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$GABA_A$ RECEPTOR α1 and β2 SUBUNIT NULL MUTANT MICE: BEHAVIORAL RESPONSES TO ETHANOL

Blednov Y.A, Walker D., Alva H., Creech K., Findlay G. and Harris R.A.

Waggoner Center for Alcohol and Addiction Research and Section of Neurobiology, University of Texas at Austin, Austin, TX 78712.

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Running title: Ethanol behavior and $GABA_A$

Address for correspondence:

Dr. Yuri A. Blednov, PhD, Waggoner Center for Alcohol and Addiction Research University of Texas A4800, 2500 Speedway MBB 1.124; Austin, TX 78712-1095.

email: yablednov@mail.utexas.edu

phone:512-232-2514 (main lab.); 512-471-2106 (office)

fax: 512-232-2525 (main lab.); 512-471-4336 (office)

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Abbreviations: THIP - 4,5,6,7-tetrahydroisoxazolo[5,4- c]pyridin-3-ol; CPP – conditioned place preference; CTA – conditioned taste aversion.

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ABSTRACT

Mice lacking either the α1 or β2 subunit of the $GABA_A$ receptor (Sur et al., 2001) were tested for ethanol, saccharin or quinine consumption, ethanol conditioned place preference, ethanol conditioned taste aversion, ethanol-simulated motor activity and handling-induced seizures following chronic consumption of an ethanol liquid diet. The α1 null mutants showed decreased ethanol and saccharin consumption, increased aversion to ethanol and a marked stimulation of motor activity after injection of ethanol. The β2 null mutants showed decreased consumption of saccharin and quinine, but not ethanol. Surprisingly, neither mutant showed marked changes in handling induced seizures before or after withdrawal of ethanol. The unique effects of deletion of these two $GABA_A$ receptor subunits on ethanol responses are discussed in terms of the distinct changes in different populations of $GABA_A$ receptors.

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A number of behavioral effects of ethanol have been attributed to actions at the $GABA_A$ receptor (for reviews Mehta and Ticku, 1999; Chester and Cunningham, 2002). Some of the evidence implicating the GABA receptor system in ethanol's motivational effects comes from studies showing that $GABA_A$ receptor antagonists (Hyytia and Koob, 1995) and benzodiazepine partial inverse agonists (Balakleevsky et al., 1990) consistently reduce ethanol self-administration in rats. In contrast, $GABA_A$ receptor agonists, such as muscimol and 4,5,6,7-tetrahydroisoxazolo [5,4- c]pyridin-3-ol (THIP), can increase voluntary ethanol intake and decrease withdrawal signs (Tomkins *et al*., 1994).

Other work proposes a role for $GABA_A$ receptors in the discriminative stimulus effects of ethanol. Thus, muscimol can substitute for ethanol when injected into the amygdala or the core of the nucleus accumbens of rats (Hodge and Cox, 1998). $GABA_A$ receptor antagonists picrotoxin and bicuculline block the stimulatory effects seen with low dose ethanol administration as well as depressant effects noted following high dose ethanol administration (Hinko, Rozanov, 1990; Koechling et al., 1991). Finally, a polymorphism of the $\gamma 2$ subunit of the $GABA_A$ receptor has recently been associated with genetic susceptibility to ethanol-induced motor incoordination and hypothermia, conditioned taste aversion, and withdrawal (Buck and Hood, 1998). Human genetic association studies have suggested that the $GABA_A$ $\beta 2$, $\alpha 6$, $\alpha 1$ and $\gamma 2$ subunit genes have a role in the development of alcohol dependence (for review see Loh and Ball, 2000). These are only examples, taken from a substantially larger literature, that implicate GABAergic neurotransmission in the in vivo actions of ethanol.

Neurons express multiple subtypes of $GABA_A$ receptors with differing subunit composition, but the physiological significance of this diversity is unknown (McKernan and Whiting, 1996). The pharmacology of a $GABA_A$ receptor is determined to some extent by its subunit composition. Thus, affinity and efficacy of benzodiazepines are influenced by α and γ subunits, but not β subunits (Wingrove et al., 1997). In contrast to benzodiazepines, effects of loreclezole and etomidate are determined by the β subunits (Stevenson et al., 1995).

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Thus, different receptor subtypes may contribute to the selective effects of drugs such as ethanol and benzodiazepines on certain types of behavior. To study the physiological role of GABAergic system mouse strains lacking individual $GABA_A$ receptor subunits have been generated. $GABA_A$ receptor α6 (-/-) knockout mice showed rather normal locomotion and exploration in drug-free situations (Homanics et al. 1997; Jones et al. 1997). However, these mice are strongly impaired by diazepam during a learned motor task on a rotarod when compared with wild-type controls, whereas ethanol sensitivity was not altered in the α6 (-/-) mutant mice, clearly indicating that α6 subunit-containing GABAA receptors are not responsible for the ethanol-induced motor impairment (Korpi et al., 1999). Wick et al. (2000) showed that compared to controls, mice carrying either the γ2L or γ2S transgene developed significantly less tolerance to the ataxic effects of ethanol without any alterations in anxiety and motor activity or acute effects of benzodiazepines and alcohol. On the contrary, mice deficient for the γ2L subunit do not differ in their behavioral or electrophysiological responses to ethanol (Homanics et al., 1999). However, this mutation increases midazolam or zolpidem sleep time about 20%, while responses to non-benzodiazepine agents such as etomidate and pentobarbital were unchanged (Quinlan et al., 2000). Mice lacking the γ2 subunit die shortly after birth (Gunther et al., 1995). Mice deficient for δ subunit showed reduced ethanol consumption, attenuated withdrawal from chronic ethanol exposure and reduced anticonvulsant effects of ethanol (Mihalek et al., 2001).

Mouse lines lacking functional $GABA_A$ receptor subunits α1or β2 were recently generated (Sur et al., 2001; Kralic et al., 2002a). In both knock-out mouse lines, ~60% of the total number of $GABA_A$ receptors were lost, consistent with the idea that many brain $GABA_A$ receptors contain these two subunits. Surprisingly, α 1(-/-) and β2 (-/-) mice do not display major phenotypic abnormalities or spontaneous seizures. α1 Null mutant mice showed compensatory over expression of α2 and α3 subunits, but β2 (-/-) mice displayed a reduction of each of the six α subunits (Sur et al., 2001; Kralic et al., 2002a). Thus, these mice provide an opportunity to study the role of $GABA_A$ receptors in alcohol action and we recently reported that deletion of these subunits reduces effects of ethanol and some other sedative hypnotic drugs on loss of righting

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reflex (Blednov et al., 2003). In the present study, we asked if these two mutations would produce similar changes in other behavioral actions of ethanol or if we could detect subunit-specific changes in actions of ethanol.

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MATERIALS AND METHODS.

Animals. Null α1 (-/-) and β2 (-/-) allele mice were created using homologous recombination and genotyped as previously described (Sur et al., 2001). Homozygote of the F2 generation were interbred avoiding any brother-sister mating and homozygous colonies of α1 (-/-), β2 (-/-) and wild type (+/+) mice were established. In this study the mice from F6-F7 generations of this interbreeding were used. Mice had mixed C57Bl6/129SvEv genetic background.

Female mice were used for all studies and were at least 14-18 weeks of age at the time of analysis; within each experiment all mice were of similar age. Mice were group housed (three to five per cage) under a 12-h light/dark cycle (lights on at 07:00 h) and provided ad lib access to food and water. All experiments were conducted in an isolated behavioral testing room in the animal facility to avoid external distractions. All mice were allowed to recover for at least 1 week between each drug treatment. All experiments were approved by the Institutional Animal Care and Use Committee.

Alcohol drinking. Mice were allowed to acclimate for 1 week to individual housing. Experiments were carried out in standard 7.5" X 12.5" cages in sliding racks. Bottles were placed vertically 3.5" from the back wall through two holes in the cage top. The distance between two bottles was about 1.0". A feeder was placed on the front wall. Two drinking tubes were continuously available to each mouse, and tubes were weighed daily. One tube always contained water. Food was available *ad libitum*, and mice were weighed every 4 d. After 4 d of water consumption (both tubes), mice were offered 3% ethanol (v/v) versus water for 4 d. Tube positions were changed every day to control for position preferences. Quantity of ethanol consumed (g/kg body weight/24 h) was calculated for each mouse and these values were averaged for every concentration of ethanol. Immediately following 3% ethanol, a choice between 6% (v/v) ethanol and water was offered for 4 d, then 9% (v/v) ethanol for 4 d and finally 12% (v/v) ethanol vs. water for 4 d.

Preference for non-alcohol tastants. Wild-type or knockout mice were also tested for saccharin and quinine consumption. One tube always contained water and the other contained

the tastant solution. Mice were serially offered saccharin (0.033% and 0.066%) and quinine hemisulfate (0.015 mM, 0.03 mM, 0.06 mM and 0.1 mM) and intakes were calculated. Each concentration was offered for 4 d, with bottle position changed every day. Within each tastant, the low concentration was always presented first, followed by the higher concentrations. Between tastants mice had two bottles with water for two weeks.

Conditioned Place Preference. Four identical acrylic boxes (30 × 15 × 15 cm) (Med Associates, St. Albans, VT) were separately enclosed in ventilated, light, and sound-attenuating chambers (Med Associates, St. Albans, VT). Each box has two compartments separated by wall with door. Compartments have a different type of floor (grid or wire mesh). Infrared light sources and photodetectors were mounted opposite each other at 2.5-cm intervals along the length of each box, 2.2 cm above the floor. Occlusion of the infrared light beams was used to measure general activity and location of the animal (left or right) within the box. Total activity counts and location of the animal (left or right compartment) within the box were recorded by a computer. The floors and the inside of the boxes were wiped with a damp sponge and the litter paper beneath the floors was changed between animals. The main principles of conditioned place preference procedure have been described earlier (Cunningham, 1993). Briefly, the place-conditioning study involved two habituation sessions, eight conditioning sessions, and one test session. For the habituation session, mice received an injection of saline immediately before being placed in the conditioning box for 5 min on a smooth paper floor. During habituation session both compartments were available for mice. The purpose of the habituation session was to reduce the stress associated with the novelty of experimental procedures and exposure to the apparatus. Mice were not exposed to the distinctive floor textures to avoid latent inhibition. For conditioning, mice were randomly assigned to two groups: saline (control) and ethanol (2.0 g/kg, i.p.) (n = 10-14/dose group). Within the ethanol group, mice were randomly assigned to one of two conditioning subgroups grid+ (GRID+) or grid- (GRID-) and exposed to a Pavlovian differential conditioning procedure. On alternating days, mice in the GRID+ group received an injection of ethanol (2 g/kg) immediately before a 5 min session on the grid floor (CS+ sessions). On intervening days, these mice received saline immediately before exposure to the wire mesh floor

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(CS- sessions). Conversely, mice in the GRID- group received ethanol paired with the wire mesh floor and saline paired with the grid floor. Mice from control group received a saline injection before being placed on either the grid floor or the wire mesh floor (alternative days). During conditioning trials, all mice had access only to one of two compartments of the apparatus. For the 30-min test session, all mice received injection of saline. Both compartments of each box were available for exploration during test session.

Conditioned taste aversion. Subjects were adapted to a water-restriction schedule (2-h water per day) over a 7-day period. At 48-h intervals over the next 10 days, all mice received 1-h access to a solution of saccharin (0.15% w/v sodium saccharin in tap water). Immediately after 1-h access to saccharin, mice received injections of saline or ethanol (2.5 g/kg). All mice also received 30-min access to tap water 5 h after each saccharin access period to prevent possible dehydration. Two-hour access to tap water was given during intervening days.

Chronic alcohol diet. Mice were individually housed and given an ethanol-containing liquid diet (BioServ, Frenchtown, NJ) (0% EtOH for 2 days, 2.3% EtOH for 2 days, 4.5% EtOH for 2 days and 6.0% EtOH for 5 days) (Snell et al., 1996). Concentrations of alcohol are expressed as volume/volume. Pair-fed control mice were given a volume of a control diet (with maltodextrin equicalorically replacing the ethanol) equal to the average volume that the ethanol-fed mice had consumed on the previous day. Because the mutant mice consumed more of the ethanol diet than the wild type mice, a 'matched' variant of the diet was used in a subsequent experiment. Mutant mice were given the average volume of the ethanol-liquid diet that the ethanol-fed wild type mice had consumed one day before. Handling-induced convulsions (HIC) were scored from 0-7 as previously described (Crabbe et al, 1991). Briefly, beginning at 7:00 A.M. on the 11th day (9-day ethanol diet), ethanol-containing diet was replaced with control diet. Animals were scored for HIC each 2 hours for the first 4 hours, then hourly for another 8 hr after withdrawal and then at 23 and 24 hr after withdrawal. Animals we weighed every other day of ethanol exposure. The volume of diet consumed was recorded daily and calculated per kg of body weight as well as amount of consumed ethanol.

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Spontaneous motor activity testing. Locomotor activity was measured in standard mouse cages in Opto-microvarimex (Columbus Instruments, Ohio, USA). Activity was monitored by 6 light beams placed along the width of the cage at 2.5 cm intervals, 1.5 cm above the floor. Each cage had bedding and food and was covered by a heavy plastic lid with holes for ventilation and bottle of water. Each mouse was prehabituated to the experimental cage for 4 h. Next, mice were removed from the experimental cages, weighed and injected with ethanol or saline (i.p.). After ethanol administration, mice were placed immediately in individually prehabituated cages and the activity was monitored every 5 min for 30 min. The activity recording system provides three different measures of activity. “Total activity” is the total number of beam breaks. “Ambulatory activity” is the number of new beam breaks. Therefore, “Ambulatory activity” will ignore the repeated breaking of the same beam, which can be caused by scratching, grooming, digging, and other stereotypic behaviors. The latter is termed “small movement” activity and is obtained by subtraction of “Ambulatory activity” from “Total activity”.

Alcohol Injections. All alcohol (Aaper Alcohol and Chemical Co., Shelbyville, KT) solutions were made in saline (20% v/v) and injected i.p. with a volume of 0.2 ml/10g of body weight. Control mice received the same volume of saline.

Alcohol Metabolism. Animals were given a dose of ethanol (4.0 g/kg i.p.) and blood samples were taken from the retro-orbital sinus in 15, 60, 120, 180 and 240 min after injection. Blood alcohol concentration (BEC) values, expressed as mg ethanol per ml blood were determined spectrophotometrically by an enzymatic assay (Lundquist, 1959).

Statistical Analysis. Data are reported as the mean ± S.E.M value. The statistics software program GraphPad Prizm (Jandel Scientific, Costa Madre, CA) was used throughout. To evaluate differences between groups, analysis of variance (one-way ANOVA or two-way ANOVA with Bonferroni post-hoc analysis) and Student’s t test with Dunnet’s correction for multiple comparisons were carried out.

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RESULTS.

Ethanol preference. We first asked if loss of α1 or β2 subunits of $GABA_A$ receptors changes alcohol consumption. In a two-bottle free-choice paradigm in which mice could drink either water or an ascending series of ethanol concentrations (0, 3, 6, 9, 12 and 15%), mice lacking α1 subunits displayed significantly reduced preference for ethanol as well as a reduction in the amount of ethanol consumed. β2 (-/-) mutant mice showed no change in ethanol consumption or preference (Fig.1a,b). Both mutants consumed more water than the control mice (Fig.1c).

Preference for non-alcohol tastants. Two weeks after the ethanol drinking study, the same mice were tested for saccharin (sweet) or quinine (bitter) consumption in a two-bottle choice paradigm using an order-balanced experimental design. α1 (-/-) and β2 (-/-) null mutant mice showed significantly reduced preference for saccharin compared to control mice (Fig.2a) as well as a reduction in consumption of the saccharin solution (Fig.2c). The water intake of both mutant mice was similar to wild type mice (Fig.2e). β2 (-/-) null mutant mice showed significantly higher avoidance to quinine and lower intake of the quinine solution than wild type or α1 (-/-) mice (Fig.2b,d). In contrast, β2 (-/-) null mutant mice showed increased intake of water compared to wild type (Fig.2f).

Place conditioning. Following the control saline injections, all three genotypes spent substantially more time on the grid floor than on the wire mesh floor (Fig.3a). Because of this preference for one type of floor, we calculated place conditioning only for the group of mice injected with ethanol paired with their less favorite type of floor - wire mesh (GRID-). The percent of time spent on the wire mesh floor by saline- and ethanol-injected mice of each genotype is shown in figure 3b. As can be seen, all three genotypes spent more time on the wire mesh floor when it was paired with ethanol than when paired with saline, reflecting development of conditioned place preference. Comparisons between the saline and ethanol subgroups showed that wild type and α1 (-/-) knockout mice developed stronger CPP than β2 (-/-) knockout mice.

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However, it should be noted that conditioned place preference for ethanol was not obtained with the GRID+ condition because of the high initial preference of the mice for this floor.

Mean activity during each 5-min ethanol (CS+) and saline (CS-) conditioning trial are depicted in Fig.3c,d. Ethanol produced an increase in activity relative to saline in α1 (-/-) knockout and wild type mice. However, in wild type mice ethanol-induced motor activation was revealed only at the 3rd and 4th conditioned trials whereas in α1 null mutant mice ethanol stimulated motor activity beginning from the 1st trial. In β2 (-/-) knockout females, the first injection of ethanol decreased the activity in comparison with saline injected group. Activity on saline trials was decreased across trials in all three genotypes. Comparison the motor activity only in CS+ trials also showed significant differences between α1 (-/-) knockout and wild type mice.

Because strain differences in basal activity complicate interpretation of the ethanol-stimulated activity, ethanol data were also analyzed as the difference between activity on each CS+ and the corresponding CS- trials. This analysis indicated a consistently greater ethanol-induced activation in α1 (-/-) knockout mice compared to wild type mice whereas β2 (-/-) null mutant females were less sensitive to ethanol-stimulated effect than wild type mice (Fig.3e).

Activity levels during the preference test mirrored genotype differences observed on saline conditioning trials. Mean ± S.E.M. activity rates were 1529 ± 151, 842 ± 111 and 3628 ± 227 for WT, α1 (-/-) and β2 (-/-) knockout mice, respectively. One-way ANOVA showed a significant genotype effect ($P<0.01$); follow-up comparisons indicated that all pair wise differences were significant (Bonferroni-corrected P's<0.01).

Conditioned Taste Aversion. Consumption of saccharin on trial 1 (before conditioning) was greatest for wild type and less for α1 and β2 null mutant mice (Fig.4a). To attempt to correct for these initial differences in saccharin intake and facilitate presentation of the data, intake was calculated as a percentage of the trial 1 consumption for each subject by dividing the amount of saccharin solution consumed on subsequent conditioning trials on amount of saccharin solution consumed on trial 1 (before conditioning). Ethanol-saccharin pairings produced reductions in

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saccharin intake across trials, indicating the development of CTA in all genotypes. However, α1 null mutant mice developed stronger CTA than wild type whereas the CTA developed by β2 null mutant mice was similar to wild type.

Spontaneous locomotion. We studied effects of ethanol on motor activity in the home cage after habituation. β2 Knockout mice demonstrated higher baseline (saline injection) motor activity than wild type and α1 null mutant mice whereas the level of basal (saline injection) motor activity of α1 (-/-) mice was lower than in wild type mice (Fig.5a,b). A range of ethanol doses enhanced motor activity in α1 (-/-) mice, but only one of these doses was effective in wild type mice and there was no effect of ethanol in β2 null mutant mice (Fig.5b). In an attempt to correct for baseline differences, ethanol effects were normalized by setting the activity after saline injection to 100% for each genotype (Fig.5c). In wild-type mice, ethanol caused only a weak increase in motor activity and only at a dose of 2 g/kg. In contrast, ethanol produced a very strong motor activation over a range of doses (1.5 – 2.5 g/kg) in α1 (-/-) knockout mice. Ethanol did not stimulate motor activity in β2 null mutant mice (Fig. 5c). Motor activity was also measured as "Ambulation" and "Small Movement", as described in the Methods section. These measures were quite similar and showed the same changes (increased basal activity in β2 (-/-) knockout mice, increased ethanol stimulation in α1 null mutant mice) as was detected by the measure of "Total activity" presented in Fig.5.

Chronic alcohol consumption. The basal HIC score measured in naive β2 (-/-) null mutant mice was significantly higher than in wild type mice (1.97 + 0.15 and 1.46 + 0.17 for mutant and wild type mice respectively). Chronic ethanol exposure followed by withdrawal of ethanol produced withdrawal seizures, as measured by the HIC score (Fig.6 a). The β2 (-/-) null mutant mice showed a significant increase in area under the HIC withdrawal curve compare with both α1 knockout and wild type mice (Fig.6 c). Pair-fed mice did not showed genotype differences in HIC score or in area under the HIC withdrawal curve (Fig.6 b,d). The intake of ethanol was higher in β2 (-/-) knockout mice than in wild type mice (Fig.7 a,b). In addition, the pattern of consumption was somewhat different, with the β2 (-/-) null mice showing their highest level of alcohol intake on the third day and consumption declined thereafter reaching levels similar to the

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wild-type group on the last day (Fig. 7). Consistent with this decrease in ethanol intake toward the end of the study period, the β2 (-/-) mutant mice showed elevated HIC scores at the time of withdrawal (Fig 6a). Taken together, these results suggest that β2 (-/-) mutant mice limited consumption during the night and beginning withdrawal before the other groups and before the alcohol-containing diet was removed.

Ethanol metabolism. There were no differences in metabolism of ethanol between wild type and either of the knockout mice (data not shown).

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DISCUSSION.

Because deletion of either the α1 or β2 subunits produced a similar loss of $GABA_A$ receptor binding (Sur et al., 2001) and function (measured in cortex by chloride flux, Blednov et al., 2002), one might expect to see similar behavioral and pharmacological phenotypes in the two lines of mutant mice. However, this is not the case; of the eleven behaviors listed in table 1, only two (ethanol loss of righting reflex and saccharin preference drinking) showed the same direction of change in both α1 and β2 null mutant mice. Thus, it is clear that differences between the two mutants are more important than the similar loss of receptor number and function. The key difference is likely the more selective changes produced by deletion of α1 as compared to the more global effects of β2 deletion. Sur et al. (2001) concluded that deletion of α1 did not alter the amount of receptors containing α2-5, and produced a 38% loss of the small population of receptors containing the α6 subunit, thus resulting in a fairly selective deletion of receptors containing α1 subunits. In contrast, deletion of the β2 subunit produced a loss of only 60% of the receptors containing α1, but additional depletion of 39-69% of receptors containing α2-6 (Sur et al., 2001). Thus, behaviors affected in the α1 mutants but not in the β2 null animals are likely mediated predominantly by α1-containing receptors whereas in β2 mutants receptors containing α2-5 may contribute significantly in addition to □1 to the altered behavior. From this classification, we propose that decreased ethanol preference drinking is caused by increased conditioned taste aversion, which is due to loss of α1 and that increased motor activity after ethanol is also caused by loss of α1. The loss of α2-5 receptors may be responsible for the high baseline activity and decreased quinine preference drinking seen in the β2 null mice (Table 1). These behavioral changes are evaluated in detail in the remainder of the Discussion.

Our demonstration of decreased alcohol preference in mice lacking α1 subunits agrees with a considerable literature on the importance of $GABA_A$ receptors in alcohol intake. For example, central injection of competitive $GABA_A$ receptor antagonists significantly decreased ethanol operant responding (Hyytia and Koob, 1995; June et al., 1998). Acute injection of negative allosteric modulators of the GABAA receptor (inverse agonists) strongly decreased

ethanol consumption in two bottle choice paradigm (Wegelius et al., 1994). Treatment with the $GABA_A$ agonist THIP was shown to enhance the acquisition of voluntary ethanol consumption in laboratory rats (Smith et al., 1992) and increase preference for ethanol over water (Boyle et al., 1993). In contrast, preference for ethanol over water was decreased following the administration of picrotoxin (Boyle et al., 1993). However, these compounds are "nonselective" GABA drugs and are therefore not capable of dissecting out potential roles of specific $GABA_A$ receptor subunits in regulating ethanol-seeking behaviors There are some recent data which suggest the importance of α1 subunit, include the demonstration that WHP (Warsaw High-Preferring) rats treated intracerebroventricularly with antisense oligodeoxynucleotide derived from α1 subunit of the $GABA_A$ receptor had decreased ethanol intake after 4–5 days of treatment (Malatynska et al., 2001). Also, Harvey et al. (2002) showed that bilateral microinfusion of 3-PBC (α1 subunit-specific mixed agonist-antagonist) in the ventral pallidum produced marked reduction in alcohol-maintained responding in alcohol-preferring (P) rats. Recently, Chester and Cunningham (2002) suggested that blockade of $GABA_A$ receptor may produce changes in the rewarding and aversive effects of ethanol concurrently by removing a normal inhibitory influence of GABA in brain areas that mediate ethanol reward and aversion.

A key question is whether differences in intake of alcohol in our mutant mice simply reflect differences in perception of tastes. Although both knockout strains showed a similar and modest decrease in consumption of sweet saccharin solutions, only the α1 (-/-) mice showed a decrease in ethanol consumption. In addition, β2 (-/-) null mutant mice (but not α1 (-/-) mice) showed avoidance for bitter quinine solutions but this mutant strain was not different from wild type mice in consumption of alcohol. Taken together, these results are consistent with our finding that deletion α1 subunit of $GABA_A$ receptor leads to avoidance of voluntarily ethanol-consumption, likely due to increase aversion to ethanol.

Some of our most striking findings are the differences in motor activity between the mutants. Consistent with previous results (Sur et al., 2001), β2 null mice demonstrated very high levels of motor activity and this high basal activity may have prevented ethanol from producing

any further enhancement of activity. In contrast, α1 knockout mice are supersensitive to the stimulant effect of ethanol but displayed lower level of basal motor activity than wild type mice. It is of interest to consider possible signaling systems that might account for the increased ethanol-stimulated motor activity seen in the α1 null mutant mice. For example, activation of $GABA_A$ receptor by THIP blocks the motor stimulant effect of ethanol (Broadbent and Harless, 1999). Thus, removal of tonic inhibitory GABAergic tone in the α1 knockout mice may enhance stimulatory effect of ethanol. The distinct effects of deletion of α1 or β2 subunits may be related to the selective expression of α subunits in the limbic and basal ganglia systems. For example, dopaminergic neurons of the ventral tegmental area and substantia nigra pars compacta contain α3 and not other α subunits; likewise the striatum and nucleus accumbens contain α2-4, but not α1 subunits. In contrast, the interneurons in these brain regions contain α1 (and β2) subunits (Schwarzer et al., 2001). This raises the possibility that reduction of GABAergic tone on dopamine neurons (produced by the β2 deletion) is required for the increase in basal activity, but $GABA_A$ receptors on interneurons are more important for the stimulatory actions of ethanol. It is of interest to note that a recent publication (Kralic et al., 2002b) found that deletion of the α1 subunit increased the sedative action of diazepam, rather than augmenting the stimulant action. This is consistent with more diverse and complex actions of ethanol in comparison with diazepam.

Deletion of $GABA_A$ receptor subunits reduced the consumption of sweet and bitter solutions and this is consistent with suggestions that activation of $GABA_A$ receptors promotes consumption of tastants. For example, benzodiazepines, apart from their anxiolytic actions, also exert effects on the affective appraisal of taste stimuli (reviewed by Berridge and Pecina, 1995). For example, Berridge and Treit (1986), using a taste reactivity method, reported that chlordiazepoxide increased the positive hedonic responses to sweet-, sour- and bitter-tasting solutions infused into the mouth; the occurrence of aversive affective reactions remained unchanged or was suppressed. These data suggest that reduction of $GABA_A$ receptors might to reduce positive hedonic responses to different tastants.

In view of the central role of $GABA_A$ receptors in suppressing seizure activity in general and alcohol withdrawal convulsions in particular (Buck and Finn, 2001), it is quite surprising that

the α1 null mutants do not show greater HIC scores than wild type controls before or after chronic alcohol consumption. The β2 null mutants show some elevation of handling induced convulsions before chronic ethanol consumption and an increase in seizures upon withdrawal from ethanol, but these effects are not marked. However, the modest increase in withdrawal seizures in the β2 mutants could be influenced by their increased consumption of the ethanol-containing liquid diet at the beginning of the experiment. This raises the important point that in addition to the changes in levels of other $GABA_A$ subunits shown by Sur et al. (2001), there are likely changes in other brain proteins in response to deletion of $GABA_A$ receptor subunits. Understanding the compensatory mechanisms and other neural strategies that allow these mutant mice to maintain near-normal brain excitability despite the loss of many $GABA_A$ receptors should provide new insight regarding genetic regulation of brain function.

In summary, these studies provide support for the importance of $GABA_A$ receptors in behavioral actions of ethanol, and emphasize that different behavioral actions of ethanol are mediated by distinct $GABA_A$ receptors.

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LEGENDS FOR FIGURES.

Fig.1. Decreased ethanol preference and consumption in α1 null mutant mice.

Panel A. Wild type mice show greater ethanol consumption than α1 null mutants ($p<0.0001$, two-way ANOVA). β2 Knockout mice were not different from wild type mice. Panel B. Wild type show greater preference for ethanol than α1 null mutants ($p=0.0015$, two-way ANOVA). β2 Knockout mice were not different from wild type mice. Panel C. β2 Knockout mice as well as α1 null mutants show greater water intake then wild type ($p<0.01$ and $p<0.05$ respectively, two-way ANOVA). n = 9 for each genotype.

Fig.2. Both mutant lines show decreased preference for saccharin and β2 knockout mice show increased avoidance of quinine. Panel A. α1 and β2 null mutant mice show lower preference for saccharin than wild type mice ($p<0.05$ for both mutant strains, two-way ANOVA). Panel B. β2 Knockout mice show greater quinine avoidance than wild type ($p<0.0001$, two-way ANOVA). α1 null mutants were not different from wild type mice. Panel C. Both mutant strains show smaller intake of saccharin solution than wild type mice ($p<0.01$ for α1 and β2 knockouts mice, two-way ANOVA). Panel D. β2 Knockout mice show smaller intake of quinine solution than wild type ($p<0.0001$, two-way ANOVA). α1 Null mutants were not different from wild type mice. Panel E. All three genotypes showed similar consumption of water in conjunction with saccharin drinking. Panel F. β2 Knockout mice consumed more water than wild type mice in conjunction with quinine drinking ($p<0.0001$, two-way ANOVA) but α1 Knockouts were not different from wild type mice.

Fig.3. α1 Knockout and wild type mice develop greater conditioned place preference with ethanol. Panel A. Percent of time spent on different floors during 30-min test session in control saline-treated groups. There was a significant effect of subgroup ($p<0.0001$) with significant interaction ($p<0.0001$, two-way ANOVA). ** - $p<0.01$, *** - $p<0.001$ – difference

between percent of time spent on grid and wire mesh floors for each genotype (two-way ANOVA, post-hoc Bonferroni analysis). Panel B. Percent of time spent on the wire mesh by saline-treated groups and GRID- conditioned subgroups (ethanol injection was paired with wire mesh). There was a significant effect of genotype ($p<0.001$, two-way ANOVA), treatment ($p<0.001$, two-way ANOVA) with significant interaction ($p<0.01$, two-way ANOVA). * - $p<0.05$, *** - $p<0.001$ – difference in percent of time spent on wire mesh between control saline-treated and ethanol-treated conditioned subgroup (GRID-) for each genotype (two-way ANOVA, post-hoc Bonferroni test). Panel C. Motor activity during each 5-min ethanol conditioned trials (CS+). There was a significant effects of genotype in comparison of α1 (-/-) knockout and wild type mice ($p<0.0001$, two-way ANOVA). Panel D. Motor activity during each 5-min saline conditioned trials (CS-). There was a significant effect of genotype and trial in comparison of α1 (-/-) as well as β2 (-/-) knockout females with wild type mice ($p<0.0001$ for genotype and trial for both mutants, two-way ANOVA). Panel E. Mean difference in activity counts between each ethanol and the corresponding saline trials. There was a significant of genotype and trial in comparison of α1 (-/-) ($p<0.0001$ for genotype and trial, two-way ANOVA) as well as β2 (-/-) knockout females ($p<0.01$ for genotype and $p<0.001$ trial, two-way ANOVA) with wild type mice. n = 10-12 for saline-injected groups of each genotype, n = 12-14 for ethanol-injected groups of each genotype.

Fig.4. α1 Knockout mice develop greater conditioned taste aversion for ethanol.

Panel A. Basal saccharin consumption in α1 knockouts was lower than in wild type ($p=0.005$) and β2 null mutant mice ($p=0.004$) (t-Student's test with Dunnet's correction for multiple comparisons). Panel B. α1 Null mutants developed stronger CTA than wild type or β2 knockout mice. *** - $p<0.001$ different from wild type mice; ### - $p<0.001$, α1 (-/-) knockout are different from β2 null mutant mice (two-way ANOVA). n = 10 for saline injection for all genotypes. n = 14 for wild type, n = 11 for α1 mutants n = 15 for β2 mutants for groups with ethanol injection.

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Fig.5. α1 Knockout mice are more sensitive to ethanol-induced motor stimulation.

Panel A shows that baseline motor activity of the three genotypes. ** - p<0.01, *** - p<0.0001, mutant mice are different from wild type (one-way ANOVA, Bonferroni post-hoc analysis). Panel B. Motor activity (number of crossed beams) for 0-10-min period. Panel C. Normalized motor activity for 0-10 min period (activity after saline injection taken as 100%). In panels B and C, significant effects of individual doses of ethanol are indicated as follows: * -P<0.05, **- P<0.01 different from saline control (one-way ANOVA, Bonferroni post-hoc analysis). Each point represents an independent group of animals. n = 15-16 per saline groups and n = 10-12 per each ethanol groups.

Fig.6. Withdrawal severity for β2 null mutant mice is greater than for wild type.

Handling induced convulsions (HIC) were measured after chronic consumption of an ethanol-containing liquid diet. Panel A. β2 (-/-) mutants showed higher withdrawal scores than wild type (p<0.0001, two-way ANOVA). Panel B. Pair-fed mice of all three genotypes showed no differences in HIC scores. Panels C and D show the area under the curve (AUC) calculated from panels A and B. AUC is greater for β2 null mutants compared with wild type (p<0.0001, t-Student's test with Dunnet's correction for multiple comparison) and α1 null mutant mice (p<0.05, t-Student's test with Dunnet's correction for multiple comparison). $^{\#}$ - p<0.05; $^{\#\#}$ - P<0.01 - two mutant strains are different; *** - p<0.001 mutant mice are different from wild type mice. Panels A, C for ethanol diet group - n = 10 for each genotype. Panels B, D for pair-fed groups n = 6 for each genotype.

Fig.7. β2 Null mutant mice consumed more but α1 knockouts consumed less ethanol than wild type mice. Panel A. β2 Null mutant mice consumed more and α1 (-/-) less of the liquid diet than wild type mice (p<0.0001 and p<0.05 respectively, two-way ANOVA). Panel B. β2 Null mutant mice consumed more and α1 knockout mice consumed less ethanol than wild type mice (p<0.0001 and p<0.05 respectively, two-way ANOVA).

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Table 1. Summary of effects of deletion of α_1 or β_2 subunits of $GABA_A$ receptors on ethanol-related behaviors.

Behavior	α_1 deletion	β_2 deletion
Ethanol loss of righting reflex *	↓	↓
Ethanol preference drinking	↓	0
Saccharin preference drinking	↓	↓
Quinine preference drinking	0	↓
Ethanol CPP	0	↓ / 0
Ethanol CTA	↑	0
Saline motor activity	↓	↑↑
Ethanol-stimulated activity	↑↑	0
Chronic ethanol consumption	↓	↑
HIC withdrawal score	0	↑
Ethanol metabolism	0	0

↓ - decreased effect; ↑ - increased effect; 0 - no difference from wild type. * - effect of deletion seen only in male mice (results for loss of righting reflex from Blednov et al., 2003).

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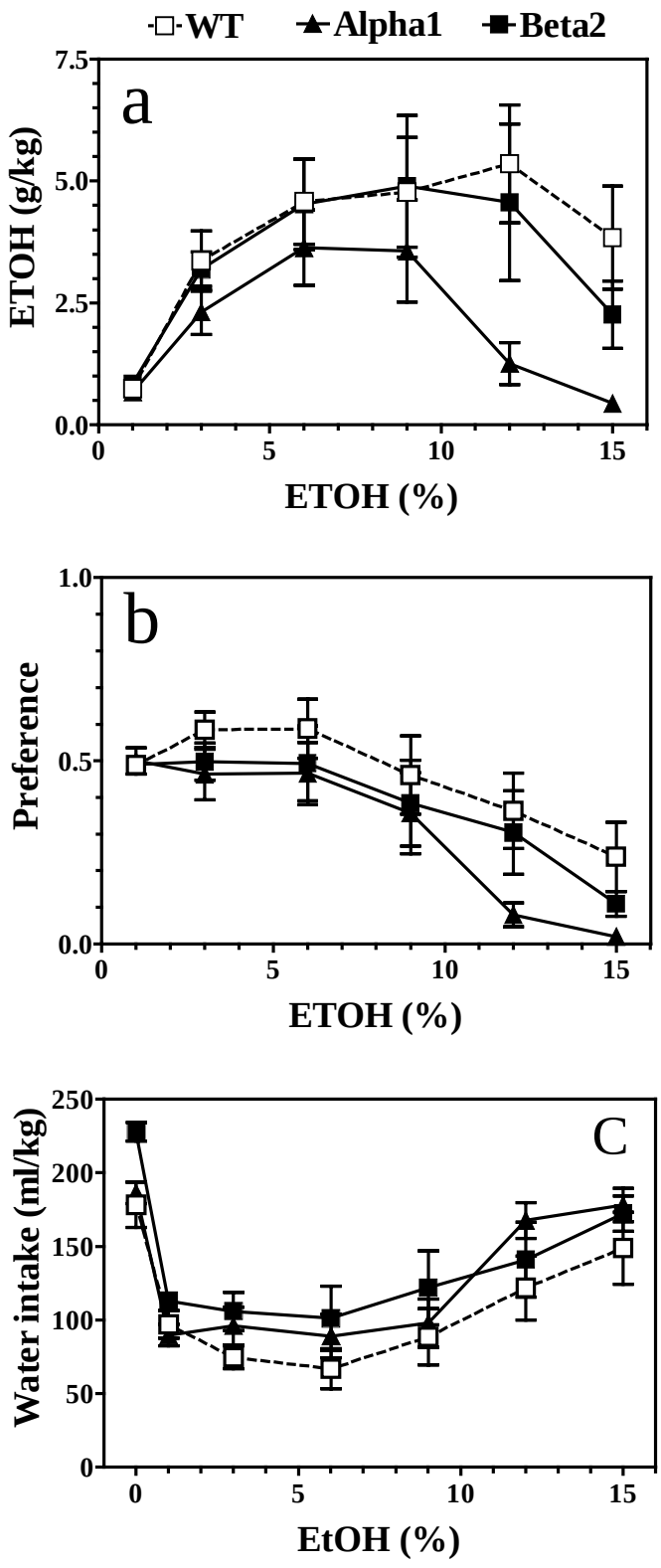

Figure 1

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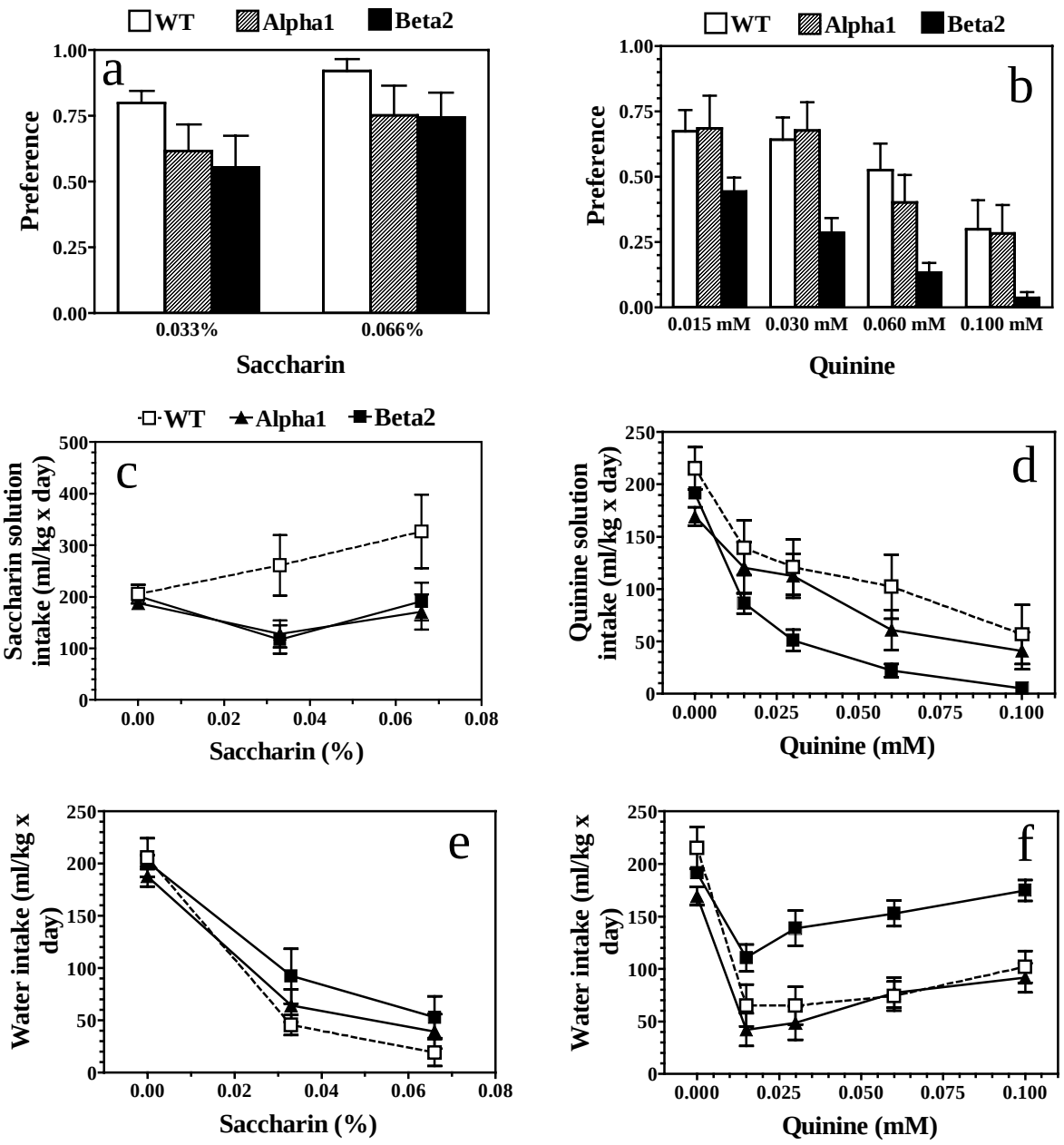

Figure 2

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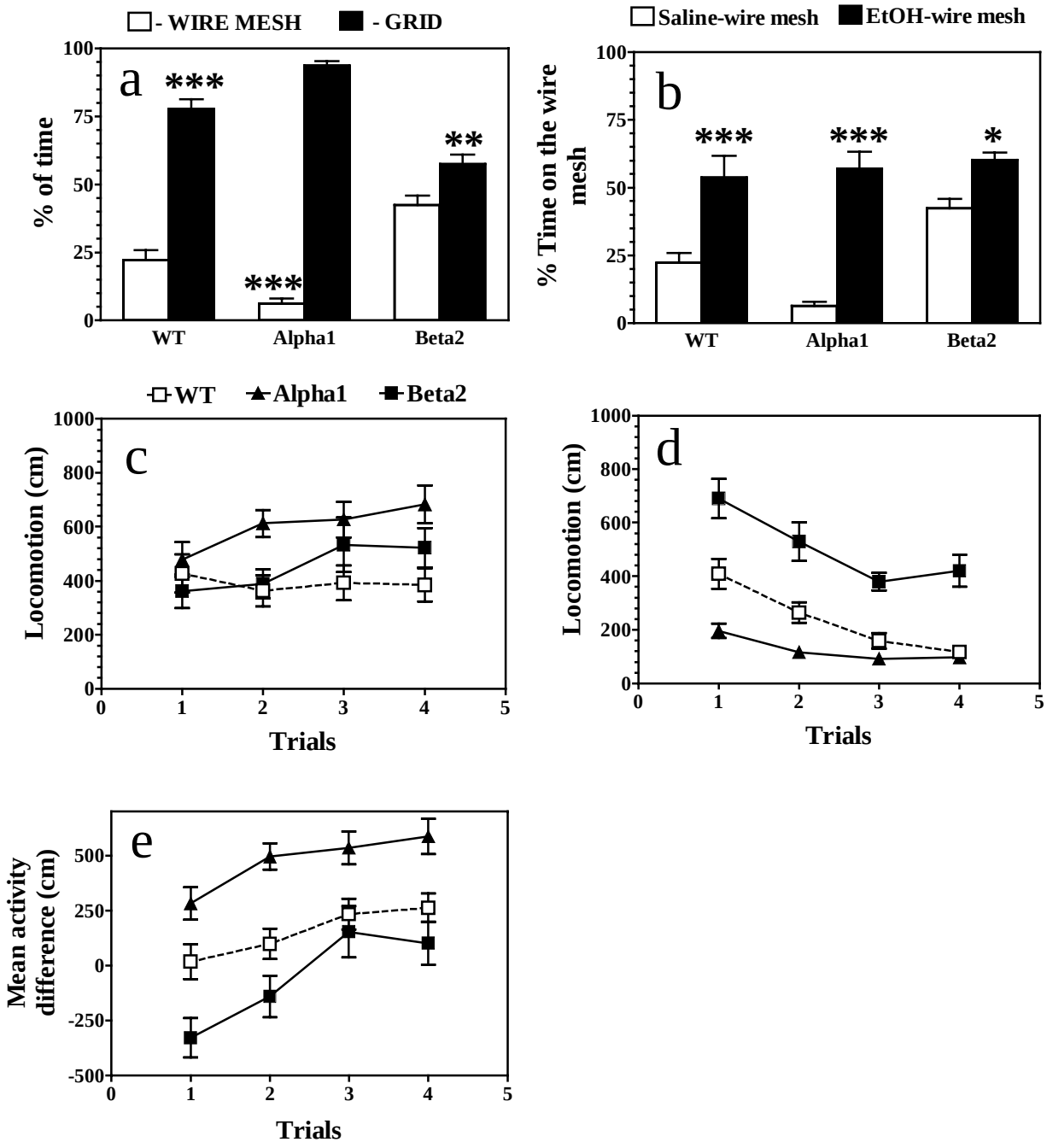

Figure 3

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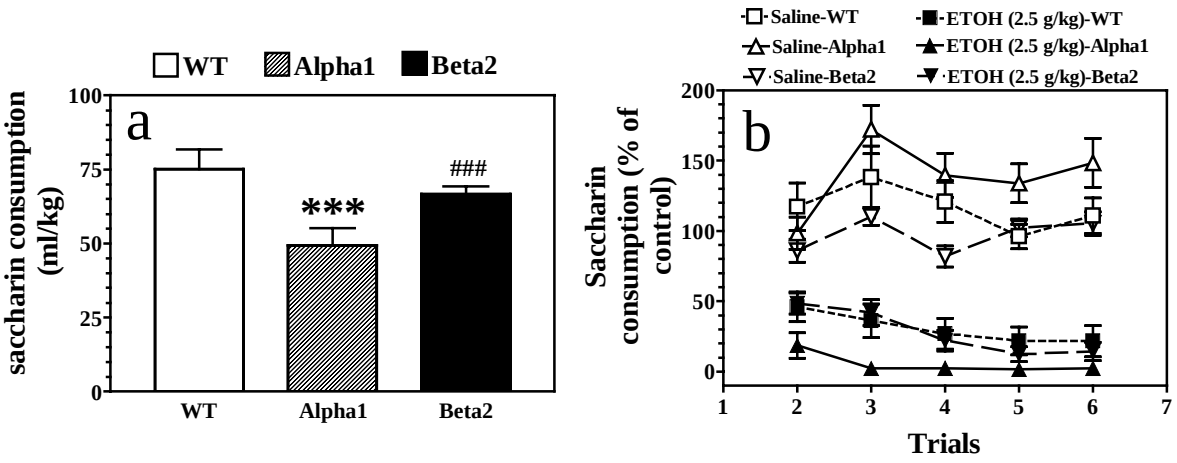

Figure 4

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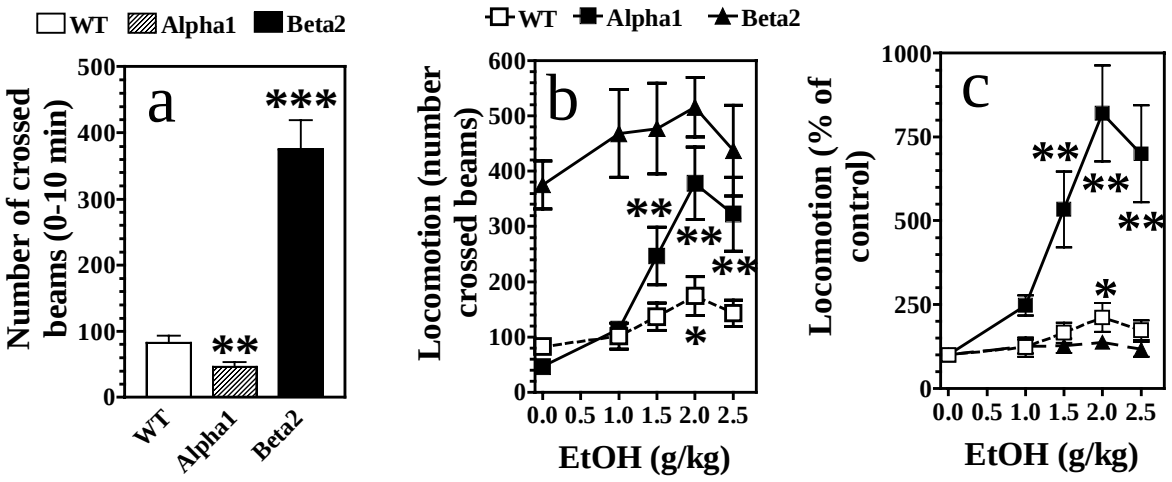

Figure 5

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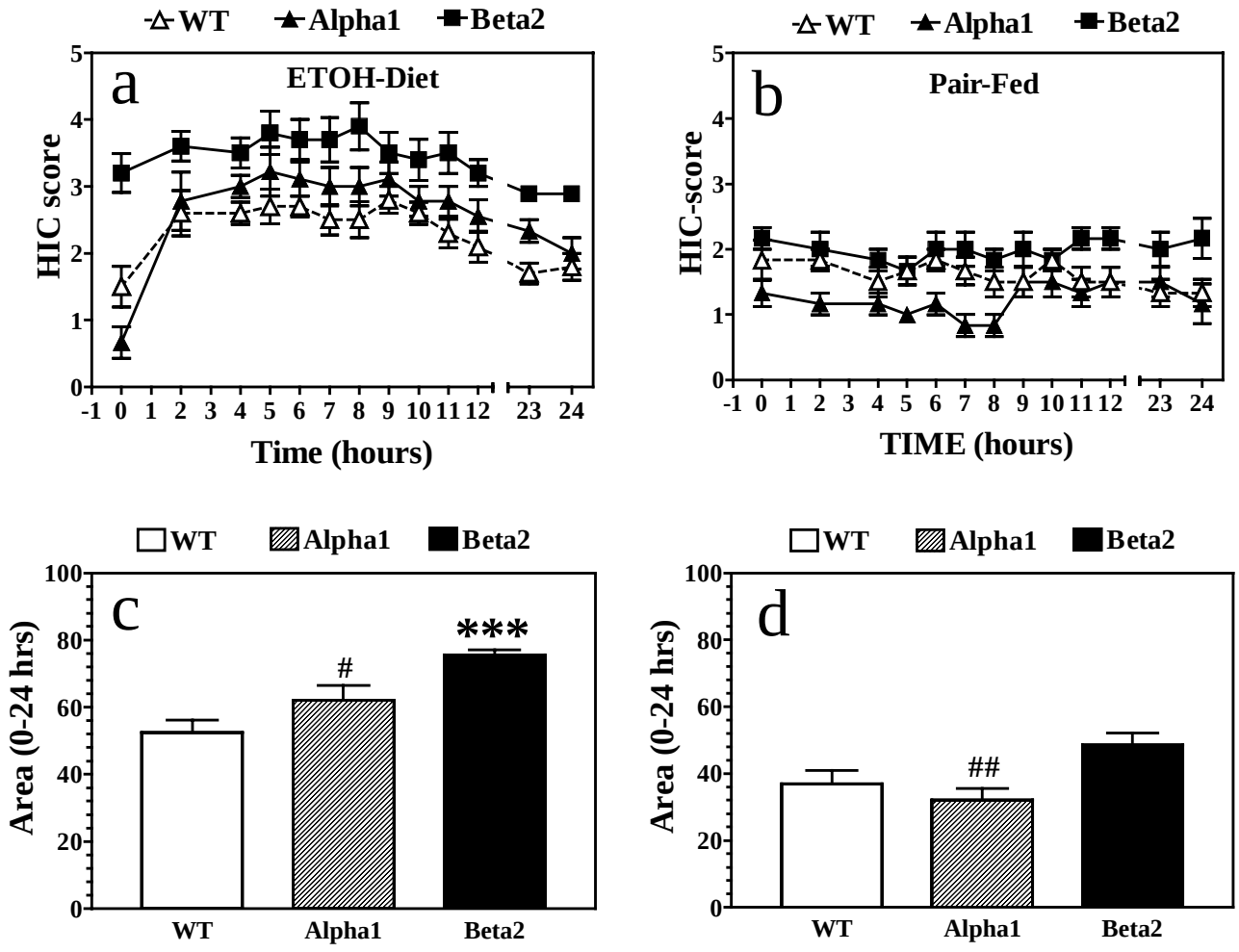

Figure 6

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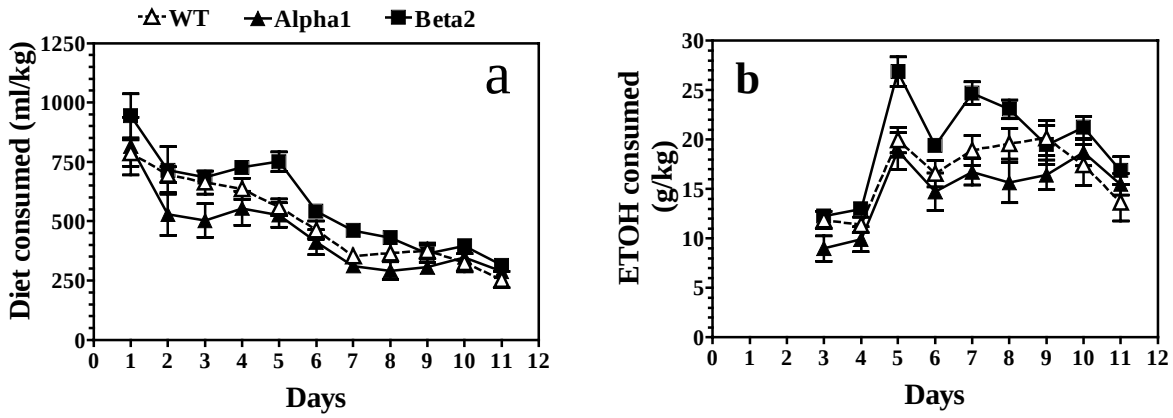

Figure 7