Published in final edited form as:

Psychopharmacology (Berl). 2015 December ; 232(23): 4359–4369. doi:10.1007/s00213-015-4069-9.

α 2-containing GABA(A) receptors: A requirement for midazolam-escalated aggression and social approach in mice

Emily L. Newman¹, Kiersten S. Smith^{2,3}, Aki Takahashi⁴, Adam Chu¹, Lara S. Hwa¹, Yang Chen¹, Joseph F. DeBold¹, Uwe Rudolph^{2,3}, and Klaus A. Miczek⁵

¹ Dept. of Psychology, Tufts University, Medford, MA 02144, USA

² Laboratory of Genetic Neuropharmacology, McLean Hospital, Belmont, MA 02478, USA

³ Dept. of Psychiatry, Harvard Medical School, Belmont, MA 02215, USA

⁴ Laboratory of Behavioral Neuroendocrinology, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki, Japan

⁵ Dept. of Neuroscience, Tufts University, Boston, MA 02111, USA

Abstract

Rationale—Benzodiazepines (BZDs) are prescribed to reduce anxiety, agitation, and muscle spasms, and for their sedative-hypnotic and anticonvulsant effects. Under specific conditions, BZDs escalate aggression in some individuals. Specific effects of BZDs have been linked to the α -subunit subtype composition of GABA_A receptors.

Objectives—Point-mutated mice rendered selectively insensitive to BZDs at α 1-, α 2-, or α 3-containing GABA_A receptors were used to determine which α -subunit subtypes are necessary for BZDs to escalate aggression and social approach and to reduce fear-motivated behavior.

Methods—During resident-intruder confrontations, male wild-type (WT) and point-mutated α 1(H101R), α 2(H101R), and α 3(H126R) mice were treated with midazolam (0–1.7 mg/kg, i.p.) and evaluated for aggression in an unfamiliar environment. Separate midazolam-treated WT and point-mutated mice were assessed for social approach toward a female or investigated in a six-day fear-potentiated startle procedure.

Results—Moderate doses of midazolam (0.3–0.56 mg/kg, i.p.) escalated aggression in WT and α 3(H126R) mutants and increased social approach in WT and α 1(H101R) mice. The highest dose of midazolam (1.0 mg/kg) reduced fear-potentiated startle responding. All mice were sensitive to the sedative effect of midazolam (1.7 mg/kg) except α 1(H101R) mutants.

Conclusions—Midazolam requires BZD-sensitive α 1- and α 2-containing GABA_A receptors in order to escalate aggression and α 2- and α 3-containing receptors to reduce social anxiety-like behavior. GABA_A receptors containing the α 1-subunit are crucial for BZD-induced sedation while

$\alpha 2$ -containing GABA_A receptors may be a shared site of action for the pro-aggressive and anxiolytic effects of BZDs.

Keywords

Benzodiazepine; midazolam; GABA_A receptor subunit; *Gabra1*; *Gabra2*; *Gabra3*; aggression; social approach; social anxiety; fear-potentiated startle

Benzodiazepines (BZDs) can have a profound calming effect on agitated non-human primates and on humans, particularly those suffering from social phobia and panic attacks (Heise and Boff 1961, Heuschele 1961, Wallace and Taylor 2009). In some individuals, however, BZDs relieve anxiety and fear but also produce impulsive aggression (in humans: Gardos et al. 1968, Weisman et al. 1998, Ben-Porath and Taylor 2002, Berman et al. 2005; in rodents: Fox et al. 1970, Miczek 1974, Miczek and O'Donnell 1980, Weerts and Miczek 1996, Ferrari et al. 1997; review: DiMascio 1973). Such outbursts of violence may be linked to reduced inhibitory control over brain regions such as the amygdala and hypothalamus which modulate aggression (Clement et al. 1987, Haller et al. 1998, Bjork et al. 2001, Halasz et al. 2002, Toth et al. 2010, Feja and Koch 2014). While low doses of BZDs may disrupt these pathways by acting on GABAergic neurons to facilitate neural disinhibition; higher doses generally inhibit neurotransmission to produce a calming or sedative effect (Rudolph and Knoflach 2011).

Functionally, BZDs increase GABA_A receptor-mediated chloride ion influx and potentiate agonist-induced inhibitory postsynaptic potentials (IPSPs; Wieland et al. 1992, Macdonald and Olsen 1994, Campo-Soria et al. 2006, Gielen et al. 2012). In the mammalian central nervous system, most GABA_A receptors are comprised of two α_{1-6} , two β_{1-3} , and one γ_{1-3} subunit. GABA_A receptors composed of $\alpha_{1-3}\beta\gamma$ subunits can be positively modulated by benzodiazepines at a binding site between α and γ subunits.

Linking the distinct behavioral effects of BZDs to GABA_A receptor subunit subtypes has been facilitated by genetic knock-in techniques that target particular subunits. In genetically modified mice, GABA_A receptors are rendered selectively insensitive to BZD binding and modulation via α -subunit-specific histidine to arginine point substitutions (e.g., $\alpha 1$ (H101R), $\alpha 2$ (H101R), or $\alpha 3$ (H126R); Wieland et al. 1992, Benson et al. 1998, Rudolph et al. 1999, Low et al. 2000, McKernan et al. 2000). Using these knock-in mice, preclinical studies suggest that $\alpha 1$ -containing GABA_A receptors mediate the sedative-hypnotic and, in part, the anticonvulsant effects of benzodiazepines; $\alpha 2$ -containing GABA_A receptors mediate their reward-enhancing and anxiolytic effects; and $\alpha 2$ - and $\alpha 3$ -containing GABA_A receptors mediate their myorelaxant activity (Low et al. 2000, Crestani et al. 2000, Crestani et al. 2001, Dixon et al. 2008, Rudolph and Knoflach 2011, Reynolds et al. 2012, Smith et al. 2012, Engin et al. 2014, Ralvenius et al. 2015). Diazepam may also attenuate risk assessment and conditioned fear responding by acting on $\alpha 2$ -containing GABA_A receptors (Morris et al. 2006, Smith et al. 2012, Koester et al. 2013).

Since reduced anxiety, fear and risk assessment may be related to behavioral disinhibition and escalated aggression, we hypothesized that BZD-sensitive $\alpha 2$ -containing GABA_A receptors may be necessary for BZD-escalated aggression. To test this hypothesis, we treated

male wild-type, $\alpha 1$ (H101R), $\alpha 2$ (H101R), and $\alpha 3$ (H126R) knock-in mice with the benzodiazepine, midazolam, and assessed their aggression, social approach, and fear-potentiated startle (FPS) responses. Identification of GABA_A receptor subtype(s) involved in BZD-escalated aggression may provide important clues for the development of subtype-specific compounds that provide the therapeutic effects of BZDs without facilitating violent outbursts.

Methods

Animals

Mutant $\alpha 1$ (H101R), $\alpha 2$ (H101R) or $\alpha 3$ (H126R) mice were homozygous for histidine to arginine point substitutions in the α subunit protein sequence rendering them selectively insensitive to benzodiazepines at $\alpha 1$ -, $\alpha 2$ -, or $\alpha 3$ -containing GABA_A receptors, respectively (Rudolph et al. 1999, Low et al. 2000). Founders were generated and bred to a C57BL/6J background (Bar Harbor, ME) for at least fifteen generations. Male and female C57BL/6J wild-type mice were purchased from Jackson Breeding Laboratories (Bar Harbor, ME) and bred to generate wild-type control mice (WT). For aggression and social approach experiments, mutant and WT mice were maintained in filial breeding pairs in clear polycarbonate cages (28×17×14 cm) with pine shavings at the Tufts Psychopharmacology Laboratory (Medford, MA). Breeding pairs generated F1-F8 offspring that were weaned on PPD21 and group-housed (>2/ cage) by sex. No more than two mice per litter were assigned to the same experimental group. Each experiment evaluated contemporary wild-type, $\alpha 1$ (H101R), $\alpha 2$ (H101R) and $\alpha 3$ (H126R) mutant mice.

For the social instigation and aggression protocols, 21-23 gram male Swiss-Webster mice (CFW; Charles River Breeding Laboratories, Wilmington, MA) were used as stimulus animals (n=80). CFW mice were group-housed in large clear polycarbonate cages (48×27×17 cm) with corn cob bedding. All mice accessed tap water and rodent chow (LabDiet 5001 Purina Rodent Diet) through wire mesh cage lids. The vivarium (21±1°C, 30-40% humidity) was kept on a 12h photocycle (lights off 0700h), and all behavioral assessments were made between 1000 and 1500h when mice were most active.

Fear-potentiated startle was assessed in adult male mutant and WT mice that were bred in the Laboratory of Genetic Neuropharmacology at McLean Hospital (Belmont, MA). After weaning, animals were group-housed by genotype in Super Mouse 750 cages containing a LifeSpan Rodent Enrichment insert (Lab Products, Seaford, DE, USA) and covered by micro-isolator non-wire bar lids. All animals had unrestricted access to food (Purina LabDiet 5P76, PMI Nutrition International, Brentwood, MO, USA) and water, and were assessed for fear-potentiated startle during the light phase of the 12h photocycle (lights off 0600h). At the conclusion of all experiments, tail samples were collected and genotypes were confirmed using PCR. Animals were cared for according to the NIH *Guide for the Care and Use of Laboratory Animals* (8th ed., 2011) and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Tufts University or the IACUC of McLean Hospital.

Home cage resident-intruder aggression

Eight-week old resident mutant or wild-type males [n=14 WT; n=16 α 1(H101R); n=12 α 2(H101R); n=14 α 3(H126R)] were housed with breeding females to foster species-typical territorial aggression. After three weeks, resident aggression was assessed using the social instigation method (Fish et al. 1999) followed by the resident-intruder protocol every 48 hours. The female and any pups were temporarily removed from the resident home cage prior to the introduction of a group-housed CFW stimulus male within a perforated, protective cage (17 × 6 × 6 cm). After five minutes, the stimulus male's cage was removed and promptly replaced with a specific, free-roaming CFW intruder male to initiate the resident-intruder confrontation. Aggressive encounters were terminated five minutes after the resident initiated the first attack bite or at five minutes if the resident failed to attack (Miczek and O'Donnell 1980).

If social instigation successfully elicited aggressive behavior in the resident male, all subsequent resident-intruder confrontations were performed in the absence of the instigation procedure. The latency to the initial attack bite and the frequency of attack bites were recorded to evaluate each resident for stable levels of aggression, operationally defined as <20% variability within the last three encounters that were not preceded by the social instigation procedure. Animals were excluded if they were not aggressive after three consecutive resident-intruder confrontations or if they were unable to achieve stable levels of aggression after 6-8 resident-intruder confrontations. There was no significant difference between genotypes in terms of the proportion of mice that exhibited stable levels of aggression: 64.3% WT; 75% α 1(H101R); 66.7% α 2(H101R); 64.3% α 3(H126R); χ^2 (2, N=56) = 0.55, p=0.91.

Aggression in a novel environment with midazolam treatment

Compared to aggression in the home cage, a novel environment reduces aggressive behavior by approximately 50% (Miczek and O'Donnell 1980, Fish et al. 1999). This suppressed level of aggression may facilitate the detection of behavioral disinhibition following treatment with benzodiazepines (Miczek and O'Donnell 1980). To assess whether midazolam can disinhibit aggressive behavior, resident males were evaluated for aggression in a large, unfamiliar environment [n=9 WT; n=12 α 1(H101R); n=8 α 2(H101R); n=9 α 3(H126R)]. The resident male was removed from his home cage and placed in one corner of an unfamiliar polycarbonate cage (84 × 51 × 42 cm) lined with fresh pine shavings. Five seconds later, the intruder was placed in the opposite corner. Like home cage encounters, confrontations in the unfamiliar environment were terminated five minutes after the first attack bite. To eliminate any phomonal cues, the unfamiliar cage was rinsed with water and lined with fresh pine shavings between each aggressive encounter. Resident-intruder confrontations occurred every 48 hours and alternated between the resident's home cage and the unfamiliar cage until the frequency of attack bites stabilized in each environmental condition (Fig. 2). Each resident then established a stable level of aggression toward a new intruder male over the course of four home-cage confrontations. These new resident-intruder pairs remained constant during the subsequent assessments of aggression after midazolam treatment.

Residents were administered midazolam HCl (0.0, 0.1, 0.3, 0.56, 1.0, 1.7 mg/kg; Sigma Aldrich) 15 minutes prior to resident-intruder confrontations in the unfamiliar environment. Vehicle (0.9% saline) and drug doses were administered intraperitoneally (i.p.) in an injection volume of 10 ml/kg of body weight. All animals received vehicle and the five randomized doses of midazolam in a mixed two-way repeated-measures experimental design. Throughout the experiment, cages were cleaned weekly at least 24 hours prior to resident-intruder confrontations.

Aggressive encounters were video-recorded with a JVC Everio GZ-MG670 digital camera and later coded by a trained observer using *The Observer XT* software (Noldus, v. 9.0.436, Wageningen, The Netherlands). A specialized keyboard was used to code the frequency of aggressive behaviors including attack bites and sideways threats and the duration of non-aggressive behaviors including grooming, walking, rearing, and allogrooming. The total duration of time that a resident spent grooming, sniffing or walking over or under his intruder male was termed allogrooming. Midazolam-induced sedation was operationally defined as a significant reduction in multiple behaviors including locomotion as compared to vehicle.

Social approach in an unfamiliar environment with midazolam treatment

Eight-week old male mutant and WT mice [n=11 WT; n=11 $\alpha 1$ (H101R); n=9 $\alpha 2$ (H101R); n=9 $\alpha 3$ (H126R)] were individually housed for one week prior to social approach testing. To minimize cues that may motivate experimental males to engage in either sexual or aggressive behavior during social approach testing, unfamiliar, group-housed, ovariectomized (OVX), WT females (n=20) served as stimulus animals. Weekly social approach assessments were conducted in a three-chamber apparatus (each chamber: 29 × 29 × 36 cm).

Prior to testing, male mice were moved from the vivarium and allowed to habituate to the procedure room for at least one hour. At that time, the central chamber doors were lowered remotely by slowly pressurizing two air pistons, one attached to the top of either door. An OVX mouse was placed in the wire stimulus cage (11 cm height, 10.5 cm base diameter; Nadler et al. 2004), leaving the opposite cage empty. The male habituated to the central chamber for five minutes, then received a systemic injection of 0.9% saline or midazolam HCl (0.1, 0.3, 0.56, 1.0, 1.7 mg/kg, i.p; Sigma Aldrich) and was returned to the central chamber. Thirteen minutes following the drug injection, the central chamber doors were lifted, allowing the male to explore the three-chamber apparatus for 10 minutes. Software (EthoVision XT 8.5) tracked the experimental male and quantified his distance travelled (cm) and his time spent within the social approach zone surrounding the base of the occupied stimulus cage. The social approach zone extended 2.25 cm past the radius of the stimulus cage. Between trials, the apparatus was thoroughly cleaned and the OVX stimulus animal was replaced to prevent the transfer of pheromonal cues between male mice. During the first two weeks, mice were administered saline and evaluated for side preference in the absence of an OVX mouse. No experimental male mice continued to express a side preference after the second assessment, and therefore all males were subsequently evaluated for social approach following systemic midazolam administration.

Fear-potentiated startle testing with midazolam treatment

Eight-week-old male mutant and WT mice [n=11-18/dose WT; n=12-13/dose $\alpha 1$ (H101R); n=10-13/dose $\alpha 2$ (H101R); n=14-15/dose $\alpha 3$ (H126R)] were habituated to the behavioral suite two hours prior to testing. Fear-potentiated startle was measured using the MedAssociates Inc. (St. Albans, VT, USA) Startle Reflex System and Advanced Startle software program according to a 6-day protocol (Smith et al. 2011, Smith et al. 2012). Prior to the presentation of auditory stimuli, mice were first allowed to habituate to the test chamber for five minutes.

Startle amplitude data from three consecutive acclimation days were used to identify an auditory stimulus intensity (dB) that would elicit a moderate, unconditioned startle response (days 1-3). As shown previously (Smith et al. 2011, Smith et al. 2012), the 20-ms 85 dB stimulus produced moderate startle amplitudes and was therefore used as the experimental auditory startle stimulus on days 4-6. During pre-conditioning (day 4), mice were habituated to the startle stimulus through the presentation of ten consecutive bursts. Subsequent bursts were intermittently preceded by a tone (3-s rise from 0-70 dB, then 27-s, 12 kHz, 70 dB). After the ten habituation bursts, startle amplitudes were measured in response to stimuli that were preceded by a tone (Tone+Startle) and to stimuli that were not preceded by a tone (Startle Only). Subjects were assigned to a midazolam group according to their matched pre-conditioning startle amplitude data (Brown et al. 1951, Smith et al. 2011). The next day, mice were conditioned to associate the tone (CS) with a subsequent foot shock (0.25-s, 0.4-mA; US) over the course of ten CS+US trials occurring at random intervals (120, 180, or 240 s). Post-conditioning (day 6), mice were treated with midazolam HCl (0.0, 0.3 or 1.0 mg/kg i.p; Ben Venue Laboratories) and 20 minutes later, the pre-conditioning test was repeated.

Statistics

A two-way mixed analysis of variance (ANOVA) was performed using SigmaPlot 13.0 to assess home cage versus unfamiliar environment attack bite frequencies (genotype x environment). Two-way mixed ANOVA were also used to evaluate the frequency and duration of aggressive and non-aggressive behaviors during resident-intruder confrontations with midazolam treatment (genotype x drug treatment); and the duration of time spent in the social approach zone along with the distance travelled during social approach testing with midazolam (genotype x drug treatment).

For FPS experiments, pre- and post-conditioning startle amplitudes were calculated for each animal by averaging within Habituation, Startle Only, or Tone+Startle trials. Two-way mixed ANOVA was used to detect genotype-associated differences in pre-conditioning responding to acoustic startle (genotype x trial type). To calculate the increase in startle response due to the pairing between CS and the US on day 5, fear conditioning scores were generated for each mouse according to trial type: (Post-conditioning startle amplitude) – (Pre-conditioning startle amplitude). A two-way mixed ANOVA was conducted on fear conditioning scores from vehicle-treated animals to assess baseline differences in fear conditioning between genotypes (genotype x trial type). For each mouse, percent FPS was used to evaluate the increase in startle response amplitude due to presentation of the Tone prior to the Startle

stimulus on the postconditioning test day: $(((\text{Tone} + \text{Startle}) - (\text{Startle Only})) / (\text{Startle Only})) * 100$. Two-way RM ANOVA was run on percent FPS in vehicle-treated mice to detect differences between pre- and post-conditioning values (genotype x test day). A two-way ANOVA was also performed on percent FPS (genotype x drug treatment). For all ANOVA, significant interactions and main effects were further analyzed using Dunnett's tests corrected for unequal n s (mutant genotypes vs. WT; midazolam doses vs. vehicle). For all analyses, $p < 0.05$ was considered statistically significant.

Results

Suppression of aggressive behavior in an unfamiliar environment

Aggressive behavior decreased when resident-intruder confrontations were in an unfamiliar environment rather than in the resident's home cage ($F(1,33)=74.80$, $p < 0.001$]; Fig. 1). There was no effect of genotype [$F=0.332$, $p=0.8$] or any effect of an interaction between genotype and cage condition on aggression [$F=0.124$, $p=0.95$].

Effects of midazolam on aggression in an unfamiliar environment

There was a significant interaction between drug treatment and genotype [$F(15, 165)=3.27$, $p < 0.001$]. Compared to vehicle, a moderate dose of midazolam (0.3 mg/kg) significantly increased aggression in WT and $\alpha 3$ (H126R) mutant mice (Fig. 2E). Conversely, midazolam-escalated aggression was absent in both $\alpha 1$ (H101R) and $\alpha 2$ (H101R) mutants (Fig. 2A and 2C). Planned contrasts also identified reduced aggression following treatment with a high dose of midazolam (1.7 mg/kg) in WT, $\alpha 2$ (H101R) and $\alpha 3$ (H126R) mutant mice (Fig. 2A and 2E). Reduced aggression was not observed in mice with benzodiazepine-insensitive $\alpha 1$ -containing GABA_A receptors (Fig. 2A). ANOVA on sideways threat frequency detected an interaction between midazolam and genotype [$F(15, 165)=2.54$, $p=0.002$]. Specifically, low to moderate doses of midazolam (0.3-1.0 mg/kg) reduced the frequency of sideways threats exhibited by $\alpha 1$ (H101R) mutants as compared to WT mice (Table 1).

Drug treatment and genotype interacted [$F(15, 165)=6.96$, $p < 0.001$] to decrease walking behavior in WT, $\alpha 2$ (H101R) and $\alpha 3$ (H126R) mutant mice administered the highest dose of midazolam (1.7 mg/kg; Table 1). Post-hoc comparisons between WT and mutant mice revealed that a moderate dose of midazolam (0.56 mg/kg) could reduce motor activity in $\alpha 2$ (H101R) mutants (Table 1). In stark contrast, $\alpha 1$ (H101R) mutants remained ambulatory even after treatment with the highest dose of midazolam (1.7 mg/kg). A main effect of drug treatment reduced the frequency of tail rattles [$F(5,165)=10.26$, $p < 0.001$] after treatment with the highest doses of midazolam (1.0 and 1.7 mg/kg). Interestingly, midazolam increased the duration of allogrooming [$F(5, 165)=12.20$, $p < 0.001$] when administered in high doses. This effect appears to be specific to social grooming considering drug treatment did not increase the duration of self-grooming (Table 1).

Effects of midazolam on social approach in an unfamiliar environment

There was a significant interaction between midazolam treatment and genotype [$F(12,144)=3.19$, $p < 0.001$]. Dunnett's post-hoc comparisons revealed increased time spent in the social approach zone by WT mice and $\alpha 1$ (H101R) mutants after treatment with a

moderate dose of midazolam (0.56 mg/kg; Fig. 2B). Additional planned contrasts identified reduced social approach behavior in $\alpha 2$ (H101R) and $\alpha 3$ (H126R) mutants administered the 1.0 mg/kg dose of midazolam (Fig. 2D and 2F).

A significant interaction between drug treatment and genotype [$F(12, 144)=2.69$, $p=0.003$] resulted in reduced locomotion as determined by two-way RM ANOVA. Post-hoc comparisons identified reduced walking by $\alpha 2$ (H101R) and $\alpha 3$ (H126R) mutants after treatment with midazolam (1.0 mg/kg; Table 2): an effect that was not detected in WT mice or $\alpha 1$ (H101R) mutants. To characterize the descending limb of the midazolam dose-effect curve in WT and $\alpha 1$ (H101R) mutant mice, these animals were assigned to an additional social approach test after treatment with 1.7 mg/kg midazolam. Extending the dose range did not alter the statistical findings reported for social approach time. However, 2-way RM ANOVA performed on distance travelled in WT mice and $\alpha 1$ (H101R) mutants revealed a significant main effect of drug dose [$F(5,100)=26.786$, $p<0.001$]. Planned post-hoc comparisons confirmed that this effect was driven by reduced distance travelled following treatment with the highest dose of midazolam (1.7 mg/kg; Table 2).

Effects of midazolam on fear-potentiated startle

Pre-conditioning startle amplitudes in response to the 85 dB acoustic Startle stimulus were similar across all genotypes and the presentation of the Tone prior to the Startle stimulus did not potentiate this response on day 4, prior to fear conditioning. Two-way ANOVA on fear conditioning scores from vehicle-treated mice identified a main effect of trial type [$F(2,102)=59.043$, $p<0.001$]; following fear conditioning, Tone+Startle trials elicited a greater increase in startle amplitude compared to Habituation trials, regardless of genotype. Likewise, two-way RM ANOVA on percent FPS data from vehicle-treated mice detected a significant main effect of the test day with post-conditioning tests eliciting greater scores than tests that occurred prior to fear-conditioning ([$F(1,51)=55.92$, $p<0.001$], Fig. 3). Two-way ANOVA (genotype x drug treatment) on percent FPS identified a main effect of drug treatment [$F(2, 148)=6.519$, $p=0.002$] with the 1.0 mg/kg dose of midazolam significantly reducing percent FPS compared to vehicle (Fig. 3). There was no significant effect of an interaction between genotype and drug treatment on percent FPS [$F=0.458$, $p=0.84$].

Discussion

We have demonstrated that GABA_A receptors containing the $\alpha 1$ - or $\alpha 2$ -subunit are essential target sites of action for midazolam (0.3 mg/kg) to escalate murine aggression toward a submissive, male intruder. In contrast, midazolam (0.56 mg/kg) may act on $\alpha 2$ - or $\alpha 3$ -containing GABA_A receptors to increase social approach and allogrooming. In a non-social setting, midazolam (1.0 mg/kg) treatment attenuated fear-potentiated acoustic startle, an effect that was most evident in WT mice. These results indicate that BZD-sensitive $\alpha 2$ -containing binding sites are crucial for midazolam to escalate aggressive and social approach behaviors. In the absence of benzodiazepine treatment, WT, $\alpha 1$ (H101R), $\alpha 2$ (H101R) and $\alpha 3$ (H126R) mutant mice showed similar levels of suppressed aggression in the unfamiliar environment and increased startle responding following fear-conditioning.

Preclinical assessments using tests of unconditioned anxiety and conditioned fear highlight the importance of $\alpha 2$ -containing GABA_A receptors for the pronounced anxiolytic-like effects of diazepam and chlordiazepoxide (Low et al. 2000, Dixon et al. 2008, Smith et al. 2012, Koester et al. 2013). The current study extends these observations and reveals that moderate doses of midazolam may act on $\alpha 2$ -containing GABA_A receptors to increase social behaviors. Additional work is necessary to determine whether this $\alpha 2$ -subunit requirement applies to any social behavior or if it is specific to behaviors suppressed within an unfamiliar environment. In contrast with diazepam and chlordiazepoxide (Smith et al. 2012), midazolam may not require wild-type-like $\alpha 2$ -containing GABA_A receptors to diminish fear-potentiated startle responding. This could reflect differential pharmacokinetics between benzodiazepines, a possibility that requires further investigation. Our results also suggest that $\alpha 1$ -containing GABA_A receptors are crucial for midazolam-escalated aggression, but not for the anxiolytic-like effect of midazolam in a social context. These findings partially dissociate escalated aggression from reduced social anxiety, suggesting that the anxiolytic effect of midazolam does not necessarily lead to heightened aggression.

Like diazepam and chlordiazepoxide, midazolam also requires BZD-sensitive $\alpha 1$ -containing GABA_A receptors in order to induce sedation (Rudolph et al. 1999, Ralvenius et al. 2015). Wild-type, $\alpha 2$ (H101R) and $\alpha 3$ (H126R) mice showed reduced aggression after treatment with the highest dose of midazolam (1.7 mg/kg). This effect may be due to non-specific suppression of motor behavior, and therefore, the absence of sedation in $\alpha 1$ (H101R) mutants may explain their high levels of aggression even after treatment with the highest dose of midazolam.

In contrast to $\alpha 1$ (H101R) and $\alpha 2$ (H101R) mice, $\alpha 3$ (H126R) mutants were sensitive to both the pro-aggressive and sedative effects of midazolam, but not to the social anxiolytic-like effect. These findings reveal a double dissociation between the effects of midazolam on social behavior: $\alpha 1$ -containing GABA_A receptors are necessary for escalated aggression but not social approach while $\alpha 3$ -containing GABA_A receptors are essential for increased social approach but not aggression. It appears that BZD-sensitive $\alpha 2$ -containing GABA_A receptors are required for midazolam-escalated aggression and social approach. It is possible that two separate, $\alpha 2$ -subunit-rich loci independently regulate aggression and social approach behaviors. Alternatively, this pattern of results may reflect two functionally distinct pathways that either receive afferents from or send efferents to a shared, $\alpha 2$ -subunit-rich region. GABA_A receptors containing the $\alpha 2$ -subunit are localized on the axon initial segment (AIS) of pyramidal cells in the hippocampus, dorsolateral prefrontal cortex, and possibly the amygdala (Nusser et al. 1996, Fritschy et al. 1998a, Fritschy et al. 1998b, Loup et al. 1998, Kempainen and Pitkanen 2000, Freund and Katona 2007, Cruz et al. 2009, Lewis et al. 2012, Ehrlich et al. 2013). Future studies should assess whether midazolam application to the AIS may affect the production and rhythmicity of action potentials, particularly in the amygdala and hippocampus. In addition, it may be feasible in the future to generate mice lacking $\alpha 2$ -containing GABA_A receptors specifically at the AIS. Such a mutation could clarify the relationship between localized GABA_A receptor modulation by benzodiazepines and aberrant social behavior.

The present work suggests that midazolam requires $\alpha 1$ - and $\alpha 2$ -containing GABA_A receptors in order to escalate aggression. Interestingly, pharmacological evaluations demonstrate that the $\alpha 1$ -containing GABA_A receptor-selective antagonist, β -CCt, can effectively block the pro-aggressive effect of midazolam (Gourley et al. 2005). These findings suggest that selective positive modulators acting on $\alpha 2$ - but not $\alpha 1$ -containing GABA_A receptors may be sufficient to reduce anxiety without promoting aggression. In addition, recent studies using mutant mice with triple histidine to arginine point substitutions [$\alpha 1$ (H101R)/ $\alpha 2$ (H101R)/ $\alpha 5$ (H105R)] show that positive allosteric modulation of $\alpha 3$ -containing GABA_A receptors may induce myorelaxation without sedation (Ralvenius et al. 2015). Selective positive modulation of $\alpha 3$ -containing GABA_A receptors may also bypass the pro-aggressive effect of some benzodiazepines. Together, these findings indicate that $\alpha 3$ -containing GABA_A receptors could serve as an ideal target for the clinical treatment of muscle spasms without an extensive side-effect profile.

Because experimental mice were generated from homozygous breeding pairs, it is possible that genotype-associated differences in parental care may yield systematic variability in baseline behavior and sensitivity to benzodiazepines during adulthood. To minimize the impact of this limitation, mutants were backcrossed to C57BL/6J mice for more than fifteen generations at which point, they are theoretically congenic with WT mice. Baseline differences between mutant and WT animals have only been identified in animals on a mixed background but not in studies that have employed a multi-generational backcrossing protocol (Rudolph et al. 1999, Low et al. 2000, Smith et al. 2012). Likewise, the present experimental work did not reveal any significant differences in aggression, social approach or fear responding in vehicle-treated animals.

In the human population, developmental and environmental conditions may interact with *GABRA2* single-nucleotide polymorphisms (SNPs) to potentially increase anxiety, impulsivity, and vulnerability to drug abuse disorders (Enoch et al. 2006, Dick et al. 2006a, Dick et al. 2006b, Dick et al. 2009, Uhart et al. 2013). Recent evaluations identify polymorphisms in subjects suffering from cocaine or alcohol addiction (Covault et al. 2004, Edenberg et al. 2004, Lappalainen et al. 2005, Drgon et al. 2006, Covault et al. 2008, Soyka et al. 2008, Dixon et al. 2010, Li et al. 2014). Therefore, ongoing experiments use point-mutated mice to evaluate the relationship between $\alpha 2$ -containing GABA_A receptor sensitivity to various GABA_A receptor ligands and escalated alcohol drinking (Newman et al. in prep).

Acknowledgements

This work was funded by NIH grants R01 AA013983 (Klaus A. Miczek, Ph.D.) and R01 MH080006 (Uwe Rudolph, M.D.). We would like to thank J. Thomas Sopko, Keisha Dodman, Darrel Gachette, Georgia Gunner, Nishani Hewage, Polly Huynh, Aida Vargas De Jesus, and Tiffany Wang for their excellent contributions.

The project described was supported by Award Numbers R01AA013983 to KAM from the National Institute on Alcohol Abuse and Alcoholism and R01MH080006 to UR from the National Institute of Mental Health. The content is the sole responsibility of the authors and does not necessarily represent the official views of the National Institute on Alcohol Abuse and Alcoholism, the National Institute of Mental Health or the National Institutes of Health.

References

- Ben-Porath DD, Taylor SP. The effects of diazepam (Valium) and aggressive disposition on human aggression - An experimental investigation. *Addict Behav.* 2002; 27:167–177. [PubMed: 11817760]
- Benson JA, Low K, Keist R, Mohler H, Rudolph U. Pharmacology of recombinant gamma-aminobutyric acid A receptors rendered diazepam-insensitive by point-mutated alpha-subunits. *Febs Lett.* 1998; 431:400–404. [PubMed: 9714551]
- Berman ME, Jones GD, McCloskey MS. The effects of diazepam on human self-aggressive behavior. *Psychopharmacology.* 2005; 178:100–106. [PubMed: 15316710]
- Bjork JM, Moeller FG, Kramer GL, Kram M, Suris A, Rush AJ, Petty F. Plasma GABA levels correlate with aggressiveness in relatives of patients with unipolar depressive disorder. *Psychiat Res.* 2001; 101:131–136.
- Brown JS, Kalish HI, Farber IE. Conditioned fear as revealed by magnitude of startle response to an auditory stimulus. *J Exp Psychol.* 1951; 41:317–328. [PubMed: 14861383]
- Campo-Soria C, Chang YC, Weiss DS. Mechanism of action of benzodiazepines on GABA(A) receptors. *Brit J Pharmacol.* 2006; 148:984–990. [PubMed: 16783415]
- Clement J, Simler S, Ciesielski L, Mandel P, Cabib S, Puglisi-Allegra S. Age-dependent changes of brain GABA levels, turnover rates and shock-induced aggressive-behavior in inbred strains of mice. *Pharmacol Biochem Behav.* 1987; 26:83–88. [PubMed: 3562502]
- Covault J, Gelernter J, Hesselbrock V, Nellisery M, Kranzler HR. Allelic and haplotypic association of GABRA2 with alcohol dependence. *Am J Med Genet B.* 2004; 129B:104–109.
- Covault J, Gelernter J, Jensen K, Anton R, Kranzler HR. Markers in the 5'-region of GABRG1 associate to alcohol dependence and are in linkage disequilibrium with markers in the adjacent GABRA2 gene. *Neuropsychopharmacol.* 2008; 33:837–848.
- Crestani F, Low K, Keist R, Mandelli MJ, Mohler H, Rudolph U. Molecular targets for the myorelaxant action of diazepam. *Mol Pharmacol.* 2001; 59:442–445. [PubMed: 11179437]
- Crestani F, Martin JR, Mohler H, Rudolph U. Mechanism of action of the hypnotic zolpidem in vivo. *Brit J Pharmacol.* 2000; 131:1251–1254. [PubMed: 11090095]
- Cruz DA, Lovallo EM, Stockton S, Rasband M, Lewis DA. Postnatal development of synaptic structure proteins in pyramidal neuron axon initial segments in monkey prefrontal cortex. *J Comp Neurol.* 2009; 514:353–367. [PubMed: 19330819]
- Dick DM, Bierut L, Hinrichs A, Fox L, Bucholz KK, Kramer J, Kuperman S, Hesselbrock V, Schuckit M, Almasy L, Tischfield J, Porjesz B, Begleiter H, Nurnberger J, Xuei XL, Edenberg HJ, Foroud T. The role of GABRA2 in risk for conduct disorder and alcohol and drug dependence across developmental stages. *Behav Genet.* 2006a; 36:577–590. [PubMed: 16557364]
- Dick DM, Jones K, Saccone N, Hinrichs A, Wang JC, Goate A, Bierut L, Almasy L, Schuckit M, Hesselbrock V, Tischfield J, Foroud T, Edenberg H, Porjesz B, Begleiter H. Endophenotypes successfully lead to gene identification: Results from the collaborative study on the genetics of alcoholism. *Behav Genet.* 2006b; 36:112–126. [PubMed: 16341909]
- Dick DM, Latendresse SJ, Lansford JE, Budde JP, Goate A, Dodge KA, Pettit GS, Bates JE. Role of GABRA2 in trajectories of externalizing behavior across development and evidence of moderation by parental monitoring. *Arch Gen Psychiat.* 2009; 66:649–657. [PubMed: 19487630]
- Dimascio A. Effects of benzodiazepines on aggression - reduced or increased. *Psychopharmacologia.* 1973; 30:95–102. [PubMed: 4576033]
- Dixon CI, Morris HV, Breen G, Desrivieres S, Jugurnauth S, Steiner RC, Vallada H, Guindalini C, Laranjeira R, Messas G, Rosahl TW, Atack JR, Peden DR, Beelli D, Lambert JJ, King SL, Schumann G, Stephens DN. Cocaine effects on mouse incentive-learning and human addiction are linked to alpha 2 subunit-containing GABA(A) receptors. *Proc Natl Acad Sci USA.* 2010; 107:2289–2294. [PubMed: 20133874]
- Dixon CI, Rosahl TW, Stephens DN. Targeted deletion of the GABRA2 gene encoding alpha-2-subunits of GABA(A) receptors facilitates performance of a conditioned emotional response, and abolishes anxiolytic effects of benzodiazepines and barbiturates. *Pharmacol Biochem Behav.* 2008; 90:1–8. [PubMed: 18313124]

- Drgon T, D'Addario C, Uhl GR. Linkage disequilibrium, haplotype and association studies of a chromosome 4 GABA receptor gene cluster: Candidate gene variants for addictions. *Am J Med Genet B*. 2006; 141B:854–860.
- Edenberg HJ, Dick DM, Xuei XL, Tian HJ, Almasy L, Bauer LO, Crowe RR, Goate A, Hesselbrock V, Jones K, Kwon J, Li TK, Nurnberger JI, O'Connor SJ, Reich T, Rice J, Schuckit MA, Porjesz B, Foroud T, Begleiter H. Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations. *Am J Hum Genet*. 2004; 74:705–714. [PubMed: 15024690]
- Ehrlich DE, Ryan SJ, Hazra R, Guo JD, Rainnie DG. Postnatal maturation of GABAergic transmission in the rat basolateral amygdala. *J Neurophysiol*. 2013; 110:926–941. [PubMed: 23719209]
- Engin E, Bakhurin KI, Smith KS, Hines RM, Reynolds LM, Tang WN, Sprengel R, Moss SJ, Rudolph U. Neural basis of benzodiazepine reward: Requirement for alpha 2 containing GABA(A) receptors in the nucleus accumbens. *Neuropsychopharmacol*. 2014; 39:1805–1815.
- Enoch MA, Schwartz L, Albaugh B, Virkkunen M, Goldman D. Dimensional anxiety mediates linkage of GABRA2 haplotypes with alcoholism. *Am J Med Genet B*. 2006; 141B:599–607.
- Feja M, Koch M. Ventral medial prefrontal cortex inactivation impairs impulse control but does not affect delay-discounting in rats. *Behav Brain Res*. 2014; 264:230–239. [PubMed: 24556205]
- Ferrari PF, Parmigiani S, Rodgers RJ, Palanza P. Differential effects of chlordiazepoxide on aggressive behavior in male mice: the influence of social factors. *Psychopharmacology*. 1997; 134:258–265. [PubMed: 9438675]
- Fish EW, Faccidomo S, Miczek KA. Aggression heightened by alcohol or social instigation in mice: reduction by the 5-HT1B receptor agonist CP-94,253. *Psychopharmacology*. 1999; 146:391–399. [PubMed: 10550489]
- Fox KA, Tuckosh JR, Wilcox AH. Increased aggression among grouped male mice fed chlordiazepoxide. *Eur J Pharmacol*. 1970; 11:119–121. [PubMed: 5464398]
- Freund TF, Katona I. Perisomatic inhibition. *Neuron*. 2007; 56:33–42. [PubMed: 17920013]
- Fritschy JM, Johnson DK, Mohler H, Rudolph U. Independent assembly and subcellular targeting of GABA(A)-receptor subtypes demonstrated in mouse hippocampal and olfactory neurons in vivo. *Neurosci Lett*. 1998a; 249:99–102. [PubMed: 9682826]
- Fritschy JM, Weinmann O, Wenzel A, Benke D. Synapse-specific localization of NMDA and GABA(A) receptor subunits revealed by antigen-retrieval immunohistochemistry. *J Comp Neurol*. 1998b; 390:194–210. [PubMed: 9453664]
- Gardos G, Dimascio A, Salzman C, Shader RI. Differential actions of chlordiazepoxide and oxazepam on hostility. *Arch Gen Psychiat*. 1968; 18:757–760. [PubMed: 4870680]
- Gielen MC, Lumb MJ, Smart TG. Benzodiazepines Modulate GABA(A) receptors by regulating the preactivation step after GABA binding. *J Neurosci*. 2012; 32:5707–5715. [PubMed: 22539833]
- Gourley SL, DeBold JF, Yin WY, Cook J, Miczek KA. Benzodiazepines and heightened aggressive behavior in rats: reduction by GABA(A)/alpha(1) receptor antagonists. *Psychopharmacology*. 2005; 178:232–240. [PubMed: 15316711]
- Halasz J, Liposits Z, Meelis W, Kruk MR, Haller J. Hypothalamic attack area-mediated activation of the forebrain in aggression. *Neuroreport*. 2002; 13:1267–1270. [PubMed: 12151784]
- Haller J, Abraham I, Zelena D, Juhasz G, Makara GB, Kruk MR. Aggressive experience affects the sensitivity of neurons towards pharmacological treatment in the hypothalamic attack area. *Behav Pharmacol*. 1998; 9:469–475. [PubMed: 9832932]
- Heise GA, Boff E. Taming action of chlordiazepoxide. *Fed Proc*. 1961; 20:393. [PubMed: 13749541]
- Heuschele W. Chlordiazepoxide for calming zoo animals. *J Am Vet Med Assoc*. 1961; 139:996–998. [PubMed: 13907058]
- Kemppainen S, Pitkanen A. Distribution of parvalbumin, calretinin, and calbindin-D-28k immunoreactivity in the rat amygdaloid complex and colocalization with gamma-aminobutyric acid. *J Comp Neurol*. 2000; 426:441–467. [PubMed: 10992249]
- Koester C, Rudolph U, Haengi T, Papilloud A, Fritschy JM, Crestani F. Dissecting the role of diazepam-sensitive gamma-aminobutyric acid type A receptors in defensive behavioral reactivity to mild threat. *Pharmacol Biochem Behav*. 2013; 103:541–549. [PubMed: 23067879]

- Lappalainen J, Krupitsky E, Remizov M, Pchelina S, Taraskina A, Zvartau E, Somberg LK, Covault J, Kranzler HR, Krystal JH, Gelernter J. Association between alcoholism and gamma-amino butyric acid alpha 2 receptor subtype in a Russian population. *Alcohol Clin Exp Res*. 2005; 29:493–498. [PubMed: 15834213]
- Lewis DA, Curley AA, Glausier JR, Volk DW. Cortical parvalbumin interneurons and cognitive dysfunction in schizophrenia. *Trends Neurosci*. 2012; 35:57–67. [PubMed: 22154068]
- Li DW, Sulovari A, Cheng C, Zhao HY, Kranzler HR, Gelernter J. Association of gamma-aminobutyric acid a receptor alpha 2 gene (GABRA2) with alcohol use disorder. *Neuropsychopharmacol*. 2014; 39:907–918.
- Loup F, Weinmann O, Yonekawa Y, Aguzzi A, Wieser HG, Fritschy JM. A highly sensitive immunofluorescence procedure for analyzing the subcellular distribution of GABA(A) receptor subunits in the human brain. *J Histochem Cytochem*. 1998; 46:1129–1139. [PubMed: 9742069]
- Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U. Molecular and neuronal substrate for the selective attenuation of anxiety. *Science*. 2000; 290:131–134. [PubMed: 11021797]
- Macdonald RL, Olsen RW. GABA(A) receptor channels. *Annu Rev Neurosci*. 1994; 17:569–602. [PubMed: 7516126]
- McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR, Whiting PJ. Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha(1) subtype. *Nat Neurosci*. 2000; 3:587–592. [PubMed: 10816315]
- Miczek KA. Intraspecies aggression in rats - effects of d-amphetamine and chlordiazepoxide. *Psychopharmacologia*. 1974; 39:275–301. [PubMed: 4476082]
- Miczek KA, Odonnell JM. Alcohol and chlordiazepoxide increase suppressed aggression in mice. *Psychopharmacology*. 1980; 69:39–44. [PubMed: 6771824]
- Morris HV, Dawson GR, Reynolds DS, Atack JR, Stephens DN. Both alpha 2 and alpha 3 GABA(A) receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. *Eur J Neurosci*. 2006; 23:2495–2504. [PubMed: 16706856]
- Nadler JJ, Moy SS, Dold G, Trang D, Simmons N, Perez A, Young NB, Barbaro RP, Piven J, Magnuson TR, Crawley JN. Automated apparatus for quantitation of social approach behaviors in mice. *Genes Brain and Behav*. 2004; 3:303–314.
- Nusser Z, Sieghart W, Benke D, Fritschy JM, Somogyi P. Differential synaptic localization of two major gamma-aminobutyric acid type A receptor alpha subunits on hippocampal pyramidal cells. *Proc Natl Acad Sci USA*. 1996; 93:11939–11944. [PubMed: 8876241]
- Ralvenius WT, Benke D, Acuna MA, Rudolph U, Zeilhofer HU. Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABAA receptor subtype. *Nat Commun*. 2015 doi:10.1038/ncomms7803.
- Reynolds LM, Engin E, Tantillo G, Lau HM, Muschamp JW, Carlezon WA, Rudolph U. Differential roles of GABA(A) receptor subtypes in benzodiazepine-induced enhancement of brain-stimulation reward. *Neuropsychopharmacol*. 2012; 37:2531–2540.
- Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature*. 1999; 401:796–800. [PubMed: 10548105]
- Rudolph U, Knoflach F. Beyond classical benzodiazepines: novel therapeutic potential of GABA(A) receptor subtypes. *Nat Rev Drug Discov*. 2011; 10:685–697. [PubMed: 21799515]
- Smith KS, Engin E, Meloni EG, Rudolph U. Benzodiazepine-induced anxiolysis and reduction of conditioned fear are mediated by distinct GABA(A) receptor subtypes in mice. *Neuropharmacol*. 2012; 63:250–258.
- Smith KS, Meloni EG, Myers KM, Van't Veer A, Carlezon WA, Rudolph U. Reduction of fear-potentiated startle by benzodiazepines in C57BL/6J mice. *Psychopharmacology*. 2011; 213:697–706. [PubMed: 20922362]

- Soyka M, Preuss UW, Hesselbrock V, Zill P, Koller G, Bondy B. GABA-A2 receptor subunit gene (GABRA2) polymorphisms and risk for alcohol dependence. *J Psychiat Res*. 2008; 42:184–191. [PubMed: 17207817]
- Toth M, Fuzesi T, Halasz J, Tulogdi A, Haller J. Neural inputs of the hypothalamic “aggression area” in the rat. *Behav Brain Res*. 2010; 215:7–20. [PubMed: 20685366]
- Uhart M, Weerts EM, McCaul ME, Guo XQ, Yan XF, Kranzler HR, Li N, Wand GS. GABRA2 markers moderate the subjective effects of alcohol. *Addiction Biology*. 2013; 18:357–369. [PubMed: 22501025]
- Wallace PS, Taylor SP. Reduction of appeasement-related affect as a concomitant of diazepam-induced aggression: evidence for a link between aggression and the expression of self-conscious emotions. *Aggress Behav*. 2009; 35:203–212. [PubMed: 19090555]
- Weerts EM, Miczek KA. Primate vocalizations during social separation and aggression: Effects of alcohol and benzodiazepines. *Psychopharmacology*. 1996; 127:255–264. [PubMed: 8912404]
- Weisman AM, Berman ME, Taylor SP. Effects of clorazepate, diazepam, and oxazepam on a laboratory measurement of aggression in men. *Int Clin Psychopharmacol*. 1998; 13:183–188. [PubMed: 9727729]
- Wieland HA, Luddens H, Seeburg PH. A single histidine in GABA-A receptors is essential for benzodiazepine agonist binding. *J Biol Chem*. 1992; 267:1426–1429. [PubMed: 1346133]

Fig. 1.

Fewer attack bites directed at the intruder male in the unfamiliar environment as compared to in the resident's home cage. Data are presented as mean $\pm$ SEM. $^{\wedge} p < 0.05$ compared to home cage.

Fig. 2.

Attack bite frequencies and time spent in the social approach zone following treatment with midazolam (0-1.7 mg/kg, i.p.) in wild-type (WT) mice compared to α1(H101R) (A, B), α2(H101R) (C, D), or α3(H126R) (E, F) point-mutated mice. All genotypes were assessed concurrently. Data are presented as mean ± SEM. * p <0.05, ** p <0.01 compared to vehicle, # p <0.05, ## p <0.01 compared to WT.

Percent Fear-potentiated Startle

Fig. 3.

Increased percent fear-potentiated startle (FPS) after fear-conditioning. Systemic treatment with midazolam (1.0 mg/kg) reduced percent FPS. Data are presented as mean $\pm$ SEM.

Table 1

Effects of midazolam and genotype on aggressive and non-aggressive behaviors

Sideways threat frequency				
Midazolam (mg/kg)	Wild type	$\alpha 1(H101R)$	$\alpha 2(H101R)$	$\alpha 3(H126R)$
veh	57.4 $\pm$ 9.1	37.8 $\pm$ 5.8	72.9 $\pm$ 11.5	41.3 $\pm$ 6.4
0.1	70.9 $\pm$ 9.3	35.8 $\pm$ 6.3 [#]	68.9 $\pm$ 14.5	48.8 $\pm$ 6.0
0.3	76.8 $\pm$ 14.7	33.9 $\pm$ 5.0 ^{##}	62.1 $\pm$ 12.9	61.0 $\pm$ 5.3
0.56	75.3 $\pm$ 14.7	31.8 $\pm$ 5.1 ^{##}	61.3 $\pm$ 20.2	57.1 $\pm$ 9.7
1.0	63.1 $\pm$ 9.0	27.9 $\pm$ 5.5 ^{##}	15.0 $\pm$ 8.3 ^{***}	24.4 $\pm$ 8.9 ^{##}
1.7	40.7 $\pm$ 12.7	28.0 $\pm$ 5.3	11.5 $\pm$ 6.1 ^{**}	14.7 $\pm$ 9.2
Walking (s)				
veh	193.7 $\pm$ 6.7	211.1 $\pm$ 6.7	185.0 $\pm$ 11.1	200.8 $\pm$ 9.5
0.1	200.2 $\pm$ 9.2	212.1 $\pm$ 8.0	187.5 $\pm$ 9.3	204.5 $\pm$ 10.1
0.3	184.7 $\pm$ 8.6	199.7 $\pm$ 6.3	169.3 $\pm$ 7.2	194.3 $\pm$ 12.1
0.56	195.7 $\pm$ 9.5	198.9 $\pm$ 7.5	143.5 $\pm$ 19.7 ^{*#}	186.2 $\pm$ 14.2
1.0	189.4 $\pm$ 6.7	195.0 $\pm$ 7.3	44.7 $\pm$ 22.5 ^{***}	152.1 $\pm$ 22.4 [*]
1.7	135.7 $\pm$ 23.4 ^{**}	194.8 $\pm$ 16.8 ^{##}	47.7 $\pm$ 17.7 ^{***}	110.9 $\pm$ 19.5 ^{**}
Tail rattle frequency				
veh	15.4 $\pm$ 6.4	13.2 $\pm$ 2.9	6.6 $\pm$ 1.8	13.9 $\pm$ 5.2
0.1	12.7 $\pm$ 6.5	13.2 $\pm$ 2.8	5.6 $\pm$ 2.4	12.7 $\pm$ 3.4
0.3	15.0 $\pm$ 7.3	8.7 $\pm$ 1.7	8.9 $\pm$ 2.7	12.5 $\pm$ 2.7
0.56	18.5 $\pm$ 10.2	12.8 $\pm$ 2.7	3.8 $\pm$ 1.4	19.1 $\pm$ 6.6
1.0	11.8 $\pm$ 5.9	5.2 $\pm$ 0.9	1.1 $\pm$ 1.1	5.4 $\pm$ 2.0
1.7	3.8 $\pm$ 3.2	5.9 $\pm$ 1.0	0.4 $\pm$ 0.3	1.3 $\pm$ 1.1
Allogrooming (s)				
veh	1.3 $\pm$ 1.2	5.6 $\pm$ 2.5	0.4 $\pm$ 0.3	1.4 $\pm$ 0.9
0.1	0.5 $\pm$ 0.4	3.6 $\pm$ 1.4	4.0 $\pm$ 3.7	1.0 $\pm$ 0.4
0.3	0.8 $\pm$ 0.8	10.7 $\pm$ 3.5	2.2 $\pm$ 1.9	7.1 $\pm$ 4.2
0.56	5.5 $\pm$ 4.4	14.9 $\pm$ 5.5	0.8 $\pm$ 0.5	10.1 $\pm$ 9.3
1.0	10.5 $\pm$ 7.8	17.5 $\pm$ 4.8	16.8 $\pm$ 9.4	36.3 $\pm$ 17.4
1.7	29.2 $\pm$ 17.5	22.5 $\pm$ 5.9	20.6 $\pm$ 12.0	37.9 $\pm$ 10.8
Grooming (s)				
veh	9.7 $\pm$ 2.8	7.1 $\pm$ 2.3	12.1 $\pm$ 4.1	5.3 $\pm$ 2.1
0.1	7.5 $\pm$ 1.7	8.2 $\pm$ 2.1	9.0 $\pm$ 2.7	7.0 $\pm$ 2.1
0.3	15.5 $\pm$ 5.2	10.6 $\pm$ 2.3	8.9 $\pm$ 2.6	10.1 $\pm$ 3.7
0.56	14.2 $\pm$ 4.7	10.6 $\pm$ 3.4	10.2 $\pm$ 4.2	9.5 $\pm$ 4.6
1.0	7.7 $\pm$ 2.3	7.8 $\pm$ 2.5	3.4 $\pm$ 2.9	7.3 $\pm$ 2.6
1.7	8.7 $\pm$ 3.1	9.6 $\pm$ 2.5	2.8 $\pm$ 1.8	7.3 $\pm$ 2.3

Mean $\pm$ SEM

*
p<0.05

**
p<0.01 compared to vehicle

p<0.05

p<0.01 compared to wild type

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Table 2

Effect of midazolam and genotype on motor activity during social approach

Midazolam (mg/kg)	Wild type	Walking (cm)		
		$\alpha 1$ (H101R)	$\alpha 2$ (H101R)	$\alpha 3$ (H126R)
veh	3743.5 $\pm$ 174.4	3531.6 $\pm$ 163.1	3022.9 $\pm$ 189.7	3009.9 $\pm$ 139.0
0.1	3651.9 $\pm$ 217.0	3614.6 $\pm$ 312.1	3006.5 $\pm$ 226.0	2512.7 $\pm$ 161.7 ^{##}
0.3	3769.2 $\pm$ 264.3	3420.4 $\pm$ 260.4	2944.7 $\pm$ 274.9 [#]	2453.2 $\pm$ 145.2 ^{##}
0.56	3763.7 $\pm$ 206.7	3591.0 $\pm$ 207.8	2992.4 $\pm$ 243.5 [#]	2504.2 $\pm$ 198.5 ^{##}
1.0	3320.1 $\pm$ 242.3	2748.8 $\pm$ 351.3	1120.5 $\pm$ 223.4 ^{***}	1460.4 $\pm$ 150.7 ^{***}
1.7	1833.8 $\pm$ 180.8 ^{**}	1286.3 $\pm$ 315.9 ^{**}	-----	-----

Mean $\pm$ SEM^{**} p<0.01 compared to vehicle[#] p<0.05^{##} p<0.01 compared to wild type