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5-HT_{3A} Receptor Subunit is Required for 5-HT_3 Antagonist-Induced Reductions in Alcohol Drinking

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The ionotropic serotonin subtype-3 (5-HT_3) receptor has emerged as a potential therapeutic target in the treatment of alcohol abuse and alcoholism because selective pharmacological antagonists reduce alcohol consumption in preclinical and clinical models. 5-HT binds to the extracellular N-terminus of the 5-HT_{3A} receptor subunit but receptor activation is also enhanced by distinct allosteric sites, which indicates the presence of other receptor subunits. It is not known if specific molecular subunits of the 5-HT_3 receptor modulate alcohol drinking. To address this issue, we characterized acute locomotor response to alcohol and alcohol consumption in a two-bottle home-cage procedure by congenic C57BL/6J mice with a targeted deletion of the 5-HT_{3A} receptor subunit gene. 5-HT_{3A}-null mice did not differ from wild-type littermate controls on measures of spontaneous locomotor activity, habituation to a novel environment, or locomotor response to ethanol (0, 0.5, 1, or 2 g/kg). Moreover, null mice did not differ from controls on measures of ethanol (2–10%) intake and preference during or after a two-bottle home-cage sucrose fading procedure. Systemic administration of the 5-HT_3 antagonist LY-278,584 (0–10 mg/kg) decreased intake of both sweetened (2% sucrose + 10% ethanol) and unsweetened (10% ethanol) ethanol in wild-type mice only. These findings indicate that reduction of alcohol drinking produced by 5-HT_3 antagonism is dependent on the presence of 5-HT_{3A}-containing receptors.
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INTRODUCTION

Serotonin (5-HT) receptors are involved in a variety of mammalian biochemical, physiological, and behavioral processes (Barnes and Sharp, 1999). 5-HT receptors are classified into seven groups (5-HT_{1-7}), comprising a total of at least 14 structurally and pharmacologically distinct receptor subtypes (Hoyer *et al*, 1994). The 5-HT_3 receptor is unique among this G-protein-coupled receptor family as the only ionotropic receptor (Derkach *et al*, 1989; Maricq *et al*, 1991). Electrophysiologically, 5-HT mediates rapid excitatory responses through ionotropic 5-HT_3 receptors (Derkach *et al*, 1989) and activation of this channel results in depolarization responses and subsequent desensitization (Lambert *et al*, 1989; Yakel and Jackson, 1988).

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The 5-HT_3 receptor is comprised of five coassembled subunits that surround a centrally gated channel (Boess *et al*, 1995). The 5-HT_{3A} subunit has been cloned (Maricq *et al*, 1991) and shown to be distributed peripherally and centrally in brain regions including the hippocampus, amygdala, and cortex (Tecott *et al*, 1993). 5-HT binds to the extracellular N-terminus of the 5-HT_{3A} subunit (Eisele *et al*, 1993), but receptor activation can be enhanced pharmacologically at allosteric sites (Lovinger and Zhou, 1993). A novel molecular subunit (eg 5-HT_{3B}) was recently found to be coexpressed with the 5-HT_{3A} subunit in human amygdala, caudate, and hippocampus (Davies *et al*, 1999) and heteromeric assemblies of human 5-HT_{3A} and 5-HT_{3B} subunits show channel conductance, calcium permeability, and current–voltage properties that closely resemble native neuronal 5-HT_3 channels (Davies *et al*, 1999). However, in rats 5-HT_{3B} subunit transcripts are not found in brain (Morales and Wang, 2002), which suggests that rodent neural 5-HT_3 receptors might be 5-HT_{3A} homomeric receptors or heteromeric receptors containing 5-HT_{3A} subunits combined with subunits other than the 5-HT_{3B} subunit (Fletcher and Barnes, 1998).

Ethanol alters the function of 5-HT$_3$ receptors (Barann *et al*, 1995; Jenkins *et al*, 1996; Lovinger *et al*, 2000; Lovinger and Zhou, 1993; Lovinger and Zhou, 1994; Lovinger and Zhou, 1998) with the most consistent finding being potentiation of receptor function by ethanol (Lovinger, 1999). Biochemically, infusion of the 5-HT$_3$ antagonist tropisetron in the nucleus accumbens decreases elevation of extracellular dopamine produced by local application of ethanol (Yoshimoto *et al*, 1992). Accordingly, a number of studies have shown that systemic administration of 5-HT$_3$ antagonists decreases voluntary alcohol drinking by rats (Fadda *et al*, 1991; Knapp and Pohorecky, 1992; Kostowski *et al*, 1993; Tomkins *et al*, 1995) and mice (Tomkins *et al*, 1995), and reduce operant ethanol self-administration by rats (Hodge *et al*, 1993). Site-specific injection of 5-HT$_3$ antagonists in the nucleus accumbens also decreases alcohol consumption (Jankowska and Kostowski, 1995). In humans, the 5-HT$_3$ antagonist ondansetron reduces alcohol-induced craving in social drinkers (Johnson *et al*, 1993) and increases abstinence among alcoholics with a biological predisposition (Johnson *et al*, 2000).

Since neural 5-HT$_3$ receptors might be homomeric or heteromeric receptors containing 5-HT$_{3A}$ subunits and 5-HT$_3$ antagonists show equal affinity for 5-HT$_{3A}$ and 5-HT$_{3A/B}$ receptors (Brady *et al*, 2001), the functional significance of specific 5-HT$_3$ receptor subunits in alcohol drinking has not been determined. To address this issue, we studied voluntary alcohol drinking both during and after a sucrose fading procedure in mice with a targeted deletion of the 5-HT$_{3A}$ receptor subunit (Kelley *et al*, 2003; Zeitz *et al*, 2002b). Moreover, to determine if the 5-HT$_3$ antagonists reduce alcohol drinking by inhibiting 3A containing receptors, we tested the effects of the 5-HT$_3$ antagonist LY-278,584 on alcohol drinking by 5-HT$_{3A}$-null mice and controls.

METHODS

Mice

5-HT$_{3A}$ receptor-null mice were derived by homologous recombination as previously reported (Zeitz *et al*, 2002a). F1 hybrid C57BL/6J × 129vJ heterozygous progeny were backcrossed to C57BL/6J mice to produce F9 generation congenics. Heterozygotes from the F9 generation were bred to generate male wild-type and 5-HT$_{3A}$-null mutants used in the present study. Genotyping was conducted in Dr David Julius' laboratory at the University of California San Francisco as described in Zeitz *et al* (2002a). All experiments were conducted in male mice. The experimenter conducting behavioral studies was blind to genotype. The mice were housed in plastic cages lined with Cell Sorb bedding and provided with food (Harlan, Indianapolis, IN) and water *ad libitum*. The vivarium was maintained on a 12 h light/dark cycle (lights on at 06 : 00) at a temperature of 22°C. All procedures were carried out in accordance with the *NIH Guide to Care and Use of Laboratory Animals* and institutional guidelines.

Locomotor Activity

Spontaneous locomotor activity and habituation of naive 5-HT$_{3A}$ (−/−, $n=16$) and 5-HT$_{3A}$ (+/+, $n=16$) mice was measured in Plexiglas chambers (43 cm^2) located in sound-attenuating cubicles equipped with exhaust fans that masked external noise (Med Associates, St Albans, VT). Two sets of 16 pulse-modulated infrared photobeams were placed on opposite walls at 1-in centers to record *x–y* ambulatory movements. Activity chambers were computer interfaced (Med Associates) for data sampling at 100-ms resolution. Mice were handled and weighed daily for 1 week before activity testing. Prior to each session, the activity chamber was wiped clean with 2.5% glacial acetic acid to limit any confounding odors. The mice were placed in the corner of the chamber and left to behave freely for 60 min.

At 1 week after baseline locomotor testing, the mice were used to determine the effects of ethanol on locomotor activity. The same procedure was used to test ethanol-induced locomotor activity except that mice were administered ethanol (0. 0.5, 1, or 2 g/kg, i.p.) in a Latin-Square randomized dose order immediately before the start of activity monitoring. Horizontal distance traveled (cm) was recorded for 60 min after ethanol injection. Tests sessions were separated by 3 days.

Alcohol Drinking

Oral alcohol drinking and preference were examined using a two-bottle choice protocol (Hodge *et al*, 1999). Eight experimentally naïve male 5-HT$_{3A}$ (+/+) and six 5-HT$_{3A}$ (−/−) mice were tested in parallel. Mice were given 1 week to acclimatize to individual housing conditions and handling. During this period, water was the only fluid available. Subsequently, two-bottle drinking sessions were then conducted 24 h per day, 7 days per week, in the home-cage. Ethanol and water solutions were in 50-ml plastic bottles (0.5 ml graduates) equipped with ball-bearing stoppers to limit spillage. The location (left or right side of the cage) of ethanol and water solutions was counter-balanced between animals to control for side preference. Volume was recorded at the beginning and end of access periods. No significant spillage was noted throughout the experiment.

To evaluate potential involvement of the 5-HT$_{3A}$ receptor in the initiation of alcohol drinking, mice were given access to a series of sweetened ethanol solutions *vs* water (eg Samson, 1986). The sucrose (% w/v) and ethanol (% v/v) content of the solutions were as follows: 10S/2E; 10S/5E; 10S/10E; 5S/10E; 2S/10E; 10E. Mice were exposed to each sweetened ethanol solution for 3 days and 10% ethanol at the end for a 6-day period.

After evaluating initiation of ethanol intake, the drinking solutions were returned to 2S/10E *vs* water for a 3-day baseline period. Then, the effects of the 5-HT$_3$ antagonist LY-278,584 (0–1 mg/kg) were evaluated on sweetened ethanol *vs* water intake. After determining the LY-278,584 dose–response curve, mice were re-exposed to 10E *vs* water for a 3-day baseline period. The effects of LY-278,584 were then redetermined on unsweetened ethanol *vs* water intake. Drug dosing was conducted in a randomized Latin-Square manner. Injections occurred immediately prior to 24-h drinking sessions no more than twice per week. The day immediately prior to saline vehicle treatment was used as a noninjection control in each experiment.

RESULTS

Locomotor Activity

To determine if any overt motor changes were produced by the targeted mutation, we first tested 5-HT$_{3A}$-null mice and wild-type littermates in a novel open-field environment for 1 h. The null mutation produced no effect on spontaneous locomotor activity (Figure 1a). Moreover, locomotor activity by both genotypes decreased as a function of time in the open-field environment ($F_{time}(5,150) = 13.55$, $p < 0.001$), which indicates normal habituation to the environment. This finding is consistent with our previous report (Kelley *et al*, 2003) and supports pharmacological evidence, which indicates that 5-HT$_3$ receptor antagonists have no effects on locomotor behavior (Jones *et al*, 1988).

The effects of ethanol on locomotor activity of the 5-HT$_{3A}$-null mice and wild-type littermates are illustrated in Figure 1b. Ethanol significantly reduced locomotor activity (F_{dose} $(3,90) = 5.08$, $p = 0.003$), which was due to reduced activity at the 2 g/kg ethanol dose as compared to the 1 g/kg dose (Tukey, $p = 0.001$). However, the ethanol-induced activity of the two genotypes did not differ. Further, planned comparisons revealed that locomotor activity after ethanol injections did not differ from the saline injection for the 5-HT$_{3A}$-null mice and wild-type littermates. These findings are consistent with pharmacological evidence showing no effect of 5-HT$_3$ receptor antagonists on ethanol-induced locomotor activity (Le *et al*, 1997), and the absence of ethanol-induced locomotor stimulation in C57BL/6J mice (Liljequist and Ossowska, 1994).

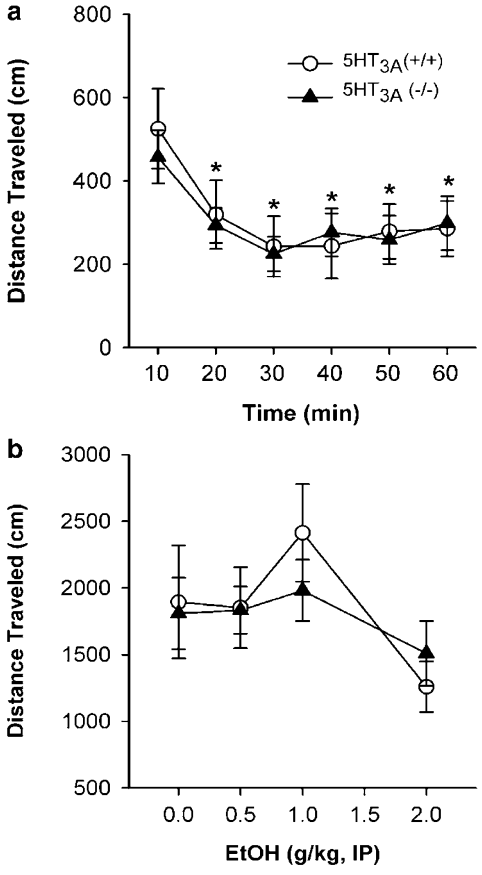

Figure 1 Deletion of the 5-HT$_{3A}$ receptor subunit produced no significant effects on motor activity or locomotor response to ethanol. (a) Spontaneous locomotor activity and habituation to a novel environment. Both null mice (−/−, $n = 16$) and wild-type controls (+/+, $n = 16$) showed normal levels of locomotor activity that decreased as a function of time in the environment. Horizontal distance traveled (cm ± standard error) was averaged for each 10 min of a 60 min exposure. (b) Locomotor response to acute ethanol administration. All data are expressed as mean (±SEM). * – indicates significantly different from initial 10-min period irrespective of genotype, $p < 0.05$, Tukey test.

Alcohol Drinking

When examined through the course of a sucrose fading procedure (eg Samson, 1986) conducted during daily 24-h periods in the home cage 5-HT$_{3A}$ receptor-null mice did not differ from wild-type controls on measures of ethanol intake or preference (Figure 2a and 2b). Repeated measures ANOVA showed that intake and preference decreased as a function of reduced sucrose and increased ethanol concentration in both genotypes (F_{dose} $(6,72) = 45.57$, $p < 0.001$). Although ethanol intake was relatively low for C57BL/6J mice (Middaugh *et al*, 1999), no differences were observed in ethanol, water, or total fluid intake between genotypes. These data indicate that deletion of the 5-HT$_{3A}$ receptor subunit does not influence the acquisition or

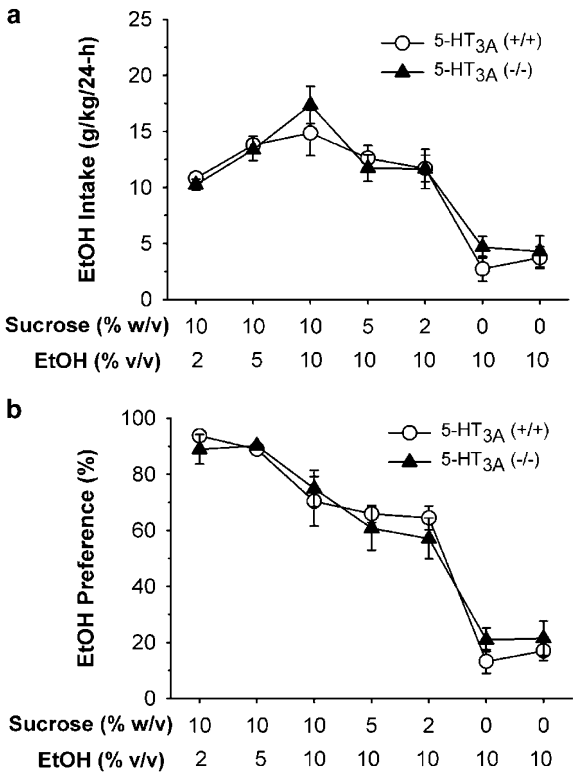

Figure 2 Initiation and maintenance of alcohol drinking by mice lacking the 5-HT$_{3A}$ receptor gene. (a) Null mice (−/−, $n = 6$) consumed the same amount of sweetened and unsweetened alcohol in a home-cage two-bottle preference test as wild-type mice (+/+, $n = 8$). (b) The null mutation had no effect on ethanol preference expressed as a percentage of total fluid intake (ethanol mls plus water mls). Each animal's alcohol intake (g/kg/24-h) or preference data were averaged over a 3-day exposure period at each ethanol/sucrose combination and then plotted as a group mean (±SEM).

maintenance of alcohol drinking under these experimental conditions.

Figure 3a shows the effects of the 5-HT$_3$ antagonist LY-278,584 on sweetened ethanol (2% sucrose + 10% EtOH) intake. LY-278,584 dose dependently decreased intake of sweetened ethanol (F_{dose} (3,33) = 4.6, $p = 0.009$), which was primarily due to a significant difference between the no-injection control and 10 mg/kg dose of LY-278,584 (Tukey, $p = 0.001$). There was no genotypic difference or interaction between LY-278,584 and genotype. Planned comparisons within the wild-type group showed that ethanol intake following no-injection differed from the 1 and 10 mg/kg doses of LY-278,584 ($p = 0.009$ and 0.01, respectively), whereas intake after saline differed only from the 10 mg/kg dose ($p = 0.013$) (Figure 3a). LY-278,584 produced no significant effects on alcohol drinking by 5-HT$_{3A}$-null mice. There was also a main effect of dose of LY-278,584 on preference for sweetened ethanol relative to water (F_{dose} (3,33) = 4.7, $p = 0.008$), which was attributable to a significant reduction in preference only in the wild-type group (Table 1). There was no effect of genotype, or a genotype by dose interaction, on sweetened ethanol preference. No differences were observed in water, or total fluid, intake.

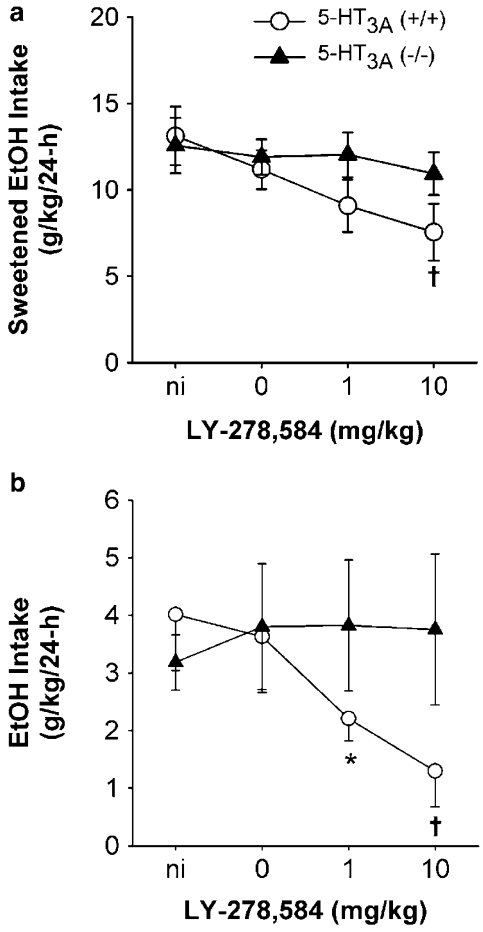

Figure 3 The 5-HT$_3$ antagonist LY-278,584 significantly decreased intake (g/kg/24 h) of sweetened (2S/10E) (a) or unsweetened (10E) (b) alcohol in a 24-h home-cage two-bottle test in 5-HT$_{3A}$ wild-type mice (+/+, $n = 8$). Data represent mean (±SEM) during a single 24-h period following i.p. administration of LY-278,584. Variability in ethanol intake by 5-HT$_{3A}$-null mice (−/−, $n = 6$) was due to a single outlier (b). Note the different *y*-axis scales on the two panels. * – indicates significantly different from no-injection control within genotype; † – indicates significantly different from both no-injection and saline vehicle control within genotype, $p < 0.05$, Tukey test.

Statistical analysis of unsweetened ethanol intake (g/kg) found no main effect of genotype or LY-278,584 (Figure 3b). However, the effects of LY-278,584 on ethanol intake (g/kg) depended on genotype as evidenced by a significant interaction ($F_{dose \times genotype}$ (3,33) = 2.9, $p = 0.04$). Planned comparisons showed that LY-278,584 (10 mg/kg) significantly decreased ethanol intake as compared to no-injection and saline controls ($p = 0.009$ and 0.01, respectively) in wild-type mice only (Figure 3b). Ethanol intake by 5-HT$_{3A}$-null mice was unchanged by any dose of the antagonist (Figure 3b). Thus, the significant interaction was due to antagonist-induced decreases in ethanol intake only within the wild-type group. RM ANOVA showed a trend toward a significant decrease in preference for unsweetened ethanol produced by LY-278,584 (F_{dose} (3,33) = 2.5, $p = 0.07$). There was no effect of genotype, and no interaction among the variables. Planned comparisons across genotype and dose of the 5-HT$_3$ antagonist showed that the 10 mg/kg dose of LY-278,584 significantly decreased preference only in the wild-type group (Table 1). No differences were observed in water, or total fluid, intake.

DISCUSSION

The 5-HT$_3$ receptor has emerged as a potential therapeutic target for the medical management of alcohol abuse and alcoholism because inhibition of the receptor by selective antagonists produces positive effects in preclinical (Costall

Table 1 Effect of LY-278,584 on Preference (mean ± SEM) for Sweetened and Unsweetened Ethanol

		LY-278,584 (mg/kg, i.p.)			
Solution	**Genotype**	**No injection**	**Saline**	**1.0**	**10.0**
2S/10E	5-HT$_{3A}$ (+/+)	0.71 (0.03)	0.74 (0.09)	0.59 (0.06)	0.46 (0.11)[a]
	5-HT$_{3A}$ (−/−)	0.74 (0.08)	0.78 (0.06)	0.70 (0.06)	0.69 (0.07)
10E	5-HT$_{3A}$ (+/+)	0.21 (0.04)	0.20 (0.04)	0.17 (0.009)	0.10 (0.04)[b]
	5-HT$_{3A}$ (−/−)	0.27 (0.06)	0.21 (0.06)	0.23 (0.06)	0.23 (0.08)

[a]significantly different from no-injection and saline controls within 5-HT$_{3A}$ (+/+) group, and from 5-HT$_{3A}$(−/−) at corresponding dose.
[b]significantly different from no-injection control.

et al, 1993) and clinical (Sellers *et al*, 1994) studies. A common finding from preclinical studies is that 5-HT$_3$ antagonists selectively decrease alcohol drinking and reinforcement (Fadda *et al*, 1991; Hodge *et al*, 1993; Jankowska and Kostowski, 1995; Knapp and Pohorecky, 1992; McKinzie *et al*, 1998; Silvestre *et al*, 1998; Tomkins *et al*, 1995), suggesting that alcohol self-administration is at least partly maintained by 5-HT$_3$ receptor activity (but see Beardsley *et al*, 1994).

Ionotropic 5-HT$_3$ receptors are thought to consist of five coassembled subunits that surround a centrally gated channel (Boess *et al*, 1995). The 5-HT$_{3A}$ subunit (Maricq *et al*, 1991) is the only known 5-HT$_3$ subunit that occurs in rodent brain (Morales and Wang, 2002). It is not known, however, if the effects of 5-HT$_3$ antagonists on alcohol drinking are mediated by this subunit or other(s), which may coexpress with the 3A subunit (eg, Fletcher and Barnes, 1998). To address this question, we examined alcohol drinking by mutant mice lacking the 5-HT$_{3A}$ receptor subunit (Zeitz *et al*, 2002a). One of the primary findings of this study is that 5-HT$_{3A}$-null mice did not differ from wild-type controls on measures of voluntary alcohol drinking either during or after a sucrose fading initiation procedure. These data are in contrast with a number of studies showing that 5-HT$_3$ antagonists decrease alcohol drinking and operant self-administration (Hodge *et al*, 1993; Knapp and Pohorecky, 1992).

To determine, therefore, if decreases in alcohol drinking produced by 5-HT$_3$ antagonists might be dependent on the 5-HT$_{3A}$ receptor subunit, we tested the effects of the 5-HT$_3$ antagonist LY-278,584 on intake (g/kg/24-h) of sweetened and unsweetened ethanol by null mice and wild types. LY-278,584 decreased intake of, and preference for, both ethanol solutions only in wild-type mice. This indicates that the 5-HT$_{3A}$ receptor subunit is required for 5-HT$_3$ antagonist-induced reductions in alcohol drinking, and strongly suggests that 5-HT$_3$ antagonist-induced reductions in ethanol drinking (Fadda *et al*, 1991; Hodge *et al*, 1993; Jankowska and Kostowski, 1995; Knapp and Pohorecky, 1992; McKinzie *et al*, 1998; Silvestre *et al*, 1998; Tomkins *et al*, 1995) are likely due to pharmacological blockade of the 5-HT$_3$ receptor subunit. Moreover, these data indicate that other 5-HT$_3$ receptor subunits, which may be expressed in brain (Fletcher and Barnes, 1998; Morales and Wang, 2002; Niesler *et al*, 2003), are not able to form functional channels that are as sensitive to 5-HT$_3$ antagonists as native receptors containing the 3A subunit.

A question that emerges from these data is why deletion of the 5-HT$_{3A}$ receptor had no effect on alcohol drinking even though the 5-HT$_3$ antagonist decreased drinking in wild-type mice. One possible explanation for the lack of agreement between targeted gene deletion and pharmacological inhibition of the 5-HT$_3$ receptor is that developmental compensation occurred in the null mutants, which may have masked any functional involvement of the receptor subunit in alcohol drinking. Compensation may be due to genetic redundancy whereby so-called 'helper' genes can take over the function of the targeted gene (Gerlai, 1996; Gerlai, 2001). Thus, it is possible that the 5-HT$_{3A}$ receptor subunit is an important mediator of alcohol drinking, but compensation by another unknown pathway concealed this involvement. In this regard, gene targeting missed the mark of revealing new information about 5-HT$_3$ receptor regulation of alcohol drinking. Whether new information could be gained by investigating the mechanisms of developmental compensation, such as uncovering novel interactions between the 5-HT$_{3A}$ receptor subunit and other genes, is a matter of debate (eg Gerlai, 2001; Routtenberg, 1995; Routtenberg, 1996).

Compensation, however, may not occur in all phenotypes associated with the targeted gene. For instance, we recently reported that 5-HT$_{3A}$-null mice exhibit less anxiety-like behavior on three validated animal models of anxiety (Kelley *et al*, 2003). This phenotypic profile corresponds with the known anxiolytic properties of pharmacological antagonists of 5-HT$_3$ receptors (eg Costall and Naylor, 1991) and suggests that the 5-HT$_{3A}$ receptor subunit is an important mediator of anxiety. One might conclude, therefore, that deletion of the 5-HT$_{3A}$ receptor subunit did not produce compensatory changes in neurobiological pathways associated with anxiety. Moreover, it seems reasonable to conclude that compensatory changes that might have masked 5-HT$_{3A}$ receptor involvement in alcohol drinking are not associated with anxiety-like behavior.

A final remark regarding potential compensatory factors merits discussion. Genetic background may interact with the mutated gene in ways that can alter phenotypic expression. For instance, protein kinase C gamma (PKCγ)-null mutants from a C57BL/6J $\times$ 129/SvJ mixed genetic background exhibited reduced ethanol sensitivity and the absence of ethanol tolerance, however, expression of the null mutation on a C57BL/6J background eliminated the phenotypes (Bowers 1999). Mice used in the present study were congenic C57BL/6J, which are widely known as a model of high alcohol preference (Belknap, 1993). Thus, it is plausible that background genes driving alcohol drinking in this mouse strain might blunt the effects of some null mutations, such as the 5-HT$_{3A}$ receptor.

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