Alpha2-containing GABA$_A$ receptors are involved in mediating stimulant effects of cocaine

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Abstract

α2 subunit-containing GABA$_A$ receptors are involved in incentive learning associated with cocaine, and in cocaine addiction. Deletion of α2-containing receptors abolishes cocaine-induced behavioural sensitisation (BS), while selective activation of α2 receptors, achieved using Ro 15-4513's agonist properties in α2(H101R) mice, induced BS. Here, we investigate further the mechanisms underlying Ro 15-4513-induced behavioural sensitisation in α2 (H101R) mice. α2(H101R) mice sensitised to Ro 15-4513 (10 mg/kg) showed an enhanced stimulant response to cocaine (10 mg/kg). In contrast, cocaine (10 mg/kg)-sensitised α2(H101R) mice did not show enhanced sensitivity to the stimulant effects of Ro 15-4513 (1, 3 and 10 mg/kg), suggesting that the neural adaptations underlying Ro 15-4513 induced BS are related to, but not identical with those associated with cocaine-induced plasticity. Secondly, we investigated whether α2-containing receptors are involved in mediating the ability of BZs to facilitate cocaine-induced activity. The non-selective (i.e., α1, α2, α3 and α5 subtype) benzodiazepine GABA$_A$ receptor agonist midazolam (10 and 30 mg/kg) potentiated cocaine (10 mg/kg) hyperactivity in wildtype mice, but not in α2(H101R) mice, in which α2-containing receptors are insensitive to benzodiazepines. To determine where α2 receptors are localised we compared BZ-insensitive sites between wildtype (α4 and α6) and α2(H101R) (α2, α4 and α6) mice, using quantitative autoradiography to estimate [^{3}H]Ro 15-4513 binding in the presence of 10 μM diazepam. α2 receptors were found in projection areas of the mesolimbic dopamine pathway including accumbens, central amygdala, and basolateral amygdala as well as CA1 and CA3 areas of the hippocampus. The involvement of the α2-containing receptor in mediating BZ's potentiating effect on cocaine hyperactivity suggests that the locomotor stimulant effects of BZs and psychostimulants may be mediated by a common neural system, but the lack of cross sensitisation to Ro 15-4513 in cocaine-sensitised α2(H101R) mice, suggests that this form of BS may occur downstream of plastic events underlying cocaine sensitisation.
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1. Introduction

The locomotor stimulant properties of drugs such as cocaine depend upon their ability to increase extracellular dopamine in the nucleus accumbens (Kelley and Iversen, 1975), while the development of behavioural sensitisation to psychomotor stimulants is associated with further increased dopamine release (Kalivas and Duffy, 1990). A primary effect of dopamine release in the NAc is modulation of GABAergic medium spiny neurones, and thus of inhibitory GABAergic outputs to the ventral pallidum (VP) (Kalivas et al., 1993; Yang and Mogenson, 1985, 1989). The VP supplies a reciprocal GABAergic innervation back to the NAc (Heimer et al., 1991), while recurrent collateral projections from medium spiny neurones provide an additional inhibitory GABAergic innervation on medium spiny neurone cell bodies (Shi and Rayport, 1994). Thus, a consequence of altered dopamine neurotransmission within accumbens is likely to be altered patterns of activity in medium spiny neurones, which are further modulated by GABAergic feedback mechanisms.

Alterations to this GABAergic circuitry downstream from the sites of action of cocaine are likely to affect the outcome of cocaine administration. In rats, behavioural sensitisation to cocaine is associated with decreased $\alpha2$ expression in nucleus accumbens (NAc) (Chen et al., 2007). Since receptors expressing $\alpha2$ subunits appear to be the dominant form of GABA$_A$ receptor in the accumbens (Schwarzer et al., 2001) (in contrast to ventral pallidum in which receptors employing $\alpha1$ subunits are the most frequent (Pirker et al., 2000; Schwarzer et al., 2001)), in the present experiments we have studied the consequences for cocaine-induced locomotor stimulation and behavioural sensitisation of manipulating $\alpha2$-containing receptor subtypes.

GABA$_A$ receptors expressing $\alpha1$, $\alpha2$, $\alpha3$ or $\alpha5$ subunits possess binding sites for benzodiazepines, whereas those expressing $\alpha4$ or $\alpha6$ subunits are insensitive to BZs, but respond to the imidazobenzodiazepine, Ro-15-4513. Ro 15 4513 acts as a partial inverse agonist at BZ-sensitive sites ($\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$) but as a partial agonist at BZ-insensitive sites ($\alpha4$ and $\alpha6$). $\alpha4$- and $\alpha6$-containing GABA$_A$ subtypes differ from BZ-sensitive subunits in that they express an arginine (R) rather than a histidine (H) residue at the drug binding domain of the receptor (Wieland et al., 1992). Introduction of a histidine to arginine point mutation at the binding site of $\alpha1$, $\alpha2$, $\alpha3$ or $\alpha5$ subunits renders the mutant GABA$_A$ receptors insensitive to BZs. Consequently, the mutated GABA$_A$ receptor adopts the same binding profile as BZ-insensitive receptors. Electrophysiology studies show that the efficacy of Ro 15-4513 is switched from inverse agonism to agonism at the mutated GABA$_A$ receptor in H101R mice (Benson et al., 1998; Crestani et al., 2002; Rudolph et al., 1999). Thus, by comparing Ro 15-4513 effects in (H101R) mutant and wildtype mice, the contribution of its agonist effects at particular receptors can be dissected out. This model has previously been used to confirm the role of $\alpha1$ GABA$_A$ receptors in mediating the sedative and anticonvulsant properties of BZs (Benson et al., 1998; Crestani et al., 2002; Rudolph et al., 1999).

Using this approach, we have recently found that whilst targeted deletion of $\alpha2$ subunits abolished behavioural sensitisation to cocaine in mice, activation of $\alpha2$-containing GABA$_A$ receptors, achieved by using Ro 15-4513 in $\alpha2$ (H101R) mice, led to the induction of behavioural sensitisation (Morris et al., submitted for publication). Here, we investigate further the mechanisms underlying Ro 15-4513-induced behavioural sensitisation in $\alpha2$(H101R) mice in an attempt to identify potential $\alpha2$-containing neural systems that might contribute to cocaine-induced behavioural sensitisation and cocaine abuse. Firstly, we investigated whether there is cross sensitisation between cocaine and Ro 15-4513 and secondly, whether the $\alpha2$-containing receptor is involved in mediating the ability of BZs to potentiate psychostimulant hyperactivity. To investigate where in the brain Ro 15-4513-induced behavioural sensitisation may potentially be mediated, we investigated expression of $\alpha2$ receptors in brain areas associated with the rewarding effects of other drugs of abuse.

BZs have been regarded as unusual among abused drugs in that they neither increase locomotor activity nor give rise to behavioural sensitisation in rodents. However, recent evidence indicates that BZs possess locomotor activating effects in the absence of modulation of GABA$_A$ receptors expressing $\alpha1$ subunits (Crestani et al., 2000, 2002; McKernan et al., 2000), presumably because, in wildtype animals, the $\alpha1$-mediated sedative effect of BZs (McKernan et al., 2000; Rudolph et al., 1999) masks the expression of enhanced locomotor response mediated by the $\alpha2$, $\alpha3$ and/or $\alpha5$ GABA$_A$ subtypes. The observation that $\alpha2$(H101R) mice are more sensitive to the sedative effects of BZs (Wafford et al., 2004), clearly implies a role for $\alpha2$-containing receptors in mediating the locomotor activating effects of BZs, which oppose $\alpha1$-mediated sedation. Consistent with that idea, facilitation of transmission at $\alpha2$-containing GABA$_A$ receptors using Ro-15-4513 in $\alpha2$(H101R) mice, induced behavioural sensitisation (Morris et al., submitted for publication), a phenomenon characteristic of other drugs of abuse. These observations open the possibility that BZs possess abuse potential because they activate mechanisms related to those associated with other drugs. To examine this idea, we investigated whether $\alpha2$(H101R) mice previously sensitised to Ro 15-4513 cross-sensitised to cocaine and vice versa.

The observation that BZs enhance the stimulant effects of psychostimulants (Sansone, 1980; Sansone et al., 1986; Sethy et al., 1970) also provides suggestive evidence that BZs act on neural systems related to those at which psychostimulants achieve their effects. We investigated the role of the $\alpha2$ receptor subtype in mediating such BZs-induced facilitation of cocaine's stimulant effects. Our approach was to treat $\alpha2$(H101R) mice, in which $\alpha2$ GABA$_A$ receptors are rendered BZ-insensitive by a point mutation (Benson et al., 1998), with combinations of midazolam and cocaine. It was hypothesised that if the $\alpha2$ subtype is involved in mediating the potentiating effects of BZs, $\alpha2$(H101R) mutant mice should be resistant to midazolam's enhancement of cocaine's stimulant effects. We measured cocaine plasma levels following cocaine treatment alone and in combination with midazolam to eliminate the possibility that BZ-facilitated psychostimulant hyperactivity was due to a pharmacokinetic effect. Otherwise it could be argued that rather than an $\alpha2$ receptor-mediated effect, this phenomena is due to the ability of BZs to reduce cocaine metabolism, resulting in greater cocaine plasma levels and facilitation of its behavioural effect.

Finally, we investigated the expression of mutated $\alpha2$-containing GABA$_A$ receptors in $\alpha2$(H101R) mice in brain areas associated with the rewarding effects of other drugs of abuse, to determine where $\alpha2$-mediated behavioural sensitisation may be mediated. Using quantitative autoradiography we assessed [^{3}H] Ro 15-4513 binding in the absence and in the presence of diazepam. Whilst diazepam binds to BZ-sensitive GABA$_A$ receptor subtypes, Ro 15-4513 binds to both BZ-sensitive and BZ-insensitive receptors. Thus, in wildtype animals, diazepam binds to the $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ subtypes, confining [^{3}H]Ro 15-4513 binding to only $\alpha4$ and $\alpha6$ subtypes. In contrast, following the point mutation of the $\alpha2$ subtype in $\alpha2$(H101R) mice, diazepam binds to only the $\alpha1$, $\alpha3$ and $\alpha5$ subtypes, enabling [^{3}H] Ro 15-4513 to bind to $\alpha2$ as well as $\alpha4$ and $\alpha6$ receptor subtypes. Increased levels of [^{3}H]Ro 15-4513 binding in $\alpha2$(H101R) mice in the presence of diazepam compared to wildtypes thus

represents the expression of the α2 receptor subtype. Using this approach we determined levels of α2-containing GABA$_A$ receptor expression in the nucleus accumbens (NAc), amygdala (basolateral nucleus (BLA), central nucleus (CEA) and the bed nucleus of the stria terminalis (BNST)), hippocampus (CA1 and CA3 areas), ventral tegmental area (VTA) and pedunculopontine nucleus (PPN) in α2(H101R) mice.

2. Methods

2.1. Animals

Wildtype and α2(H101R) mice, bred in the Department of Psychology at the University of Sussex, from homozygous mutant and wildtype parents were used for the behavioural experiments. Approximately equal numbers of male and female mice were used in each group. Mice were housed in groups of two or three under a 12:12-hour light/dark cycle (lights on at 7:00 AM). Room temperature was maintained at 19–21 °C and humidity was kept at $50 \pm 10\%$. All mice had *ad libitum* access to food and water. Wildtype and α2(H101R) mice (Dias et al., 2005; Morris et al., 2006), bred in the Neuroscience Research Centre at Merck, Sharp and Dohme (Harlow, Essex) from heterozygous parents, were used in the quantitative autoradiography experiment. All experiments were carried out under the UK Animal (Experimental Procedures) Act 1986.

3. Materials

Cocaine hydrochloride and midazolam hydrochloride, purchased from MacFarlane Smith (Edinburgh, UK) and Sigma-Aldrich (Dorset, UK), respectively, were dissolved in sterile 0.9% saline. Ro 15-4513 purchased from Sigma-Aldrich was suspended in a saline solution containing 0.2% Tween 80. All drugs were administered via the i.p route at a volume of 10 ml/kg.

The buffer used for autoradiography was made from 10 mM KH$_2$PO$_4$ and 100 mM KCl at a pH of 7.4 (using KOH), stored and used at 4 °C. [^{3}H]Ro 15-4513, 6.1 nM (NEN Life Science Products, Boston, USA), 10 μM diazepam (Sigma-Aldrich, Poole, UK) and 10 μM bretazenil (supplied by Merck Sharp and Dohme, Harlow, UK) were used.

4. Locomotor activity

To measure locomotor activity, 16 black perspex circular runways (diameter=24 cm, runway width=6.5 cm) were used, each containing eight infra-red photobeams equally spaced at 45 (Mead and Stephens, 1998). Forward activity was counted as each beam break following the breaking of 3 consecutive beams.

4.1. Procedure

Locomotor activity was measured in a 2.5-hour session. Each session began with a 1-hour habituation period, following which each animal was dosed with the assigned drug and immediately returned to the runways for a further 1.5 h. Behavioural sensitisation was induced by administering Ro 15-4513 and cocaine repeatedly and intermittently, at 2–3 days intervals. We will refer to this treatment schedule as 'repeated treatment' for the remaining manuscript.

4.2. Cocaine (10 mg/kg) challenge in Ro 15-4513 (10 mg/kg) sensitised α2(H101R) mice

α2(H101R) and wildtype mice sensitised to Ro 15-4513 (following repeated treatment over a total of 14 treatments, administered at 2–3 days interval) or given repeated vehicle, were dosed with vehicle and 10 mg/kg cocaine. Following the establishment of behavioural sensitisation (see (Morris et al., submitted for publication), the animals were dosed with vehicle, 2 days after receiving their last repeated treatment and, 1 week later, with cocaine, instead of the sensitising dose of Ro 15-4513. Treatment groups ($n=14–16$) included approximately equal numbers of male and female mice.

4.3. Conditioned activity in Ro 15-4513 sensitised α2(H101R) mice

The Ro 15-4513 treated α2(H101R) and wildtype mice received vehicle in 1) the locomotor runways and 2) novel locomotor boxes (2 days later). Each test session comprised a 1-hour habituation session, dosing and then immediate exposure to the apparatus for a further 1.5 h. Again, treatment groups ($n=14–16$) included approximately equal numbers of male and female mice.

4.4. Ro 15-4513 (3 and 10 mg/kg) challenge in cocaine (10 mg/kg) sensitised α2(H101R) mice

Wildtype and α2(H101R) mice received repeated treatment with 10 mg/kg cocaine or with vehicle. A total of 13 treatments were administered at 2–3 days interval over a 7-week period. Two days after receiving their last vehicle or cocaine treatment each animal received a vehicle injection, followed in subsequent sessions by ascending doses of Ro 15-4513, 3 mg/kg and 10 mg/kg. Treatment groups ($n=6–8$) included male and female mice.

4.5. Effect of co-administration of cocaine and midazolam on locomotor activity in α2(H101R) and their wildtype mice

An independent groups design was used in which α2(H101R) and wildtype mice were dosed with 10 mg/kg cocaine in addition to 0, 1, 3, 10 and 30 mg/kg of midazolam (group sizes, 9–13, males and females).

4.6. Plasma levels of cocaine and its metabolites following treatment with cocaine alone and in combination with midazolam

Wildtype mice were dosed with 10 mg/kg cocaine in addition to 10 ml/kg saline ($n=6$) or 10 mg/kg midazolam ($n=6$). Fifteen minutes after drug treatment (at which peak locomotor activity levels were shown following the co-administration of 10 mg/kg cocaine and 10 mg/kg midazolam, data not shown) mice were killed by stun and decapitation and trunk blood was collected in sodium heparinized tubes and spun in a bench top Sorval

centrifuge at 3000 rpm for 10 min. The plasma was removed, frozen on dry ice and stored at -80 °C for later analysis. Levels of cocaine and its metabolites, benzoylecgonine and ecgonine methylester, were analysed by Dr Andrew Smith using HPLC at the Clinical Chemistry department of the Royal Sussex hospital (Brighton, East Sussex).

5. Quantitative autoradiography

5.1. Tissues for autoradiography

Wildtype and α2(H101R) mice, 4 males and 4 females of each genotype, were sacrificed by stun and decapitation and brains were rapidly removed and frozen in cold *iso*-pentane $(-40$ °C) and stored at $(-80$ °C) until further use. The mouse brain was mounted onto a plate embedded in dry ice and covered with Thermo Shandon M-1 embedding matrix to fix its position and allow frozen sectioning. 12 μM coronal sections were cut using a cryostat. The brain slices were thaw mounted and air-dried and then stored at -80 °C until use. Data from 1 α2(H101R) male was excluded due to poor brain slices.

5.2. Autoradiography procedure

The slides were left at room temperature for approximately 15 min to thaw and then placed in ice-cold buffer and shaken at 100 rpm for 15 min and in deionised water for a further 15 min. Slides from each animal were incubated for 60 min at 0° in [3H] Ro 15-4513 (to measure both BZ-sensitive sites and BZ-insensitive sites), [3H] Ro 15-4513+100 μM diazepam (to measure BZ-insensitive sites) and [3H] Ro 15-4513+10 μM bretazenil (to measure non-specific binding). Slides were then washed (3×30 s) in ice-cold buffer and dipped briefly in ice-cold deionised water and rapidly cold air-dried. The slides were placed onto [3H] hyperfilm, and were developed 7 weeks later to allow subsequent quantitative analysis using commercial software (MCID). All slides were corrected for non-specific binding.

6. Statistical analysis

In all experiments, where sphericity assumptions were violated, the Greenhouse–Geisser correction was applied. There were no main effects of sex in any of the experiments, so male and female data were combined.

6.1. Locomotor activity

To identify whether cocaine-induced behavioural sensitisation, activity levels on the first and final treatment days were compared, using a three-way mixed factor ANOVA (between-subjects factors: genotype and repeated drug treatment and within-subjects factor: session).

A three-way mixed factor ANOVA (between-subjects factors: genotype and repeated treatment and within-subjects factor: drug challenge) was used to identify whether there were any differences in the locomotor response to cocaine (in Ro 15-4513 sensitised mice) or Ro 15-4513 (in cocaine-sensitised mice)

between control and sensitised groups. The behavioural responses to vehicle in the drug-paired and non-drug-paired environments, in Ro 15-4513 treated α2(H101R) (sensitised) and wildtype mice were compared using independent samples *t*-tests.

The effects of the co-administration of cocaine and midazolam were analysed using a 2-way mixed design ANOVA (between-subjects factor: genotype and within-subjects factor: midazolam). One-way repeated measures ANOVA were used for post-hoc analysis.

6.2. Blood plasma levels

Blood plasma levels of cocaine, and its metabolites, benzoylecgonine and ecgonine methylester, were compared between mice treated with cocaine alone and in combination with midazolam using an independent measures *t*-test.

6.3. Quantitative autoradiography

The average [3H] Ro 15-4513 concentration, following incubation with [3H] Ro 15-4513 alone and in combination with diazepam, was calculated from 2 brain slices from each area for each animal. The percentage of BZ-insensitive binding sites were calculated by dividing [3H] Ro 15-4513 binding following incubation with diazepam (BZ-insensitive GABA$_A$ subtypes) by [3H] Ro 15-4513 binding following incubation with [3H] Ro 15-4513 alone (All GABA$_A$ subtypes, BZ-insensitive and BZ-sensitive) using the equation (diazepam+[3H] Ro 15-4513 binding/[3H] Ro 15-4513 binding)*100.

Independent samples *t*-tests were used to compare 1) BZ-insensitive and -sensitive sites, 2) BZ-insensitive sites, and 3) percentage of BZ-insensitive sites. To calculate the percentage of α2 GABA$_A$ subtype expression, the percentage of BZ-insensitive sites following incubation with [3H] Ro 15-4513 in the presence of diazepam in wildtype mice (α4 and α6) was subtracted from the percentage of BZ-insensitive sites following incubation with [3H] Ro 15-4513 in the presence of diazepam in α2(H101R) mice (α2, α4 and α6).

7. Results

7.1. Cocaine (10 mg/kg) challenge in Ro 15-4513 (10 mg/kg) sensitised α2(H101R) mice

Fig. 1A shows that Ro 15 4513 increased activity levels in α2(H101R) mutant mice but not wildtypes, and that this stimulant effect increased with repeated treatment. Fig. 1B shows that cocaine-induced activity levels [main effect of cocaine, $F(1,57)=26.02$, $p<0.001$, $e=1.00$], differed between genotypes [main effect of genotype, $F(1,57)=6.25$, $p=0.015$, $e=1.00$ and a significant genotype by repeated treatment, $F(1,57)=4.55$, $p=0.037$, $e=1.00$]. A two-way mixed design ANOVA showed that cocaine produced an increase in locomotor activity in Ro 15-4513 sensitised α2(H101R) mice but not Ro 15-4513 treated wildtype mice [significant cocaine by genotype interaction, $F(1,27)=7.88$, $p=0.009$, $e=1.00$] (see Fig. 1B). Fig. 1B also shows that vehicle and cocaine-induced
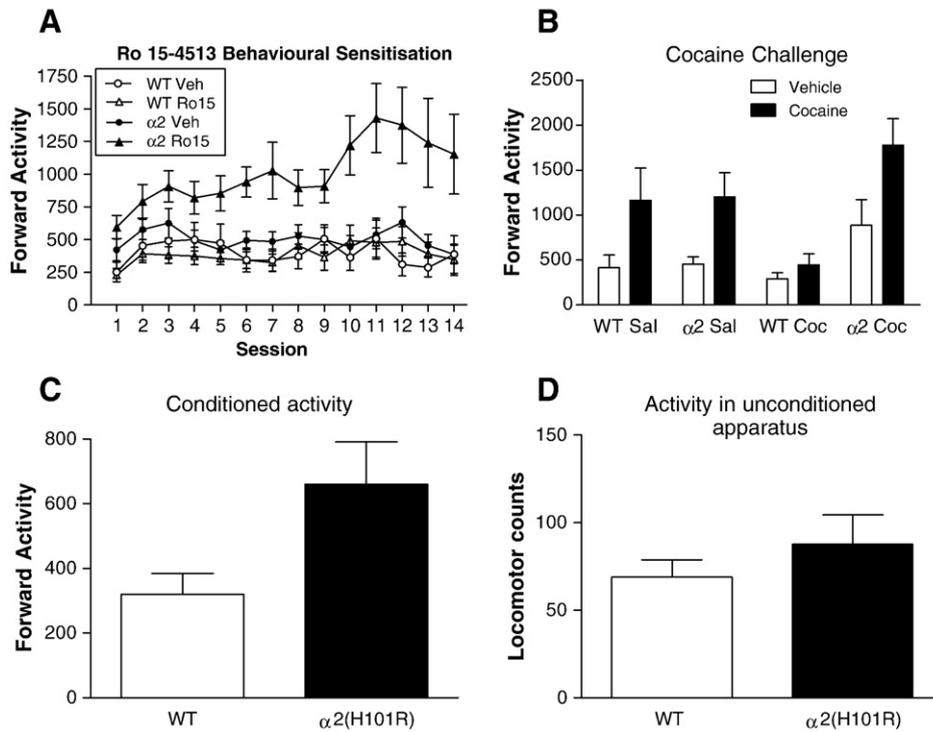
Fig. 1. Cocaine challenge and conditioned activity in α2(H101R) and wildtype mice exposed to repeated vehicle or Ro 15-4513 treatment (see Morris et al., submitted for publication) A. α2(H101R) mice sensitised to Ro 15-4513 following repeated treatment (see Morris et al., submitted for publication). B. Cocaine challenge. Mice were dosed with vehicle 2 days after chronic treatment and 10 mg/kg cocaine 1 week later. Cocaine induced a locomotor stimulant effect in all groups except wildtype mice previously treated with Ro 15-4513 (significant genotype by repeated treatment interaction, $p=0.037$). There were no differences between cocaine-induced activity levels in control and Ro 15-4513 sensitised α2(H101R) mice ($p=0.87$). C. Conditioned activity. Ro 15-4513 sensitised α2(H101R) mice showed enhanced activity levels in response to vehicle in the runways which had previously been paired with Ro 15-4513, compared to their wildtype counterparts ($p=0.031$). D. In contrast, there were no differences between vehicle-induced activity levels in these mice when tested in an environment which had not been paired with Ro 15-4513 ($p=0.193$).

locomotor activity were greater in Ro 15-4513-sensitised α2 (H101R) mice, than in α2(H101R) mice that had received only a few Ro 15 4513 pairings, however, this effect was non-significant [non-significant main effect of repeated pre-treatment, $F(1,29)=2.13$, $p=0.156$].

7.2. Conditioned activity in Ro 15 4513 sensitised α2(H101R) mice

Following repeated pairings of Ro 15 4513 with the locomotor runways, the locomotor response to vehicle in α2 (H101R) mice was increased in the apparatus, compared to
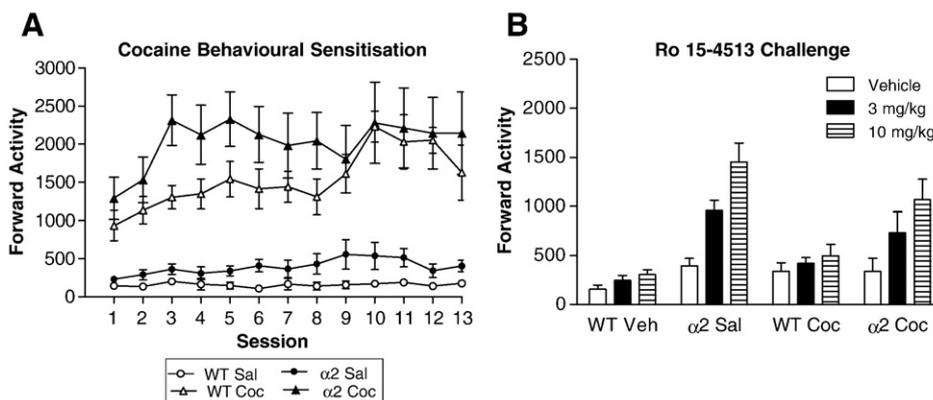

Fig. 2. Cocaine-induced behavioural sensitisation and Ro 15-4513 challenge. α2(H101R) and wildtype mice were exposed to repeated vehicle or cocaine treatment and then challenged with vehicle, 3 mg/kg and 10 mg/kg Ro 15-4513 in ascending order. A. Cocaine induced a locomotor activating effect in wildtype and α2(H101R) mice (non-significant genotype by repeated treatment interaction, $p=0.678$). Wildtype and α2(H101R) mice showed greater cocaine-induced hyperactivity on day 13 compared to day 1 (significant session by cocaine interaction, $p=0.054$; non-significant session by cocaine by genotype interaction, $p=0.556$). B. Ro 15-4513 induced similar levels of hyperactivity in cocaine-sensitised and control α2(H101R) mice (non-significant Ro 15-4513 by genotype by repeated treatment interaction, $p=0.406$).
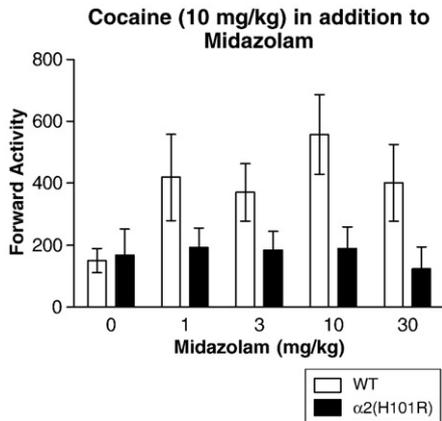
Fig. 3. Locomotor activity levels induced by cocaine (10 mg/kg) alone and in combination with midazolam (vehicle, 1, 3, 10 and 30 mg/kg) and Ro 15-4513 (vehicle, 3 and 10 mg/kg) in wildtype and α2(H101R) mice. Midazolam potentiated cocaine hyperactivity in wildtype but not α2(H101R) mice (main effect of genotype, $p = 0.041$), at 1 mg/kg ($p = 0.054$), 3 mg/kg ($p = 0.027$), 10 mg/kg ($p = 0.009$) and 30 mg/kg ($p = 0.054$).

wildtype mice that had received repeated Ro 15 4513 in the runways (Fig. 1C) [$t(19.06) = -2.32$, $p = 0.031$]. Since there was no difference between genotypes when the same animals were tested in a novel apparatus following vehicle administration (Fig. 1D) [$t(28) = -1.33$, $p = 0.193$], this finding indicates the ability of the runway to elicit conditioned activity in Ro 15 4513-sensitised knockin mice relative to the non-sensitised wildtypes.

7.3. Ro 15-4513 (1, 3 and 10 mg/kg) challenge in cocaine (10 mg/kg) sensitised α2(H101R) mice

Fig. 2A shows that cocaine-induced hyperactivity in both wildtype and α2(H101R) mice [main effect of cocaine, $F(1,25) = 52.07$, $p < 0.001$; non-significant genotype by repeated treatment interaction, $F(1,25) = 0.18$, $p = 0.678$]. An enhanced response to the locomotor activating effect of cocaine (indicating behavioural sensitisation) was shown on day 13 compared to day 1 in both genotypes [significant session by cocaine interaction, $F(1,26) = 4.07$, $p = 0.05$; non-significant session by cocaine by genotype interaction, $F(1,26) = 0.36$, $p = 0.56$].

Fig. 2B shows that Ro 15-4513 produced a locomotor stimulant effect in α2(H101R), but not wildtype mice [significant Ro 15-

4513 by genotype interaction, $F(2,48) = 17.23$, $p < 0.001$, $e = 0.785$]. Post-hoc analysis revealed that 3 mg/kg [$t(11) = -5.16$, $p < 0.001$] and 10 mg/kg [$t(11) = -5.78$, $p < 0.001$] Ro 15-4513 enhanced activity levels in α2(H101R) mice. There were no significant differences between levels of Ro 15-4513-induced hyperactivity in cocaine-sensitised and control α2(H101R) mice [non-significant Ro 15-4513 by genotype by treatment interaction, $F(2,48) = 0.86$, $p = 0.406$, $e = 0.785$].

7.4. Effect of co-administration of cocaine and midazolam on locomotor activity in α2(H101R) and their wildtype mice

Fig. 3 shows that cocaine alone induced similar levels of hyperactivity in wildtype and α2(H101R) mice [$t(23) = -0.28$, $p = 0.784$]. Midazolam potentiated cocaine hyperactivity in wildtype but not α2(H101R) mice [main effect of genotype, $F(1,22) = 4.73$, $p = 0.041$]. Post-hoc analysis demonstrated that 1 mg/kg [$t(11) = -2.16$, $p = 0.054$], 3 mg/kg [$t(11) = -2.54$, $p = 0.027$], 10 mg/kg [$t(11) = -3.14$, $p = 0.009$] and 30 mg/kg [$t(11) = -2.16$, $p = 0.054$] midazolam potentiated cocaine hyper-activity in wildtype mice.

7.5. Plasma levels of cocaine and its metabolite following treatment with cocaine alone and in combination with midazolam

There were no differences between blood plasma levels of cocaine [$t(4.47) = 0.82$, $p = 0.455$] (Fig. 4A) and its metabolites, benzoylecgonine [$t(9) = -0.15$, $p = 0.881$] (Fig. 4B) and ecgonine methylester [$t(9) = -0.25$, $p = 0.808$] (Fig. 4C), in mice treated with cocaine alone and in combination with midazolam.

7.6. Quantitative autoradiography

There were no significant differences between levels of [^{3}H] Ro 15-4513 binding in wildtype and α2(H101R) mice in any brain area studied (Table 1), indicating that there are no differences in total GABA$_A$ receptor expression (α1–α6) between genotypes.

A significantly greater number and percentage of diazepam-insensitive receptors in α2(H101R) compared to wildtype mice, following incubation with diazepam in addition to [^{3}H] Ro 15-4513, represents the expression of mutated α2 receptors. Table 2 shows that significantly higher levels of BZ-insensitive
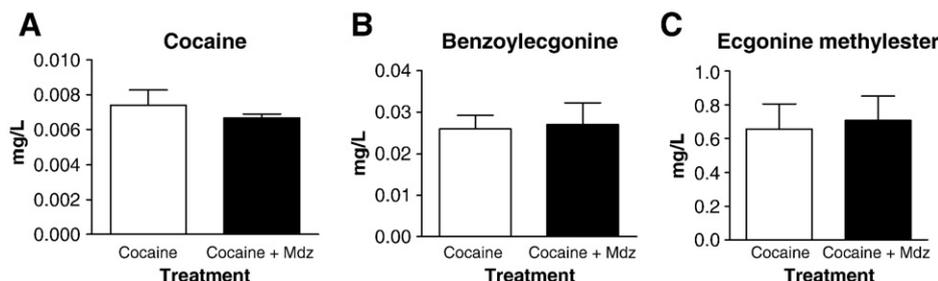

Fig. 4. Blood plasma levels of cocaine and its metabolites following cocaine alone and in combination with midazolam. There were no differences between blood plasma levels of A. cocaine ($p = 0.455$) or its metabolites, B. benzoylecgonine ($p = 0.881$) and C. ecgonine methylester ($p = 0.808$) in wildtype mice treated with 10 mg/kg cocaine alone and in combination with 10 mg/kg midazolam.

Table 1
Mean (standard error) levels of [³H] Ro 15-4513 binding (nCi/mg) in wildtype and α2(H101R) mice following incubation with [³H] Ro 15-4513 alone (column 2) and [³H] Ro 15-4513 with 5 nM of diazepam

Brain area	[³H] Ro 15-4513 binding		[³H] Ro 15-4513 binding following incubation with diazepam		% diazepam-insensitive receptors		% α2 expression
	WT	α2(H101R)	WT	α2(H101R)	WT	α2(H101R)	
NAc	34.53 (2.05)	31.73 (3.65)	2.78 (0.17)	8.21 (1.39)	9.44 (1.10)	23.57 (3.32)	14%
CEA	68.06 (4.32)	60.06 (8.71)	3.09 (0.05)	5.48 (0.96)	4.67 (0.32)	12.03 (4.06)	7%
BLA	105.22 (3.01)	87.44 (10.50)	3.27 (0.08)	5.90 (0.57)	3.13 (0.13)	8.03 (1.90)	5%
BNST	34.79 (3.14)	41.21 (1.63)	2.61 (0.15)	8.17 (1.55)	7.79 (0.57)	19.95 (3.78)	12%
CA1	81.85 (5.65)	77.81 (5.82)	3.25 (0.11)	11.13 (0.90)	4.08 (0.26)	14.77 (1.57)	11%
CA3	74.40 (5.72)	82.87 (5.17)	3.32 (0.09)	15.59 (1.60)	4.73 (0.50)	18.93 (1.68)	14%
VTA	44.72 (4.70)	40.12 (4.07)	3.02 (0.04)	3.27 (0.18)	7.41 (0.93)	8.66 (0.98)	–

The % of diazepam-insensitive receptors was calculated by using the equation (diazepam + [³H] Ro 15-4513 binding)/[³H] Ro 15-4513 binding * 100 (column 4). The % expression of α2-containing GABA$_A$ receptors was calculated by subtracting the % of BZ-insensitive sites in wildtype mice (α4 and α6) from the % of BZ-insensitive sites in α2(H101R) mice (α2, α4 and α6) (column 5).

sites were apparent in the NAc, amygdala: BLA, CEA and BNST, hippocampus: CA1 and CA3 but not in the VTA or PPN. Our results (see Table 1) show that α2 receptors are expressed at varying levels in different brain areas: NAc, 14%; BLA, 5%; CEA, 7%; BNST, 12%; CA1, 11%; CA3, 14% (Table 1).

8. Discussion

We have previously reported that α2(H101R), but not wildtype mice develop behavioural sensitisation when given repeated administrations of the atypical benzodiazepine, Ro 15-4513 (Morris et al., submitted for publication). Here we show that the genotypes do not differ from each other in sensitisation to cocaine. Sensitisation to cocaine was accompanied by the ability of the drug-paired apparatus to increase activity in its own right, a phenomenon known as conditioned activity

Table 2
t-tests comparing diazepam-insensitive sites and percentage of diazepam-insensitive sites, betweeen wildtype (α4 and α6) and α2(H101R) (α2, α4 and α6) mice

Brain area	Diazepam-insensitive sites	% Diazepam-insensitive sites
NAc	$t(6.18) = -3.88, p = 0.008$	$t(13) = -4.27, p = 0.001$
Amygdala		
BLA	$t(6.24) = -4.54, p = 0.004$	$t(6.05) = -2.57, p = 0.042$
CEA	$t(6.04) = -2.45, p = 0.047$	$t(6.07) = -1.80, p = 0.121$
BNST	$t(6.12) = -3.56, p = 0.012$	$t(6.28) = -3.18, p = 0.018$
Hippocampus		
CA1	$t(6.17) = -8.68, p < 0.001$	$t(6.33) = -6.72, p < 0.001$
CA3	$t(6.04) = -7.65, p < 0.001$	$t(7.08) = -8.10, p < 0.001$
VTA	$(t(13) = -1.45, p = 0.170)$	$(t(13) = -0.92, p = 0.370)$
PPN	$(t(13) = -0.53, p = 0.606)$	$(t(13) = -0.82, p = 0.427)$

Diazepam-insensitive sites were determined by using quantitative autoradiography to estimate [³H]Ro 15-4513 binding in the presence of 10 μM diazepam. The percentage of diazepam-insensitive sites were significantly greater in α2 (H101R) compared to wildtype mice in the NAc, BLA, BNST and CA1 and CA3 areas of the hippocampus, but not in the VTA or PPN.

(Stewart, 1983), while the sensitised animals did not differ from controls when tested in a non-conditioned environment.

Induction of behavioural sensitisation to one drug of abuse frequently results in increased locomotor stimulant effects not only of that drug, but also of other drugs of abuse, even of other pharmacological classes. Thus, animals sensitised to cocaine show increased stimulant effects of morphine and vice versa (Cunningham et al., 1997; McDaid et al., 2005), suggesting that common mechanisms underlie sensitisation to all drugs of abuse. In the current experiments we investigated the extent to which mice sensitised to Ro 15-4513 showed cross sensitisation to cocaine, and also whether cocaine sensitisation cross sensitises to Ro 15-4513. α2(H101R) mutant mice sensitised to Ro 15-4513 showed some evidence of an enhanced response to the locomotor activating effects of cocaine, though the effect was not marked.

In contrast, cocaine-sensitised α2(H101R) mutant mice did not show enhanced stimulant effects of Ro 15-4513. Cross sensitisation between drugs of abuse has been associated with neurochemical sensitisation of the mesolimbic dopamine system (Akimoto et al., 1990; Kalivas and Duffy, 1990; Kalivas and Stewart, 1991; Kazahaya et al., 1989); adaptations in the NAc (Cador et al., 1995; Dougherty and Ellinwood, 1981) mediate the expression of behavioural sensitisation, whilst neuroadaptations in the VTA mediate its induction (Cador et al., 1995; Perugini and Vezina, 1994; Stewart and Vezina, 1989; Vezina and Stewart, 1990). The lack of cross sensitisation between cocaine and Ro 15-4513 suggests that α2-mediated behavioural sensitisation may not involve adaptations of the mesolimbic dopamine pathway, consistent with Ro 15 4513's stimulant effects in α2(H101R) mutant mice not being associated with increases in dopamine turnover in the NAc (Morris et al., submitted for publication).

An alternative account of cross sensitisation posits that behavioural sensitisation consists of two components, conditioned activity occasioned by repeated pairings of an environment with unconditioned rewarding or stimulant effects of drug, combined with the ability of abused drugs to induce unconditioned increases in activity (Le Merrer and Stephens, 2006),

presumably as a consequence of their common ability to increase dopamine availability. According to this account, behavioural sensitisation, then, results from a drug's stimulant properties acting as a gain amplifier of conditioned activity. In keeping with this account, the stimulant effect of cocaine was greater in mice previously sensitised to Ro 15 4513, and which showed conditioned activity. That Ro 15-4513 was no more effective in cocaine-sensitised mice than controls might then relate to its inability to affect dopamine turnover, and thus act as a gain amplifier to the cocaine-conditioned behaviour.

Interestingly, wildtype mice treated with repeated Ro 15-4513 were insensitive to the stimulant properties of cocaine, suggesting that repeated reduction of transmission through α2-containing GABA$_A$ receptors in the wildtype may have opposite effects to facilitated transmission through the same receptors in the α2 (H101R) mutant mice. Alternatively, it can be speculated that the pro-convulsant effects of Ro 15-4513 (Corda et al., 1989; Crestani et al., 2002; Lister and Nutt, 1988) may be mediated by inverse agonism of α2-containing receptors; in that case, wildtype mice treated repeatedly with Ro 15-4513 may sensitise to its pro-convulsant effects, consequently resulting in an enhanced sensitivity to the pro-convulsant effects of cocaine (Ritz and George, 1997).

We further investigated the role of α2-containing receptors in mediating the behavioural effects of cocaine by testing whether α2-containing receptors are involved in mediating the phenomenon by which BZs facilitate the locomotor activating effects of psychostimulants (Sansone, 1980; Sansone et al., 1986; Sethy et al., 1970). We were able to rule out the possibility that such facilitation reflected simply a pharmacokinetic interaction between cocaine and midazolam resulting from them sharing a common metabolic pathway involving CYP3A enzymes (Ladona et al., 2000), as no differences in plasma levels of cocaine and its metabolites were found between mice treated with cocaine (10 mg/kg) alone and in combination with midazolam (10 mg/kg).

The ability of midazolam (1, 3, 10 and 30 mg/kg) to enhance the stimulant properties of cocaine (10 mg/kg) in wildtype mice, was abolished in α2(H101R) mice, indicating that this interaction is mediated by α2-containing receptors. This observation suggests a mechanism by which facilitated GABA transmission at α2-containing receptors forms part of a chain of neural events downstream of the facilitated dopamine release in the NAc, thought to underlie drug-induced hyperactivity (Wise and Bozarth, 1987), allowing the neuronal consequences of dopaminergic activation to be facilitated.

In a final experiment, we used quantitative autoradiography to investigate the expression of α2-containing receptors in additional brain areas associated with the mesolimbic dopamine system. Although, α2-containing receptors have been previously studied in these areas using immunohistochemical methods, recent evidence suggests that the behavioural effects induced by Ro 15-4513 may not be mediated by all α2-containing receptors. GABA$_A$ receptors are pentameric protein structures assembled from two α1, α2, α3 or α5 subunits (homologous) or two different α subunit forms (heterologous) (Benke et al., 2004), in combination with a β variant and the γ2 subunit (Benke et al.,

2004; Pritchett et al., 1989). Although it is believed that a histidine to arginine point mutation renders all α2-containing GABA$_A$ receptors BZ-insensitive, at heterologous receptors, the presence of an alternative α isoform may allow residual benzodiazepine facilitation (Benke et al., 2004). Conversely, the ability of Ro 15 4513 to exert agonist properties at H101R-mutated α2-containing receptors may be limited to homologous α2-containing receptors. In this case, Ro 15-4513-induced behavioural sensitisation in α2 (H101R) mice may be mediated by homologous α2 receptors, which account for only 46% of all α2-containing GABA$_A$ receptors (Benke et al., 2004).

Homologous α2 receptor expression was identified by increased levels of [^{3}H] Ro 15-4513 binding in the presence of diazepam in α2(H101R) compared to wildtype mice. Of the areas studied, expression of the α2 homologous receptors was identified in the NAc, BLA, BNST, CEA and the CA1 and CA3 areas of the hippocampus. In contrast, there were no significant differences between genotypes in [^{3}H]Ro 15-4513 binding in the presence of diazepam, in the VTA, or PPN. The α2 GABA$_A$ subtype accounted for approximately 14% in the NAc, 7% in the CEA, 5% in the BLA, 12% in the BNST, 11% in the CA1 and 14% in the CA3, consistent with the identification of α2 subunits in the BLA, CEA, BNST, NAc, CP and CA1 and CA3 areas of the hippocampus using immunohistochemical methods. α2 subtype expression has been shown in the VTA using other techniques.

The identification of high levels of α2-containing receptors in the NAc, consistent with previous observations (Schwarzer et al., 2001), suggests that these receptors are a likely candidate for mediating the midazolam-induced potentiation of cocaine hyperactivity, possibly by reducing the inhibitory output of GABA neurones projecting to the VP (Alheid and Heimer, 1988; Kalivas et al., 1993), an action that is known to play an important role in mediating drug-induced hyperactivity (Austin and Kalivas, 1988; Bourdelais and Kalivas, 1990; Gong et al., 1997; Mogenson and Nielsen, 1983; Wallace and Uretsky, 1991). It is thought that psychostimulant enhanced dopamine release in the NAc (Di Chiara and Imperato, 1988; Kelley and Iversen, 1975) reduces GABA transmission in the VP by inhibiting NAc to VP GABAergic projection cells (Bourdelais and Kalivas, 1990; Ferre et al., 1994; Yang and Mogenson, 1985, 1989), resulting in a locomotor stimulant effect. We speculate that the Ro 15-4513-induced stimulant effect may also be mediated by decreased GABA transmission in the VP, but via a mechanism other than increased accumbal dopamine.

It is of interest to note that BZ facilitation of psychostimulant hyperactivity in rodents is used as a model of mania associated with bipolar disorder. Amphetamine in combination with the BZ, chlordiazepoxide (CDP), induces repetitive locomotor activity in rats exposed to a novel environment, which is thought to represent excessive activity characteristic of episodes of mania in humans (Aylmer et al., 1987; Rushton and Steinberg, 1966; Vale and Ratcliffe, 1987). Drugs used to treat mania in humans are able to reduce the heightened levels of activity induced by the amphetamine–CDP combination; lithium, lamotrigine and valproate have all been shown to attenuate amphetamine–CDP induced hyperactivity in rats (Arban et al.,

2005; Aylmer et al., 1987; Cao and Peng, 1993; Dencker et al., 2008; Rushton and Steinberg, 1966; Vale and Ratcliffe, 1987). Consequently, this animal model has been used to assess the anti-manic properties of novel compounds (Dencker et al., 2008; Lamberty et al., 2001). Our observation that selective agonism of α2-containing GABA$_A$ receptors mediate benzodiazepine potentiation of cocaine hyperactivity, may suggest that an inverse agonist at α2-containing GABA$_A$ receptors would produce an attenuation of cocaine hyperactivity, and thus a reduction of manic-like symptoms in humans.

In conclusion, the asymmetrical pattern of cross sensitisation between cocaine and Ro 15-4513 suggests that the neuroadaptations underlying Ro 15-4513-induced behavioural sensitisation are not identical to those associated with cocaine and other drugs of abuse. Our observation that the BZ-induced potentiation of cocaine hyperactivity was abolished in the absence of α2-containing GABA$_A$ receptor modulation, suggests that the locomotor stimulant effects of BZs and psychostimulants are mediated by a common neural system. However, this effect may simply be due to an additive effect of the stimulant properties of both drugs. Together, our observations that Ro 15-4513 does not increase dopamine release in the NAc and Ro 15-4513 does not decrease dopamine release in the PFc (effects thought to underlie the stimulant properties of other abused drugs), suggests that the α2 mediated stimulant effect, subsequent induction of behavioural sensitisation and the potentiation of cocaine hyperactivity may be mediated by non-dopaminergic neural systems.

Acknowledgements

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