Available online at www.sciencedirect.com
ScienceDirect
Pharmacology, Biochemistry and Behavior 90 (2008) 1 – 8
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Targeted deletion of the *GABRA2* gene encoding α2-subunits of $GABA_A$ receptors facilitates performance of a conditioned emotional response, and abolishes anxiolytic effects of benzodiazepines and barbiturates

C.I. Dixon [a], T.W. Rosahl [b], D.N. Stephens [a,*]

[a] *Department of Psychology University of Sussex, Falmer, Brighton, BN1 9QG, UK*
[b] *Genetically Engineered Models, Merck Research Laboratories, Rahway, NJ, USA*

Available online 31 January 2008

Abstract

Mice with point-mutated α2 $GABA_A$ receptor subunits (rendering them diazepam insensitive) are resistant to the anxiolytic-like effects of benzodiazepines (BZs) in the conditioned emotional response (CER) test, but show normal anxiolytic effects of a barbiturate. We investigated the consequence of deleting the α2-subunit on acquisition of the CER with increasing intensity of footshock, and on the anxiolytic efficacy of a benzodiazepine, diazepam, and a barbiturate, pentobarbital. α2 knockout (KO) and wildtype (WT) mice were trained in a conditioned emotional response (CER) task, in which lever pressing for food on a variable interval (VI) schedule was suppressed during the presentation of a compound light/tone conditioned stimulus (CS+) that predicted footshock. The ability of diazepam and of pentobarbital to reduce suppression during the CS+ was interpreted as an anxiolytic response. There were no differences between the genotypes in shock sensitivity, as assessed by their flinch responses to increasing levels of shock. However, α2 KO mice showed a greater suppression of lever pressing than WT littermates in the presence of a compound cue signalling footshock. Diazepam (0, 0.5, 1 and 2 mg/kg) induced a dose-dependent anxiolytic-like effect in WT mice but no such effect was seen in KO mice. Similarly, although pentobarbital (20 mg/kg) reduced the ability of the CS+ to reduce lever pressing rates in WT mice, this effect was not seen in the KO. These findings suggest that α2-containing $GABA_A$ receptors mediate the anxiolytic effects of barbiturates, as well as benzodiazepines, and that they may be involved in neuronal circuits underlying conditioned anxiety.

Keywords: $GABA_A$; α2 subunit; Knockout; Anxiety; Diazepam; Barbiturate; Conditioned emotional response

1. Introduction

Benzodiazepines achieve their effects by binding at a distinct site on $GABA_A$ receptors, and modulating the frequency of GABA-induced channel opening, and resultant chloride flux (Bormann and Kettenmann, 1988; McDonald, 1979). $GABA_A$ receptors are pentameric protein structures, benzodiazepine-sensitive subtypes being assembled from two α1, α2, α3 or α5-subunits or two different α subunit forms (Benke et al., 2004), in combination with a β variant and the γ2-subunit (Benke et al., 1991; Pritchett et al., 1989). The α4- and α6-subunits convey insensitivity to benzodiazepines, as the essential arginine residue within the benzodiazepine-binding site, is substituted with histidine (Wieland et al., 1992). Pharmacological and genetic studies have suggested that distinct receptor subtypes play distinct functional roles. Thus, studies in which histidine-to-arginine point mutations have been introduced into the benzodiazepine-binding domain of members of the α subunit family to make them insensitive to benzodiazepines, have highlighted that the sedative and amnesic properties of benzodiazepines are mediated by the α1-subunit (McKernan et al., 2000; Rudolph et al., 1999), whilst both targeted deletion of α5-subunits (Collinson et al., 2002), and pharmacological inhibition of α5-containing receptors (Collinson et al., 2006) facilitate learning and memory. α2- and α3-subunit-containing receptors have been implicated in the myorelaxant properties of benzodiazepines (Crestani et al., 2001), while α3-containing receptors have also been related to the anxiolytic effects (Atack

* Corresponding author. Tel.: +44 1273 678638; fax: +44 1273 876619.
E-mail address: d.stephens@sussex.ac.uk (D.N. Stephens).

0091-3057/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2008.01.015

et al., 2005; McKernan et al., 2000; Morris et al., 2006), though deletion of α3-subunits does not compromise the anxiolytic effects of diazepam in mice with intact α2-containing receptors (Yee et al., 2005). Most importantly for the present manuscript, a mutation rendering α2-containing $GABA_A$ receptors insensitive to benzodiazepines results in loss of anxiolytic effects of diazepam (Low et al., 2000; Morris et al., 2006), strongly suggesting that diazepam's anxiolytic effects are mediated by their action at α2-containing $GABA_A$ receptors, though α3-containing receptors also play a role (Dias et al., 2005; Morris et al., 2006).

Although these kinds of experiments provide strong evidence that α2-containing receptors are involved in the anxiolytic properties of benzodiazepines, they leave open questions of whether the same receptors mediate anxiolytic properties of other drugs, such as barbiturates, and whether α2-containing $GABA_A$ receptors are not only involved in mediating drug responses, but also contribute to neural circuitries underlying aspects of anxiety itself. Studies using the histidine-to-arginine point mutation are unable to address these questions, as they leave the binding domains for barbiturates and alcohol unaffected, and these drugs consequently retain their anxiolytic properties in the point-mutated mice (Morris et al., 2006). Although we found the point-mutated mice to be more anxious in the CER (Morris et al., 2006), interpretation of that observation is difficult as such mice are reported to retain normal $GABA_A$ receptor expression (Wafford et al., 2004) and transmission (Low et al., 2000; Marowsky et al., 2004) and thus provide a poor test of whether disruption of this circuitry contributes to anxiety.

We therefore investigated the effect of a targeted deletion of the gene encoding the α2-subunit (*GABRA2*) in a CER test of conditioned anxiety and subsequent pharmacological manipulation of this effect by diazepam and the non-benzodiazepine anxiolytic, pentobarbital. The CER test was used so as to provide a direct comparison between this study and the previous work conducted using the α2(H101R) mutant mouse (Morris et al., 2006). We predicted that the KO mouse would show an enhanced suppression of lever pressing in response to fear-conditioned stimuli and a loss of the anxiolytic effect of diazepam, similar to the effect seen in the α2(H101R) mutant. We also predicted that deletion of the *GABRA2* gene would abolish the anxiolytic effect of pentobarbital, implying a more general role of the $GABA_A$ α2-subunit in anxiety behaviour rather than only benzodiazepine anxiolysis.

2. Materials and methods

2.1. Animals

α2-subunit KO mice: an α2 subunit-specific cDNA probe was used to screen a bacterial artificial chromosome (BAC) library containing genomic mouse DNA (Research Genetics, Huntsville, AL). The targeting vector was generated from overlapping BAC-derived subclones in pBlue-script covering 12 kb of genomic DNA containing exons 4 and 5 of the α2 gene. The targeting vector contained a 6 kb ApaI-XbaI long arm including exon 5, the 1.55 kb SphI-SacI short arm, the neomycin resistance gene, and the *HSV-TK* gene and resulted in a complete deletion of exon 4. The linearized targeting vector was introduced into AB2.2 embryonic stem (ES) cells in several independent experiments. Targeting frequency, which was confirmed by PCR and Southern blot analysis, was 1:100. Three independent ES cell clones gave rise to highly chimeric males and germ-line transmission. Chimeric mice were bred with deleter mice (Schwenk et al., 1995) to eliminate the neomycin resistance gene in the genome, to establish breeding colonies. For the present experiments, homozygous and wild-type littermate controls were obtained from breeding heterozygous parents.

Although early on there was about a 30% decrease in the number of mutants surviving to weaning, α2-subunit KO mice now appear to breed and develop normally. α2-subunit KO mice are now maintained on a mixed C57BL6J/129SvEv genetic background, and a colony has been established at the University of Sussex. It is currently not known whether genetic deletion of this subunit altered the expression of other $GABA_A$ receptor subunits.

Animals were housed two or three to a cage under a 12 h light/dark cycle, (lights on at 7.00 am) in a holding room with controlled temperature (≈21 °C) and humidity (≈50%). Food and water were available *ad libitum* until commencement of the experiment. Mice weighed between 20 and 30 g at the beginning of the study and were subsequently food restricted throughout to maintain approximately 85% of baseline body weight. All experiments were performed between 9 am and 6 pm and were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986, following ethical review.

2.2. Drugs

Diazepam (Hoffman LaRoche, Basel, Switzerland) was suspended in a 0.9% saline solution containing 10% cremophor. Pentobarbital hydrochloride (Sigma–Aldrich, Poole, UK) was dissolved in 0.9% saline solution. Both were administered i.p. at a volume of 10 ml/kg.

2.3. Apparatus

Mouse operant chambers (Med Associates, Vermont, USA) containing two levers were used. Stimulus lights were situated above each lever, with a food magazine between the levers and a speaker located above the magazine. The floor of the chamber consisted of steel rods connected to a shock generator (Med Associates, Vermont, USA). The chambers were connected to a computer, which recorded behaviour using Med-PC software.

2.4. Conditioned emotional response

2.4.1. Food shaping

During 15 daily 1-hour sessions, $GABA_A$ α2-subunit KO mice ($n=10$) and WT littermates ($n=10$) were trained to operate one of two levers to obtain a food pellet (20 mg; Noyes Precision Pellets, Formula P; Research Diets, New Brunswick,

NJ). The active lever was counterbalanced across the experiment and the houselight was off throughout. A response on the alternative lever had no programmed consequence, whilst a response on the active lever initially elicited food delivery on a VI (variable interval) 1 s reinforcement schedule. The VI schedule escalated though the following values; 5, 10, 30, 60, 90 and 120 s. Ten reinforcers were to be obtained at each level before advancing to the next VI schedule. Food was delivered on the first lever response after the specified time had elapsed. The animals were trained until a stable rate of responding was evident on a VI 120 s schedule. Subsequently, mice were habituated to the light (30 s) and tone (2.9 kHz for 30 s, 0.5 s pulses) cues during a single 30 min session.

2.4.2. Shock sensitivity

To ascertain any differences in sensitivity to the footshock between genotypes, flinch thresholds were measured. Mice were placed in the operant chambers and exposed to a 0.5 s footshock starting at 0.1 mA and increasing by 0.05 mA to a maximum of 0.5 mA, until a flinch was observed, by an observer unaware of genotype.

2.4.3. CER training

During daily 35 min sessions, food pellets were available on a VI 120 s schedule. The light and tone cues were assigned as CS+ and CS− in a counterbalanced design. Each session included ten cue presentations; 5 CS+ and 5 CS−, each presented for 30 s. The cues were activated by a lever press after a variable time period (mean value 120 s; range 30–210 s). The CS+ was immediately followed by a 0.5 s unavoidable footshock whilst the CS− had no consequence. The footshock was initially delivered at an intensity of 0.1 mA and increased by 0.05 mA every 3 days up to a shock intensity of 0.5 mA.

The rate of lever pressing during these sessions was recorded for 30 s prior to CS onset, the 30 s period during CS presentation and for 60 s following CS presentation. The degree of suppression of lever pressing behaviour to the CS+ was used as a measure of anxiety. The suppression ratio was calculated using the equation $A/(A+B)$, where A is the number of lever presses during the CS and B is the number of presses during the 30 s period prior to CS presentation. The average suppression ratio to CS+ and CS− was calculated for each session. A ratio of 0.5 indicated no suppression to the CS, whilst a value of 0 denotes complete suppression. The average number of lever presses per minute was also calculated from the rate of lever presses made during the 60 s following CS presentation. Since each shock level consisted of 3 sessions, the mean value across sessions was used to give a single measure of activity per shock intensity.

2.5. CER test sessions

The CER test sessions were implemented as described above, with all animals receiving a footshock of 0.5 mA intensity. Suppression ratios to this shock intensity were equal for both genotypes. Pentobarbital (0 and 20 mg/kg; WT $n=8$ and KO $n=7$) was administered 20 min before testing in a latin square design with at least one training session between each drug-test day. Following a further 3 days training, diazepam (0, 0.5, 1 and 2 mg/kg; WT $n=7$ and KO $n=8$) was administered 30 min prior to testing using a latin square design. Animals were excluded at this point in the study either due to a failure to lever press or a failure of the CS to continue to produce a suppression in lever responding.

2.6. Statistical analysis

All data were log transformed to achieve homogeneity of variance. To assess the ability of both genotypes to acquire a lever press response to obtain a food reward, a two-way mixed design ANOVA was used to analyse the percentage of active lever presses, with training session as a within-subject factor and genotype as between-subject factor. The number of lever presses on both levers was measured at session 20 to identify any baseline differences in responding. These data were

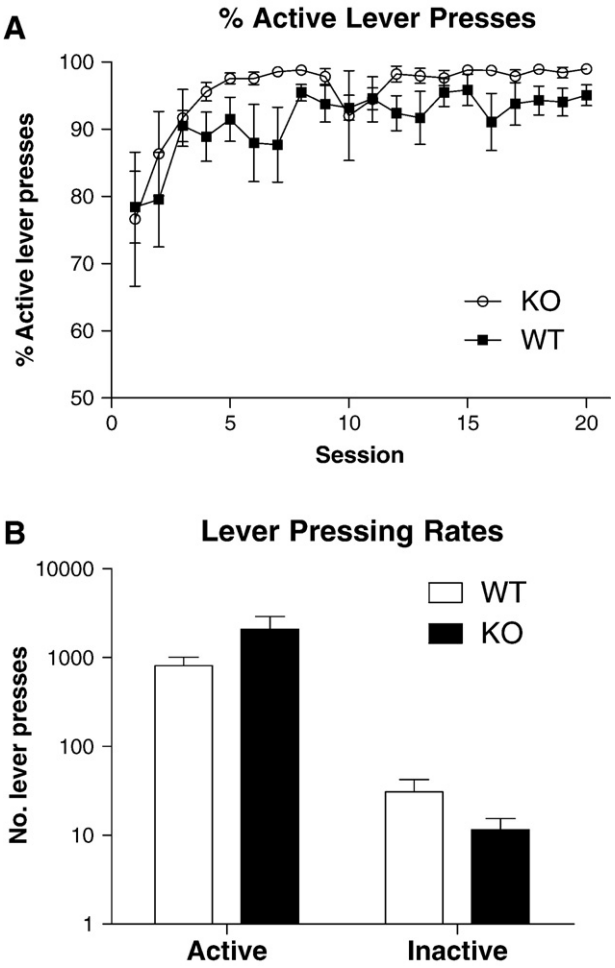

Fig. 1. Food shaping. Data represent mean and SEM. (A) Active lever presses as percentage of total, showing acquisition of operant responding to acquire food reward. Percentage of active lever presses increases with session ($p<0.001$) and does not differ between genotypes. (B) Active and inactive lever pressing rates during session 20. Responding is higher on the active lever ($p<0.001$) but does not significantly differ between genotypes, although a trend is evident for KO mice to show a higher rate of responding on the active lever. (Note that the y-axis of Fig. 1B is using a Log $_{10}$ scale).

analysed using a two-way mixed design ANOVA with lever as within-subject and genotype as between-subject factor. To assess the sensitivity of each genotype to the shock, the flinch threshold was analysed using an independent samples *t*-test.

A two-way mixed design ANOVA was used to test the ability of differing shock levels to alter the suppression ratio during the

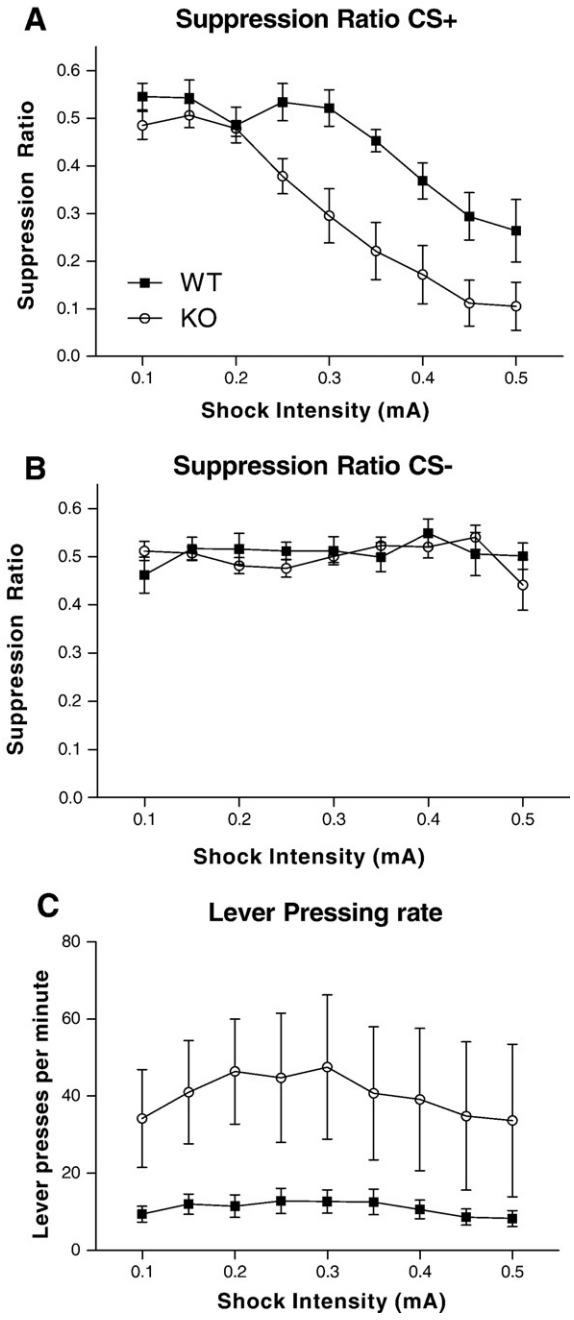

Fig. 2. CER training. Data represent mean and SEM. During 35 min sessions, mice were exposed to 5 CS+ presentations which were followed immediately by footshock and 5 CS− presentations which had no programmed consequence. The intensity of the footshock increased by 0.05 mA every 3 sessions, with the mean value across the 3 sessions taken to give a single measure per shock intensity. (A) Suppression of responding to CS+ presentation increased with footshock intensity ($p<0.001$). (B) The degree of suppression was not affected after CS− presentation (NS) and did not differ between genotypes (NS). (C) Baseline responding on the active lever varied with footshock intensity ($p<0.01$).

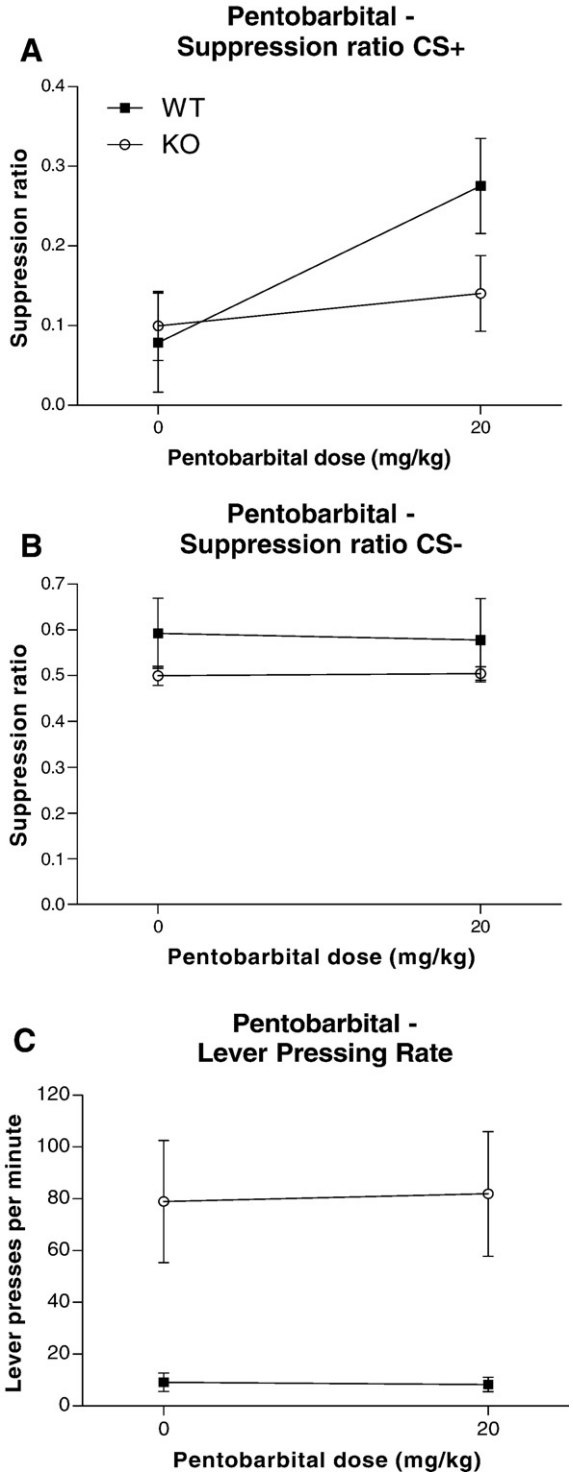

Fig. 3. Data represent mean and SEM. Effect of pentobarbital on conditioned suppression of lever pressing. WT ($n=8$) and KO ($n=7$) mice were administered with pentobarbital (20 mg/kg) 20 min prior to CER testing. (A) Pentobarbital significantly increased the suppression ratio during the CS+ ($p<0.01$) in WT ($p<0.01$) but not KO animals. (B) Administration of pentobarbital had no effect on suppression of responding during CS− presentation. (C) Rates of lever pressing on the active lever were higher in the KO mice ($p<0.01$) but were not altered by pentobarbital dose in either genotype.

CS+ and CS− presentations, and the baseline lever pressing rate during the 60 s post CS presentation. Shock level was treated as a within-subject factor, with genotype as a between-subject factor.

To test the ability of pentobarbital and diazepam to reduce suppression to the CS+ on the active lever, a two-way mixed design ANOVA was conducted for each compound, with dose as within-subject factor and genotype as between-subject factor. Suppression ratios for both the CS+ and CS− were tested, along with lever pressing rate to assess any possible effects of the compounds on lever responding. Where significant dose by genotype interactions were found, *post hoc* analyses were conducted using one-way repeated measures ANOVA for each genotype.

3. Results

3.1. Conditioned emotional response

3.1.1. Food shaping

During the 20 food shaping sessions, both genotypes acquired the ability to respond on the active lever to receive a food reward, on a VI 120 s schedule (Fig. 1A). A significant effect of session ($F_{(19,342)}=2.945$, $p<0.001$) on the percentage of active lever presses shows that an increase in responding on the active lever across the training sessions. This increase did not differ between genotypes (non-significant session by genotype interaction, $F_{(19,342)}=0.854$, NS; non-significant effect of genotype $F_{(1,18)}=0.494$, NS).

Fig. 1B shows the rate of responding on both active and inactive levers on day 20 of food training. Responding was higher on the active lever (main effect of lever; $F_{(1,18)}=149.882$, $p<0.001$), a trend that is evident across both genotypes (non-significant main effect of genotype, ($F_{(1,18)}=0.030$, NS). There was a trend for the KO animals to have a higher rate of responding on the active lever but this was not significant (lever by genotype interaction, ($F_{(1,18)}=3.046$, $p=0.098$).

3.1.2. Footshock sensitivity

Flinch thresholds were similar for both genotypes (WT mean 0.22±0.01 mA; KO mean 0.20±0.01 mA) indicating a similar sensitivity to the shock ($t(18)=2.060$, NS).

3.1.3. CER training sessions

Fig. 2A shows the suppression ratio of WT and KO mice in response to CS+ presentation paired with footshock. As shock intensity increased from 0.1 to 0.5 mA, both genotypes showed a suppression in lever responding, as demonstrated by a significant effect of shock level ($F_{(8,144)}=25.107$, $p<0.001$). However, KO animals showed a greater suppression than WT (significant shock level by genotype interaction, $F_{(8,144)}=2.830$, $p<0.01$; significant main effect of genotype, $F_{(1,18)}=15.953$, $p<0.01$). In contrast, CS− presentation had no effect on lever pressing behaviour as shown by the lack of suppression of responding by both genotypes (Fig. 2B; non-significant effect of shock level, $F_{(8,144)}=1.249$, NS; non-significant shock level by genotype interaction, $F_{(8,144)}=1.217$, NS).

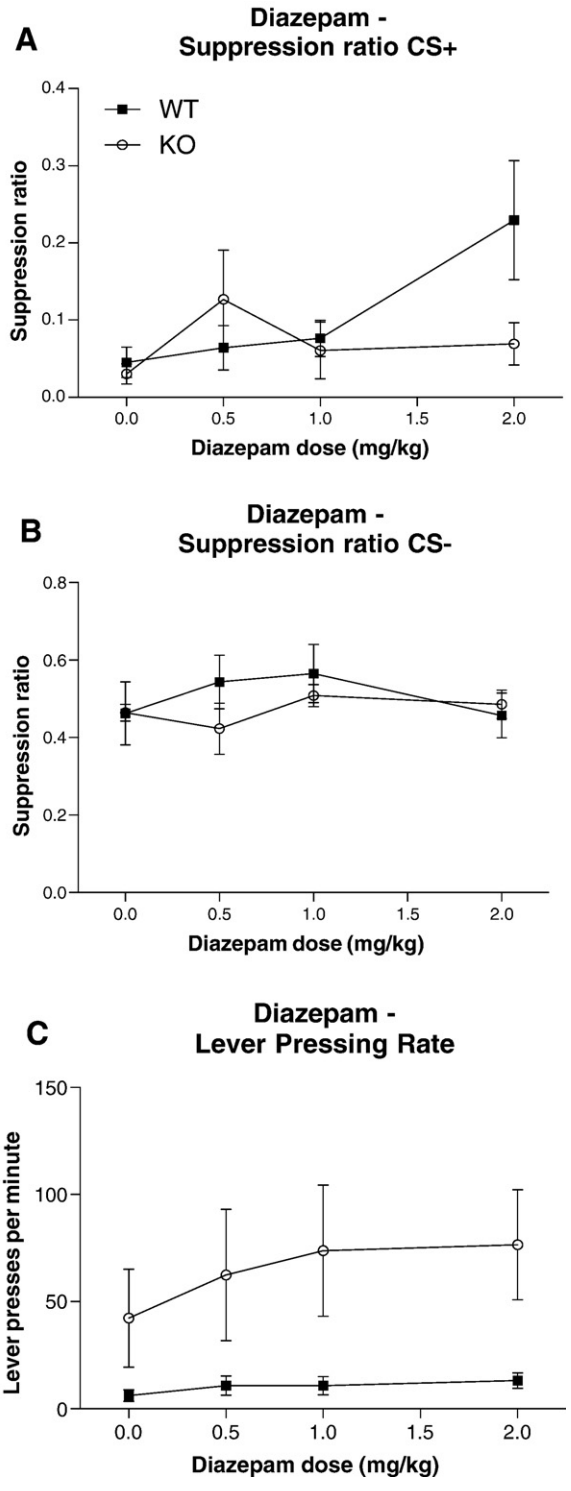

Fig. 4. Data represent mean and SEM. Effect of diazepam on conditioned suppression of lever pressing. WT ($n=7$) and KO ($n=8$) mice were administered with diazepam (0.5, 1 and 2 mg/kg) 30 min prior to CER testing. (A) Diazepam significantly increased the suppression ratio during CS+ ($p<0.05$) in WT ($p<0.01$) but not KO animals. (B) Administration of diazepam had no effect on suppression of responding during CS− presentation. (C) Rates of lever pressing on the active lever were generally higher in the KO mice ($p<0.01$). Increasing doses of diazepam increased lever pressing ($p<0.01$) but did so in both genotypes.

Analysis of active lever pressing rates at each shock level indicated that KO animals were more active than WT animals at lower shock levels (Fig. 2C; significant effect of shock level, $F_{(8,144)}=2.671$, $p<0.01$; significant shock level by genotype interaction, $F_{(8,144)}=2.709$, $p<0.01$). Both genotypes showed a small increase of baseline active lever pressing at mid-range shock intensities (WT main effect of session, $F_{(8,72)}=2.473$, $p<0.05$; KO main effect of session, $F_{(8,72)}=2.690$, $p<0.05$).

3.2. CER drug-test sessions

3.2.1. Pentobarbital

Fig. 3A shows that pentobarbital (20 mg/kg) increased the suppression ratio in response to CS+ presentation, in WT but not KO mice (significant genotype by dose interaction, $F_{(1,13)}=4.824$, $p<0.05$). Pentobarbital administration had no effect in either genotype on responding during CS− presentation when no shock was administered (Fig. 3B; non-significant effect of dose, $F_{(1,13)}=0.010$, NS; non-significant dose by genotype interaction, $F_{(1,13)}=0.033$, NS).

Whilst a main effect of genotype ($F_{(1,13)}=12.708$, $p<0.05$) on baseline active lever presses rates was evident, highlighting that KO mice have a higher lever pressing rate (Fig. 3C), this was not affected by administration of pentobarbital. Non-significant effects of dose ($F_{(1,13)}=0.282$, NS) and dose by genotype interaction ($F_{(1,13)}=0.122$, NS) indicate that pentobarbital caused an increase in the suppression ratio in WT mice at a dose which does not cause sedation in either genotype.

3.2.2. Diazepam

Fig. 4A shows that the administration of diazepam caused an increase in the suppression ratio (main effect of dose, $F_{(3,39)}=3.061$, $p<0.05$) but this effect differed between WT and KO mice (significant dose by genotype interaction, $F_{(3,39)}=2.726$, $p<0.05$). ANOVA conducted on each genotype showed the anxiolytic effect was evident in WT (main effect of dose, $F_{(3,18)}=6.063$, $p<0.01$) but not KO mice (non-significant effect of dose, $F_{(3,18)}=0.945$, NS).

Diazepam had no effect on lever pressing rate during CS− presentation, which was not paired with shock (Fig. 4B; non-significant effect of dose, $F_{(3,39)}=0.793$, NS; non-significant interaction $F_{(3,39)}=0.662$, NS). Fig. 4C shows the rate of active lever pressing for each dose of diazepam. A main effect of genotype ($F_{(1,13)}=5.001$, $p<0.05$) showed that lever pressing was higher in KO mice than WT mice, as in the previous stages of the experiment. Diazepam increased lever pressing (main effect of dose, $F_{(3,39)}=7.250$, $p<0.01$). However, a non-significant dose by genotype interaction ($F_{(3,39)}=0.587$, NS) implies that this stimulant effect is similar between genotypes and therefore does not account for the genotype differences in behaviour after administration of diazepam.

4. Discussion

The current experiments show that deletion of the *GABRA2* gene encoding the $GABA_A$ α2-subunit resulted in an exaggerated suppression of lever pressing in the presence of fear-conditioned stimuli, and, furthermore, prevented the anxiolytic effects of diazepam and a drug acting at another site on the $GABA_A$ receptor, pentobarbital. Previous experiments investigating the role of this subunit in anxiety have concentrated on its ability to mediate the anxiolytic properties of benzodiazepine compounds. In particular, the α2(H101R) mutant mouse strain has been used in common tests of unconditioned (Low et al., 2000) and conditioned (Morris et al., 2006) anxieties to demonstrate that modulation of this subunit is a major mechanism whereby benzodiazepines achieve their anxiolytic effects. These tests, however, whilst providing information about benzodiazepine modulation of anxiety, do not allow investigation of the role of the α2-subunit in anxiety processing in general or the modulation of anxiety by compounds interacting with the receptor at a site other than the benzodiazepine-binding site.

During the food shaping phase of the present experiment, mice were trained to respond on a VI 120 s schedule to obtain food reward. Both WT and KO mice achieved stable lever pressing with similar rates of acquisition. At this stage, there was a trend for KO animals to show higher responding on the active lever only, although this was not significant. This increased rate of lever pressing in the KO animals was evident throughout the study, implying that the KO animals have a tendency towards hyperactivity, although this was subject to high variability within the group. During the CER training phase, both genotypes showed suppression of lever pressing in response to the CS+, which was paired with increasing amplitude of footshock; presentation of the CS−, which had no programmed consequence, had no effect on lever responding for food in either genotype. Interestingly, the KO mice showed an increase in suppression of lever pressing to CS+ presentation when compared to WT counterparts. To control for differences in sensitivity to footshock, a flinch threshold test was conducted (Crawley, 1999). Since the threshold was similar for both genotypes, it seems that this increased suppression is not accounted for by an increased sensitivity to shock. Similarly, although KO mice showed a tendency towards higher rates of responding on the active lever, the suppression ratio calculation controls for such differences in response rate, so that differences in lever pressing rates are also unlikely to be the cause of the exaggerated suppression. A further possible interpretation is that the KO animals, rather than showing increased anxiety, show facilitated learning. This explanation appears, however, to be unlikely since KO mice show normal acquisition of operant lever pressing behaviour in the food shaping phase of the current study, while in tests of pavlovian association, including discriminated approach, pavlovian approach and pavlovian-to-instrumental transfer (see (Mead and Stephens, 2003) for description of tests), these animals perform at a level comparable to that of WT litter mates (Dixon and Stephens, unpublished data). It is therefore likely that the facilitated conditioned emotional response reflects an increased level of anxiety of the KO, suggesting that α2-containing $GABA_A$ receptors play a role in modulating conditioned anxiety. Whether other forms of anxiety are also modulated by these receptors remains to be examined.

An important consideration when working with KO mice is that compensatory changes may take place in response to the deletion, and that such compensatory changes, rather than the targeted deletion, may be responsible for any behavioural changes. Certainly, in the case of deletion of the *GABRA1* gene, resulting in loss of α1-subunits, there is evidence for increased expression of α3, and decreased expression of γ2-subunits, as well as alterations in receptor clustering and distribution (Kralic et al., 2006). Whether related changes occur in the α2 KO is not known at present. However, it is worth noting that the anxiogenic phenotype of the KO, and its loss of response to the anxiolytic effect of diazepam resembled that of the H101R mutant (Morris et al., 2006), which, because this mutation does not alter $GABA_A$ receptor response to GABA (Low et al., 2000; Marowsky et al., 2004), is thought not to be subject to compensatory changes in expression of other subunits.

In the drug testing phase of the experiment, the suppression ratio had reached an approximate value of 0.15 at a shock level of 0.5 mA in both genotypes. Administration of pentobarbital caused a reduction in the degree of suppression in WT animals at a dose of 20 mg/kg but not in KO littermates. In the CER test, a reduction in the suppression of lever pressing in response to fear-conditioned stimuli is interpreted as evidence for anxiolytic activity (Millan, 2003). This dose did not induce sedation in either genotype and the lever pressing rate remained unaffected. A similar effect was seen after administration of diazepam, with a dose-dependent increase in lever pressing, reaching significance at 2 mg/kg. Diazepam also caused an increase in lever pressing, but the "anxiolytic" effect in the CER is unlikely to simply reflect increased responding as lever pressing during the baseline period outside of CS presentation is considered in the suppression ratio calculation. Importantly, the diazepam-induced increase in responding was observed in both genotypes and so cannot account for the loss of the anxiolytic effect of diazepam in the KO, and indicates that the ability of diazepam to facilitate operant responding is not mediated by α2-containing receptors.

It should be noted that although drug testing was carried out at a shock level (0.5 mA) giving rise to similar levels of suppression in both genotypes, it cannot be ruled out that the α2 KO mice may have been more anxious at this shock level, and that the failure to find a statistically reliable difference may have reflected the already low suppression ratios of both groups, but in particular of the KO (i.e. a floor effect). Thus, an alternative explanation of the ineffectiveness of anxiolytic drugs to exert an anxiolytic effect in the KO mice may be that the level of anxiety was too high to be overcome. A similar consideration might be applied to our previous report (Morris et al., 2006) in which α2 (H101R) mutants also showed an increased level of anxiety, and a decreased anxiolytic effect of diazepam. However, in that experiment, pentobarbital gave a similar size of anxiolytic effect. Such a control anxiolytic drug was not available in the present experiments.

Further evidence for the role of α2-containing receptors in anxiety lies within the anatomical location of the subunit within the rodent amygdala, a structure known to play a crucial role in anxiety (LeDoux, 2000). Since the clinical use of benzodiazepines has implicated a role for $GABA_A$ receptors in anxiety processing, GABA transmission within the amygdala has been of particular interest. Benzodiazepine administration into the amygdala results in anxiolysis (Harris and Westbrook, 1995; Nagy et al., 1979). Furthermore, administration of a GABA antagonist into the amygdala elicits anxious behaviour (Sanders and Shekhar, 1995), implicating a role of GABA transmission within the amygdala irrespective of benzodiazepine modulation. The potential importance of the α2-subunit in mediating this process has been highlighted by the localisation of specific alpha subunits within the amygdala. The two predominant subunits within the rodent amygdala are the α1- and α2-subunits (Pirker et al., 2000). The α2-subunit is highly expressed in the central nucleus of the amygdala (CeA) whilst the α1-subunit shows little or no expression in this nucleus (Kaufmann et al., 2003; Marowsky et al., 2004). The α1-subunit predominates in the basolateral nucleus (BLA) with moderate α2-subunit expression, but the α2-subunit has been reported to prevail pharmacologically over the α1-subunit when the two subunits are co-assembled (Kaufmann et al., 2003). The strong prevalence of the α2-subunit and its predominant role in mediating the inhibitory effects of diazepam within the amygdala (Marowsky et al., 2004) provide a strong correlation with behavioural observations that the subunit possesses an important role in mediating anxiolytic responses.

Whilst the α2-subunit is often discussed as the major subunit involved in anxiolysis, recent studies have additionally implicated a role for the α3-subunit in the anxiolytic effect of benzodiazepines. Thus, an inverse agonist selective for the α3-subunit shows an anxiogenic effect (Atack et al., 2005) whilst an α3-subunit-selective agonist is anxiolytic in the rat elevated plus maze (Dias et al., 2005). Furthermore, L838 417, an α2-, α3- and α5-subunit-selective agonist, retains its anxiolytic activity in the α2(H101R) mutant mouse strain in the CER test of conditioned anxiety (Morris et al., 2006), in which benzodiazepine action at α2-containing $GABA_A$ receptors is abolished. Since the α5-subunit has not previously been implicated in anxiety processing and mutation of the α2-subunit renders receptors benzodiazepine insensitive, the anxiolytic effect can be attributed to receptors expressing the α3-subunit. In light of this evidence, it is perhaps surprising that no anxiolytic effects of diazepam or pentobarbital, which might reflect a residual action through α3-containing receptors, were found in the α2-subunit KO mouse. The anxiolytic effect of L838-417 as described by Morris et al. (2006) was achieved in the α2(H101R) mutant mouse at a dose of 30 mg/kg whilst a similar anxiolytic effect was seen in the WT at 10 mg/kg. This observation suggests that a higher occupancy may be required at receptors containing the α3-subunit to achieve the same anxiolytic behavioural effect. With regard to diazepam, the binding affinity for the α3-subunit is higher than that of the α2-subunit, but the modulation of GABA-induced chloride influx is similar (Smith et al., 2001), suggesting a lower efficacy at α3-containing receptors. Pentobarbital also has a lower efficacy at receptors containing the α3-subunit, as assessed by a decreased ability to increase GABA-mediated chloride influx when compared to other α subunits (Smith et al., 2001). It is therefore

possible that the doses used in the current experiment were not sufficient to observe an anxiolytic effect mediated by α3-containing receptors. It also cannot be ruled out that deletion of the *GABRA2* gene has resulted in compensatory changes in the expression of the α3-subunit. Benke et al. (2004) have shown that only 46% of $GABA_A$ receptors containing an α2-subunit are homologous whilst 19% are in combination with the α3-subunit. Therefore, it is possible that a downregulation of the α3-subunit has occurred due to an inability to form heterologous receptors with the α2-subunit.

In conclusion, our studies show that targeted deletion of the *GABRA2* gene provides further evidence for the role of the α2-subunit in mediating the anxiolytic effects of benzodiazepine compounds, as shown by an insensitivity of the KO mouse to the anxiolytic effects of diazepam in the CER test of conditioned anxiety. Furthermore, the inability of pentobarbital to demonstrate anxiolytic effects in the KO mouse implicates a potential role for the subunit in anxiety processing beyond the scope of benzodiazepine compounds.

Acknowledgements

This study is supported by MRC Grant G0600874 to DNS. CID was supported by a BBSRC studentship, supplemented by a British Pharmacological Society training grant. Thanks to Dr Sarah King for help and advice with genotyping the $GABA_A$ α2 KO mice.

References

Atack JR, Hutson PH, Collinson N, Marshall G, Bentley G, Moyes C, et al. Anxiogenic properties of an inverse agonist selective for alpha3 subunit-containing GABA A receptors. Br J Pharmacol 2005;144:357–66.

Benke D, Fakitsas P, Roggenmoser C, Michel C, Rudolph U, Mohler H. Analysis of the presence and abundance of GABAA receptors containing two different types of alpha subunits in murine brain using point-mutated alpha subunits. J Biol Chem 2004;279:43654–60.

Benke D, Mertens S, Trzeciak A, Gillessen D, Mohler H. GABAA receptors display association of gamma 2-subunit with alpha 1- and beta 2/3-subunits. J Biol Chem 1991;266:4478–83.

Bormann J, Kettenmann H. Patch-clamp study of gamma-aminobutyric acid receptor Cl− channels in cultured astrocytes. Proc Natl Acad Sci U S A 1988;85:9336–40.

Collinson N, Atack JR, Laughton P, Dawson GR, Stephens DN. An inverse agonist selective for alpha5 subunit-containing GABAA receptors improves encoding and recall but not consolidation in the Morris water maze. Psychopharmacology (Berl) 2006;188:619–28.

Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, et al. Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA receptor. J Neurosci 2002;22:5572–80.

Crawley JN. Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res 1999;835:18–26.

Crestani F, Low K, Keist R, Mandelli M, Mohler H, Rudolph U. Molecular targets for the myorelaxant action of diazepam. Mol Pharmacol 2001;59:442–5.

Dias RSWFA, Fradley RL, Garrett EM, Stanley JL, Tye SJ, Goodacre S, et al. Evidence to show a significant role for a3-containing GABAA receptors in mediating the anxiolytic effects induced by benzodiazepines. J Neurosci 2005;25:10682–8.

Harris JA, Westbrook RF. Effects of benzodiazepine microinjection into the amygdala or periaqueductal gray on the expression of conditioned fear and hypoalgesia in rats. Behav Neurosci 1995;109:295–304.

Kaufmann WA, Humpel C, Alheid GF, Marksteiner J. Compartmentation of alpha 1 and alpha 2 GABA(A) receptor subunits within rat extended amygdala: implications for benzodiazepine action. Brain Res 2003;964:91–9.

Kralic JE, Sidler C, Parpan F, Homanics GE, Morrow AL, Fritschy JM. Compensatory alteration of inhibitory synaptic circuits in cerebellum and thalamus of gamma-aminobutyric acid type A receptor alpha1 subunit knockout mice. J Comp Neurol 2006;495:408–21.

LeDoux JE. Emotion circuits in the brain. Annu Rev Neurosci 2000;23:155–84.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, et al. Molecular and neuronal substrate for the selective attenuation of anxiety. Science 2000;290:131–4.

Marowsky A, Fritschy JM, Vogt KE. Functional mapping of GABA A receptor subtypes in the amygdala. Eur J Neurosci 2004;20:1281–9.

McDonald JRab, J. Enhancement of GABA-mediated postsynaptic inhibition in cultured mammalian spinal cord neurons: a common mode of anticonvulsant action. Brain Research 1979:167:323.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, et al. Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. Nat Neurosci 2000;3:587–92.

Mead AN, Stephens DN. Selective disruption of stimulus-reward learning in glutamate receptor gria1 knock-out mice. J Neurosci 2003;23:1041–8.

Millan MJ. The neurobiology and control of anxious states. Pro neurobiol 2003;70:83–244.

Morris HV, Dawson GR, Reynolds DS, Atack JR, Stephens DN. Both alpha2 and alpha3 GABAA receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. Eur J Neurosci 2006;23:2495–504.

Nagy J, Zambo K, Decsi L. Anti-anxiety action of diazepam after intra-amygdaloid application in the rat. Neuropharmacology 1979;18:573–6.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 2000;101:815–50.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, et al. Importance of a novel GABAA receptor subunit for benzodiazepine pharmacology. Nature 1989;338:582–5.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, et al. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature 1999;401:796–800.

Sanders SK, Shekhar A. Anxiolytic effects of chlordiazepoxide blocked by injection of GABAA and benzodiazepine receptor antagonists in the region of the anterior basolateral amygdala of rats. Biol Psychiatry 1995;37:473–6.

Schwenk F, Baron U, Rajewsky K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res 1995;23:5080–1.

Smith AJ, Alder L, Silk J, Adkins C, Fletcher AE, Scales T, et al. Effect of alpha subunit on allosteric modulation of ion channel function in stably expressed human recombinant gamma-aminobutyric acid(A) receptors determined using (36)Cl ion flux. Mol Pharmacol 2001;59:1108–18.

Wafford KA, Macaulay AJ, Fradley R, O'Meara GF, Reynolds DS, Rosahl TW. Differentiating the role of gamma-aminobutyric acid type A (GABAA) receptor subtypes. Biochem Soc Trans 2004;32:553–6.

Wieland HA, Luddens H, Seeburg PH. A single histidine in GABAA receptors is essential for benzodiazepine agonist binding. J Biol Chem 1992;267:1426–9.

Yee BK, Keist R, von Boehmer L, Studer R, Benke D, Hagenbuch N, et al. A schizophrenia-related sensorimotor deficit links alpha 3-containing GABAA receptors to a dopamine hyperfunction. Proc Natl Acad Sci U S A 2005;102:17154–9.