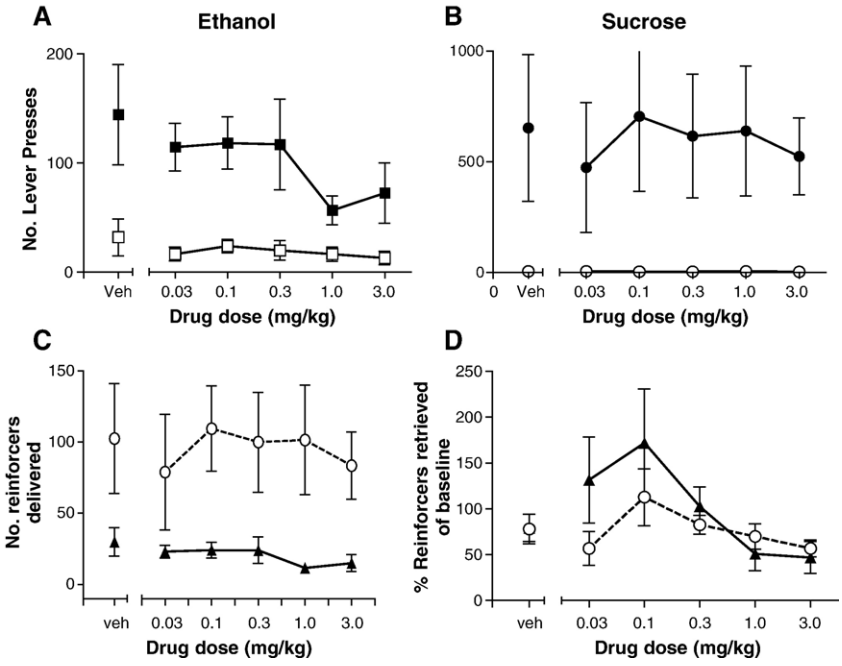


Fig. 6. Effects of the α5 selective inverse agonist α5IA-II on operant performance of mice responding for 10% ethanol or 4% sucrose reinforcers, available on a fixed ratio 4 (FR4) schedule. A. Effects on numbers of presses on the active (closed symbols) and inactive (open symbols) levers when ethanol was the reinforcer. B. Effects on numbers of presses on the active (closed symbols) and inactive (open symbols) levers when sucrose was the reinforcer. Note different scales of *Y*-axes in these two figures. α5IA-II treatment reduced lever pressing ($F_{5,7}=9.64$; $p<0.01$). A significant dose by reinforcer interaction ($F_{5,7}=12.06$; $p<0.01$) indicated that the drug effect on lever presses depended on the reinforcer. C. Effect of α5IA-II on numbers of reinforcers delivered, showing an effect when ethanol (filled symbols), but not sucrose (open symbols), was the reinforcer ($F_{5,7}=8.44$; $p<0.01$). D. Effect of α5IA-II on retrieval of earned reinforcers of ethanol (filled symbols) and sucrose (open symbols). 2-way ANOVA revealed a main effect of dose ($F_{5,7}=10.45$; $p<0.01$) and of reinforcer ($F_{1,11}=11.19$; $p<0.01$) and an interaction ($F_{5,7}=8.72$; $p<0.01$). There were no effects of drug on the number of sucrose reinforcers retrieved (one-way ANOVA $F_{5,42}=1.42$; ns), but at higher doses, the drug reduced the percentage of ethanol reinforcers retrieved ($F_{5,42}=4.01$; $p<0.01$).

ns), but that at higher doses, the drug reduced the percentage of ethanol reinforcers retrieved ($F_{5,42}=4.01$; $p<0.01$).

There was no effect of drug on the total number of magazine entries during a session ($F_{5,7}=1.13$; ns), a marginal effect of reinforcer ($F_{1,11}=4.9$; $p=0.05$), and no interaction ($F_{5,7}=1.46$; ns).

4. Discussion

Although several reports indicate that drugs acting at the benzodiazepine site of α5-containing $GABA_A$ receptors to reduce chloride flux (inverse agonists) reduce self-administration of ethanol (Bao et al., 1992; Glowa et al., 1988; June et al., 2001; McKay et al., 2004; Rassnick et al., 1993; Samson et al., 1989, 1987) in the present experiments we found no differences between female mice in which the α5 subunit had been deleted, and wildtype mice in the self-administration of ethanol over a range of concentrations, when combined with sucrose. Although, when given free access to ethanol-containing solutions, at low ethanol concentrations the knockouts did not differ from wildtype mice in the amount of ethanol consumed, and in preference for ethanol relative to water, at higher concentration the knockout showed reduced avidity for ethanol in a number of measures. Thus, targeted deletion of α5 subunits had little effect on the rewarding effects of ethanol as measured in the operant paradigm, though it did affect consumption. We found no evidence in the rotarod, or loss of righting reflex procedure that the sedative/ataxic effects of ethanol differed between the genotypes, nor that they differed in a simple measure of tissue ethanol concentrations following a larger dose.

Nevertheless, α5 knockout mice differed from wildtype mice in several tests. Firstly, although they did not differ in their rates of responding for ethanol/sucrose mixtures, they responded at lower rates on a FR4 schedule to obtain sucrose reinforcers. Rates of responding of the knockouts for the ethanol/sucrose mixture were similar to those for sucrose alone, whereas the wildtype animals showed higher rates of responding for the 10% sucrose reinforcer than for the mixture. Two potential explanations of this pattern readily come to mind. Firstly, the knockouts may be insensitive to the presence of ethanol in the reinforcer, so that response rates do not differ between sucrose alone, and sucrose with added ethanol. This account implies that responding in both genotypes is maintained by the sucrose reinforcer, and that ethanol reduces the effectiveness of the sucrose reinforcer in the wildtype. Since it was not possible to train this background strain of mice (derived from a C57Bl/6×129SVcross) to perform an operant schedule to obtain ethanol alone, this seems possible. That the α5 knockout appears to be insensitive in this measure to the presence of ethanol in the solution may be consistent with the ability of an α5 inverse agonist to prevent the ability of ethanol to act as a discriminative stimulus (Platt et al., 2005), since both manipulations will reduce functionality of α5-containing $GABA_A$ receptors.

However, in a free choice test, over a wide range of concentrations encompassing that used in the operant tests, we found that, although the genotypes did not differ in their ethanol preference at low ethanol concentrations, at higher concentrations they had a lower preference. These data resemble observations of (Boehm et al., 2004) in male mice, and suggest that deletion of the α5 subunit results in reduced preference for higher ethanol concentrations (though it should be noted that Boehm et al. (2004) did not find effects of the α5 deletion in female mice). In the present experiments, differences began to emerge between the genotypes at an ethanol concentration of about 8%, suggesting that at higher concentration the α5 KO mice found ethanol to be less rewarding, or that at higher intakes, the knockouts were more sensitive to the sedative effects of ethanol. Since the self-administration experiments used concentrations of 10%, this former possibility is difficult to reconcile with the lack of effect of the gene deletion on self-administration, The latter seems unlikely since the knockouts did not differ from wildtype mice in the ataxic effects of ethanol in the rotarod or righting reflex tests, nor in the effects of ethanol on operant responding, nor in the development of tolerance to the sedative effects. However, a relative insensitivity to the stimulus properties of ethanol could also potentially account for these results.

An alternative explanation of the operant data might be that under the ethanol/sucrose condition, the mice performed for ethanol, and that the genotypes did not differ. The difference that emerged with the sucrose reinforcer would then indicate that deletion of the α5 subunit resulted in sucrose becoming relatively less reinforcing, so that this genotype maintained lower rates of responding for sucrose alone. The mechanism whereby α5 subunits might contribute to signaling the reinforcing properties of sucrose, but not ethanol is not clear, but either explanation is incompatible with α5 subunits playing an important role in signaling the positive reinforcing properties of ethanol. Lastly, it should be considered that in these experiments, the mice we had available had been bred from homozygous wildtype and knockout stock, so that, in principle, the background strains may have differed sufficiently to give rise to some of the behavioural differences observed. However, it seems unlikely that in three generations of separate breeding that significant genetic drift should occur between the populations.

A second aspect of these experiments was the investigation of the putative role of α5-containing receptors in mediating the effects of the benzodiazepine receptor inverse agonist, Ro 15-4513 on ethanol's reinforcing properties. In wildtype mice, Ro 15-4513 decreased the rate of lever pressing for ethanol/sucrose mixtures, but not sucrose alone, suggesting a specific role of α5 subunits in the effect of the inverse agonist on ethanol reward, in keeping with previous observations. The drug was effective in the wildtype mice at doses of 9 and 27 mg/kg, but in the knockout at only the 27 mg/kg dose. These data suggest that at the lower dose, Ro 15-4513 acts in wildtype mice at α5-containing receptors to reduce the rewarding effects of ethanol, but that at a higher dose other mechanisms, perhaps involving other $GABA_A$ receptor subtypes, may come into play.

How might α5 play a role in the ability of Ro 15-4513 to reduce the reinforcing effects of ethanol while its deletion has little effect on ethanol's reinforcing properties? As already mentioned, Platt and colleagues (Platt et al., 2005) have recently suggested that the discriminative stimulus properties of ethanol are mediated by α5-containing $GABA_A$ receptors. Presumably, interoceptive cues provided by ethanol contribute to instrumental performance for ethanol reward by providing feedback that the instrumental response is successful. Thus, the ability of inverse agonists to suppress ethanol-reinforced responding in the wildtype may reflect a deficit in identifying the interoceptive effects of the drug, rather than an effect on reward per se.

In experiments with constitutive deletion of the targeted gene, the knockout procedure results in global loss of the functional protein throughout the body, so that the neural substrates of behavioural changes are difficult to identify. In the case of the α5 subunit of $GABA_A$ receptors, which is localised in only few brain areas, all effects of the knockout procedure are likely attributable to a single role for the protein, but the possibility that compensations for the loss of the α5 protein occur during development must be considered. The α5 KO mouse shows surprisingly few spontaneous behavioural alterations (Collinson et al., 2002), so that it seems possible that changes in expression of other $GABA_A$ subunits may compensate for the lack the α5 subunit.

We therefore complemented our knockout studies by using a novel compound, α5IA-II, that acts selectively as an inverse agonist at α5-containing $GABA_A$ receptors, while, although showing similar affinity, displays either no, or very weak negative intrinsic efficacy for other receptor subtypes (Street et al., 2004). This substance thus allows an examination of the role of α5 subunits in alcohol reward, without the potential complications of interpretation imposed by constitutive knockouts, and offering an alternative approach to inverse agonists with partially selective affinity for α5-containing receptors. Additionally, using this approach, we were able to test the drug in a mouse strain that shows instrumental responding for unsweetened ethanol solutions, removing a potential confound.

As has been shown in rats for non-selective (Ro 15 4513; (Bao et al., 1992; Glowa et al., 1988; Rassnick et al., 1993; Samson et al., 1989, 1987) and partially selective inverse agonists (June et al., 2001), α5IA-II reduced lever pressing, and the numbers of reinforcers received, when a 10% ethanol solution was the reinforcer, but was ineffective in reducing responding for a 4% sucrose reinforcer, though it is important to note that response rates for the sucrose reinforcer were considerably higher. A tendency of α5IA-II to increase responding for ethanol at a low dose was not statistically reliable. These effects of α5IA-II on operant responding for ethanol appeared to be dependent on its action at benzodiazepine receptors since they were antagonised by the non-specific antagonist, flumazenil (data not shown). In addition to α5IA-II reducing lever pressing for ethanol solution, and thus the number of reinforcers earned, this compound also reduced the efficiency with which earned ethanol (but not sucrose)

reinforcers were retrieved (and presumably consumed) by the mice. These effects were observed at doses above 1 mg/kg. In separate experiments, we measured the ability of α5IA-II , at a range of doses, to displace flumazenil from brain binding sites. From these data, we calculate that the behavioural effects of α5IA-II seen at doses of 1 and 3 mg/kg correspond to central benzodiazepine binding site occupancies of over 90%.

Thus, in summary, the ability of benzodiazepine inverse agonists to suppress instrumental responding for ethanol appears to be mediated by their action at α5-containing $GABA_A$ receptors, though the role of the same receptors in signaling ethanol reward is less convincing, since targeted deletion of α5 subunits did not decrease instrumental responding for ethanol, though it did reduce ethanol preference in a two-bottle choice experiment. In the present experiments, the effect of an α5-selective inverse agonist on instrumental responding for ethanol, which can be construed as drug seeking, was achieved only at dose giving rise to receptor occupancies greater than 90%. Nevertheless, these results suggest that clinical studies to determine whether α5 inverse agonists suppress ethanol consumption are warranted. In this regard, it is interesting to note that two different α5 subtype selective compounds have been described as entering clinical development (Chambers et al., 2004; Xue et al., 2004).

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