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Neural Basis of Benzodiazepine Reward: Requirement for α2 Containing $GABA_A$ Receptors in the Nucleus Accumbens

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Despite long-standing concerns regarding the abuse liability of benzodiazepines, the mechanisms underlying properties of benzodiazepines that may be relevant to abuse are still poorly understood. Earlier studies showed that compounds selective for α1-containing $GABA_A$ receptors (α1$GABA_A$Rs) are abused by humans and self-administered by animals, and that these receptors may underlie a preference for benzodiazepines as well as neuroplastic changes observed in the ventral tegmental area following benzodiazepine administration. There is some evidence, however, that even L-838, 417, a compound with antagonistic properties at α1$GABA_A$Rs and agonistic properties at the other three benzodiazepine-sensitive $GABA_A$ receptor subtypes, is self-administered, and that the α2$GABA_A$Rs may have a role in benzodiazepine-induced reward enhancement. Using a two-bottle choice drinking paradigm to evaluate midazolam preference and an intracranial self-stimulation (ICSS) paradigm to evaluate the impact of midazolam on reward enhancement, we demonstrated that mice carrying a histidine-to-arginine point mutation in the α2 subunit which renders it insensitive to benzodiazepines (α2(H101R) mice) did not prefer midazolam and did not show midazolam-induced reward enhancement in ICSS, in contrast to wild-type controls, suggesting that α2$GABA_A$Rs are necessary for the reward enhancing effects and preference for oral benzodiazepines. Through a viral-mediated knockdown of α2$GABA_A$Rs in the nucleus accumbens (NAc), we demonstrated that α2 in the NAc is necessary for the preference for midazolam. Findings imply that α2$GABA_A$Rs in the NAc are involved in at least some reward-related properties of benzodiazepines, which might partially underlie repeated drug-taking behavior.
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INTRODUCTION

Benzodiazepines have been prescribed as anxiolytics and sleep aids for over five decades and are still listed among the most commonly prescribed drugs in the United States (Salzman, 1998; Tan *et al*, 2011). Benzodiazepines remain the second most-commonly abused prescription drugs following opioid pain relievers (http://www.samhsa.gov/data/DAWN.aspx) and abuse by patients and polydrug users remains a significant health concern, particularly as vulnerable individuals may develop an addiction to benzodiazepines.

Benzodiazepine abuse follows a few different patterns, based on the reason for use and the characteristics of the users. One common type of user is patients who are initially prescribed benzodiazepines for legitimate use for a temporary period, but who later become abusers by extending the use period and increasing the dosage, and reaching and surpassing cumulative drug doses that are defined as 'addiction' (O'Brien, 2005; Salzman, 1998; Griffiths and Weerts, 1997; Busto *et al*, 1986). For many of these users, the basic reason for abuse is physical dependence, as defined by the withdrawal symptoms following cessation. However, moderate increases in doses over time reported by these users suggest that there is an additional drug effect component that is separate from simply avoiding withdrawal symptoms (Busto *et al*, 1986; McCabe, 2007). As tolerance develops to this effect over time, the dose needs to be increased proportionally to achieve the same subjective effect.

The second group of abusers is polydrug users. This group often uses benzodiazepines to fight off the unpleasant effects of other drugs, such as irritability and anxiety, or to amplify the 'high' from other drugs such as opioids. There are, nevertheless, reports of benzodiazepines being used for the sake of their own 'high' without being combined with other drugs, and some polydrug users define benzodiazepines as their primary drug of abuse (Griffiths and Weertz, 1997; Busto *et al*, 1986). Thus, there are several reasons for

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benzodiazepine abuse, such as physical dependence, management of the adverse effects of other drugs, or the 'high' or other positive subjective effects of the benzodiazepine itself. Although the relationship between the pleasurable effects of drugs and the development of addiction is not clear (de Wit and Phillips, 2012), these positive subjective effects may comprise a form of positive reinforcement (ie, increase the likelihood of repeated use).

All known clinical actions of benzodiazepines are mediated by positive allosteric modulation of GABA$_A$ receptors, specifically of GABA$_A$ receptors containing the α1, α2, α3, or α5 subunits. Work with gene-targeted mice, in particular knock-in mice in which the benzodiazepine site of the respective GABA$_A$ receptors was rendered insensitive to classical benzodiazepines by a histidine to arginine point mutation at a conserved residue (α1(H101R), α2(H101R), α3(H126R), and α5(H105R)), and studies using subtype-selective compounds have allowed the mapping of defined benzodiazepine actions to specific GABA$_A$ receptor subtypes, as defined by their α subunits (eg, Rudolph *et al*, 1999; McKernan *et al*, 2000; Low *et al*, 2000). The mechanisms by which benzodiazepines exert the positively reinforcing effects that induce repeated drug-taking or maintain long-term drug-taking, however, remain poorly understood.

A few lines of evidence point to the α1-containing GABA$_A$ receptors (α1GABA$_A$Rs) as the likely substrate for the abuse-related effects of benzodiazepines. First, there is evidence of abuse of the α1-preferring compound zolpidem by polydrug users (eg, Hajak *et al*, 2003; Evans *et al*, 1990) and second, some evidence that this drug might also have at least mild positive subjective effects in drug-naïve subjects (Licata *et al*, 2011). This drug has also been shown to maintain self-administration in primates (eg, Rowlett and Lelas, 2007; Ator, 2002; Griffiths *et al*, 1992, Rowlett *et al*, 2005; see also Ator *et al*, 2010). In mice, the positive modulation of α1GABA$_A$Rs was found to be necessary for midazolam preference in a two-bottle choice drinking paradigm, which has also been called 'oral midazolam self-administration' (Tan *et al*, 2010). Taken together, these findings suggest that the activation of α1GABA$_A$Rs may be sufficient to produce the positively reinforcing properties of benzodiazepines that may lead to repeated drug-taking. Moreover, two electrophysiological studies (Heikkinen *et al*, 2009; Tan *et al*, 2010) have found that benzodiazepines cause long-term adaptations in the reward circuits similar to those caused by other drugs of abuse via the α1GABA$_A$Rs on the GABAergic interneurons of the ventral tegmental area (VTA). Thus, α1GABA$_A$Rs may also be involved in the long-term plastic changes induced by drugs of abuse, in addition to the positive reinforcement that leads to self-administration.

It should be noted though that although the α1GABA$_A$Rs appear to mediate some properties of benzodiazepines that may be involved in abuse-related processes, there are a few lines of evidence that suggest that other GABA$_A$R subtypes may also be involved. First, although tranquilizers with binding preference for α1GABA$_A$Rs are abused, both the estimated relative abuse liability and the reports of actual abuse were found to be higher for the nonselective benzodiazepine diazepam compared with zolpidem (Griffiths and Johnson, 2005). Nonselective benzodiazepines such as diazepam and midazolam are also self-administered in primates (eg, Griffiths *et al*, 1981, 1991), although at a lower rate than zolpidem (eg, Griffiths *et al*, 1992; Rowlett *et al*, 2005). However, L-838, 417, an antagonist at α1GABA$_A$Rs with agonistic properties at α2GABA$_A$Rs, α3GABA$_A$Rs and α5GABA$_A$Rs, was also self-administered (Rowlett *et al*, 2005). Thus, a drug can maintain self-administration even if it is an antagonist at α1GABA$_A$Rs. In addition, it has been reported that benzodiazepines lead to reward enhancement in intracranial self-stimulation (ICSS) studies in rodents (Olds, 1970; Straub *et al*, 2010). We have recently demonstrated that reward enhancement by diazepam was completely abolished and even led to aversive-like effects at doses that do not impair responding (as measured by maximal response rates) in α2(H101R) mice (Reynolds *et al*, 2012). Taken together, these findings suggest that although α1GABA$_A$Rs are involved in the positively reinforcing effects of benzodiazepines, other subtypes, especially α2GABA$_A$Rs, may also have a role.

On the basis of the interesting finding that the α2GABA$_A$Rs are essential for the reward-enhancing actions of benzodiazepines, and other evidence pointing to the possibility of the involvement of non-α1 subunits in drug reinforcement, our goals in this study were two-fold. First we aimed to investigate whether the α2GABA$_A$Rs were required for the reward-enhancing actions of and preference for benzodiazepines. To this end, we tested α2(H101R) mice, employing wild-type, α1(H101R), and α3(H126R) mice as controls, in a two-bottle choice midazolam drinking paradigm. In this paradigm, mice consumed 0.8–1.1 mg/kg/day midazolam, which represents a pharmacologically relevant concentration (Tan *et al*, 2010). We also tested the reward-enhancing effects of midazolam in the ICSS paradigm in a different group of wild-type, α1(H101R), α2(H101R), and α3(H126R) mice. These studies revealed that both α1GABA$_A$Rs and α2GABA$_A$Rs are required for the reward-enhancing actions of the benzodiazepine midazolam.

Our second goal was to identify the anatomical location of the α2GABA$_A$Rs involvement in the preference for this benzodiazepine. Although the α1GABA$_A$Rs are abundant in the VTA and ventral pallidum, α2GABA$_A$Rs are expressed very sparsely in those structures. Instead, the α2GABA$_A$Rs are expressed very densely in another component of the brain reward circuitry, the medium spiny neurons (MSNs) of the nucleus accumbens (NAc). To test the hypothesis that the α2GABA$_A$Rs in the NAc may be important for the preference for midazolam, we specifically knocked down the α2 subunit in the NAc using cre-*lox*P-mediated recombination, and found that this manipulation indeed resulted in the abolishment of the preference for midazolam in the two-bottle choice task without affecting behavior in tests for anxiolytic-like action (elevated plus maze) and behavioral despair (forced swim test and tail suspension test).

MATERIALS AND METHODS

All experiments and procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee following guidelines in the NIH Guide for the Care and Use of Laboratory Animals. All mice were bred in the C57Bl/6J background (Source: Jackson Laboratory,

Bar Harbor, ME) and housed individually. All experimental mice were bred in the same animal room at McLean Hospital. Food and water were available *ad libitum*.

Experiments with H-R Point Mutant Mice

Subjects. A total of 15 wild-type, 16 α1(H101R), 16 α2(H101R), and 16 α3(H126R) male mice were used for the midazolam drinking, and 7 wild-type, 8 α1(H101R), 7 α2(H101R), and 8 α3(H126R) male mice were used for the ICSS experiment. Point-mutant mice were bred as homozygous pairs. The mutations (for generation, see Rudolph *et al*, 1999; Low *et al*, 2000) were backcrossed for 27 [α1(H101R)], 16 [α2(H101R)], and 20 [α3(H126R)] generations on the C57BL/6 J background. Midazolam drinking, ICSS, and open field (please see Supplement) tests were conducted on separate cohorts of animals aged 17–25 weeks for ICSS, 8–12 weeks for the other tests.

Drugs. Midazolam (Bedford Laboratories, Bedford, OH) was mixed in a 4% sucrose solution (0.004 mg/ml) for the two-bottle choice drinking experiment. For ICSS, it was diluted in 0.9% saline at concentrations of 0.1 mg/ml and 0.2 mg/ml and was administered intraperitoneally at a volume of 10 ml/kg.

Midazolam drinking. Mice were initially habituated to two bottles both containing water for 2 days, and then both bottles containing 4% sucrose for 2 days. Starting from Day 5, the two-bottle choice procedure was started with one bottle containing 4% sucrose and the other containing 4% sucrose with midazolam (0.004 mg/ml). The animals were given continuous access to midazolam for 6 days. Sides were switched daily such that the midazolam-containing bottle was on a different side every day. Consumption from each bottle was measured and the liquids were topped off every 24 h. Two bottles were kept in the same configuration in a separate cage to measure liquid loss due to dripping and this volume was subtracted from the recorded consumption from each bottle. Relative midazolam consumption was calculated as (midazolam solution consumption)/(sucrose only solution consumption).

Intracranial self-stimulation. All procedures were described in detail previously (Carlezon and Chartoff, 2007; Reynolds, 2012). Monopolar electrodes were implanted in the right medial forebrain bundle at the level of the lateral hypothalamus. Mice were trained initially on a constant frequency and then on a rate-frequency schedule. Drug testing days involved initial testing to establish a baseline, and then post-injection testing which was compared with this baseline. Please see Supplementary Methods for details of the training and testing procedures.

Experiments with NAc Knockdown Mice

Subjects. A total of 31 *Gabra2$^{f/f}$* and 27 wild-type male mice aged 8–12 weeks at the time of surgery were used for the NAc α2 knockdown experiments. For the generation of the Gabra2 floxed allele in C57BL/6 N ES cells see, Witschi *et al* (2011) (Bred for 13 generations on C57BL/6 J).

Drugs. Midazolam was prepared as described above. Ethanol (200 proof; Pharmaco-Aaper, Brookfield, CT) was mixed in distilled water in a concentration of 6% (v/v). Cocaine (Sigma, St Louis, MO) was dissolved in 0.9% saline (1 mg/ml, 2 mg/ml) and was administered at a volume of 10 ml/kg.

Virus injection surgery. Recombinant adeno-associated virus (rAAV) expressing improved-Cre (iCre) and an enchanced-YFP variant (Venus) under the control of a single neuron-specific synapsin promoter (Tang *et al*, 2009) was used for knockdown surgeries. Heterologous protein expression from a single open reading frame was achieved with the use of viral 2A peptide bridge separating the two protein-coding sequences. rAAVs serotype 1 and 2 were generated as described (Tang *et al*, 2009), and purified by AVB Sepharose affinity chromatography (Smith *et al*, 2009, GE Healthcare). For each virus, the infectious titer was determined by rat primary neuron cultures (about 1.0×10^8 infectious virus particle/ml).

rAAV-iCre was microinjected bilaterally (0.3 μl/side) to NAc in wild-type and α2floxed (Gabra2$^{f/f}$) animals aged 8–10 weeks. Animals were anesthetized with a ketamine/xylazine cocktail and the infusion was made through 30 G cannulae at +1.7 mm AP, +/− 2.3 mm ML, −4.5 mm DV from bregma at a 20° lateral cannula angle. Experiments started 3 weeks after the injection of the AAV vector.

Immunohistochemistry. Animals ($n = 6$ Wt + *rAAV-Syn-iCre-2AVenus* and $n = 6$ *Gabra2$^{f/f}$* + *rAAV-Syn-iCre-2AVenus*) were transcardially perfused with a periodate–lysine–paraformaldehyde solution 3 weeks after surgery. Forty micrometer thick sections were stained free floating with anti-GFP (chicken – Chemicon), and anti-GABA$_A$ α2 (rabbit – Synaptic Systems); or with anti-GABA$_A$ α2 alone for diaminobenzidine (DAB) staining. Images were taken in the NAc using a ×60 objective on a Nikon confocal microscope. For fluorescent imaging, masks were drawn based on Venus filling of cells, and the intensity, number and size of GABA$_A$ α2-positive puncta were quantified using Metamorph. For DAB, GABA$_A$ α2 staining integrated intensity was quantified from bright field images across regions of the NAc using imageJ.

Behavioral tests. Subjects were divided into two cohorts: Cohort 1 and Cohort 2. The behavioral tests were conducted in the following order with at least a one-week break between each test: Cohort 1: Cocaine locomotor sensitization, midazolam self-administration test, the tail suspension test; Cohort 2: the elevated plus maze test, FST and the ethanol preference test. All testing was conducted during the light phase of the light/dark cycle.

Elevated plus maze. Behavior in the elevated plus maze was measured at 30 lux light conditions using the EthoVision XT (Noldus Information Technology, The Netherlands) tracking system. Animals were placed in the center zone of the maze facing one of the open arms for a total testing period of 5 min (see Smith *et al*, 2012 for details).

Forced swim test. A clear Plexiglas cylinder (diameter: 20 cm) was filled with water (23–25 °C). The mice were placed in the water for a 6 min test session carried out under ~100 lux room-lighting conditions. Movement was video recorded and latency to immobility and total time immobile was scored manually (see Vollenweider *et al*, 2011 for details).

Tail suspension test. Mice were suspended by the tail from a table edge (70 cm high) for a test session of 6 min. Movement was video recorded and latency to immobility and total time immobile was scored manually (see Vollenweider *et al*, 2011 for details).

Please see Supplementary Information for further Methods.

RESULTS

ICSS in Point-Mutant Mice

The results of the ICSS experiment are depicted in Figure 1a–c, as well as in Supplementary Figures 1 and 2. The different genotypes did not differ in their pre-drug baseline threshold values (Supplementary Figure 1). As seen in Figure 1a, the administration of midazolam caused a leftward shift in the frequency-response functions for wild-type and α3(H126) mice, indicative of a reward-enhancing effect, while such a shift was not apparent in α1(H101R) and α2(H101R) mice (see Supplementary Figure 2 for the depiction of threshold values for baseline and post-drug passes). A two-way ANOVA employing genotype and midazolam dose as factors revealed a significant main effect of genotype ($F(3, 86) = 16.10$; $P < 0.01$), a significant main effect of midazolam dose ($F(2, 86) = 15.92$, $P < 0.01$) and a significant genotype × midazolam dose interaction ($F(6, 86) = 3.80$, $P < 0.01$) on reward threshold in ICSS (Figure 1b). Post hoc Dunnett's test using the vehicle group in each genotype as the comparison group revealed that both doses of midazolam caused a significant decrease in reward thresholds in wild-type and α3(H126) mice ($P < 0.01$ for each comparison), while there was no effect of midazolam in α1(H101R) and α2(H101R) mice. The analysis of the maximum response data revealed a significant midazolam dose ($F(3, 86) = 7.83$, $P < 0.01$) main effect, where the 1 mg/kg dose of midazolam caused an increase in maximum responding compared with the vehicle control (*post hoc* Dunnett's test, $P < 0.01$; Figure 1c). The lack of reductions in maximum response rates suggests that the doses of midazolam employed do not impair the animals' ability to respond (ie, spin the wheel) in this test. The level of sedation with 1 mg/kg midazolam was also measured in an open field test (see Supplementary Methods and Supplementary Figure 3), where a trend toward sedation was observed in wild-type, α2(H101R), and α3(H126) mice, but a two-way ANOVA revealed no significant main effects or interactions. The abolishment of the reduction in reward thresholds in α1(H101R) and α2(H101R) mice reveals that the positive modulation of both the α1GABA$_A$Rs and α2GABA$_A$Rs is required for the reward-enhancing effects of midazolam in the ICSS paradigm.

Midazolam Drinking in the two-Bottle Choice Task in Point Mutant Mice

The results of the midazolam two-bottle choice experiment are depicted in Figure 2a–d. As seen in Figure 2a, all genotypes showed similar levels of total fluid consumption on the first 4 days of the experiment, and a similar increase in consumption when the drinking liquid was switched from water to sucrose, suggesting comparable baseline liquid consumption and appetitive reaction to sucrose. The average total daily liquid consumption remained in the 14–18ml range for the rest of the duration of the experiment. A mixed design two-way ANOVA with day as the within subjects and genotype as the between subjects factor revealed a main effect of genotype ($F(3,185) = 20.52$, $P < 0.01$) and no significant effect of day (Figure 2b). As the experiment day was not a significant factor, the midazolam consumption ratios were averaged for the duration of the experiment. As seen in Figure 2c, α3(H126R) mice behaved similarly to wild types in this test, while the preference for midazolam was abolished in α1(H101R) and α2(H101R) animals (Post hoc Dunnett's tests comparing α1(H101R) and α2(H101R) mice to wild types: $P < 0.01$ and $P < 0.05$, respectively). The daily consumption per body weight was between 0.81 and 1.14 mg/kg for wild-type and α3(H126R) mice, whereas it remained between 0.48 and 0.70 mg/kg for α1(H101R) and α2(H101R) animals (Figure 2d). A two-way mixed ANOVA revealed a significant effect of day ($F(5,167) = 19.88$, $P < 0.01$), a significant effect of genotype ($F(3,167) = 42.45$, $P < 0.01$), and a significant interaction effect ($F(15,167) = 6.22$, $P < 0.01$). α1(H101R) and α2(H101R) mice consumed significantly less midazolam compared with controls throughout the experiment, while α3(H126R) also consumed less midazolam than controls on Days 5, 7, and 8. This finding indicates that the positive allosteric modulation of both α1GABA$_A$Rs and α2GABA$_A$Rs is necessary for midazolam preference in this paradigm, but the positive modulation of either receptor subtype alone is not sufficient to maintain midazolam preference. Thus, the results from the ICSS paradigm and the oral midazolam self-administration experiments both point to α1GABA$_A$Rs and α2GABA$_A$Rs, but not α3GABA$_A$Rs, being required for at least some of the reward-related actions of midazolam.

NAc-Specific Knockdown of α2GABA$_A$Rs

Approximately equivalent numbers of cells in the NAc of rAAV-Syn-iCre-2AVenus-injected WT (*Gabra2*$^{+/+}$:: NAc_rAAV-Syn-iCre-2AVenus) and floxed α2 (*Gabra2*$^{f/f}$:: NAc_rAAV-Syn-iCre-2AVenus) mice were observed to express GFP. As shown in Figure 3, the expression of the α2GABA$_A$Rs was reduced in the NAc of *Gabra2*$^{f/f}$:: NAc_rAAV-Syn-iCre-2AVenus mice (3C and 3D) compared with *Gabra2*$^{+/+}$:: NAc_rAAV-Syn-iCre-2AVenus mice (3A and 3B). There was a significant decrease in the α2 DAB ($t(10) = 3.51$, $P < 0.01$) and fluorescent ($t(10) = 10.57$, $P < 0.01$) staining intensity in the NAc of *Gabra2*$^{f/f}$:: NAc_rAAV-Syn-iCre-2AVenus compared with controls (Figure 3e and f, respectively). Similarly, the quantification of α2 puncta on the GFP-positive cells showed a significant reduction of both the number ($t(10) = 4.88$, $P < 0.01$) and size ($t(10) = 3.88$, $P < 0.01$) of α2 puncta in the NAc of *Gabra2*$^{f/f}$:: NAc_rAAV-Syn-iCre-2AVenus

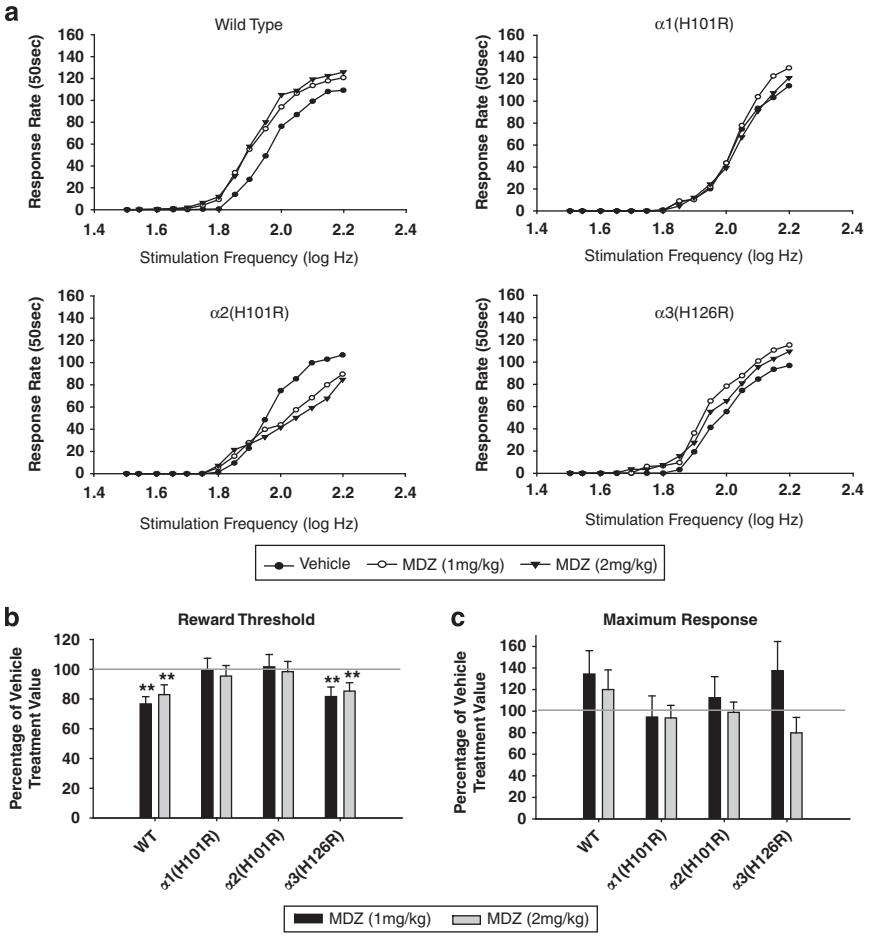

Figure 1 Intracranial self-stimulation in wild-type and α1(H101R), α2(H101R), and α3(H101R) point-mutant mice. (a) Average rate-frequency functions plotted for each genotype show a leftward shift for wild-type and α3(H101R) mice, whereas no such effect was observed for α1(H101R) and α2(H101R) mice. (b) Reward thresholds in ICSS expressed as the mean (±SEM) percentage of vehicle injection threshold values. A reduction in midazolam-injected animals compared with vehicle-injected animals of the same genotype in reward thresholds is indicative of reward enhancement. The symbol ** indicates different from the corresponding vehicle group in a *post hoc* Dunnett's test at $P<0.01$. (c) Maximum response rates in ICSS expressed as mean (±SEM) percentage of vehicle injection maximum response values.

mice compared with controls (Figure 3g and h, respectively). The percentage of GFP-positive cells that expressed α2 above threshold was also significantly reduced in the NAc of the floxed α2 mice ($t(10)=10.87$, $P<0.01$) compared with WT controls (not shown).

Midazolam Drinking in the Two-Bottle Choice Task in NAc α2-Knockdown (*Gabra2$^{f/f}$*:: NAc_rAAV-Syn-iCre-2AVenus) and Control (*Gabra2$^{+/+}$*:: NAc_rAAV-Syn-iCre-2AVenus) Mice

The results of the midazolam two-bottle choice experiment are depicted in Figure 4a–c. As seen in Figure 4a, the control and NAc α2-knockdown mice showed similar levels of water consumption and a comparable increase in liquid consumption upon switching from water to sucrose. A two-way ANOVA with day as the within-subjects factor and genotype as the between-subjects factor revealed a significant main effect of genotype ($F(1, 139)=24.89$, $P<0.01$) and a significant genotype by day interaction ($F(5,139)=2.42$, $P<0.05$) effect on relative midazolam consumption. Further analysis with Fisher LSD *post hoc* tests indicated that the wild-type control mice had higher relative midazolam consumption ratios than NAc α2 knockdown mice on Days 7–10 of testing ($P<0.05$ for Day 9 and $P<0.01$ for the remaining days; Figure 4b). Figure 4c depicts the daily midazolam consumption per weight. In a two-way mixed ANOVA, there was a significant main effect of genotype ($F(1,131)=27.31$, $P<0.01$), but no effect of day. The control animals consumed on average 0.79–1.41 mg/kg/day of midazolam, whereas the NAc α2-knockdown animals consumed significantly less, 0.59–0.86 mg/kg/day. Thus, the binding to positive modulation by midazolam of the α2GABA$_A$Rs in the NAc is required for the preference for midazolam.

Elevated Plus Maze, Forced Swim Test, and Tail-Suspension Test in NAc α2-Knockdown Mice

Mice were tested in the elevated plus maze, a test used to assess anxiolytic- or anxiogenic-like effects of drugs or genetic alterations, and two tests of behavioral despair, the forced swim test and the tail suspension test, to investigate

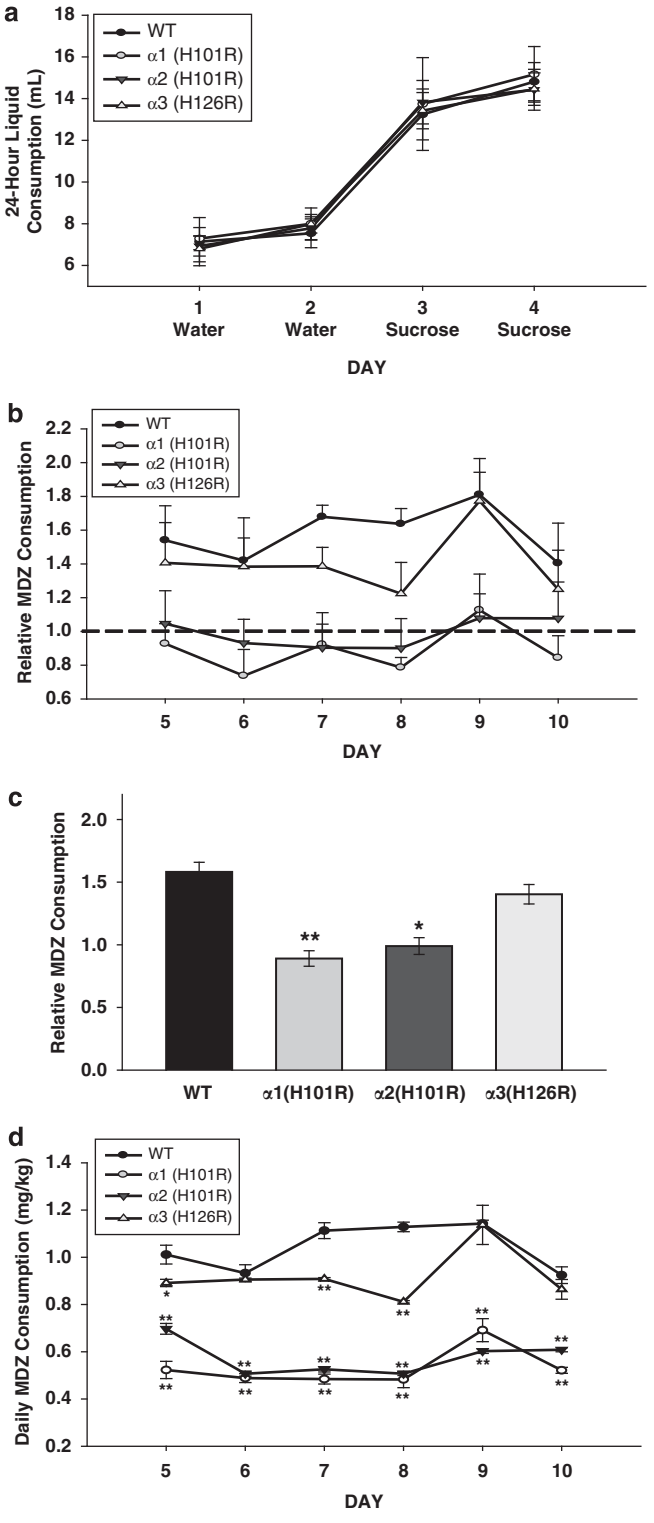

Figure 2 Two-bottle choice midazolam drinking in wild-type and α1(H101R), α2(H101R), and α3(H101R) point-mutant mice. (a) Habituation days for the two bottle choice experiment, where the mice were presented with water in both bottles or 4% sucrose in both bottles. (b) Relative mean (±SEM) midazolam consumption over the 6 test days in the two-bottle choice midazolam drinking paradigm. (c) Relative mean (±SEM) midazolam consumption averaged through the test period in the oral midazolam self-administration paradigm. (d) Daily mean (±SEM) midazolam consumption per kilogram body weight. The symbol * indicates different from wild-type controls in a *post hoc* Dunnett's test at $P<0.05$, ** indicates different from wild-type controls at $P<0.01$.

possible baseline behavioral differences between wild-type control and NAc knockdown mice. There was no difference in percent open arm time ($t(20)=0.52$, $P=0.60$) or percent open arm entries ($t(20)=1.02$, $P=0.31$) in the elevated plus maze test (Figure 5a). There was also no difference in the time-to-first immobility ($t(20)=0.10$, $P=0.92$) and total time spent immobile ($t(20)=0.47$, $P=0.65$) in the forced swim test (Figure 5b) or in the tail suspension test (Figure 5c; $t(20)=0.26$, $P=0.80$ and $t(18)=0.75$, $P=0.47$, respectively). Thus, the baseline behavior of the NAc α2 knockdown mice was indistinguishable from wild-type mice in tests of anxiolytic-like action and behavioral despair.

Ethanol Preference and Locomotor Sensitization to Cocaine in NAc α2-Knockdown Mice

Preference for ethanol, a drug that exerts its effects at least partially through GABA$_A$ receptors, and locomotor sensitization to the dopaminergic drug cocaine were measured in order to investigate whether the NAc α2-knockdown mice had an overall impairment in their general responses to drugs with high abuse liability (see Supplementary Methods). NAc α2-knockdown mice were not different from controls in their preference for ethanol, general ethanol consumption levels, and in terms of locomotor sensitization to cocaine (Supplementary Figure 4A, B and C, respectively).

DISCUSSION

Despite the well-recognized abuse liability of benzodiazepines, investigations of the GABA$_A$ receptor subtypes and specific neuronal mechanisms involved in this abuse potential started only recently. Initial studies, as well as evidence for abuse of α1-preferring compounds, have highlighted the role of the α1GABA$_A$Rs in the self-administration of and the preference for benzodiazepines, as well as in the plastic changes in the VTA following benzodiazepine administration (eg, Hajak *et al*, 2003; Rowlett *et al*, 2005; Heikkinen *et al*, 2009; Tan *et al*, 2010). Here, we present evidence that the α2GABA$_A$Rs may also contribute to some positively reinforcing properties of benzodiazepines, as measured by preference for midazolam in a two-bottle choice paradigm and by reward enhancement in the ICSS. Moreover, we report that the α2GABA$_A$Rs in the NAc mediate midazolam preference.

The ICSS paradigm is based on the operant response of the animals to brain stimulation and can be viewed as the animals' willingness to work to obtain a certain level of stimulation. Although animals will learn operant responses that elicit stimulation in a large number of different brain areas (Zacharko *et al*, 1990), the medial forebrain bundle was selected in the current study because of the relative lack of motor artifacts upon stimulation in this region, as well as for comparability to earlier studies from our laboratory using other benzodiazepines (Straub *et al*, 2010; Reynolds *et al*, 2012). Responding to lower stimulation frequencies after the administration of a drug than those that maintained responding previously is interpreted as 'reward enhancement', and is commonly observed after the administration of drugs of abuse (Wise, 1996).

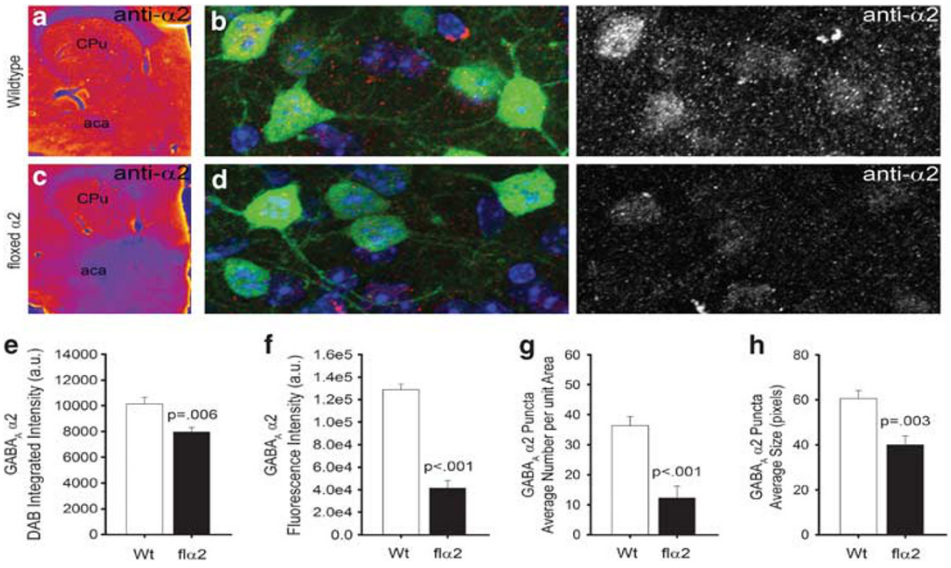

Figure 3 Expression of rAAV-Syn-iCre-2AVenus in the nucleus accumbens of wild-type and *Gabra2$^{f/f}$* mice. (a) DAB staining of GABA$_A$ receptor α2 subunit in a brain section from a representative wild-type mouse injected with rAAV-Syn-iCre-2AVenus. (c) Same as a in a rAAV-Syn-iCre-2AVenus injected *Gabra2$^{f/f}$mouse* A, c. Warm colors (red/yellow) represent areas of high staining intensity, whereas cooler colors (purple/blue) represent areas of lower staining intensity. (b) Left panel: fluorescent staining of Venus (green) and GABA$_A$ α2 (red) in the nucleus accumbens of a representative wild-type mouse injected with rAAV-Syn-iCre-2AVenus, cell nuclei labeled with DAPI (blue); right panel: GABA$_A$ α2 staining only. (d) Same as b in *Gabra2$^{f/f}$* mouse injected with rAAV-Syn-iCre-2AVenus B, d. (e) Quantification of α2 DAB staining intensity (a, c) in the nucleus accumbens of rAAV-Syn-iCre-2AVenus -injected wild-type and floxed α2 mice. (f) Quantification of α2 fluorescence staining intensity (b, d) on Venus-positive cells in the nucleus accumbens of rAAV-Syn-iCre-2AVenus-injected wild-type and floxed α2 mice. (g) Quantification of the number of α2 puncta on GFP-positive cells in the nucleus accumbens of rAAV-Syn-iCre-2AVenus-injected wild-type and *Gabra2$^{f/f}$* mice. (h) Quantification of the size of α2 puncta on Venus-positive cells in the nucleus accumbens of rAAV-Syn-iCre-2AVenus-injected wild-type and *Gabra2$^{f/f}$*mice.

It should be noted, however, that a few other factors other than reward enhancement can possibly affect responding in ICSS. The first one is the animals' ability to perform the required response (in this case, wheel-spinning), which can be affected by sedative, muscle-relaxant, and/or ataxic effects of drugs. Although benzodiazepines are known to have sedative and muscle-relaxant effects, the open-field test (Supplementary Figure 3) data suggest that the level of sedation is low with the 1 mg/kg dose of midazolam, the dose at which we observed the larger effects on reward threshold. In α1(H101R) mice, midazolam led to an increase in general activity levels (consistent with previous observations; McKernan *et al*, 2000; Crestani *et al*, 2000); however, such a general locomotor effect was not observed in α2(H101R) mice. The maximal responding data from the ICSS test suggest that there was no impairment of responding even at the highest dose of midazolam employed in this study in any of the genotypes. Taken together, these findings make it unlikely that the reduction in reward thresholds was confounded by unspecific locomotor effects, especially in α2(H101R) mice.

Second, it has been suggested that even at locations that are considered to be positively reinforcing, ICSS produces some aversive-like effects because of the peripheral excitation of fear-related brain regions (Liebman, 1985). This leads to a conflict-like situation where the animals want to perform the operant behavior to receive the rewarding effect, but feel 'fear' after the stimulation is given. Although this is more of a concern in areas closer to 'fear' regions, such as lateral hypothalamus, the possibility still exists for medial forebrain bundle, especially taking into account the ascending fibers from the amygdala passing through this region. Benzodiazepines were hypothesized to increase ICSS responding by reducing this ambivalence rather than enhancing reward *per se*. Such an explanation would be in line with our finding that the α2GABA$_A$Rs are required for the reward-enhancing actions of benzodiazepines in ICSS, as this subunit has previously been shown to mediate the anxiolytic-like action of benzodiazepines (Low *et al*, 2000; Smith *et al*, 2012; see Engin *et al*, 2012 for a review). However, it should be noted that the use of reward thresholds rather than total response rates as the measure for reward enhancement makes alternative interpretations such as motor effects or conflict effects relatively unlikely, as motor effects and the excitation of structures further away through volume conductance and the activation of nearby fibers are both more likely to occur at higher stimulation frequencies, whereas reward thresholds mostly depend on responding at lower frequencies. In addition, such an 'anti-conflict' interpretation of midazolam effects would mean that α2(H101R) mice, where there is no longer an anxiolytic-like effect of midazolam, would show significantly reduced levels of maximal response rates compared with controls following midazolam administration. Such a reduction is also not evident from our findings. Thus, although it is not possible to completely eliminate this alternative interpretation of 'reduction of aversion', it is unlikely to be the sole source of the reported findings.

In an earlier study, we showed that zolpidem did not cause reward enhancement in ICSS (Reynolds *et al*, 2012), whereas our current findings show that the α1GABA$_A$Rs are required for the reward-enhancing actions of midazolam.

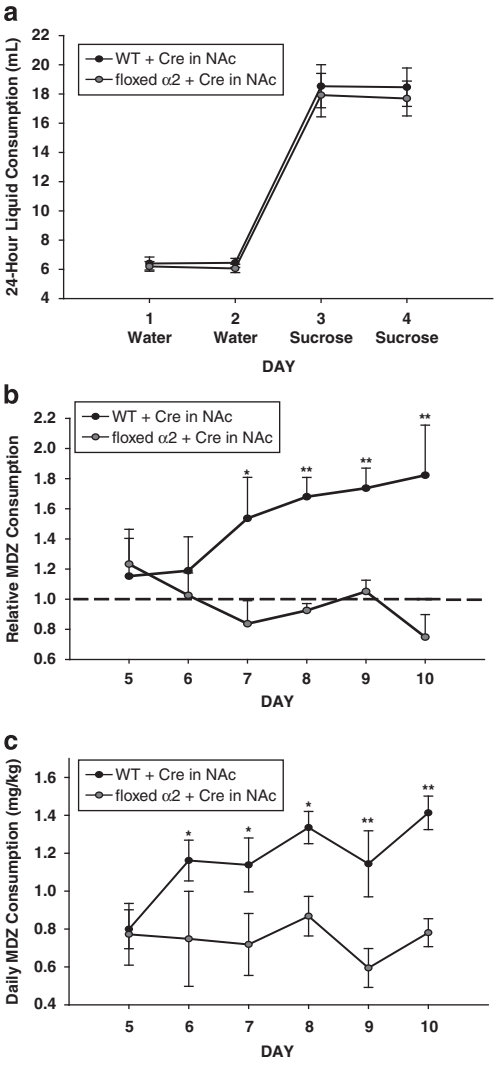

Figure 4 Two-bottle choice midazolam in wild-type and nucleus accumbens α2 knockdown mice. (a) Habituation days for the two-bottle choice experiment, where the mice were presented with water in both bottles or 4% sucrose in both bottles. (b) Relative mean (±SEM) midazolam consumption over the 6 test days in the two-bottle choice midazolam drinking paradigm (c) Daily mean (±SEM) midazolam consumption per kilogram body weight. The symbol * indicates different from wild-type controls at $P<0.05$, ** indicates different from wild-type controls at $P<0.01$.

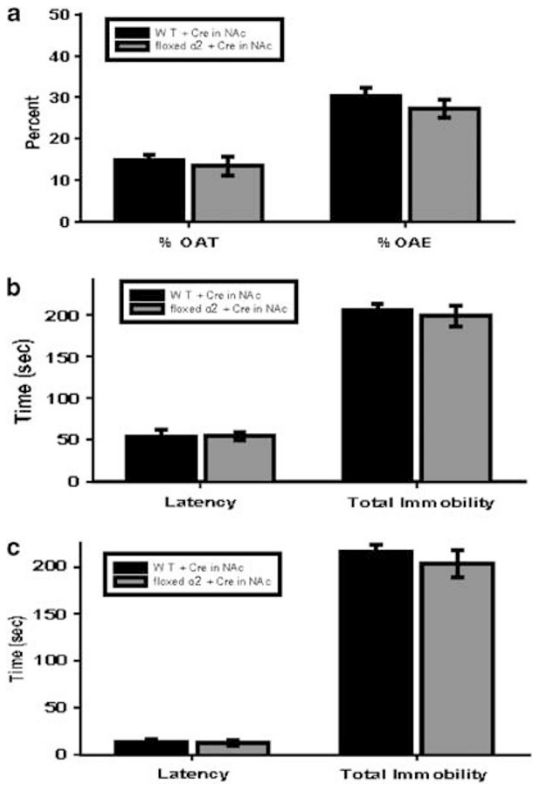

Figure 5 Behavior of wild-type and nucleus accumbens α2 knockdown mice in the elevated plus maze, the forced-swim test, and the tail suspension test. (a) Mean (±SEM) percentage of time spent in the open arms (%OAT) during the test period and mean (±SEM) percentage of total arm entries (open + closed) made into the open arms (%OAE) by wild-type (black) and nucleus accumbens α2 knockdown (gray) mice in the elevated plus maze. (b) Mean (±SEM) latency to first immobility (left) and total time spent immobile (right) by wild-type (black) and nucleus accumbens α2 knockdown (gray) mice in the forced swim test. (c) Mean (±SEM) latency to first immobility (left) and total time spent immobile (right) by wild-type (black) and nucleus accumbens α2 knockdown (gray) mice in the tail suspension test.

This could be interpreted as the α1GABA$_A$Rs being required, but not sufficient for, reward enhancement. However, it should be noted that the reward-enhancing effects of diazepam are reduced but still present in α1(H101R) mice, whereas they are abolished in α2(H101R) animals (Reynolds *et al*, 2012). Thus, the ICSS studies point to a complex picture where both α1 and α2 GABA$_A$Rs are probably involved in reward enhancement, but the role of one receptor subtype may be more dominant than the other depending on the drug in question.

Our experiments using α2 NAc knockdown mice demonstrate that the preference for midazolam depends on the positive modulation of the α2GABA$_A$Rs in NAc, possibly on MSNs. As the midazolam is dissolved in a 4% sucrose solution to fight off the possible effects of its bitterness, one interpretation could be that the animals have different reactions to the palatable taste of the sucrose solution. It should, however, be noted that as seen in Figures 2a and 4a, the sucrose consumption level on Days 3 and 4 of the experiments where the animals were presented with just the sucrose solutions in both bottles, were comparable between groups. Another possibility is that the hyperphagic effects of the benzodiazepine (Cooper, 2005) increases the consumption of the palatable sucrose solution. This is, however, unlikely to be the cause of the preference for midazolam, as the midazolam consumption is compared with another bottle that also contains 4% sucrose, and thus, it would be expected that the hyperphagic effects would lead to increased drinking from both bottles, which would leave the midazolam consumption ratio unaffected. It has been shown using point mutant and global knockout mice that the hyperphagic effects of benzodiazepines do not depend on α1- or α2GABA$_A$Rs (Morris *et al*, 2009). Thus, the abolishment of midazolam preference in the α1(H101R), α2(H101R), and NAc α2 knockdown animals cannot be explained by the simple abolishment of hyperphagic effects.

As previous studies have shown some possible differences in the behavior of α2 global knockout animals compared with controls in tests of behavioral despair (Vollenweider *et al*, 2011), and unconditioned anxiety-like behavior such as light/dark box and free-choice exploration (Koester *et al*, 2013), alcohol preference (Boehm *et al*, 2004), and locomotor sensitization to cocaine (Dixon *et al*, 2010), we wanted to test whether the NAc α2 knockdown resulted in baseline differences in any of these tests (see Supplementary Information for Methods). Although some of these previously reported phenotypic differences are small and sex-specific (eg, changes in alcohol preference were observed only in females and the effects sizes were small; Boehm *et al*, 2004; Dixon *et al*, 2012), or task-specific (eg, locomotor sensitization to cocaine was abolished in knockout mice without any effects in conditioned place preference to cocaine; Dixon *et al*, 2010; 2011), in some cases effects that are masked in global knockout animals due to compensations may be more easily observed in conditional or inducible knockout animals where compensations are smaller or absent. However, our experiments revealed no differences between NAc knockdown and control animals on any of these variables (Figure 5 and Supplementary Figure 4). Thus, the robust difference observed between the control mice and NAc α2 knockdown mice is relatively specific to midazolam preference, rather than being secondary to changes in other behaviors or general differences in drug-induced behaviors.

Although our studies provide evidence for the involvement of the α2GABA$_A$Rs in the NAc in some of the reward-related effects of benzodiazepines, they do not specify an exact mechanism for this involvement. All known drugs of abuse act on VTA dopaminergic neurons and/or NAc, typically leading to increased dopamine levels in NAc (Wise *et al*, 1996; Luscher and Ungless, 2006; see however, Berridge and Robinson (1998) for a critical evaluation of this view). This initial increase in NAc extracellular dopamine levels seems to be critical for the initial rewarding properties of drugs and is also common to natural rewards (Avena *et al*, 2008). Interestingly, so far benzodiazepines have not been shown to increase dopamine levels in the NAc as determined by dialysis, at least after acute administration. On the contrary, a number of studies showed a reduction in the extracellular dopamine concentrations in NAc following the systemic administration of benzodiazepines (Invernizzi *et al*, 1991; Finlay *et al*, 1992; Takada *et al*, 1993), a paradoxical finding considering the modest but well-documented reinforcing actions of benzodiazepines in humans and laboratory animals (see Licata and Rowlett (2008) for a review). This could indicate that benzodiazepines affect the mesolimbic dopamine system differently than other drugs of abuse, for example, by simultaneously modulating multiple sites in this circuit, and/or that benzodiazepines may exert their effects through routes that do not directly involve dopamine signaling. It should also be noted that certain drugs can exert disinhibition and addiction-like plasticity of the VTA dopaminergic neurons without having positive reinforcing effects in behavioral tests (eg, Vashchinkina *et al*, 2012), demonstrating that there is not a one-to-one relationship between the electrophysiological signature of a drug in the mesolimbic dopaminergic circuits and its reinforcing behavioral effects.

In either case, it is likely that the involvement of the α2GABA$_A$Rs in benzodiazepine reward comprises a complex mechanism. It has been shown that the D1- and D2-expressing MSNs have opposite effects on behavioral sensitization to and the development of conditioned place preference to cocaine (Lobo *et al*, 2010). Dense staining in earlier studies (Fritschy and Mohler 1995; Hörtnagl *et al*, 2013; Pirker *et al*, 2000) and our immunohistochemical analysis of the virally-infected cells in wild-type mice suggest that a large population of MSNs in the NAc expresses α2GABA$_A$Rs. D2-positive MSNs project to the VP (Smith *et al*, 2013), which in turn sends GABAergic projections onto the VTA (Kalivas *et al*, 1993), at least partially onto GABAergic interneurons (Kaufling *et al*, 2009). Thus, it is conceivable that the positive modulation of the α2GABA$_A$Rs on D2-positive MSNs ultimately contributes to the disinhibition of the VTA dopaminergic neurons.

α2GABA$_A$Rs are potential drug targets for anxiety disorders, depression, and improvement of cognition in schizophrenia (Engin *et al*, 2012). The finding that α2GABA$_A$Rs may be involved in the reward-enhancing effects and preference for benzodiazepines opens up questions in two directions. The first question is whether the positive modulation of α2GABA$_A$Rs may be responsible for some of the positive reinforcing effects of benzodiazepines responsible for their abuse. This is a complex question, as it is not known exactly how large of a role the rewarding properties of these drugs have in abuse, as opposed to simple physical dependence or reward secondary to anxiety relief. The second question is if the positive modulation of α2GABA$_A$Rs indeed creates a reward state, whether this can be utilized in a therapeutic setting, for example, in the alleviation of anhedonia symptoms. Combined with previous findings that the α2GABA$_A$Rs are required for the anxiolytic-like effects of benzodiazepines (Low *et al*, 2000; Smith *et al*, 2012; Morris *et al*, 2008) and that the genetic deletion of α2GABA$_A$Rs can lead to behavioral despair (Vollenweider *et al*, 2011), this possibility points to the potential utility of α2GABA$_A$R-specific compounds in the treatment of anxiety and mood disorders.

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Author Contributions

EE conceptualized the experiments, conducted behavioral studies, analyzed data, wrote the manuscript; KIB conducted animal surgeries and behavioral experiments, analyzed data; KSS conceptualized and supervised experiments; RMH did immunofluorescence staining and analysis on NAc knockdown animals; LMR did surgeries and training on ICSS experiments; WT and RS designed and provided a novel viral construct for cre-*lox*P-mediated knockdown; SJM supervised immunofluorescence experiments; UR conceptualized the study and experiments, supervised the overall work, wrote the manuscript.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)