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Journal Title: Journal of Neuroscience Nursing
Volume: Volume 30, Number 21
Publisher: Lippincott, Williams & Wilkins | 2010-05-26, Pages 7139-7151
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1523/JNEUROSCI.0693-10.2010
Permanent URL: http://pid.emory.edu/ark:/25593/ffdfc

Final published version: http://www.jneurosci.org/content/30/21/7139

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Accessed October 9, 2023 11:02 AM EDT
Amygdala-specific reduction of α1-GABA<sub>A</sub> receptors disrupts the anticonvulsant, locomotor, and sedative, but not anxiolytic effects of benzodiazepines in mice

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Abstract

The heterogeneity and distribution of GABA<sub>A</sub> receptor subunits mediates differential roles in behavior. It is thought that particular behavioral responses to benzodiazepine (BZ) ligands might be associated with an action at a regionally defined receptor subtype. However, the role of specific GABA<sub>A</sub> receptor subtypes in particular brain regions is less clear. Such detailed knowledge of regional α1-GABA<sub>A</sub> receptor function will advance our understanding of the neural circuitry underlying the role of GABA<sub>A</sub> receptors and the effects of GABA<sub>A</sub>-modulating drugs on behavior. By combining inducible, site-specific α1 subunit deletion, using a lentivirus expressing Cre-recombinase in mice with the α1 subunit gene flanked by loxP sites, we examine baseline and pharmacological effects of deletion of amygdala α1-GABA<sub>A</sub> receptors. We find that amygdala-specific reduction of α1 receptor subunits does not affect mRNA or protein levels of amygdala α2 or α3 subunit receptors. Nor does this inducible reduction affect baseline locomotion or measures of anxiety. However, we also find that this inducible, site-specific deletion does disrupt the normal sedative-locomotor inhibition as well as the anticonvulsive effects, of two distinct benzodiazepine-site ligands, diazepam and zolpidem, which is relatively α1-subunit selective. These data, utilizing inducible, region and subunit-specific deletion, combined with pharmacogenetic approaches, demonstrate that amygdala expression of the α1-GABA<sub>A</sub> receptor subunit is required for normal benzodiazepine effects on sedation, locomotion, and seizure inhibition, but not for anxiolysis.

Introduction

Fast synaptic inhibition in the mammalian forebrain is mediated by GABA interacting with post-synaptic GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs), which are pentameric complexes composed of multiple subunits (α1–6, β1–3, γ1–3, ρ1–3, ε, π, and δ) (Burt and Kamatchi, 1991; Barnard et al., 1998; Mehta and Ticku, 1999). The various combinations of subunits result in a heterogeneous population of GABA<sub>A</sub>Rs, although most contain at least one member of the α, β, and γ subunit classes in a proposed stoichiometry of two α-, two β-, and one γ-subunit (Backus et al., 1993; Sieghart et al., 1999; Mohler et al., 2002; Sieghart and Sperk, 2002). Defined subunit compositions display specific regional and cellular distribution (Fritschy and Mohler, 1995) which together confer distinct electrophysiological and pharmacological properties (Barnard et al., 1998; Sanger, 2004; Mohler, 2006; Nutt, 2006; Sieghart, 2006). Receptors containing the α1, α2, α3, or α5 subunits in combination with any of the β-subunits...
and the γ2-subunit are sensitive to benzodiazepine (BZ) modulation (Wisden et al., 1991; Hadingham et al., 1996; Wafford et al., 1996).

By far the largest population of benzodiazepine-sensitive (BZ)-GABA<sub>A</sub>R subtypes contain α1 subunits (McKernan and Whiting, 1996). The behavioral and pharmacological functional roles of α1-GABA<sub>A</sub>R subtypes have been revealed using subtype-selective drugs (for review see, Atack, 2003). One such agent is the imidazopyridine zolpidem, among the most common clinically used sedative-hypnotics. Studies using recombinant GABA<sub>A</sub>Rs demonstrate zolpidem has a high affinity for the GABA<sub>A</sub>Rs containing α1 subunits, an approximately 20-fold-lower affinity at α2- and α3-containing GABA<sub>A</sub>Rs, and no affinity at α5-containing GABA<sub>A</sub>Rs (Pritchett and Seeburg, 1990; Sanger et al., 1996b). When compared to nonselective benzodiazepines, zolpidem and other agents with preferential affinity for α1-GABA<sub>A</sub>R subtypes (e.g., zaleplon, zopiclone) show great potency in inducing motor-sedative effects in rodents (Griebel et al., 1996; Sanger et al., 1996a; Drover, 2004). α1-selective agonists also possess anticonvulsant properties (Sanger et al., 1996a; Veliskova et al., 1998; Drover, 2004). For example, zolpidem reduces pentylenetetrazole (PTZ) and electroconvulsive shock induced convulsions (Depoortere et al., 1986; Sanger et al., 1996a; Crestani et al., 2000).

The behavioral and pharmacological roles of α1-GABA<sub>A</sub> Rs have been further elucidated by pharmacological studies using mice with targeted point mutations rendering them insensitive to allosteric modulation by BZ-site ligands due to the replacement of a conserved histidine residue with arginine at position 101 of the α1-GABA<sub>A</sub> subunit gene (Benson et al., 1998; Rudolph et al., 1999a; Crestani et al., 2000; Marowsky et al., 2004). These corresponding α1 (H101R) mice fail to show a motor-sedative effect to diazepam or zolpidem (Rudolph et al., 1999a; McKernan et al., 2000). The ability of diazepam and zolpidem to prevent PTZ-induced seizures was also reduced in α1(H101R) mice (Rudolph et al., 1999a; Crestani et al., 2000). In contrast, α2 and α3 point mutated mice display dose-dependent increases in the seizure thresholds which are comparable to wild type controls (Low et al., 2000).

Evidence from mice with deletion of the α1 subunit gene also supports the view that α1-containing receptors in part mediate the anticonvulsant effect of diazepam. Global knockouts lack all α1-containing receptors as assessed by high-affinity [3H]zolpidem-binding sites (Kralic et al., 2002A), yet exhibit a viable phenotype despite a resulting loss of a majority of GABA<sub>A</sub>Rs in the brain (Kralic et al., 2002a, 2005; Sur et al., 2001; Vicini et al., 2001). Behavioral studies reveal these mutants exhibited a tremor when handled and were more susceptible to bicuculline-induced seizures (Sur et al., 2001; Kralic et al., 2002a). Furthermore, while diazepam dose-dependently increased the seizure threshold of wild-type mice, it failed to increase the seizure threshold in α1 knockouts (Kralic et al., 2002a).

While the use of conventional knockout and point-mutation strategies have provided vital insights into the various roles of α1-GABA<sub>A</sub> Rs, there are a number of limitations with traditional knockout strategies. Relatively little is known about the role of this subtype in regionally defined areas of the brain (c.f. Kralic et al., 2006; Zeller et al., 2008). In addition, there are often compensatory changes with other GABA<sub>A</sub>R subtypes (Sur et al., 2001; Kralic et al., 2002c; Rudolph and Mohler, 2004). In certain instances, the compensatory changes are unexpected based on known pharmacology. For example, benzodiazepines and α1-selective compounds show great potency for suppressing locomotor activity (Crestani et al., 2000; McKernan et al., 2000); however, due to compensation or developmental effects, α1 KO mice are more sensitive to the sedative effects of diazepam (Kralic et al., 2002c; Zeller et al., 2008). Such complex compensatory changes make it difficult to assess whether phenotypic consequences result from the lack of normal gene expression in the adult or compensatory abnormalities caused by α1-GABA<sub>A</sub>R gene deletion during early pre- or post-natal periods.
There is a need to examine the role of different GABA$_A$R genes with an inducible method, allowing time-, regional-, and subunit-specific deletion of specific receptor subunits. The lateral and basolateral nuclei of the amygdala express high levels of $\alpha_1$-GABA$_A$Rs, and are primary sites involved in many behavioral responses induced by BZs (Hevers and Luddens, 1998; Teuber et al., 1999; Pirker et al., 2000; Smith, 2001; Kaufmann et al., 2003; Kopp et al., 2004; Savic et al., 2005; Heldt and Ressler, 2006; Engin and Treit, 2008). To address these issues, the current study examined the behavioral, pharmacological and compensatory consequences of targeted $\alpha_1$-GABA$_A$Rs deletion in the amygdala of adult mice.

**Methods**

**Animals**

Adult *Gabra1-tm1Geh* male mice (9–15 weeks) weighing 23–28 g were used in this study. Mice were housed in standard group cages (<6/cage) and given ad libitum access to food and water on a 12-hr light/dark cycle. All experiments were performed during the light cycle and were conducted on mice between 5 and 10 weeks of age. The experiments were approved by our Institutional Protocol Approval Committee and were in accordance with Yerkes Primate Research Center Regulations.

The *Gabra1-tm1Geh* targeted knock-in mice (Jackson Laboratories, Maine) possess loxP sites on both side of the $\alpha_1$ exon encoding an essential transmembrane domain. Normal expression of the $\alpha_1$ gene is observed in mice that are homozygous for this “floxed” allele, and these mice do not display any deficiencies in $\alpha_1$-GABA$_A$. In the presence of Cre recombinase (CRE), the transmembrane domain of the $\alpha_1$-GABA$_A$ gene is deleted, resulting in a non-functional $\alpha_1$ subunit (Vicini et al., 2001).

**Lentivirus production**

Localized deletion of $\alpha_1$-GABA$_A$Rs was accomplished by microinjections of a CRE recombinase-expressing lentivirus vector. This vector was derived from the HIV-based lentivirus backbone pLV-CMV-GFP-U3Nhe (Tiscornia et al., 2003) which allows for virally mediated expression of green fluorescent protein (GFP) driven by a cytomegalovirus (CMV) promoter. The Cre-recombinase expressing viral vector (LV-Cre) was created by replacing the GFP coding sequence in pLV-CMV-GFP-U3-Nhe with the coding sequence for Cre-recombinase. Viral particles were produced by co-transfecting these lentiviral packaging constructs with plasmids coding for delta 8.9 and VSV-G into HEK-293T cells following standard methods (Tiscornia et al., 2006; Heldt et al., 2007; Jasnow et al., 2009). The packaged, unconcentrated virus was collected over a period of 5 days post-transfection and then concentrated using ultracentrifugation and resuspended in sterile PBS/1% BSA. The resulting titer was assessed in HEK-293T cells. The observed titer of the GFP and Cre-expressing viruses typically ranged from 1×10$^8$ to 1×10$^9$ infectious particles per ml.

**Cell culture Experiments**

Primary cultures of hippocampal neurons from Gabra1-tm1Geh floxed mice (P10) were prepared using a modified protocol based on Brewer et al. (1997). Briefly, the hippocampus was dissected on ice and dissociated in Hibernate A medium (BrainBits, Springfield, IL, USA). Neurons were plated onto poly-D-lysine-pre-coated 24-well plates at a density of 2.5 × 10$^5$ cells/cm$^2$ in Neurobasal A media (Invitrogen, Carlsbad, CA, USA). The cultures were kept in a humidified incubator at 37C and 5% CO$_2$. Two weeks after plating, wells received either 1 µl of LV-Cre, LV-GFP or were left untreated followed by 3 days of incubation at 37C and 5% CO$_2$. Neurons were then fixed with 4% Paraformaldehyde in PBS and blocked for non-specific binding in PBS containing 1% BSA and 3% normal goat serum, $\alpha_1$-GABA$_A$R subunits were stained with rabbit anti-$\alpha_1$-GABA$_A$R subunits antibody (1:500; Fisher, Pittsburgh, PA, USA)
and goat anti-rabbit Alexa Fluor 568 (1:1000; Invitrogen). After PBS rinse, cells were treated with Hoechst 33342 stain (10 μg/ml) for 10 min to identify nuclei of both neuronal and glial cells. Wells were viewed using a Leica DMRA microscope and random images from LV-GFP, LV-Cre (n=8), and untreated wells (n=8, area=0.175cm²/each) were captured using a Nikon Digital Sight DS-U1 camera system and NIS Elements BR2.30 Software. Hoechst and α1-GABA̶AR positive neurons from the same area were counted, and the level of α1-GABA̶AR subunit expression was calculated as a percentage of the total Hoechst-stained nuclei.

**Surgery and Histology**

Gabra1-tm1Geh mice received bilateral amygdala microinjections of either the CRE lentivirus or the control pLV-CMV-GFPV3-Nhe lentivirus that does not produce CRE. Mice were anesthetized by injections of Ketamine-Metomidine (80 mg/kg: 1.0 mg/kg, i.p.), then mounted in a stereotaxic apparatus. Small holes were drilled in the skull above the injection site, and a 30-gauge Hamilton microsyringe was lowered to the following coordinates from bregma based on the mouse brain atlas of Paxinos and Franklin (2001): AP=−1.4, ML=±3.3, DV=−5.0. The microsyringe was left in place 10 min before and after each injection, and a total volume of 1.0 μl LV-CRE or LV-GFP was administered at a rate of 0.05μl/min at each site. After surgery the incision was closed with cyanoacrylate glue and each mouse was placed on a heated pad after a post-surgical i.p. injection of Antisedan (4.0 mg/kg). The narcotic analgesic, buprenorphine (0.05 mg/kg, s.c.), was administered to mice after recovery from anesthesia. Post-surgical monitoring was performed prior to testing.

One week after behavioral testing, mice were anesthetized with isoflurane and the brains were rapidly dissected and frozen on crushed dry ice. Coronal sections (20 μm) of brains were cut on a Leica cryostat (Nussloch, Germany) at −20°C, mounted on gelatin-coated slides, and stored at −80°C until processed for histochemistry. For each brain, sections were placed on 10 consecutive sets of slides such that each set contained similar sequential sections of the brain. Four sets of slides from each brain were used for *in situ* hybridization analyses of mRNA (Cre, α1, α2, and α3), and 1 set of slides was stained with cresyl violet.

**Control LV-Cre and LV-GFP mice behavioral experiments**

To control for any actions of Cre other than specific cre-loxP-mediated recombination (Schmidt-Supprian and Rajewsky, 2007), we examined the sedative-locomotor inhibition and the anticonvulsive properties of zolpidem after LV-GFP and LV-Cre microinjections in mice without the loxP-flanked target gene. In this experiment, C57BL/6J received bilateral LV-GFP (n=10) or LV-Cre (n=10) amygdala microinjections (ns=8) as outlined Surgery and Histology section. 14 days after surgery, all mice were exposed for 30 min to a novel open-field apparatus acclimation session. One day later, mice received Session 1 of a 2-session random crossover experimental designed to evaluate the effect of zolpidem treatment on open-field activity. On Session 1, approximately one-half of the LV-GFP and LV-Cre mice were administered a low-dose pretreatment 30 min before testing (0.25 mg/kg); the remaining mice were given a high-dose (0.5 mg/kg). Test Session 2 was conducted following a seven-day drug washout period to limit pretreatment crossover effects. On this session, drug pretreatment for each mouse was reversed. Analyses excluded one LV-Cre animal due to poor injection. Two weeks after open-field testing mice the anticonvulsant effects of zolpidem were tested by administration of 2.5 mg/kg/i.p. 30 min before PTZ administration (85 mg/kg). Eight mice from each surgery group were used for the anticonvulsant test.

**Test drugs and doses**

Zolpidem, a preferential GABA̶ARα1 subtype modulator, and diazepam, a nonselective BZ-site agonist (Sigma, St Louis, MO) were dissolved in a 15% DMSO/saline solution. The chemoconvulsant pentylenetetrazole (PTZ, Sigma) was dissolved in saline. All drugs were...
administered in a volume of 8 ml/kg after animal weights were recorded. Test drug doses levels for examining their motor-impairing effects were based on preliminary showing that the lower test drug dose (diazepam, 5.0 mg/kg; zolpidem, 0.25 mg/kg) reliably decreases activity from baseline levels, and the high dose (diazepam, 10.0 mg/kg; zolpidem, 0.5 mg/kg) causes a significant decrease in activity in comparison to the low dose (Figure S1, supplemental data). Thus, for the motor-impairing effects, mice received either diazepam (5.0 or 10 mg/kg;) or zolpidem (0.25 or 0.5 mg/kg). Examination of potential group differences in the convulsant action of PTZ was conducted with two different doses (55 and 85mg/kg/i.p.). A PTZ dose of 85 mg/kg was used to examine the anticonvulsive effects based on preliminary data showing highly reliable decreases in both myoclonic and tonic seizure latencies during a 20min observation period (Figure S2). For drug-induced seizure inhibition, mice were pretreated with either diazepam (0.25 or 1.0 mg/kg) or zolpidem (2.5 or 4.0 mg/kg). Dose levels were based on preliminary showing that the lower test drug dose (diazepam,0.25 mg/kg; zolpidem, 2.5 mg/kg) was effective at inhibiting tonic seizures, whereas the high dose (diazepam, 1.0 mg/kg; zolpidem, 4.0 mg/kg) was effective at inhibiting both myoclonic and tonic seizures (Figures S3 & S4).

**In situ hybridization**

In situ hybridization was performed to examine the expression of mRNA using procedures and probes previously described (Ressler et al., 2002; Heldt and Ressler, 2007). Briefly, hybridization riboprobes are prepared from linearized clones using appropriate RNA polymerase and radioactive $^{35}$S-UTP in the polymerase reaction. Radiolabeled antisense RNA strands are base hydrolyzed and isolated using a sephadex spin column (Roche Quick Spin, Indianapolis, IN). Probes are diluted to a concentration of 100,000 cpn/ul in hybridization buffer, and sections are incubated overnight in humid chambers at 52°C with 75 μl of probe-buffer solution covered with a Parafilm coverslip. Slides are then washed, air dried, and apposed to Biomax MR autoradiography film (Eastman Kodak, NY) for 1–5 days.

The alpha1 subunit plasmid was constructed by TOPO subcloning of a cDNA PCR product using the following customized primers: a1 sense, GGAGTGACGACTGTTCTGACTATG; a1 antisense, TTCTGGAACCACGCTTTTG (Sigma Chemical, St. Louis, MO USA). Plasmid DNA sequencing (ISU, Ames, IA, USA) confirmed subclone sequence and orientation. The resulting a1 PCR transcript corresponds to nucleotides 1307–1509 of the a1 cDNA which is flanked by loxP sites and deleted by CRE recombinase (Keir et al., 1991). These nucleotides encode amino acids included in the second transmembrane domain and intercellular loop between transmembranes 3 and 4. Previous analyses have demonstrated signal specificities and density distributions using both sense and antisense RNA riboprobes (Heldt and Ressler, 2007).

**Image Analysis**

Each film was scanned using a high-resolution Epson 3700 flat-bed scanner (3000 dpi) and saved in JPEG format at a size of 32000 × 18000 pixels. To estimate levels of receptor mRNA transcript, the total area of each target site subregion (in pixels) was first outlined by freehand tool of Adobe Photoshop with reference to Nissl-stained section from the same mouse and the atlas of Paxinos and Franklin (2001). The hybridization signal intensities of brain regions were defined on the basis of grey values (GVs) between 0 (brightest) and 255 (darkest) obtained from the luminosity histogram feature of Adobe Photo. For each section, GVs were recorded for the regions of interest, as well as an adjacent background area with little or no hybridization signal. For the quantification, signal intensities were computed by subtracting background area GVs from region of interest GVs. Independent statistical comparisons were carried out for LA and BL regions of the amygdala. Statistical analyses were performed with independent samples.
Western Blots

Unilateral amygdala punches were obtained with a 1 mm brain punch tool, suspended in 25 μl homogenized lysis buffer (5 mM HEPES, 1 μM EDTA) with a protease inhibitor cocktail kit (Roche, IN, USA), then homogenized with a sonicator on ice. The protein concentrations were measured using 2.5 μL samples of homogenates with BCA protein assay reagent and bovine serum albumin (BSA) as the reference standard (Pierce, Rockford, IL) (Bradford, 1976). Equal amounts of protein (20 μg) per animal were boiled for 5 min and loaded on 7.5% polyacrylamide gels (Bio-Rad). After electrophoresis, the samples were transferred to nitrocellulose membrane (0.45 μm, Bio-Rad Laboratories) at 30 mA for 2 h at 4°C (25 mM Tris-HCl, 190 mM glycine, and 20% methanol). After transfer, the membranes were washed three times with blocking buffer (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM HEPES, pH 7.4) with 5 min intervals. Membranes were then incubated with a primary antibody at 4°C overnight. Following three washes with blocking buffer with 5 min intervals, membranes were incubated with a corresponding HRP-coupled secondary antibody at room temperature for 60 min. The membranes were subsequently washed with water to remove the substrate solution and visualized by SuperSignal West chemiluminescence substrate system (Pierce, Rockford, IL). Reactive protein bands were visualized and analyzed using an Alpha Innotech Fluorchem imaging system (Alpha Innotech, San Leandro, CA). Rabbit polyclonal antibody to mouse α1-GABA_A (1:1000) and α3-GABA_A (1:1000) were obtained from Abcam (Cambridge, MA). Rabbit polyclonal antibody to mouse α2-GABA_A (1:1000) was obtained from Alomone labs (Jerusalem, Israel). Monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH, 1:3000) was obtained from Fitzgerald Industries (Concord MA). Total blotted protein levels were normalized to levels of GAPDH to control for variations in protein loading. As such, the relative values were quantified as the protein of interest divided by the loading control. Statistical analyses were performed with independent samples t-tests (LSD). For ease of presentation, figures are presented as percentages of control group (LV-GFP).

Behavioral and histological group assignments

All mice (N=144) were tested in the open-field apparatus 14 days after surgery to evaluate baseline motor behavior in a novel environment. Baseline anxiety-like behavior, as assessed by evaluating the percent of time mice spent in the central zone of the open field, was obtained from 105 of these mice. Time spent in the central zone was omitted from the remaining 39 animals due to data recovery difficulties. Fifteen LV-GFP mice and 16 LV-Cre mice were used to examine the convulsant action of PTZ (55 and 85 mg/kg/i.p.). Western blot analyses were performed on homogenized amygdala punches collected from LV-GFP mice (n=10) and LV-Cre mice (n=10). The effect of test-drug treatment on open-field activity was evaluated using a 2-session random crossover experimental design using the remaining animals (N=93). Separate animals were used to examine the behavioral effects of diazepam and zolpidem at each of two dose levels. On Session 1, approximately one-half of the LV-GFP and LV-Cre mice were administered a low-dose pretreatment 30 min before testing (diazepam, 5 mg/kg; zolpidem, 0.25 mg/kg); the remaining mice were given a high-dose (diazepam, 10 mg/kg; zolpidem, 0.5 mg/kg). Test Session 2 was conducted following a seven-day drug washout period to limit pretreatment crossover effects. On this session, drug pretreatment dose level for each mouse was reversed. Two weeks after open-field testing mice were assigned to one of eight groups to test the anticonvulsant effects of test drugs. Mice in the diazepam groups were pretreated with either 0.25 mg/kg/i.p. (LV-GFP, n=10; LV-Cre, n=12) or 1.0 mg/kg (LV-GFP, n=10; LV-Cre, n=12) 30 min before PTZ administration. Mice in the zolpidem groups were pretreated with either 2.5 mg/kg/i.p. (LV-GFP, n=12; LV-Cre, n=16) or 4.0 mg/kg (LV-GFP,
n=11; LV-Cre, n=10) before PTZ administration. Histological examination and in situ hybridization analyses of mRNA levels were performed on coronal brain sections of the remaining animals one week after behavioral testing.

Separate animals were used to examine the behavioral effects of diazepam on elevated plus maze performance (LV-GFP, n=9; LV-Cre n=10). On Session 1, approximately one-half of the LV-GFP and LV-Cre mice were administered diazepam (1.5 mg/kg/i.p.) 30 min before testing; the remaining mice were given vehicle. Test Session 2 was conducted following a two-week drug washout period to limit crossover effects. On this session, drug pretreatment dose level for each mouse was reversed.

Motor Activity and motor-impairing effects of test drugs

Baseline motor activity and the motor-impairing effects of test drugs were measured by examining the total ambulatory distance (in cm) during the 30-min open field test session. In rodents, drug-induced changes in both ambulatory distance and counts represent a standard behavioral assay for testing the motor effects of drugs (Vogel and Vogel, 2002). The anxiety-like behaviors were evaluated by computing percent of time mice spent in the central zone of the open field. An increase in the percent of central zone time is indicative of an anxiolytic-like phenotype and best takes into account potential confounding changes in locomotive activity (Prut and Belzung, 2003). The central zone was defined as the central compartment of the floor centrally located 6 cm from the perimeter of the chamber walls. Activity for each mouse was measured 30 min after test drug injection using individual activity chambers constructed from clear polycarbonate and equipped with four 24-beam infrared arrays across the base of each chamber wall (MED Associates, Model, OFA-MS). Activity data is collected via computer and was analyzed with the MED Associates’ Activity Monitor Data Analysis software. All testing was conducted under standard room lighting.

Assessing the anxiolytic-like activity of diazepam on elevated plus-maze

The elevated plus maze consisted of two open arms (50 × 6.5 cm) and two closed arms with a wall (50 × 6.5 × 15 cm) attached to a common central platform (6.5 × 6.5 cm) to form a cross. The maze was elevated 65 cm above the floor. Test sessions were conducted under standard room lighting (100 lux) where behaviors were continuously videotaped by a video camera placed over the apparatus. Activity was collected, and analyzed via computer using automated Limelight software (Coulbourn, PA). Before each test, the plus maze was cleaned with Quatricide (Pharmacal, Waterbury, CT).

Elevated plus-maze testing was conducted with a separate cohort of animals. Thirty minutes before elevated plus-maze testing, each animal was weighed then injected i.p. with diazepam (1.5 mg/kg). At the start of each session, a mouse was placed in the central “hub” of the maze with its head facing an open arm and was allowed to freely explore for 5 min. The percentage of open arm entries [open arm/(open + closed arm) entries] × 100 and percentage time in open arms [time in open arms/(time in open + closed arms)] × 100 were computed. Both of these parameters are indicators of anxiolytic-like activity (Pellow and File, 1986; Hogg, 1996). The total number of arm entries (open+closed) was used as an indicator of locomotor activity (Rodgers and Dalvi, 1997). Arm entry was considered complete if all four paws entered a closed or open arm from the central platform.

PTZ-induced seizures and anticonvulsant effects of test drugs

The systemic administration of PTZ for induction of generalized clonic seizures in rodents is widely employed to identify potential anticonvulsants (Loscher and Schmidt, 1994; Meldrum, 2002). Immediately following PTZ injection, mice were placed individually in acrylic observation chambers cage (450 mm ×350 mm×300 mm) for a 20 min (1200s) observation
period. Because the appearance of myoclonic and clonic seizures indicate the initiation and spreading of the seizures, respectively, we evaluated both parameters in the present study. The time between the injection of PTZ and the appearance of myoclonic jerks or “jumps” was defined as the myoclonic seizure-onset time (Kaputlu and Uzbay, 1997). Clonic seizures were defined as convulsions involving the whole body and loss of righting ability. Mice were given an “anticonvulsant rescue” dose of diazepam (5mg/kg) 5 s after clonic seizure onset or immediately after the observation period, whichever came first. To examine the anticonvulsant effects of test drugs, mice were given test drugs 30 minutes before administration of PTZ (85 mg/kg PTZ). If no seizure occurred during the observation period, the mice were considered protected. Each mouse was used only once in an experiment. Duration of 1200s was taken as a cutoff time in calculation of the onset time of PTZ-induced seizures. Data were analyzed with One-Way ANOVA to examine relationships between experimental groups and seizure onset latency. Pearson correlations were used to examine relationships between $\alpha_1$-GABA$\_A$R mRNA level and response to diazepam (0.25mg) in delaying myoclonic seizure onset latencies. Note that one animal was removed from the correlation analyses due to a latency that was > 3 S.D. above the mean.

**Statistical Analyses of Behavioral Measures**

Group differences in baseline motor activity and percent central zone time were examined using two-sample $t$-tests. Two-way ANOVAs were used to examine group differences in seizures latencies, ambulatory distances, and elevated plus-maze dependent measures. The motor-impairing effects of test drugs, as measured by ambulatory distance (in cm), were analyzed by means of a 2 × 2 ANOVA with group (LV-Cre, LV-GFP) as between-subjects factor and drug level as the within-subjects factors. For diazepam drug levels were 5.0 and 10 mg/kg; for zolpidem, drug levels were 0.25 and 0.5 mg/kg. Group differences in PTZ-induced myoclonic and clonic latency to seizures were each assessed using a 2 × 2 ANOVA with group (LV-Cre, LV-GFP) and PTZ drug level (55 or 85 mg/kg) as between-subjects factors. To examine the anticonvulsant effects of test drugs, myoclonic and clonic seizures latencies (in sec) were analyzed separately by means of 2 × 2 ANOVAs with group (LV-Cre, LV-GFP) and drug level as between-subjects factors. Drug levels for the zolpidem were 2.5 and 4.0 mg/kg; levels for diazepam were 0.25 and 1.0 mg/kg. Elevated plus-maze dependent measures, including total arm entries and percentages of open arm entries and open arms time were analyzed separately by means of a 2 × 2 ANOVA with group (LV-Cre, LV-GFP) as the between-subjects factor and treatment (Vehicle, Diazepam) as the within-subjects factor. Follow-up comparisons between dose levels or group were completed with pairwise or two-sample $t$-test as needed. The level of significance was set at $p=0.05$.

**Results**

**Robust deletion of $\alpha_1$-GABA$\_A$R subunits in vitro**

Figure 1 images represent the same field visualized separately illustrating bright-field with Hoechst-stained nuclei (top panels) and $\alpha_1$-GABA$\_A$R subunits antibody (lower panels) in primary cultures of hippocampal neurons. As seen in Figures 1a–b, the number of Hoechst stained neurons in both LV-Cre and untreated cells were similar, $t(14) = 0.36, p > 0.05$). The mean (sem) number of total Hoechst stained neurons was 52.5(4.0) in untreated wells and 55.1 (5.9) in LV-Cre treated wells. In addition, the average number of Hoechst stained neurons with primary dendrites was similar between wells of untreated and LV-Cre neurons (untreated: 19.9 (3.9); LV-Cre: 20.6(2.0); $t(14) = 0.17, p > 0.05$). Because Hoechst stains the condensed chromatin in apoptotic cells more than normal chromatin, the similarity in number, intensity and density of staining suggests an absence of apoptosis in LV-Cre infected cells. As seen in Figures 1c–d, the number $\alpha_1$-GABA$\_A$AR positive neurons were significantly higher in vehicle treated cells. In untreated wells, the level of $\alpha_1$-GABA$\_A$R subunit expression as a percentage
of the total Hoechst-stained nuclei was 35%(3%). In contrast, no α1-GABAAR positive neurons were identified in LV-Cre treated wells, p < 0.01.

**Amygdala specific reduction of α1-GABAAR mRNA, with no affect on α2 or α3 levels**

Qualitatively, the selective decrease in α1-GABAAR subunit expression within the amygdala was observed following LV-Cre infection. Shown in Figures 1a–f are representative photomicrographs of parallel sections following LV injection into mouse amygdala. Figures 2a–c show the decreased expression of α1 subunit mRNA and Cre recombinase expression pattern in LV-Cre mice. In comparison, Figure 2d shows the α1-GABAAR mRNA expression pattern in LV-GFP mice. Photomicrographs revealed no change in the expression of α2 or α3 subunits in LV-Cre mice with decreased α1 subunit mRNA (Figure 2e–f). Nissl-stained sections also revealed no discernable histological abnormalities in the amygdala from animals infected with LV-Cre (Figure S5).

Quantitative analyses of mRNA expression levels reveal that the LV-Cre approach to site-specific GABA subunit deletion resulted in statistically significant reduction of functional α1-GABAAR subunits that was largely limited to the amygdala. Comparisons of α1-subunit mRNA transcript levels identified significantly less α1-GABAAR subunit expression in LV-Cre mice in comparison with LV-GFP mice. In particular, reliable mRNA changes were noted for α1 within both the LA, t(38) = 4.17, p < 0.01, and BL, t(38) = 6.13, p < 0.01. With reference to GFP animals, the relative level of α1-GABAAR mRNA expression within the LA and BL were 52.2% and 44.7%, respectively, in LV-Cre-infected animals (Figure 3a). No significant group differences were identified for α2 or α3 subunits in either the LA or BL (p > 0.05).

**Amygdala specific reduction in α1-GABAAR protein, with no affect on α2 or α3 levels**

Previous studies have revealed that both global and targeted deletion of α1 subunits during development leads to compensatory increases in the expression of α2- and α3-GABAAR subtypes that may function to protect against the α1-GABAAR loss (Sur et al., 2001; Kralic et al., 2002c; Kralic et al., 2006; Zeller et al., 2008). Thus, we performed western blot analyses of tissue homogenates to examine α1-, α2-, and α3-GABAAR subunit protein expression in mice given either LV-Cre or LV-GFP microinjections (Figures 3b–c). A comparison of α1-subunit protein levels in the amygdala revealed significantly less α1-GABAAR protein expression in LV-Cre mice in comparison with LV-GFP mice, t(12) = 7.62, p < 0.01. No group differences were detected in α2 or α3 subunit protein levels t(12) = 0.22, p > 0.05 and t(12) = 0.98, p > 0.05, respectively. With reference to GFP control animals, the level of α1-GABAAR amygdala protein expression in LV-Cre-infected animals was 62.4%. The relative levels of α2- and α3-GABAAR protein were 99.3% and 93.1%, respectively. Together with the mRNA in situ findings, these results indicate that Cre-mediated inducible gene reduction of the α1-GABAAR subunit in adult mice leads to no significant compensatory increases in the expression of α2- and α3-GABAAR subtypes within the timeframe of these experiments.

**No difference in baseline and central zone locomotion in LV-Cre and LV-GFP mice**

Two weeks after microinjection surgery, the sedative effects of diazepam and zolpidem were examined in both α1-GABAAR deleted mice (LV-Cre) and controls (LV-GFP). As seen in Figure 4, when LV-Cre and LV-GFP mice were tested in open-field, both groups showed similar levels of locomotor activity as measured by ambulatory distance, t(142) = 0.81, p > 0.05. Thus, in the open field, a reduction of α1-GABAARRs located within the amygdala does not critically affect baseline motor behavior as assessed by percent central zone time.
LV-Cre infected mice have decreased sensitivity to diazepam on motor-sedative behavior

Following baseline locomotion testing in the open-field, LV-Cre and LV-GFP mice were treated with diazepam and subsequently tested in the open-field. At doses of 5 and 10 mg/kg, diazepam produced a decrease in locomotor activity in both groups when compared to previous drug-free open-field testing, ps > 0.05. However, as seen in Figure 4a, LV-GFP mice showed significantly less motor activity than LV-Cre mice as reflected by a significant main effect of group, $F(1,42) = 4.49, p < 0.05$. Direct comparisons between groups at each dose level revealed that the motor activity of LV-GFP mice was significantly lower than LV-Cre mice at 10 mg/kg but not 5 mg/kg dose level, $t(42) = 2.26, p < 0.05, t(42) = 1.39, p > 0.05$, respectively. These findings indicate that the reduction of amygdala $\alpha_1$-GABA$\mathrm{A}$Rs impairs the motor-impairing effects of diazepam at 10 mg/kg.

LV-Cre infected mice have decreased sensitivity to $\alpha_1$-selective zolpidem on motor-sedative behavior

To examine whether deficits in diazepam-induced motor deficits seen in LV-Cre mice were due to a reduction in $\alpha_1$-GABA$\mathrm{A}$Rs, we examined the motor-impairing effects of zolpidem in LV-Cre and LV-GFP mice. Overall, the administration of zolpidem at doses of either 0.25 or 0.5 mg/kg induced a reduction in motor activity in both LV-Cre and LV-GFP mice when compared to drug-free novel open-field testing, ps < 0.05. As seen with diazepam, zolpidem induced a greater motor activity reduction in LV-GFP mice than in LV-Cre mice as reflected by a significant main effect of group, $F(1,47) = 13.76, p < 0.01$, (Figure 4b). The zolpidem-induced motor impairment was significantly greater in LV-GFP mice compared to LV-Cre mice at both 0.25 mg/kg, $t(47) = 2.25, p < 0.05$, and 0.5 mg/kg $t(47) = 3.72, p < 0.01$, dose levels as measured by ambulatory distance. Together these findings suggest that the motor-impairing actions of zolpidem are in part mediated by $\alpha_1$-GABA$\mathrm{A}$Rs located within the amygdala.

No difference in the proconvulsant effect of pentylenetetrazole in LV-Cre and LV-GFP mice

To assess in vivo the consequences of reduced amygdala $\alpha_1$-GABA$\mathrm{A}$Rs on the convulsant actions of PTZ, the latency to myoclonic and clonic seizures was examined in LV-Cre and LV-GFP mice following intraperitoneal injections (55 or 85 mg/kg). As shown in Figures 5a–b, no group differences in latency to myoclonic and clonic seizures were detected at either dose of PTZ (ps > 0.05). These findings indicate that a localized decrease of amygdala $\alpha_1$-GABA$\mathrm{A}$Rs does not affect PTZ-induced latency to seizures.

LV-Cre infected mice have decreased sensitivity to diazepam with pentylenetetrazole seizures

One week after open-field testing, the anticonvulsant effects of diazepam were tested in LV-Cre and LV-GFP mice. Mice were first given systemic injections of diazepam (0.25 or 1.0 mg/kg) followed 30 min later by administration of PTZ (85mg/kg). With the combined LV-Cre and LV-GFP groups, there was a significant relationship between lateral amygdala (Figure 5c; $r=0.61, p<0.05$) and basolateral amygdala (Figure 5d; $r=0.81, p<0.005$) $\alpha_1$-GABA$\mathrm{A}$R mRNA level and response to 0.25mg diazepam in delaying myoclonic seizure onset. This suggests that animals with reduced $\alpha_1$-GABA$\mathrm{A}$R mRNA following LV-Cre infection would have shorter seizure onset latencies.

In comparison to 0.25mg/kg, the pretreatment of 1mg/kg diazepam increased the latency to the first myoclonic episode in both LV-Cre and LV-GFP mice as reflected by a significant main effect of dose, $F(1,40) = 12.42, p < 0.01$, (Figures 6a–b). Overall, diazepam increased myoclonic latency in LV-GFP mice significantly more than in LV-Cre mice as reflected by a significant main effect of group, $F(1, 40) = 23.35, p < 0.01$. Comparisons between groups at...
each dose level revealed the anticonvulsant effect of 0.25 mg/kg was greater in LV-GFP mice than LV-Cre as measure by latency to myoclonic onset, \( t(20) = 2.44, p < 0.05 \). LV-GFP mice also showed significantly greater myoclonic latency at the 1.0 mg/kg dose level in comparison to LV-Cre mice, \( t(20) = 4.71, p < 0.01 \).

For clonic seizure episodes, the LV-GFP group displayed a higher mean latency than the LV-Cre group after pretreatment of diazepam, as reflected by a significant group effect, \( F(1,40) = 9.67, p < 0.01 \). Direct comparisons revealed that the clonic latencies of LV-GFP mice were significantly higher at both 0.25 mg/kg and 1.0 mg/kg dose level, \( t(20) = 2.21, p < 0.05 \) and \( t(20) = 2.26, p < 0.05 \), respectively. Together, these results suggest that the anticonvulsant effects of diazepam are blunted in mice with decreased \( \alpha_1 \)-GABA\(_A\)Rs in the amygdala.

**LV-Cre infected mice have decreased sensitivity to zolpidem with pentylentetrazole seizures**

To test the anticonvulsant effect of zolpidem, mice were first given systemic injections of the zolpidem (2.0 or 4.0 mg/kg) followed 30 min later by the administration of PTZ (85 mg/kg). A significant main effect of dose, \( F(1, 45) = 52.83, p < 0.01 \), revealed that the anticonvulsant effect of 4.0 mg/kg was significant greater than 2.5 mg/kg as measured by the latency to myoclonic episode. The ability of zolpidem to prevent PTZ-induced myoclonic seizures was reduced in LV-Cre mice as reflected by a significant group effect, \( F(1, 45) = 11.70, p < 0.01 \). As seen in Figure 6c, this group main effect was largely influenced by the greater anticonvulsant effect on myoclonic seizure at the zolpidem dose level of 2.5 mg/kg in LV-GFP mice, \( t(26) = 3.26, p < 0.01 \). LV-Cre and LV-GFP mice pretreated with 4 mg/kg showed a similar latency to the first myoclonic jerk episode following the PTZ injection, \( t(19) = 1.79, p > 0.05 \).

An examination of clonic seizure latencies indicated that overall, the anticonvulsant effects of zolpidem were greater in LV-GFP mice, as exhibited by a reliable group effect, \( F(1, 45) = 7.88, p < 0.01 \). As seen in Figure 6d, when compared to LV-GFP mice, the latency to clonic seizure onset was significantly lower in LV-Cre at both 2.5 mg/kg and 4.0 mg/kg dose levels, \( t(26) = 2.30, p < 0.05 \) and \( t(19) = 2.55, p < 0.05 \), respectively. Together, these findings are consistent with the view that \( \alpha_1 \)-GABA\(_A\)Rs in the amygdala play an important role in mediating the anticonvulsant effect of zolpidem.

**No difference in anxiolytic effect of diazepam in LV-Cre and LV-GFP mice**

In addition to their motor-sedative and anticonvulsant effects, benzodiazepines are renowned for their capacity to reduce anxiety. There is considerable evidence indicating that the amygdala is a major site of action. In rodents, the anxiolytic effects of benzodiazepines in the amygdala have been demonstrated on a number of animal models of anxiety, including the elevated-plus maze (Green and Vale, 1992; Pesold and Treit, 1995; Nunes-de-Souza et al., 2000). On the other hand, microinjections of benzodiazepine antagonists and inverse agonists into the amygdala induce anxiogenic-like behaviors (Hodges et al., 1987; Da Cunha et al., 1992) and infusions of antagonists can also prevent the anxiolytic effects of peripheral injections of benzodiazepine agonists (Petersen et al., 1985; Sanders and Shekhar, 1995). Because past pharmacological and behavioral studies suggest that \( \alpha_2 \)-, but not \( \alpha_1 \)-containing receptors, mediate the anxiolytic effect of diazepam (Rudolph et al., 1999a; Low et al., 2000; McKernan et al., 2000; Atack, 2003), we examined whether the amygdala-specific lack of \( \alpha_1 \)-GABA\(_A\)R expression leads to differential responsiveness of diazepam’s anxiolytic effects.

As seen in Figures 7a–b, diazepam produced an anxiolytic-like effect in both LV-GFP and LV-Cre mice. An evaluation of the percentage time in open arms revealed a significant treatment effect \( F(1,17)=15.83, p<0.01 \). Neither the group effect nor Group × Treatment interaction were significant, \( F_{s}(1,17)<1.21, ps>0.28 \). Paired \( t \)-test indicated that both LV-GFP mice (\( p = 0.03 \))
and LV-Cre mice ($p = 0.02$) displayed an increased percentage time in open arms after diazepam administration. Likewise the evaluation of the percentage open arm entries also revealed a significant treatment effect $F(1,17) = 12.78$, $p < 0.01$ but no reliable group effect nor Group × Treatment interaction, $F_s(1,17)<1.24$, $ps>0.28$. Diazepam reliably increased the percentage open arm entries in both LV-GFP ($p = 0.04$) and LV-Cre ($p = 0.03$) groups. As seen in Figure 7c, assessment of total arm entries (open + closed) indicated no significant group differences in number of entries during drug or vehicle testing, $t(17) < 2.1$, $p > 0.06$.

Open field exploratory behavior (as examined for locomotion in Figure 4), was also examined for anxiety-like behavior with regards to percent time in center vs. surround of the open field. Assessment of the percent central zone time revealed no differences between groups, (LV-GFP, 59.5%; LV-Cre, 59.3%; $t(103) = 0.94$, $p > 0.05$).

**Motor and anticonvulsant effects of zolpidem in control LV-Cre and LV-GFP mice**

We next examined the sedative-locomotor inhibition and the anticonvulsive properties of zolpidem after LV-GFP and LV-Cre microinjections in wild-type mice without the loxP-flanked target gene. Overall, the administration of zolpidem induced a reduction in motor activity similarly in both LV-Cre and LV-GFP mice when compared to drug-free novel open-field testing ($p < 0.05$; LV-Cre: Mean=4284, sem=±847; LV-GFP: mean=4477, sem=±601).

As seen in Figure S5, no differences in zolpidem-induced motor impairment were identified in control LV-GFP and LV-Cre mice at either 0.25 or 0.5 mg/kg dose levels, $t(17) < 0.14$, $p > 0.05, 0.01$, as measured by ambulatory distance. For the anticonvulsant effect of zolpidem, an examination of seizure latencies revealed no differences control LV-GFP and LV-Cre mice in myoclonic onset ($t(14) = 0.468$, $p > 0.05$, or clonic seizure onset ($t(14) = 0.139$, $p > 0.05$ (Figure S6). Together with the results from Gabra1-tm1Geh floxed mice, these findings suggest the deficits in the zolpidem-induced motor and anticonvulsive effects were not due to actions of Cre other than specific cre-loxP-mediated recombination.

**Discussion**

**Baseline activity and the motor sedation effects of test drugs**

The present studies were undertaken in order examine the role of amygdala $\alpha_1$-GABA$_\text{A}$Rs in the motor-sedative and anticonvulsant actions of diazepam. The reduction of amygdala $\alpha_1$-GABA$_\text{A}$Rs in LV-Cre mice produced no differences in baseline activity in the open field. Likewise, global deletion of the $\alpha_1$ subunit reportedly does not alter baseline locomotor activity despite the reported existence of tremors (Sur et al., 2001; Kralic et al., 2002c). However the motor-sedative effects of diazepam were blunted at a dose level of 10 mg/kg in LV-Cre mice in comparison to LV-GFP mice. The motor-impairing effects of zolpidem were reduced at both 0.25 mg/kg and 0.5 mg/kg in LV-Cre mice. This finding is consistent with past reports indicating that a point mutation of the $\alpha_1$ subunit resulted in the complete loss of diazepam- and zolpidem-induced motor impairment (Rudolph et al., 1999a; Crestani et al., 2000; McKernan et al., 2000). In contrast, the motor-impairing/sedative effects of diazepam were not impaired in either $\alpha_2$- or $\alpha_3$-point mutated mice (Low et al., 2000). In fact, when tested in a novel environment, McKernan et al. (2000) found that diazepam (3 mg/kg) increased locomotor activity considerably in $\alpha_1$(H101R) mice. In contrast, global deletion of the $\alpha_1$ subunit increased the sensitivity to the motor-impairing effects of diazepam in the open field (Kralic et al., 2002a; Kralic et al., 2002c). This latter effect was possibly caused by the compensatory increase in levels of $\alpha_2$ and/or $\alpha_3$ subunit peptide expression in forebrain glutamateergic cells (Zeller et al., 2008), which was notably not seen in our current in vivo study. Together, these findings indicated that the motor-sedative effects of diazepam and zolpidem are partially due to their actions on amygdala $\alpha_1$-GABA$_\text{A}$Rs. The sedative effects which remained after test drug injections are likely mediated by $\alpha_1$-GABA$_\text{A}$Rs located in other neuronal regions.
or possibly by any remaining $\alpha_1$-GABA$_A$Rs within the amygdala. Notably, since no effects were seen when the same viruses were injected into wild-type, non-floxed mice, as discussed previously by Schmidt-Supprian and Rajewsky (2007), we can be reasonably assured that the behavioral phenotypes observed were in fact specifically due to the targeted deletion of the $\alpha_1$-GABA$_A$R within the amygdala.

Seizure susceptibility to PTZ

The susceptibility to PTZ-induced convulsions was unaltered in LV-Cre mice in this study. Both LV-GFP and LV-Cre groups displayed similar latencies to myoclonic and clonic PTZ-induced seizures at 55 and 85mg/kg dose levels. The lack of group differences in our study suggests that amygdala $\alpha_1$-GABA$_A$Rs are not critically involved in the proconvulsant actions of PTZ. Past evidence, however, suggests that $\alpha_1$-GABA$_A$Rs likely mediate the proconvulsant effects of BZ-site convulsants and GABA$_A$R antagonists. In wildtype mice, BZ-site inverse agonists such DMCM and Ro 15-4513 display proconvulsant properties (Crestani et al., 2002) whereas they produce no proconvulsant properties in the $\alpha_1$(H101R) mice. Global deletion of the $\alpha_1$-GABA$_A$Rs results in an increased seizure susceptibility to the GABA$_A$R antagonist bicuculline which competes with GABA for its binding site (Kralic et al., 2002a). The lack of group differences may likely be due to the mechanisms involved in PTZ seizure initiation. In addition to the disinhibition of the GABA system (Macdonald and Barker, 1977), PTZ-induced activation of the glutamatergic system is involved in seizure initiation (Nevins and Arnold, 1989). Thus, in the absence of $\alpha_1$GABA$_A$R subtypes, glutamatergic-induced over-excitation is likely contained by the synaptic inhibition provided by remaining GABA$_A$Rs. Consistent with this interpretation, the sensitivity to kainic acid induced seizure activity is unaltered in global $\alpha_1$ KOs (Schneider Gasser, 2007). Furthermore the sensitivity to PTZ-induced seizure activity is unaltered in mutant $\alpha_1$(H101R) mice (Crestani et al., 2002). Thus it appears that the role of the $\alpha_1$-GABA$_A$Rs in mediating the proconvulsant actions is restricted to benzodiazepine site ligands. To the degree that the proconvulsant actions of bicuculline and inverse agonists are due to their action on amygdala $\alpha_1$-GABA$_A$Rs, we would predict that the LV-Cre mice may also show an increased seizure susceptibility to these compounds. Additional studies are underway to compare the proconvulsant effects of a variety of GABAergic ligands.

Anticonvulsant effects of test drugs

In contrast to the similar susceptibility to PTZ-induced convulsions, significant group differences were detected in the anticonvulsant effects of test drugs. In comparison to LV-GFP mice, LV-Cre mice showed shorter myoclonic and clonic latencies when either 0.25 or 1.0 mg/kg of diazepam was administered 30min before PTZ (85mg/kg). The ability of zolpidem to inhibit convulsant actions of PTZ was also blunted in LV-Cre mice. At the dose of 2.5 mg/kg, LV-Cre mice showed shorter myoclonic latencies, and at both 2.5 and 4.0 mg/kg of zolpidem, clonic seizure latencies were longer in LV-GFP mice as compared to LV-Cre mice. Our results are consistent with reports from KO and point mutated studies. For example, in contrast to wild type mice, $\alpha_1$-GABA$_A$ knockout mice also show attenuated diazepam-dependent increases in seizure threshold levels (Kralic et al., 2002a). In point-mutated $\alpha_1$(H101R) mice, the ability of zolpidem to prevent PTZ-induced seizures is eliminated (Crestani et al., 2000), and the anticonvulsant activity of diazepam is partially lost in mutant $\alpha_1$(H101R) mice (Rudolph et al., 1999a). This later finding indicates that diazepam’s anticonvulsant effects are also mediated by other GABA$_A$R subtypes ($\alpha_2$-, $\alpha_3$-, $\alpha_4$, $\alpha_5$), and indeed can be antagonized by the benzodiazepine antagonist, flumazenil (Rudolph et al., 1999a). However, Low et al (2000) have reported no changes in the anticonvulsant activity of diazepam in point-mutated mice with $\alpha_2$- or $\alpha_3$-GABA$_A$Rs that have been rendered diazepam-insensitive. Likewise, the anticonvulsant effects of diazepam are unaffected in point-mutated $\alpha_5$(H101R) mice (Crestani, et al 2002) and diazepam’s anticonvulsant effects are unaltered by pretreatment with RO4938581, a novel $\alpha_5$-GABA$_A$R inverse agonist (Ballard et al., 2009).
Specificity and efficiency of α1-GABA_A subunit deletions within the amygdala

Genetic ablation of α1-GABA_A subunits using the Cre/loxP system in combination with a Cre recombinase expressing lentivirus allowed us to address the question of whether compensatory changes result from diminished gene expression in adult animals or from the lack of normal expression during development. In the current study, quantitative analyses of mRNA expression levels in LV-Cre mice revealed significantly less α1-GABA_A subunit expression in LV-Cre mice in comparison with LV-GFP mice, whereas no differences were identified for α2 or α3 subunits. Thus, the reduction of α1-mRNA levels does not result in increased transcription levels of α2 or α3 subunits. At the translational level, the expression of α1-GABA_A amygdala protein expression in LV-Cre-infected animals was also decreased. Thus, the partial α1-mRNA deficit in LV-Cre mice was sufficient to disrupt protein production. The α1-GABA_A mRNA expression levels in the amygdala represented 45–52% of control values whereas western blot analysis indicated that α1-GABA_A subunit expression was reduced by only 40% relative to LV-GFP mice. It is worth noting that western blot results were conducted on tissue homogenates tissue punches centered at the level of the amygdala and likely included non-infected tissue located outside the lateral and basolateral amygdala. The relative levels of α2- and α3-GABA_A protein were unchanged. Thus, our results indicate that Cre-mediated gene deletion of the α1-GABA_A subunit in adult mice leads to no significant compensatory increases in the expression of α2- and α3-GABA_A subtypes. It seems unlikely that alterations in the motor and anticonvulsant actions of test drugs seen in the present study can be attributed to an up-regulation of α2- and/or α3-GABA_A subtypes. However, it cannot be ruled out that regional changes in subtypes other than α2 and α3 may have influenced our behavioral results.

Previous studies have revealed that both global and targeted deletion of α1 subunits can lead to compensatory changes that may function to protect against the α1-GABA_A loss. For example, in vivo studies conducted with global KO strains displayed increased expression of α2 and α3 subunit peptides in brain regions where the α1 subunit is absent (Sur et al., 2001; Kralic et al., 2002a; Schneider Gasser, 2007), which in some cases are gradually lost in successive generations of mice (Sur et al., 2001). Likewise forebrain-specific α1 knockout and heterozygote mutants also show increased levels of α3 subunit peptide expression (Zeller et al., 2008), indicating that the reduction of mRNA caused by the loss of a single α1 subunit allele can also cause compensatory changes. The up-regulation of GABA_A subunits is not the result of increased mRNA expression and likely takes place at the level of translation or post-translationally, at the level of receptor assembly or receptor trafficking (Bosman et al., 2005; Ogris et al., 2006). In contrast, Kralic et. al, revealed that α1 subunit gene-deficient heterozygous mice show an approximate 40% decrease in α1 subunit expression yet no significant changes in α2 or α3 peptide in spite of significant decrease in β2/3 peptide. Hence, the vital factors which dictate when and whether such compensatory changes occur are unclear. The conditional KO approach used in this study may be particularly useful for
investigating the fundamental temporal and spatial processes that govern normal brain development.

**Baseline anxiety-like behavior and anxiolytic-like effects of diazepam**

In the elevated plus maze, diazepam produced an anxiolytic effect as indexed by an increase in the percent time in the open arms and percent of open arm entries in both LV-GFP and LV-Cre mice. Vehicle treated animals from both groups displayed similar plus maze behavior, suggesting no differences in baseline anxiety-like behavior. In support, we observed similar percent of central time between groups in the open field test. Together the findings indicate that a reduction in amygdala α1-GABA_A Rs does not affect baseline anxiety-like behavior or the anxiolytic-like activity of diazepam. These findings are consistent with experiments showing that diazepam effectively reduces anxiety in both α1 knockout and α1 point mutated mice as measured in the elevated plus maze (Rudolph et al., 1999b; Kralic et al., 2002a). Diazepam also reliably reduces anxiety-related behaviors in these global α1 point mutated mice as measured in the light-dark choice test (Rudolph et al., 1999a). In contrast, the anxiolytic effect of diazepam was undetected in α2 point mutated mice, suggesting that α2-, but not α1-containing receptors, mediate the anxiolytic effect of diazepam (Low et al., 2000). Indeed, subtype selective agonists that have relatively strong effects at receptors containing the α2, α3 and α5 subunits when compared to receptors containing the α1 subunits show anxiolytic-like activity. For example, SL651498, a novel pyridoindole derivative, behaves as a full agonist at GABA_A Rs containing α2 and α3 subunits and as a partial agonist at GABA_A Rs containing α1 subunits (Griebel et al., 2001). Similar to diazepam, SL651498 induces anxiolytic-like activity in a variety of behavioral tests including the elevated plus-maze, light/dark test, and punished lever pressing (Griebel et al., 2001; Atack, 2003 #80; Griebel et al., 2003). Likewise, the benzodiazepine-site ligand L-838,417, which preferentially activates GABA_A Rs containing the α2, α3 and α5 subunits, also shows potent anxiolytic action in animal models of anxiety (McKernan et al., 2000). However, unlike α1-selective agonists, both L-838,417 and SL651498 produce little or no sedation at doses which produce anxiolytic-like activity (McKernan et al., 2000; Griebel et al., 2003).

It has been suggested that the compensatory increases in the α2 and/or α3 subunits of α1 KO mice may mask the lack of anxiety phenotype and altered sensitivity to BZ-induced anxiolytic-like effect (Sur et al., 2001; Kralic et al., 2002a). For example, homozygous α1 KO mice appear more sensitive to the anxiolytic effects of BZs when compared to heterozygous mice, possibly due the differential developmental upregulation of α2 and/or α3 subunits. Our results suggest that compensatory changes in the amygdala are not responsible for the normal BZ-induced anxiolytic-like effect seen in α1 KO mice.

In conclusion, these data demonstrate that the α1-GABA_A R within the amygdala is required for sedative-locomotor inhibition and anticonvulsive effects, but not anxiolytic actions of benzodiazepines. We have shown that temporally and spatially restricted inducible reduction of the α1 subunit results in reduced agonist-induced motor-sedative and anticonvulsant effects without affecting baseline motor and anxiety levels or diazepam-induced anxiolytic behavior. Further research using region- and cell type-selective manipulations of GABA receptors should further elucidate GABAergic mechanisms underlying complex behavior.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

Support was provided by NIH (DA019624, and F32 MH073389-01), the Center for Behavioral Neuroscience (NSF agreement IBN-987675), the Burroughs Wellcome Fund, and by an NIH/NCRR base grant (P51RR000165) to Yerkes National Primate Research Center. We thank Dr. Liping Mou for her excellent technical assistance with primary hippocampal neuronal cultures.

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Figure 1. $\alpha_1$-GABA$_A$R deletion in cultured mouse neurons with Cre and in vivo lentivirus infectivity of mouse amygdala

Panels a–f demonstrate the level of $\alpha_1$-GABA$_A$R subunit expression visualized with immunohistochemistry in LV-GFP infected, untreated (no LV), and LV-Cre infected primary mouse neurons from the floxed mouse line used for the remaining in vivo studies. (a) Overlay of green fluorescent and Hoechst stain (blue) image depicting LV-GFP infection and chromatin-positive nuclei of neuronal cells. (b) Immunocytochemistry for $\alpha_1$-GABA$_A$R (red) in same field as in (a). (c) Overlay of bright field Hoechst stain (blue) image of primary neuronal culture with no infection. (d) Immunocytochemistry for $\alpha_1$-GABA$_A$R (red) in same field as in (c). (e) Overlay of bright field Hoechst stain (blue) image of primary neuronal culture with LV-Cre infection. (f) Immunocytochemistry for $\alpha_1$-GABA$_A$R (red) in same field as in (e). These data demonstrate the complete removal of $\alpha_1$-GABA$_A$R signal from cells infected with LV-Cre from this targeted, inducible, knockout mouse strain. (g–i) low power micrograph of cresyl violet staining (g), GFP fluorescence (h), and GFP fluorescence overlayed with Hoechst stain (i) in mouse with LV-GFP infection, demonstrating the dense infectivity of lentivirus in the mouse basolateral amygdala (BLA). Large arrow demonstrates target of infection in BLA. Also shown are central nucleus of amygdala (CeA) and lateral amygdala (LA).
Figure 2. Region- and subunit-specific reduction of α1-GABA<sub>A</sub>R subunit mRNA in the adult brain

In situ hybridization analyses of mRNA expression indicated that local LV-Cre injections (a) decreased the expression of α1 subunit relative to LV-GFP infected mice and (b) produced reliable expression of Cre recombinase. (c) Overlay of Cre recombinase with α1-GABA<sub>A</sub>R pmRNA expression. Local LV-GFP injections produced (d) no discernable decreases in α1-GABA<sub>A</sub>R subunit mRNA expression. LV-Cre injections produced no evident effect on (e) the expression of the α2 subunit or (f) the expression of the α3 subunit. Arrows denote basolateral amygdala in all sections.
Figure 3. mRNA and protein analyses of GABA<sub>A</sub>R subunit expression

(a) mRNA levels are expressed as percentages with reference to LV-GFP control animals. Lateral (LA) and basolateral (BLA) nuclei of the amygdala. (b) Western blot analysis of α1-, α2-, and α3-GABA<sub>A</sub>R protein levels from amygdala tissue homogenates. (c) Digital image example of the western blot membrane showing protein bands of α1-, α2-, and α3-GABA<sub>A</sub>Rs as well as GAPDH protein in LV-Cre (+) and LV-GFP (−) mice. Protein amounts determined from these western blots were normalized to levels of a GAPDH to loading control. Protein levels are expressed as percentages with reference to LV-GFP control animals. Error bars denote 1 standard error of the mean (SEM). Stars indicated that the difference was statistically significant, ps<0.05.
Figure 4. Baseline activity and motor effects of test drugs in LV-Cre and LV-GFP mice
(a) Diazepam effects on locomotor activity. Baseline motor activity (control) was measured by examining the total ambulatory distance (in cm) during the 30-min open field test session. To examine the motor-impairing effects of diazepam, mice were given test drug injection (i.p.) 30 minutes before open field testing. The motor-impairing effects of diazepam are significantly blunted in mice with decreased α1-GABAA Rs in the amygdala at the 10mg/kg dose. (b) With zolpidem, motor-impairing effects are significantly blunted at both the 0.25mg/kg and 0.5mg/kg dose. Total ambulatory distances are expressed as mean + SEM. Star indicates that the difference between virus groups was statistically significant (p<.05).
Seizure onset latencies were defined as the time (in sec) between the injection of PTZ and the first appearance of myoclonic and clonic seizure activity. Behaviors were assessed during 20 min (1200s) observation period. (a) Local amygdala deletion of α1-GABA_{A}Rs does not affect PTZ-induced myoclonic seizure onset time. (b) Local amygdala deletion of α1-GABA_{A}Rs does not affect PTZ-induced clonic seizure onset time. (c) Despite the lack of effect on PTZ-induced seizure latency, there was a significant correlation between Lateral Amygdala α1-GABA_{A}R mRNA level and response to diazepam (0.25mg) in delaying seizure onset (n=12, \( r=0.61, p<.05 \)). (d) Basolateral Amygdala α1-GABA_{A}R mRNA level also correlates with response to diazepam in delaying seizure onset (n=12, \( r=0.81, p<.005 \)). Error bars denote 1 standard error of the mean (SEM).
Figure 6. The anticonvulsant effects of benzodiazepines on PTZ-induced seizures in LV-Cre and LV-GFP mice

Seizure onset latencies were defined as the time (in sec) between the injection of PTZ and the first appearance of (a, c) myoclonic and (b, d) clonic seizure activity assessed during 20 min (1200s) observation period. Diazepam or zolpidem was given 30 minutes before administration of PTZ. The anticonvulsant effects of (a–b) diazepam and (c–d) zolpidem are blunted in mice with decreased α1-GABA\(_A\)Rs in the amygdala. In bar graphs, latencies to seizure onset are expressed as means. Error bars denote 1 standard error of the mean (SEM). Stars indicated that the difference was statistically significant, \(p<0.05\).
Figure 7. The effects of diazepam on elevated plus-maze (EPM) performance
Mice were given 1.5 mg/kg of diazepam (DZP) or vehicle (VEH) 30 min before testing. Bar graphs show the (a) percentage of open arm entries, (b) percentage of open arm time, and (c) total (open + closed) number of arm entries on the elevated plus-maze. Error bars denote 1 standard error of the mean (SEM). Stars indicate significant difference from vehicle treatment, ps<0.05.