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Ethanol-induced locomotor sensitization in DBA/2J mice is associated with alterations in $GABA_A$ subunit gene expression and behavioral sensitivity to $GABA_A$ acting drugs

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ABSTRACT

Repeated exposure to ethanol may produce increased sensitivity to its acute locomotor stimulant actions, a process referred to as locomotor sensitization. Neuroadaptation within certain brain circuits, including those possessing $GABA_A$ receptors, may underlie locomotor sensitization to ethanol. Indeed, $GABA_A$ receptors are documented mediators of ethanol's cellular and behavioral actions. Moreover, because subunit composition of this receptor is predictive of its pharmacology, it is possible that alterations in subunit composition contribute to the expression of locomotor sensitization to ethanol. The goal of the present study was to determine if alterations in $GABA_A$ subunit composition are associated with the expression of locomotor sensitization in DBA/2J mice, a strain known to be particularly susceptible to the development of this behavioral phenomenon. Following a modified 14 day sensitization procedure (Phillips et al., 1994) relative changes in $GABA_A$ subunit gene expression were assessed in discrete mesolimbic brain regions. To determine if the observed changes in gene expression produced functional changes in the locomotor responses to drugs known to either preferentially or generally activate $GABA_A$ receptors normally possessing the significantly altered subunits, separate cohorts of animals were challenged with one of several low doses of zolpidem (α1-selective), etomidate (β2/3-selective), or flurazepam (γ2-directed) and assessed for locomotor alterations. Sensitized animals displayed increased expression of the α1, β2, and γ2 (v1) subunits in the Nucleus Accumbens (NAc) but not Ventral Tegmental Area (VTA). Additionally, sensitized animals displayed altered sensitivity to the locomotor actions of etomidate and flurazepam. These results support the hypothesis that neuroadaptive changes in $GABA_A$ subunit composition participate in the expression of locomotor sensitization.

Enhanced sensitivity to the acute locomotor stimulant actions of alcohol (ethanol) following repeated administrations, referred to as locomotor sensitization, is thought to represent an increase in the positive subjective effects of this substance (Robinson and Berridge, 1993). The enhanced vulnerability of certain mouse strains to the development of this sensitization has been compared to similar observation in high risk human populations (Newlin and Thomson, 1991). Although the development of locomotor sensitization is not fully understood, it is believed to be mediated by distinct neuroplastic changes associated with repeated ethanol exposure. A number of neurotransmitter systems have been implicated in the behavioral phenomenon, including dopamine (Palmer et al., 2003; Souza-Formigoni et al., 1999), glutamate (Broadbent and Weitemier, 1999), and GABA (Broadbent and Harless, 1999). The $GABA_A$ receptor system is of particular interest to our understanding of ethanol sensitization because of the large GABA presence in brain regions of the mesolimbic system thought important in the induction and expression of the behavioral phenomenon (for rev. see Kumar et al., 2009; Phillips and Shen, 1996), and because the function of $GABA_A$ receptors is positively modulated by ethanol (Allan and Harris, 1987).

Because $GABA_A$ subunit composition is critical for its pharmacology, changes in composition, rather than strictly increases or decreases in $GABA_A$ receptor numbers, may be responsible for behavioral sensitization. To date, there have been 8 classes of $GABA_A$ receptors subunits characterized (α1–6, β1–3, γ1–3, θ1–3, ρ1–3, δ, ε, and π). The considerable flexibility in the expression and function of this 5 subunit (pentameric) complex has been shown to alter distinct ethanol-related behavioral responses (Boehm et al., 2004). For example, insights from knockout studies have indicated that α1 knockout mice display increased sensitivity to the acute stimulant response to ethanol (Blednov et al., 2003; Boehm et al., 2004; June et al., 2007; Kralic et al., 2003). Although no studies have looked directly at the development or expression of sensitization in

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these knockouts, the data suggest that α1 subunit-associated composition may play an important role in enhanced locomotor sensitivity to ethanol, and perhaps locomotor sensitization to the drug.

The goal of the present study was to determine whether locomotor sensitization to ethanol is associated with changes in $GABA_A$ receptor subunit mRNA expression in brain regions believed important in the induction and expression of the behavioral phenomenon. Additionally, we wanted to determine if any such subunit alterations might be associated with changes in the locomotor responses to drugs known to preferentially activate $GABA_A$ receptors normally possessing these subunits. Based on the recent knockout literature suggesting a role for the α1 subunit in the acute stimulant response to ethanol (Blednov et al., 2003; June et al., 2007; Kralic et al., 2003), we hypothesized that ethanol sensitization would result in a relative decreased expression of this subunit in areas of the brain thought to be involved in the phenomenon; namely the Ventral Tegmental Area (VTA) and Nucleus Accumbens (NAc), and a significant relative increase in the abundantly expressed α2 subunit in these areas due to compensatory regulation (Kralic et al., 2002; Sur et al., 2001). Furthermore, we predicted that these changes would lead to altered sensitivity to drugs that act either preferentially or generally at receptor complexes containing the above mentioned subunits.

1. Methods

1.1. Animals

DBA/2J (D2) mice bred in our colony at the Binghamton University animal facility were used for each experiment. Due to constraints on availability, female mice were used in experiments 1 and 2, and male mice were used in experiments 3 and 4. All animals were housed 2–4 to a cage in standard, clear, polycarbonate shoebox mouse cages and had ad lib access to standard rodent chow and tap water except during behavioral testing which occurred during the light phase of the vivarium light/dark cycle. Mice were naïve and at least 60 days of age at the time of testing. Vivarium lighting was maintained on a 12/12 h cycle with the lights turning on at 7:00 AM, and the temperature and humidity were held at approximately 21 °C and 50%, respectively. All of the procedures were approved by the Binghamton University Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (The National Academic Press, 2003).

1.2. Locomotor activity testing chambers

Locomotor activity testing was conducted using the VersaMax Animal Activity Monitoring System (Accuscan Instruments Inc., Columbus, OH). Locomotor activity was detected by interruption of intersecting photocell beams evenly spaced along the walls of the 40×40 cm test chamber. This equipment was situated in sound-attenuating box chambers (inside dimensions, 53 cm across×58 cm deep×43 cm high) equipped with a house light and fan for ventilation and background noise. The locomotor activity testing equipment was interfaced with a Dell computer. Testing continued for 10–15 min during which time consecutive photocell beam interruptions were translated into distance traveled in cm by the VersaMax computer program. Data were collected in 1-min time intervals.

1.3. Alcohol and drug administration

200 Proof alcohol was purchased from Pharmco, Inc (Brookfield, CT) and diluted in 0.9% physiological saline. Ethanol (20% v/v) and saline were administered by intraperitoneal (i.p.) injection in a volume of 0.2 ml per 0.01 kg of body weight. Zolpidem, etomidate, and flurazepam (Sigma Aldrich) were suspended in approximately 30 μl of Tween 80, diluted in 0.9% physiological saline and injected in a volume of 0.1 ml per 0.01 kg of body weight.

1.4. Sensitization procedure

We used an established mouse model of ethanol sensitization developed by Phillips et al. (1994), details of which are shown in Table 1. The mice in each cage were randomly assigned to the repeated saline group (RS), or the repeated ethanol group (RE) for each experiment. The first two days of testing served to habituate the mice to the i.p. injections and testing in the locomotor activity chambers. On these days mice were habituated to the testing room for 45–60 min, weighed, and then injected with sterile 0.9% saline, and immediately placed in the center of the activity testing chambers for 10 (experiment 1)–15 (experiments 2–4) minutes. On day 3 mice were again moved to the experimental room and allowed to habituate for 45–60 min, only on this day mice were given injections according to their assigned group. Mice in the RE group received 2.0 g/kg ethanol whereas those assigned to the RS group received an isovolumetric saline injection. On days 4–13 mice assigned to the RE group received a 2.5 g/kg ethanol dose once daily and mice in the RS group continued to receive an equivalent volume of saline each day. This 0.5 g/kg higher dose was chosen based on literature demonstrating its effectiveness at inducing strong locomotor sensitization in mice using similar paradigms (Lessov and Phillips, 1998; Meyer et al., 2005), including data from our lab (Boehm et al., 2008). None of the mice were tested in the locomotor activity testing chambers following injection on days 4–13; mice were placed immediately into their home cage following injection. On day 14 mice were again allowed to habituate to the testing room for 45–60 min. However, all mice received a challenge injection of 2.0 g/kg ethanol (experiment 1) or drug (experiments 2–4) on this day and were immediately tested in the locomotor activity testing chambers for 10–15 min. The final day (15) animals were either euthanized for tissue collection (experiment 1) or given injections of 2.0 g/kg ethanol to verify between- and within-groups sensitization in groups that had received drug challenges the previous day (experiments 2–4). All pilot experiments performed to choose optimal drug doses (see exp 2–4 methods below) were performed using the same apparatus, test duration, and pretreatment time used in experiments 1–4.

1.5. Tissue collection and processing

Whole brains were quickly removed from animals, chilled in physiological saline, and cut into 1 mm coronal slices using an acrylic brain matrix. The NAc and VTA were microdissected bilaterally and immediately placed in RNA later (Qiagen) for preservation of mRNA. Tissue extraction for each animal took between 5 and 7 min from the time of cervical dislocation to final collection of tissue. Preserved microdissected tissue was homogenized using a rotor-stator

Table 1
Locomotor sensitization paradigm.

Treatment groups	Habituation	Acute EtOH	Daily treatments (induction)	Challenge (expression)	Sensitization (verification)
	Days 1–2	Day 3	Days 4–13	Day 14	Day 15
Repeated ethanol (RE)	Saline	EtOH (2.0 g/kg)	EtOH (2.5 g/kg)	EtOH or drug	Ethanol (2.0 g/kg)[a]
Repeated saline (RS)	Saline	Saline	Saline	EtOH or drug	Ethanol (2.0 g/kg)[a]

[a] In experiment 1 all animals were euthanized for tissue collection in lieu of ethanol challenge.

homogenizer and RLT lysis buffer (Qiagen). mRNA was then isolated using Qiagen 'Qiashreddar' and 'Minikit' spin columns and was analyzed for integrity and concentration using an Experion microphoresis system (BioRad) following manufacturer's instructions. Normalized mRNA was then Reverse-Transcribed for cDNA synthesis using a DNase treatment and first-strand kit (Invitrogen). A 1:4 cDNA dilution was then used as a template for all real-time PCR reactions.

1.6. Real-time PCR

$GABA_A$ subunit specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm) or taken from a Harvard mouse primer database (http://pga.mgh.harvard.edu/primerbank/) and ordered from IDT (Coralville, IA). Primers were designed to span an intron of the desired gene product and were subject to a BLAST search to ensure that they were specific to the target of interest. Using the IQ5 system (BioRad), 40–50 cycle real-time reactions were performed using SYBR green/TAQ master-mix (BioRad). After an initial 3 min hot start (95 °C), each cycle consisted of a 30 s denaturation (95 °C), a 30 s annealing (60 °C), and then a 30 s extension (55 °C). Immediately prior to the melt-curve analysis, an additional 1 min annealing (60 °C) cycle was performed to ensure product alignment. Melt-curve analysis was performed to verify the presence of a single primer directed product. This analysis incorporated increases in temperature in 5 °C increments every 15 s starting at 55 °C and ending at 95 °C. The presence of a single peak, expressed as the negative first derivative of the changes in fluorescence as a function of temperature, was indicative of primer directed amplification specificity. All values were normalized to β-Actin as an internal control housekeeping gene using the ΔΔ CT method (Pfaffl, 2001) and are expressed as percent change from RS group.

1.7. Experiment 1 — alterations in $GABA_A$ subunit mRNA expression

The goal of experiment 1 was to determine whether locomotor sensitization to ethanol was associated with changes in $GABA_A$ receptor subunit mRNA expression in brain regions believed important in the induction and expression of the behavioral phenomenon. Animals were subjected to the above mentioned sensitization procedure, challenged with 2.0 g/kg ethanol solution on day 14 after which they were immediately placed into the activity chambers for 10 min. On day 15 all animals were sacrificed by cervical dislocation and brain tissue was collected as described above.

1.8. Experiment 2 — zolpidem challenge

The goal of experiment 2 was to evaluate possible differences in the locomotor properties of low doses of the α1-selective benzodiazepine agonist zolpidem (Faure-Halley et al., 1993; Sieghart, 1995). All mice were assigned to either the RE or RS groups. These groups were further divided into three groups prior to day 14 challenge; one of two doses of zolpidem (0.4 or 0.2 mg/kg) or ethanol (2.0 g/kg). These zolpidem doses were chosen based on pilot work indicating that whereas the 0.2 mg/kg dose was at the threshold of eliciting an acute locomotor depressant response, the 0.4 mg/kg dose was high enough to produce this response compared to vehicle injected controls (data not shown). On day 14 of the sensitization procedure mice were injected with zolpidem or ethanol and immediately placed in the locomotor activity chambers for 15 min. Day 15 served to confirm the development of ethanol sensitization (or lack thereof; RS group) in mice that had received zolpidem the previous day. Treatment on this day was identical to day 14 except that all animals received 2.0 g/kg ethanol.

1.9. Experiment 3 — etomidate challenge

Experiment 3 was conducted to examine the locomotor properties of low doses of the β2/3-subunit selective agonist etomidate (Smith *et al.*, 2004) in sensitized vs. non-sensitized mice. This experiment was identical to experiment 2 except that RE and RS groups were further divided into one of three doses of etomidate (1.6, 3.2 and 6.4 mg/kg) or ethanol (2.0 g/kg) for day 14 challenge. These doses were chosen based on pilot work indicating dose-dependent decreases in locomotion over time as well as an initial stimulant response in the first 5 min immediately following administration of the highest etomidate dose (data not shown).

1.10. Experiment 4 — flurazepam challenge

Experiment 4 was conducted to examine the locomotor properties of low doses of the non-specific benzodiazepine flurazepam (Pritchett et al., 1989) in sensitized vs. non-sensitized mice. This experiment was identical to the previous 2 experiments except that the RE and RS groups were further divided into one of three doses of flurazepam (1, 2, and 4 mg/kg) or ethanol (2.0 g/kg) for day 14 challenge. Pilot work indicated that compared to vehicle, these doses initially produce acute stimulation followed by dose-dependent reductions in locomotor activity over time (data not shown).

1.11. Statistical analysis

Locomotor data collected prior to tissue harvesting in experiment 1 were analyzed by a mixed factor two-way analysis of variance (ANOVA) with repeated treatment (RS vs. RE) as the between groups factor and day as the within subjects factor. Analyses of relative changes in gene expression on day 15 were calculated using the ΔΔ CT method (Pfaffl, 2001). Because data transformed in this way are not normally distributed, results were compared using a non-parametric Mann–Whitney tests and are expressed as percent change from the RS control group.

All drug doses used in experiments 2–4 were chosen based on pilot work demonstrating their time dependent alterations of locomotor activity as previously described. Due to these dose and time dependent alterations in locomotor activity, all day 14 analyses were separated by dose. Thus, total distance traveled and time course effects were analyzed by mixed two-way ANOVA with time bin (5 min) as the within subjects factor and repeated treatment (RS vs. RE) as the between subjects factors for each drug/alcohol dose. Locomotor activity data in experiments 2–4 were also analyzed to confirm the development of locomotor sensitization following ethanol challenge on day 15. Because there were no significant interactions with day 14 drug dose assignments in any experiment, we collapsed on this factor for graphical presentation and analysis of these data. Total distance traveled on days 1, 2, 3 and 15 were analyzed by mixed two-way within subjects ANOVA with day as the within subjects factor and repeated treatment (RE/RS) as the between subjects factors.

Follow-up post-hoc tests (Newman–Keuls) were carried out where appropriate for all the above analyses. Results were considered significant at $P<.05$. All statistical analyses were conducted using the Statistica Version 7 statistical package (Tulsa, OK).

2. Results

2.1. Experiment 1 — alterations in $GABA_A$ subunit mRNA expression

Locomotor activity data from days 1, 2, 3 and 14 for experiment 1 can be seen in Fig. 1. Repeated measures analysis indicated significant main effects of treatment [$F(1, 22) = 95.22$ $P<.001$] and day [$F(3, 66) = 294.82$ $P<.001$] as well as a significant treatment * day

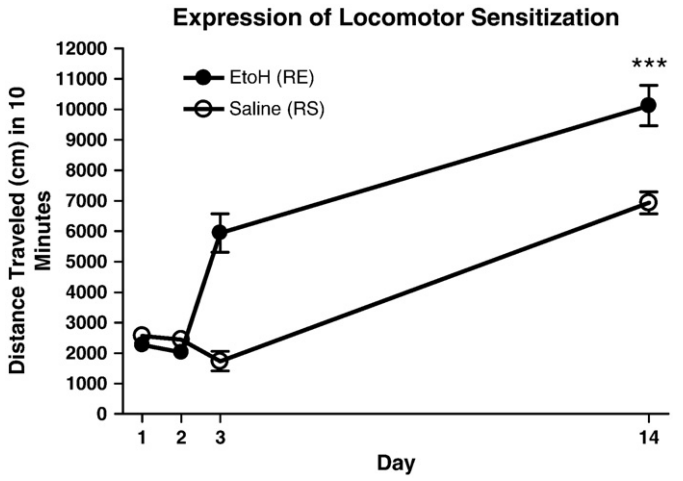

Fig. 1. Ethanol-induced locomotor sensitization in DBA/2J mice ($n = 10$–12 per group). Asterisks indicate between (day 14; RE vs. RS) and within (day 3 vs. day 14; RE) groups sensitization following low-dose (2.0 g/kg) ethanol challenge in RE group (***P<.001 — between and within subjects). Data illustrates mean ± SEM.

interaction [$F(3, 66) = 75.04$ P<.001]. Post-hoc tests confirmed that this was due to the development of significant day 14 locomotor sensitization in the RE group compared to their own acute ethanol challenge response on day 3 (P<.001), as well as to the acute response of the RS group on day 14 (P<.001).

Relative changes in gene expression following the 14 day sensitization procedure can be seen in Table 2. Analysis indicated that there were significant increases in the α1 (74%; $z = 3.78$; $p = .00016$), β2 (32%; $z = 3.18$; $p = .0014$), and γ2L (variant 1 — (Wang and Burt, 1991)) (29%; $z = 2.63$ $p = .0086$) subunits in the NAc of RE mice compared to that of the RS group. There were no statistically significant differences in any of the examined subunits in the VTA between the RE and RS groups.

2.2. Experiment 2 — zolpidem challenge

Day 14 locomotor results for the α1-selective drug zolpidem can be seen in Fig. 2A–C. Analysis revealed a significant main effect of repeated treatment for the ethanol challenged animals only [$F(1,18) = 7.33$ P<.05; Fig. 2A]. There were no differences between the RE and RS treatment groups on this day at either of the zolpidem doses (Fig. 2B–C).

On day 15, all groups were challenged with ethanol to confirm the development of locomotor sensitization (Fig. 5A). Analysis revealed significant differences across days (interaction) between the RE and RS groups [$F(3, 162) = 39.16$ P<.001]. Post-hoc results confirmed both within (day 15 vs. day 3; RE mice only) and between groups (day 15; RE vs. RS mice) sensitization to ethanol (Ps<.001; Fig. 5A).

Table 2
Values represent percent change from saline injected (RS) control group. A value of 100% indicates no change whereas values above or below 100% indicate relative increases or decreases in gene expression respectively (Pfaffl, 2001). *s indicate significant difference from RS controls (**P<.01; ***P<.001). Values are mean ± SEM.

$GABA_A$ subunit	NAc (RE) ($N = 12$)	NAc (RS) ($N = 12$)	VTA (RE) ($N = 10$)	VTA (RS) ($N = 10$)
α1	***174 ± 14	102 ± 6	90 ± 5	100 ± 3
α2	105 ± 4	101 ± 5	103 ± 6	101 ± 4
α4	106 ± 4	102 ± 7	105 ± 8	103 ± 9
β2	**132 ± 5	101 ± 5	94 ± 5	102 ± 8
γ2 (v1)	**129 ± 8	102 ± 6	94 ± 5	105 ± 10
γ2 (v2)	131 ± 11	104 ± 10	79 ± 11	104 ± 10
γ3	121 ± 14	104 ± 10	87 ± 12	102 ± 8

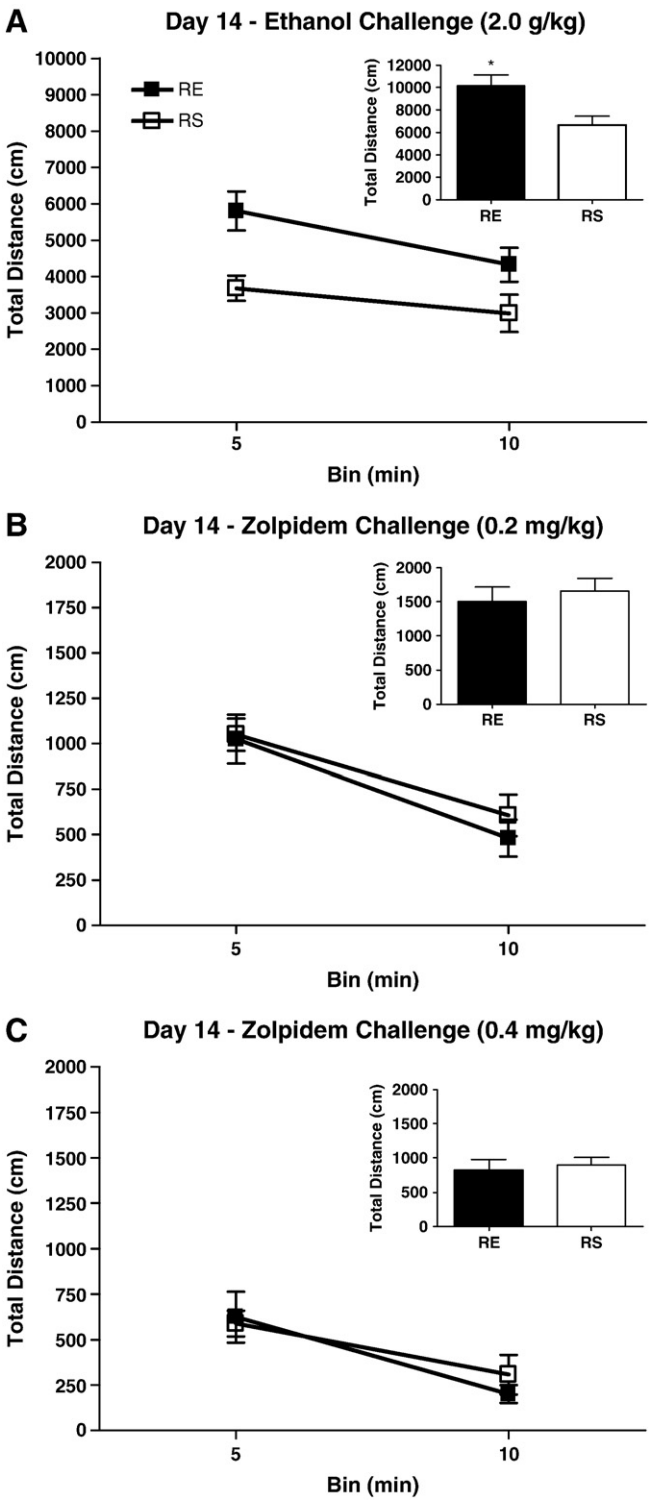

Fig. 2. Time course of locomotor response to zolpidem on day 14 ($n = 9$–10 per dose group). Insets reflect total cumulative distance traveled during 15 min session. A. Acute response to 2.0 g/kg ethanol in RE and RS groups. B. Acute response to 0.2 mg/kg zolpidem in RE and RS groups. C. Acute response to 0.4 mg/kg zolpidem in RE and RS groups (*P<.05). Data illustrates mean ± SEM.

2.3. Experiment 3 — etomidate challenge

Day 14 locomotor results following challenge with the β2/3-selective compound etomidate can be seen in Fig. 3A–D. Analysis revealed significant differences (main effects) between the RE and RS treatment groups at the 2.0 g/kg ethanol dose [$F(1, 20) = 40.79$ P<.001],

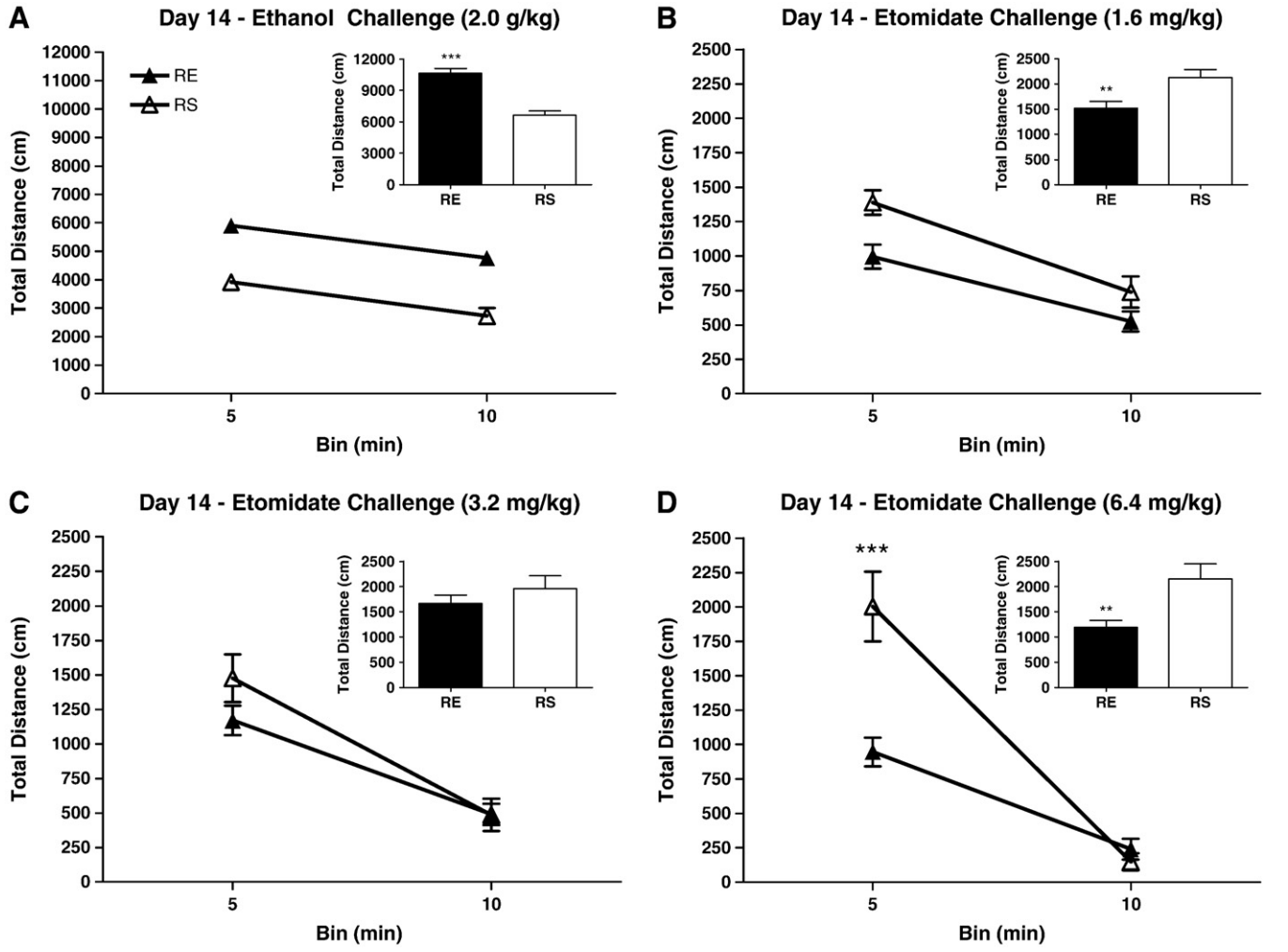

Fig. 3. Time course of locomotor response to etomidate ($n = 10$–12 per dose group). Insets reflect total cumulative distance traveled during 15 min session. A. Acute response to 2.0 g/kg ethanol in RE and RS groups. B. Acute response to 1.6 mg/kg etomidate in RE and RS groups. C. Acute response to 3.2 mg/kg etomidate in RE and RS groups. D. Acute response to 6.4 mg/kg etomidate in RE and RS groups (**P<.01, ***P<.001). Data illustrates mean ± SEM.

as well as the 1.6 [$F(1, 19) = 8.62$ P<.01], and 6.4 [$F(1, 20) = 9.34$ P<.01] mg/kg etomidate doses. These results are shown in Fig. 3A, B and D insets. Time course analysis revealed a significant treatment*bin interaction [$F(2, 40) = 25.37$ P<.001] following the highest (6.4 mg/kg) etomidate dose which post-hoc tests confirmed was due to a significant difference between RS and RE groups at the 1st 5 min bin (P<.001; Fig. 3D).

Analysis of day 15 data revealed a significant interaction of repeated ethanol treatment and day [$F(3, 255) = 49.36$ P<.001; Fig. 5B], with post-hoc results confirming both within (day 15 vs. day 3; RE mice only) and between groups (day 15; RE vs. RS mice) locomotor sensitization to ethanol (Ps<.001).

2.4. Experiment 4 — flurazepam challenge

Day 14 locomotor results following challenge with the γ2-directed drug flurazepam can be seen in Fig. 4A–D. Analysis revealed significant differences (main effects) between the RE and RS groups following the 2.0 g/kg ethanol challenge [$F(1, 17) = 58.64$ P<.001], and the 1 [$F(1, 18) = 10.99$ P<.01] and 2 mg/kg [$F(1, 18) = 26.75$ P<.001] flurazepam challenge doses (Fig. 4A, B, and C insets). There was also a significant time*treatment interaction following the 4 mg/kg [$F(1, 18) = 38.18$ P<.001; Fig. 4D] flurazepam dose. Post-hoc tests confirmed locomotor differences between the RS and RE groups at the 1st 5 min bin for this flurazepam dose (P<.05).

Ethanol challenge on day 15 again confirmed that repeated daily ethanol injections produced locomotor sensitization (Fig. 5C). Analysis detected a significant interaction of repeated treatment and day [$F(3, 231) = 44.66$ P<.001] which post-hoc results confirmed was due to both within (day 15 vs. day 3; RE mice only) and between groups (day 15; RE vs. RS mice) sensitization (Ps<.001).

3. Discussion

In order to examine our hypothesis that changes in $GABA_A$ subunit composition may relate to ethanol-induced locomotor sensitization we set out to explore possible gene expression and behavioral differences to drugs known to act preferentially at receptors possessing the significantly altered $GABA_A$ subunits. Specifically, we hypothesized that there would be relative decreases in α1 gene expression and compensatory α2 increases, and that these changes would confer increased sensitivity to the locomotor actions of subunit-preferring and generally acting $GABA_A$ compounds. Contrary to our hypothesis, we observed that repeated ethanol exposure capable of inducing locomotor sensitization significantly *increased* relative $GABA_A$ α1, as well as β2 and γ2, subunit gene expression in the NAc compared to repeated saline injected animals. We also observed dose-dependent alterations in locomotor responses to β2 (etomidate) and γ2 (flurazepam) acting drugs. These data provide evidence that neurobiological adaptations at the level of specific

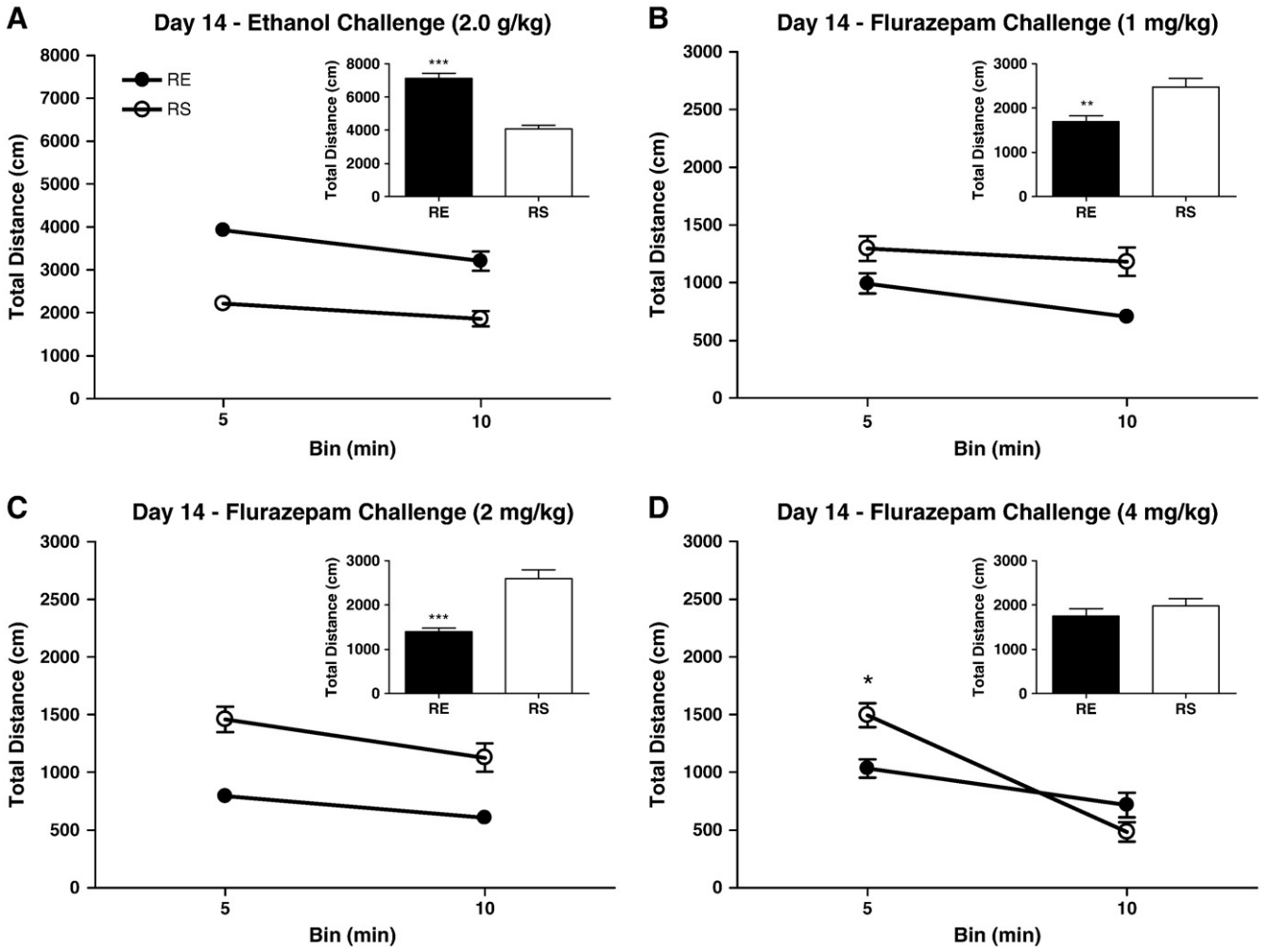

Fig. 4. Time course of locomotor response to flurazepam ($n = 9$–10 per dose group). Insets reflect total cumulative distance traveled during 15 min session. A. Acute response to 2.0 g/kg ethanol in RE and RS groups. B. Acute response to 1 mg/kg flurazepam in RE and RS groups. C. Acute response to 2 mg/kg flurazepam in RE and RS groups. D. Acute response to 4 mg/kg flurazepam in RE and RS groups (*P<.05, **P<.01, ***P<.001). Data illustrates mean ± SEM.

$GABA_A$ receptor subunit composition may relate to the observation of ethanol-induced locomotor sensitization.

The results of the current work are similar to those reported previously by Meyer et al. (2005). Their data suggest the development of tolerance to the robust stimulant effects elicited by the $GABA_A$ acting compounds pentobarbital and allopregnanolone. Overall, our data are also indicative of various degrees of tolerance. However, to maximize our subunit selectivity and examine upward and downward shifts in drug responses, we intentionally used low doses which were at the threshold of creating a locomotor response, elicited a subtle locomotor depressing response, or a stimulant response typically followed by decreases in locomotion (biphasic response). Therefore, although purely speculative, some of the observed effects may have been be due to some degree of sensitization to the sedative effects of these compounds rather than strictly tolerance to their acute stimulant actions. One way to address this possibility is to run treatment group (RE vs. RS) × drug dose ANOVAs for each drug. Results of such analysis [$F(2, 54) = 4.19$ $P \leq .05$] indicated that the highest dose of flurazepam did induce a significant decrease in locomotion compared to the 2 lower doses ($P \leq .05$). Thus, it is possible that lower doses of flurazepam were sufficient to induce its sedative properties in the RE group. Indeed, if particular subunits of the $GABA_A$ receptor are in fact differentially increased in the brains of sensitized mice as our gene expression data suggests, then one might expect heightened sensitivity to either or both of these behavioral actions. Of course, our ethanol-induced $GABA_A$ receptor subunit gene expression data have only begun to unravel this complicated phenomenon.

Considerable literature exists in support of subunit specific alterations following repeated ethanol exposure using various other rodent models and ethanol exposure paradigms. For example, subunit specific changes in the $GABA_A$ receptor expression have been reported in mice (Buck et al., 1991; Reilly and Buck, 2000; Sheela Rani and Ticku, 2006) and rats (Charlton et al., 1997; Chen et al., 1998; Sanna et al., 2003; Sarviharju et al., 2006), with results varying across brain region and ethanol exposure paradigm. In support of our data, Reilly and Buck (2000) reported significant increases in β2 subunit mRNA in cerebellar tissue of DBA/2J mice at doses of ethanol similar to those used in our studies. Similarly, α1 subunit gene expression has been shown to be down regulated in whole mouse brain following a chronic liquid diet ethanol exposure paradigm (Buck et al., 1991). α1 subunit protein expression has also been shown to be decreased in the VTA of rats exposed to 12 weeks (but not 1–4 weeks) of ethanol liquid diet (Charlton et al., 1997; Ortiz et al., 1995). These α1 gene expression and protein findings confirm that ethanol is capable of inducing changes in this subunit, albeit in a different direction than reported in the current work. However, the length and duration of ethanol exposure, time of tissue collection following ethanol challenge (24 h vs. during intoxication), as well as our decision to evaluate alterations within discrete brain regions are but three procedural differences that may have contributed to these differential effects.

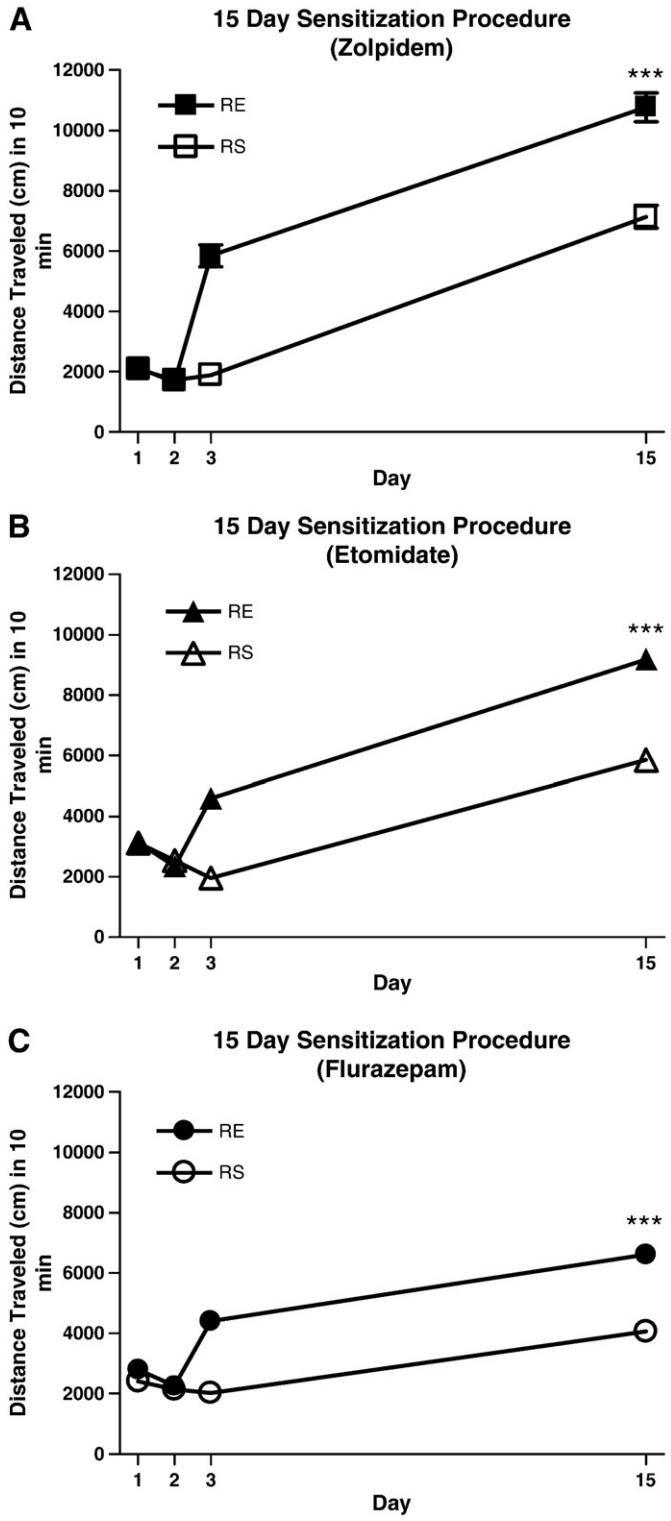

Fig. 5. Total distance traveled over days by repeated treatment group. Data are shown collapsed on individual drug doses and represent response to ethanol challenge (final $n = 28–47$ per group). A. Distance traveled in 10 min for experiment 1 (zolpidem). B. Distance traveled in 10 min for experiment 2 (etomidate). C. Distance traveled in 10 min for experiment 3 (flurazepam). Asterisks indicate between (day 14; RE vs. RS) and within (day 3 vs. day 14; RE) groups sensitization following low-dose (2.0 g/kg) ethanol challenge in RE group. (***$P<.001$ — between and within subjects). Data illustrates mean ± SEM.

Another important consideration relates to possible sex differences in 1) ethanol-induced locomotor sensitization and 2) $GABA_A$ subunit composition. There have been reports that female animals develop more robust locomotor sensitization than do males (Forgie and Stewart, 1994; Robinson, 1984). In fact, we have specifically chosen to use female animals in past experiments for this reason (Boehm et al., 2008). Indeed, in the current experiments female animals appeared to display more robust stimulant and sensitized locomotor responses. There have also been reports of neurosteroids having direct influence on $GABA_A$ receptor subunit composition (S. S. Smith et al., 2007). One notable example reported that the phase of estrous in female animals is associated with the expression of $GABA_A$ δ subunits in the dentate gyrus of the hippocampus (Maguire et al., 2005). More work is clearly necessary to better characterize ethanol-induced changes in $GABA_A$ subunit expression and the specific brain regions within which such changes lead to alterations in locomotor sensitivity to ethanol and other drugs of abuse.

Although we believe these data may relate directly to GABAergic neuroadaptations, there are several alternative explanations that are worth discussing. First, our rationale for testing animals on the 14th day and then verifying the development of sensitization on day 15 was due to literature from our lab (Boehm et al., 2008) and others (Meyer et al., 2005) that suggests greater degrees of behavioral sensitization when exposed to the relatively more novel test chamber. Our goal was to take advantage of this observation in order to maximize group differences on day 14. However, it is possible that this relative novelty combined synergistically with the drugs to contribute, at least in part, to the observed differences in locomotion between the two groups. Related to this, there have been reports of both increases and decreases in spontaneous locomotor behaviors during periods of ethanol withdrawal in rats and mice (Kliethermes, 2005). For example, Kliethermes et al. (2005) directly evaluated differences in locomotion induced by withdrawal from vapor inhalation in several strains of mice. Although the authors observed large decreases in locomotor behavior during peak withdrawal (7–10 h after removal from vapor chambers), these differences disappeared following 24 h of abstinence. More work is necessary to determine the extent to which ethanol withdrawal may interact with spontaneous locomotor and/or GABA-acting drug responses, but we cannot rule this out as a possible contribution to our observations.

The results of our gene expression data also possess several interpretational caveats that merit discussion. First and foremost, our goal was to evaluate relative changes in $GABA_A$ subunit gene expression between animals that did (RE) or did not (RS) display locomotor sensitization. To that end, our behavioral data support the use of directly comparing the acute and chronic ethanol treatment groups. However, because our data are expressed as relative changes in gene expression compared to the acute ethanol exposed (RS) group, it is possible that the perceived increases in gene expression in the RE group reflect significant *decreases* elicited by a single acute ethanol exposure in the RS group. Additionally, the ability to detect significant *decreases* in gene expression using the current method is complicated by the fact that values expressed as percent change from a control group can never drop below zero (floor effect). Although purely speculative, this may have contributed to the lack of statistically significant changes in $GABA_A$ subunit expression in the VTA; non-significant reductions in the expression of α1, β2, and γ2 subunits were observed in RE mice (see Table 2). Future experiments that include ethanol naïve saline injected controls and/or known subunit mRNA standards will be needed to evaluate these possibilities; particularly those related to specific directional hypothesis.

We did not set out to determine if significant increases in gene expression in the NAc of RE mice were directly related to the *degree* of expression of locomotor sensitization. Future experiments using heterogeneous populations of mice that develop varying degrees of ethanol exposure may be one way to determine the strength of such relationships if any exist. Similarly, because 1) the relationship between mRNA and protein is not necessarily linear and 2) the systemic drug studies did not preferentially target the brain regions

assayed for gene expression alterations, we cannot conclude that the observed behavioral and molecular results are directly related.

In conclusion, the current data add to the growing literature implicating brain region-specific alterations in $GABA_A$ subunit composition as influential to ethanol-related behavioral phenotypes. In particular, the current work demonstrates the plasticity of accumbal $GABA_A$ receptor systems in response to repeated ethanol exposures that result in the expression of locomotor sensitization. Future work will be necessary to determine the extent to which such brain region-specific changes contribute to the induction and expression of ethanol sensitization.

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References

Allan AM, Harris RA. Involvement of neuronal chloride channels in ethanol intoxication, tolerance, and dependence. Recent Dev Alcohol 1987;5:313–25.
Blednov YA, Walker D, Alva H, Creech K, Findlay G, Harris RA. Gabaa receptor alpha 1 and beta 2 subunit null mutant mice: behavioral responses to ethanol. J Pharmacol Exp Ther 2003;305(3):854–63.
Boehm 2nd SL, Ponomarev I, Jennings AW, Whiting PJ, Rosahl TW, Garrett EM, et al. Gamma-aminobutyric acid a receptor subunit mutant mice: new perspectives on alcohol actions. Biochem Pharmacol 2004;68(8):1581–602.
Boehm 2nd SL, Goldfarb KJ, Serio KM, Moore EM, Linsenbardt DN. Does context influence the duration of locomotor sensitization to ethanol in female DBA/2J mice? Psychopharmacology (Berl) 2008;197(2):191–201.
Broadbent J, Harless WE. Differential effects of gaba(a) and gaba(b) agonists on sensitization to the locomotor stimulant effects of ethanol in DBA/2J mice. Psychopharmacology (Berl) 1999;141(2):197–205.
Broadbent J, Weitemier AZ. Dizocilpine (mk-801) prevents the development of sensitization to ethanol in DBA/2J mice. Alcohol Alcohol 1999;34(3):283–8.
Buck KJ, Hahner L, Sikela J, Harris RA. Chronic ethanol treatment alters brain levels of gamma-aminobutyric acida receptor subunit mRNAs: relationship to genetic differences in ethanol withdrawal seizure severity. J Neurochem 1991;57(4): 1452–5.
Charlton ME, Sweetnam PM, Fitzgerald LW, Terwilliger RZ, Nestler EJ, Duman RS. Chronic ethanol administration regulates the expression of gabaa receptor alpha 1 and alpha 5 subunits in the ventral tegmental area and hippocampus. J Neurochem 1997;68(1):121–7.
Chen F, Rezvani A, Jarrott B, Lawrence AJ. Distribution of gabaa receptors in the limbic system of alcohol-preferring and non-preferring rats: in situ hybridisation histochemistry and receptor autoradiography. Neurochem Int 1998;32(2):143–51.
Faure-Halley C, Graham D, Arbilla S, Langer SZ. Expression and properties of recombinant alpha 1 beta 2 gamma 2 and alpha 5 beta 2 gamma 2 forms of the rat GABAA receptor. Eur J Pharmacol 1993;246(3):283–7.
Forgie ML, Stewart J. Effect of prepubertal ovariectomy on amphetamine-induced locomotor activity in adult female rats. Horm Behav 1994;28(3):241–60.
June Sr HL, Foster KL, Eiler 2nd WJ, Goergen J, Cook JB, Johnson N, et al. Dopamine and benzodiazepine-dependent mechanisms regulate the EtOH-enhanced locomotor stimulation in the GABAA alpha1 subunit null mutant mice. Neuropsychopharmacology 2007;32(1):137–52.
Kliethermes CL. Anxiety-like behaviors following chronic ethanol exposure. Neurosci Biobehav Rev 2005;28(8):837–50.
Kliethermes CL, Cronise K, Crabbe JC. Home cage activity and ingestive behaviors in mice following chronic ethanol vapor inhalation. Physiol Behav 2005;85(4): 479–88.
Kralic JE, Korpi ER, O'Buckley TK, Homanics GE, Morrow AL. Molecular and pharmacological characterization of GABA(A) receptor alpha1 subunit knockout mice. J Pharmacol Exp Ther 2002;302(3):1037–45.
Kralic JE, Wheeler M, Renzi K, Ferguson C, O'Buckley TK, Grobin AC, et al. Deletion of gabaa receptor alpha 1 subunit-containing receptors alters responses to ethanol and other anesthetics. J Pharmacol Exp Ther 2003;305(2):600–7.
Kumar S, Porcu P, Werner DF, Matthews DB, Diaz-Granados JL, Helfand RS, et al. The role of gaba(a) receptors in the acute and chronic effects of ethanol: a decade of progress. Psychopharmacology (Berl) 2009;205(4):529–64.
Lessov CN, Phillips TJ. Duration of sensitization to the locomotor stimulant effects of ethanol in mice. Psychopharmacology (Berl) 1998;135(4):374–82.
Maguire JL, Stell BM, Rafizadeh M, Mody I. Ovarian cycle-linked changes in gaba(a) receptors mediating tonic inhibition alter seizure susceptibility and anxiety. Nat Neurosci 2005;8(6):797–804.
Meyer PJ, Palmer AA, McKinnon CS, Phillips TJ. Behavioral sensitization to ethanol is modulated by environmental conditions, but is not associated with cross-sensitization to allopregnanolone or pentobarbital in DBA/2J mice. Neuroscience 2005;131(2):263–73.
Newlin DB, Thomson JB. Chronic tolerance and sensitization to alcohol in sons of alcoholics. Alcohol Clin Exp Res 1991;15(3):399–405.
Ortiz J, Fitzgerald LW, Charlton M, Lane S, Trevisan L, Guitart X, et al. Biochemical actions of chronic ethanol exposure in the mesolimbic dopamine system. Synapse 1995;21(4):289–98.
Palmer AA, Low MJ, Grandy DK, Phillips TJ. Effects of a Drd2 deletion mutation on ethanol-induced locomotor stimulation and sensitization suggest a role for epistasis. Behav Genet 2003;33(3):311–24.
Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.
Phillips TJ, Shen EH. Neurochemical bases of locomotion and ethanol stimulant effects. Int Rev Neurobiol 1996;39:243–82.
Phillips TJ, Dickinson S, Burkhart-Kasch S. Behavioral sensitization to drug stimulant effects in C57BL/6J and DBA/2J inbred mice. Behav Neurosci 1994;108(4):789–803.
Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, et al. Importance of a novel gabaa receptor subunit for benzodiazepine pharmacology. Nature 1989;338(6216):582–5.
Reilly MT, Buck KJ. GABA(A) receptor beta(2) subunit mRNA content is differentially regulated in ethanol-dependent DBA/2J and C57BL/6J mice. Neurochem Int 2000;37(5–6):443–52.
Robinson TE. Behavioral sensitization: characterization of enduring changes in rotational behavior produced by intermittent injections of amphetamine in male and female rats. Psychopharmacology (Berl) 1984;84(4):466–75.
Robinson TE, Berridge KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev 1993;18(3):247–91.
Sanna E, Mostallino MC, Busonero F, Talani G, Tranquilli S, Mameli M, et al. Changes in GABA(A) receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal. J Neurosci 2003;23(37): 11711–24.
Sarviharju M, Hyytia P, Hervonen A, Jaatinen P, Kiianmaa K, Korpi ER. Lifelong ethanol consumption and brain regional gabaa receptor subunit mrna expression in alcohol-preferring rats. Alcohol 2006;40(3):159–66.
Sheela Rani CS, Ticku MK. Comparison of chronic ethanol and chronic intermittent ethanol treatments on the expression of GABA(A) and NMDA receptor subunits. Alcohol 2006;38(2):89–97.
Sieghart W. Structure and pharmacology of gamma-aminobutyric acida receptor subtypes. Pharmacol Rev 1995;47(2):181–234.
Smith AJ, Oxley B, Malpas S, Pillai GV, Simpson PB. Compounds exhibiting selective efficacy for different beta subunits of human recombinant gamma-aminobutyric acid a receptors. J Pharmacol Exp Ther 2004;311(2):601–9.
Smith SS, Shen H, Gong QH, Zhou X. Neurosteroid regulation of GABA(A) receptors: focus on the alpha4 and delta subunits. Pharmacol Ther 2007;116(1):58–76.
Souza-Formigoni ML, De Lucca EM, Hipolide DC, Enns SC, Oliveira MG, Nobrega JN. Sensitization to ethanol's stimulant effect is associated with region-specific increases in brain D2 receptor binding. Psychopharmacology (Berl) 1999;146(3): 262–7.
Sur C, Wafford KA, Reynolds DS, Hadingham KL, Bromidge F, Macaulay A, et al. Loss of the major GABA(A) receptor subtype in the brain is not lethal in mice. J Neurosci 2001;21(10):3409–18.
The National Academic Press. Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research 2003.
Wang JB, Burt DR. Differential expression of two forms of GABAA receptor gamma 2-subunit in mice. Brain Res Bull 1991;27(5):731–5.