

Etomidate and propofol-hyposensitive GABA_A receptor $\beta 3(N265M)$ mice show little changes in acute alcohol sensitivity but enhanced tolerance and withdrawal

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Abstract

Gamma-aminobutyric acid-A (GABA_A) receptors are ligand-gated ion channels comprised of subunits from several classes (alpha, beta, gamma, delta). Recent studies have clearly demonstrated that the functional properties of GABA_A receptors are altered following chronic ethanol administration that could provide the molecular basis for the previously proposed role of these receptors in ethanol tolerance and dependence. Because the subunit composition of GABA_A receptors determines receptor pharmacology, the present study was devoted to assess if the behavioral sensitivity after acute and chronic ethanol exposure depends on $\beta 3$ -containing GABA_A receptors. In the present study, we used knock-in mice harboring a point mutation (N265M) in the second transmembrane region of the $\beta 3$ subunit of the GABA_A receptor in order to study acute and chronic behavioral effects of ethanol. More specifically, we tested tolerance to loss of righting reflex (LORR) and the development of withdrawal signs after chronic ethanol exposure using ethanol vapor chambers. Our results show that the $\beta 3(N265M)$ mutation does not play a major modulatory role of acute ethanol-induced LORR. However, following repeated LORR testing, enhanced tolerance to the intoxicating effects of ethanol was observed—a finding which was unrelated to the pharmacokinetics of ethanol as both genotypes had the same blood alcohol concentrations following repeated LORR testing. In addition, following chronic alcohol vapor exposure, mouse mutants displayed increased handling-induced convulsions during withdrawal. The results of the present study suggest that the alcohol effects abolished by the $\beta 3(N265M)$ mutation do not play a dominant role in acute alcohol intoxication but influence ethanol tolerance and withdrawal.

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On the behavioral level, GABA_A receptors have been implicated in mediating acute ethanol effects such as motor impairment [17]. Development of tolerance and expression of withdrawal symptoms associated with chronic alcohol exposure is also influenced by the activity of GABA_A receptors [4,5]. On the molecular level, acute ethanol enhances GABA_A-mediated inhibition, whereas following chronic exposure inhibition is reduced

[6,10]. However, ethanol's action on GABA_A receptor activity strongly depends on the species, brain area or cell type studied, suggesting that the GABA_A receptor subunit composition is crucial in this respect [9]. Several subunits have been identified with the majority of GABA_A receptors being composed of α , β , γ , and δ subunits forming a pentameric ligand-gated ion channel [1]. While most types of GABA_A receptors display responses to ethanol only at high concentrations (>60 mM) *in vitro*, it has been found that low concentrations (1–3 mM) of ethanol increase GABA-induced chloride channels in $\alpha 4\beta 2\delta$ receptors [15]. Indeed, there is a general agreement that the tonic GABA currents mediated by $\alpha 4\beta \delta$ and $\alpha 6\beta \delta$ GABA_A receptors are uniquely sensitive to low ethanol concentrations [18]. These effects produced by low ethanol doses on those δ subunit-containing GABA_A receptors seem to be specifically related to the presence of the $\beta 3$ subunit as, in $\alpha 4\beta \delta$ subunit

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combinations, receptors containing the $\beta 3$ subunit have been found to be almost 10 times more sensitive than receptors containing the $\beta 2$ subunit [16]. These findings on the molecular level do not match perfectly well with the behavioral phenotype, as mouse models in which either the $\beta 3$ or δ subunit was genetically deleted failed to exhibit dramatic changes in sensitivity to the depressant effects of alcohol, although they did show alterations regarding other ethanol-induced effects [2,11,12]. However, the conclusions that may be drawn from $\beta 3$ knockout mice are rather limited, as these animals display epilepsy, cleft palate, and hyper-sensitive behavior, leading to the death of approximately 90% of the homozygotes [2,7]. We have recently generated a knock-in mouse model harboring a point mutation in the asparagine-265 residue located in the transmembrane domain 2 of the GABA_A receptor $\beta 3$ subunit (N265M). These mice do not show the physiological or behavioral alterations that characterize $\beta 3$ knockout mice but are equally less sensitive to the action of the intravenous anesthetics etomidate and propofol [8]. Furthermore, previous work has demonstrated that the $\beta 3$ (N265M) mutation abolishes the effects of high ethanol concentrations on the activity of recombinant GABA_A receptors [12,16], thus suggesting that mice bearing this mutation might be less sensitive to the effects of high ethanol doses. Thus, $\beta 3$ (N265M) mice provide a unique model to further test the hypothesis of a critical role for $\beta 3$ subunits in mediating acute and chronic effects of ethanol. Therefore, based on these premises, we designed the present study in order to assess the initial sensitivity and the development of tolerance to ethanol-induced loss of righting reflex (LORR).

The generation of $\beta 3$ (N265M) mice has been recently described by us in detail [8]. Twenty-four age-matched male homozygous mutants and 32 wild-type mice of a 129/Sv \times 129/SvJ (statistically 12.5%/87.5%) background were used in the present study. All animals were singly housed in standard hanging cages at 21 ± 1 °C and $50 \pm 5\%$ relative humidity on a 12-h light/dark cycle with lights on at 7 a.m. Animals were provided with standard rodent food and tap water *ad libitum*. All experimental procedures were approved by the Committee on Animal Care and Use, and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Mice were IP injected with appropriate volumes of a 20% (v/v) ethanol solution so as to attain doses of 3.5, 4.0 or 4.5 g/kg ($n = 8$ –14 per group). Immediately after injection, the mice were placed in a plastic cage devoid of nesting material. A mouse was considered to have had lost the righting reflex, when having stopped moving for more than 30 s in this “waiting cage”. Ten minutes later, the mice were placed on their backs. LORR duration was calculated as the time elapsed until the mice were able to right themselves three times within a minute. LORR tolerance was assessed by re-testing the same mice under identical conditions 24 h later.

$\beta 3$ (N265M) and wild-type mice were chronically exposed to ethanol using alcohol vapor chambers (La Jolla Alcohol Research Inc.; La Jolla, CA, USA). Ethanol exposure conditions were set according to the standard operating procedure for chronic ethanol exposure via inhalation route and dependence

testing recommended by the Integrative Neuroscience Initiative in Alcoholism (INIA). Thus, mice were initially IP challenged with a 1.5 g/kg ethanol dose and then housed ($n = 2$ per chamber) in four vapor chambers for 72 h. Ethanol vapor was delivered to the chambers at a rate of 5 l/min, which maintains the ethanol concentration at 16–18 mg/l-air in the chambers. We did not use pyrazole injections because our own pilot studies showed that they were not needed to ensure constant blood alcohol levels of 150 mg/dl (32 mM). Six hours after the cessation of ethanol exposure, handling-induced convulsions were evaluated using a four-point qualitative scale by an observer blind to the subjects' genotype [19]. Also in agreement with our pilot studies, control (air exposed) mice ($n = 2$ per chamber) during 72 h showed no handling-induced convulsions (data not shown).

Immediately after righting reflex recovery, a blood sample (15 μ l) was taken from the peri-orbital sinus of each mouse injected with the 4.5 g/kg. BALs were spectrophotometrically determined by using a commercially available kit (Ethanol FS; DiaSys International, Cologne, Germany). BALs during ethanol vapor chamber exposure were assessed daily in two randomly chosen mice (one per genotype).

All data are presented as means \pm the standard error of the mean (S.E.M.) and a significance level of $p \leq 0.05$ was used throughout this study. BALs, LORR duration and development of tolerance to the ethanol effect were analyzed by means of two- or three-way ANOVAs, with a repeated measures factor, when necessary. Tukey H.S.D. tests for unequally sized samples were used for post-hoc comparisons. Pearson's index was used for correlation analysis between BALs and LORR duration. Because withdrawal signs were scored using a qualitative ordinal scale, data were analyzed by means of a Mann–Whitney *U*-test.

In the LORR/tolerance experiment, a three-way ANOVA (factors: genotype \times ethanol dose \times days) did not yield a significant three-way interaction but a significant effect of the factors ethanol dose and days [$F(2,66) = 38.96$, $p < 0.001$ and $F(1, 66) = 19.53$, $p < 0.001$; respectively] as well as a significant interaction of the factors genotype and days [$F(1, 66) = 5.11$, $p < 0.05$] were observed. Thus, in the first acute test (test 1), a significant increase in the duration of LORR was observed across the applied dose range with no differences between genotypes (Fig. 1A). This dose-effect relationship was also observed in test 2, however, LORR duration was significantly reduced in $\beta 3$ (N265M) mutants but not in control mice (Fig. 1A). Thus, based on the significant interaction between the genotype and days factors, it can be concluded that both genotypes differed in the development of tolerance to ethanol-induced LORR ($p < 0.05$). Indeed, as revealed by Tukey HSD, $\beta 3$ (N265M) mutants ($p < 0.001$), but not control mice ($p > 0.26$), displayed a shorter LORR duration on day 2. Further, and although the three-way interaction did not yield a significant effect, the development of tolerance was not independent of the ethanol dose. Thus, a series of Student's *t* tests comparing the reduction of LORR duration between tests (tests 2 and 1) on both genotypes revealed that the higher the ethanol dose, the higher the significance of these differences (data not shown). On the other hand, the shorter duration of LORR in $\beta 3$ (N265M) mutants as

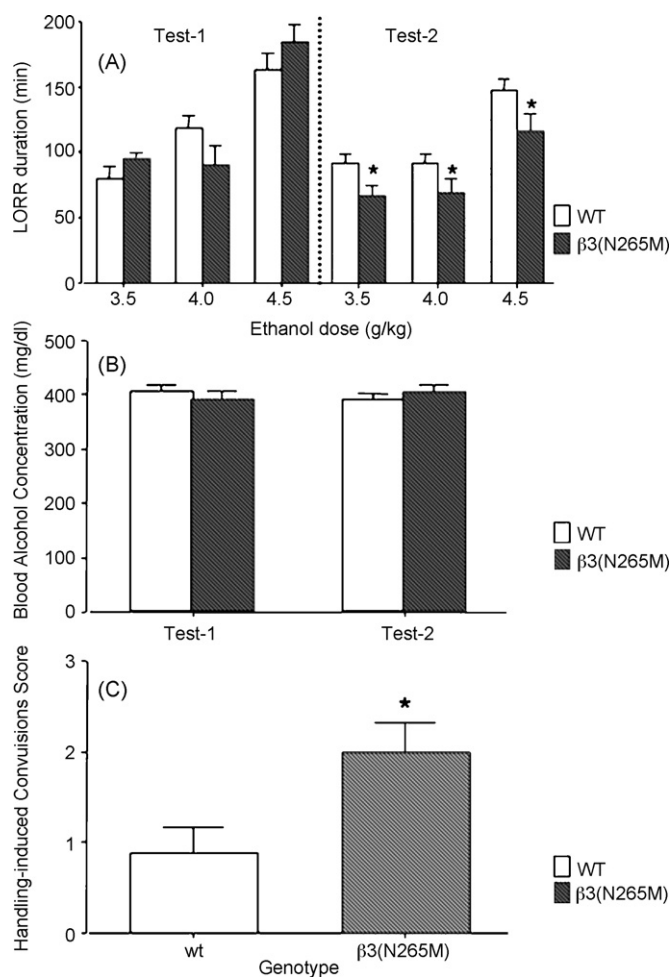


Fig. 1. Ethanol tolerance and withdrawal but not initial sensitivity to ethanol-induced LORR is enhanced in GABA_A receptor $\beta 3(N265M)$ mutant mice. (A) The duration of LORR in minutes is shown following IP administration of high intoxicating ethanol doses. A genotype difference is observed in test 2, a fact unrelated to alcohol pharmacokinetics (B). Handling-induced convulsions after chronic ethanol vapor exposure were also enhanced in $\beta 3(N265M)$ mutant mice (C), * $p < 0.05$. All values are given as the mean of $n = 8-14 + S.E.M.$

compared to control mice does not seem to depend on pharmacokinetic differences as BALs were identical during testing and did not differ between genotypes (Fig. 1B). Indeed, a two-way ANOVA (day \times genotype) failed to yield any significant effect and no significant correlation between BALs and LORR duration was found. Finally, a Mann–Whitney U -test revealed that $\beta 3(N265M)$ mutants showed more intense ethanol withdrawal signs (handling-induced convulsions) than control mice [$U = 12.5$, $p < 0.05$]. These results are shown in Fig. 1C.

$\beta 3$ GABA_A receptor subunit expression is especially prominent in the olfactory bulb, the cortex, the hypothalamus and the amygdala, but it is its strong expression in the cerebellum that points to a role of this subunit in mediating some forms of behavioral sensitivity to alcohol [2]. However, here we show that high intoxicating doses of ethanol leading to LORR do not produce altered behavioral responses in knock-in mice harboring a single point mutation in a specific residue (N265) of the $\beta 3$ subunit of the GABA_A receptor when compared to wild-type littermate control mice. This observation is in agreement with a previous

study by Quinlan et al. [13] reporting that $\beta 3$ knockout mice show no alterations in the duration of LORR following alcohol or enflurane administration but exhibit a reduced LORR duration after midazolam or etomidate administration. This pattern of results clearly parallels those obtained in $\beta 3(N265M)$ mutants [8], which show reduced LORR duration in response to propofol and etomidate but not to enflurane or, as shown in the present study, to ethanol administration. Therefore, it seems that a single point mutation (N265M) in the $\beta 3$ subunit of the GABA_A receptor results in a very similar functional outcome than a global $\beta 3$ knockout, suggesting that $\beta 3$ -containing GABA_A receptors do not influence the ability of a single ethanol injection to produce LORR. These results are not unexpected, since recent studies have shown that there are two alcohol sites associated to highly alcohol sensitive δ -containing GABA_A receptors and that mutations at the transmembrane domains of this receptor subunit (i.e. the $\beta 3(N265M)$ mutation) would only affect the “high dose” (>100 mM) site [16], which seems to be activated on response to ethanol concentrations higher than those required to produce LORR. Therefore, our results support the notion that this subunit may play a minor role in acute alcohol intoxication. Additional studies are needed to determine, if the same conclusions might be extended to mutations at other β (i.e. $\beta 1$ and $\beta 2$) GABA_A receptor subunits.

In contrast to the acute alcohol effects, some of the behavioral changes observed after repeated/chronic alcohol exposure do seem affected by mutations at the $\beta 3$ subunit. More specifically, we observed that $\beta 3(N265M)$ mutants do not differ from control mice in the duration of LORR after an acute ethanol injection, irrespective of the alcohol dose administered. In ‘test 2’ of LORR, $\beta 3(N265M)$ mutants displayed significantly shorter latencies in regaining the righting reflex, when retested 24 h later with an identical ethanol dose. This effect was not observed in control mice, a fact that is in agreement with our previous pilot studies using non-mutant mice that often require a larger number of alcohol injections to produce a significant reduction in LORR duration. This reduction of LORR duration in $\beta 3(N265M)$ mutants does not seem to be related to any pharmacokinetic difference, as similar BALs were observed after each LORR test and no differences between genotypes were seen. These results suggest that the $\beta 3$ subunit facilitates some of the neuroadaptive changes that result in behavioral tolerance to this particular effect of ethanol. Another set of results from our study seems compatible with this conclusion. Thus, in the present study it was also observed that the sudden interruption of sustained ethanol exposure produced more intense withdrawal signs (i.e. handling-induced convulsions) in $\beta 3(N265M)$ mutants as compared to control mice. These results seem to point again to a higher sensitivity of the functional outcome of chronic alcohol exposure in $\beta 3(N265M)$ mutant mice. Therefore, it is plausible that the N265M mutation in the $\beta 3$ subunit facilitates some of the neural adaptations resulting from the alcohol administration and further enhances the effects of subsequent alcohol re-exposures (i.e. tolerance) and the consequences of abrupt alcohol removal (withdrawal signs).

Our findings seem to be in agreement with other studies assessing the possible subunit dependency of the functional

outcomes associated with alcohol tolerance and withdrawal. Thus, chronic alcohol exposure results in a significant reduction of the mRNA encoding for the $\beta 3$ GABA_A receptor subunit and this reduction is associated with changes of GABA_A receptor mediated Cl⁻ currents related to both chronic alcohol exposure and withdrawal [14]. In addition, Buckley et al. [3] have recently reported that there is a trend towards lower $\beta 3$ protein levels in human alcoholic brain, whereas the $\beta 2$ subunit shows a trend to higher protein levels, while total β subunit protein levels remain roughly the same. This observation suggests that alcohol acts predominantly on the $\beta 3$ -containing GABA_A receptors, which get downregulated after chronic alcohol exposure. This fact seems in line with our findings that the N265M mutation in the $\beta 3$ subunit facilitates some of the neuroadaptive changes induced by chronic alcohol exposure.

The results of the present investigation suggest a modulatory role for $\beta 3$ -containing GABA_A receptors in alcohol tolerance and withdrawal. Thus, the N265M mutation in the $\beta 3$ subunit facilitates some of the neuroadaptive changes involved in the development of alcohol tolerance and withdrawal. However, the alcohol effects abolished by the $\beta 3$ (N265M) mutation do not seem to play a dominant role in acute alcohol intoxication.

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