

A Null Mutation of the Serotonin 6 Receptor Alters Acute Responses to Ethanol

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The anatomical distribution and pharmacology of serotonin 6 receptors (5-HT₆R) implicate them as contributors to the serotonergic regulation of complex behavior. To complement the limited range of pharmacological tools available to examine 5-HT₆R function, we have generated a mouse line bearing a constitutive null mutation of the 5-HT₆R gene. No perturbations of baseline behavior were noted in a wide array of assays pertinent to multiple neurobehavioral processes. However, 5-HT₆R mutant mice demonstrated reduced responses to the ataxic and sedative effects of ethanol. No differences in ethanol metabolism were evident between wild-type and 5-HT₆R mutant mice. These findings implicate 5-HT₆R in the serotonergic modulation of responses to ethanol.

Neuropsychopharmacology (2006) **31**, 1801–1813. doi:10.1038/sj.npp.1301030; published online 1 February 2006

Keywords: receptors; serotonin; 5-HT₆; 'knockout' mouse; ethanol tolerance; loss of righting reflex; ataxia

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is an important regulator of many complex neuropsychological processes, including affect, exploration, motivation, feeding, and responses to substances of abuse (Aghajanian and Sanders-Bush, 2002; Barnes and Sharp, 1999). To date, 14 serotonin receptor subtypes that mediate the CNS actions of 5-HT have been identified (Hoyer *et al*, 1994; Barnes and Sharp, 1999). The functional significance of a number of these receptor subtypes remains unclear. This is the case for the 5-HT₆R, a G-protein-coupled receptor that is positively linked to the adenylate cyclase second messenger system (Monsma *et al*, 1993; Ruat *et al*, 1993; Kohen *et al*, 1996; Branchek and Blackburn, 2000). Receptor expression in rats, guinea pigs, and humans appears CNS-limited and most prominent in the caudate-putamen, nucleus accumbens, and olfactory tubercle, with lesser expression in the hippocampus, cortex, and amygdala (Monsma *et al*, 1993; Ruat *et al*, 1993; Ward *et al*, 1995; Kohen *et al*, 1996; Roberts *et al*, 2002). Several classes of psychotropic drugs such as atypical antipsychotics, tricyclic antidepressants, and

hallucinogens bind with high affinity to 5-HT₆R (Monsma *et al*, 1993; Roth *et al*, 1994; Glatt *et al*, 1995). Thus, the expression pattern and pharmacological properties of 5-HT₆R indicate that they may contribute to serotonergic modulation of clinically relevant neuropsychological processes and psychopharmacological responses. Administration of 5-HT₆R antagonists and antisense oligonucleotides have been reported to produce several behavioral effects in rodents, including feeding suppression (Svartengren *et al*, 2004), yawning and stretching (Bourson *et al*, 1995; Sleight *et al*, 1996; Sleight *et al*, 1998; Bentley *et al*, 1999), decreased anxiety-related behaviors (Hamon *et al*, 1999), and improved performance in learning and memory tasks (Meneses, 2001; Rogers and Hagan, 2001; Woolley *et al*, 2001; Russell and Dias, 2002; Lindner *et al*, 2003; Riemer *et al*, 2003).

The prominent expression of 5-HT₆R within dopaminergic terminal fields (Boess *et al*, 1998; Gérard *et al*, 1997; Hamon *et al*, 1999), including the nucleus accumbens, raises the possibility that these receptors may also play a role in the serotonergic modulation of responses to substances of abuse. Rats treated with 5-HT₆R antagonists showed greater sensitivity to the locomotor and reinforcing effects of amphetamine, and elevated extracellular dopamine in the prefrontal cortex compared to those treated with vehicle (Frantz *et al*, 2002).

Genetic association studies also suggest that specific 5-HT₆R polymorphisms may be associated with several major neuropsychiatric diseases, including schizophrenia (Tsai *et al*, 1999a; however, see Shinkai *et al*, 1999; Ohmori *et al*, 2001) and Alzheimer's disease (Tsai *et al*, 1999b;

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Received 29 March 2005; revised 12 October 2005; accepted 26 October 2005

Online publication: 23 December 2005 at <http://www.acnp.org/citations/Npp122305050211/default.pdf>

however, see Thome *et al*, 2001). 5-HT₆R polymorphisms may also influence clinical responses to atypical antipsychotic drugs (Lane *et al*, 2004).

To examine functional roles of 5-HT₆R, we used a gene targeting approach to produce mice constitutively lacking these receptors. Although extensive assessment of these animals revealed no abnormalities of baseline behaviors, marked abnormalities were observed in behavioral responses of these animals to ethanol. We report that mice lacking 5-HT₆R have decreased sensitivity to ethanol-induced ataxia and sedation, and increased ethanol-induced open field locomotion. These findings suggest that 5-HT₆R activity might influence susceptibility to the effects of ethanol.

MATERIALS AND METHODS

Targeting Vector

An 11 kb DNA fragment encoding the first exon of the *htr6* gene was obtained from a 129 mouse P1 bacteriophage library (gift from Drs Mark Hamblin and Ruth Kohen, Seattle, WA). Within this fragment, a 300 bp *Pst*I-*Pst*I segment encoding a portion of the 5-HT₆R protein from the middle of the third to the end of the fifth transmembrane domain was deleted and replaced by a neomycin-resistance gene cassette (NEO) driven by a phosphoglycerate kinase (PGK) promoter (PGK/NEO). A herpes simplex virus thymidine kinase (TK) gene driven by a PGK promoter (PGK/TK) was placed at the 3' end of the genomic sequence to complete the p5HT6TKN targeting construct (Figure 1a).

Generation of Homologous Recombinant Clones

The p5HT6TKN construct was linearized and electroporated into 129-derived JM1 embryonic stem (ES) cells (gift of Dr Roger Pedersen), followed by application of a standard positive-negative selection strategy (Mansour *et al*, 1988) to enrich for targeted clones. The resulting ES cell colonies were screened for homologous recombination by Southern blot analysis. A genomic fragment (*Hind*III-*Xba*I) corresponding to a region 3' to the expected integration site was used to probe genomic DNA digested with *Xba*I. Wild-type and mutant alleles were indicated by 11 and 7.5 kb fragments, respectively (Figure 1a and b). Greater than 6% of drug-resistant clones had undergone homologous recombination, as confirmed by Southern blotting using the indicated 5' probe with *Xba*I-digested genomic DNA (data not shown).

Generation of 5-HT₆R Null Mutant Mice

Male chimeras produced by injection of targeted ES cells into C57BL/6J blastocysts were bred with C57BL/6J females. Germline transmission of the targeted mutation was verified by Southern blot analysis of tail DNA from F1 offspring. Experimental animals were derived from heterozygote crosses to produce wild-type, heterozygous, and homozygous mutant mice (Figure 1c). All mice used in these studies had a C57BL/6J allelic contribution of $\geq 75\%$. Animal care and handling procedures were in accordance with the UCSF institutional guidelines and federal regulations.

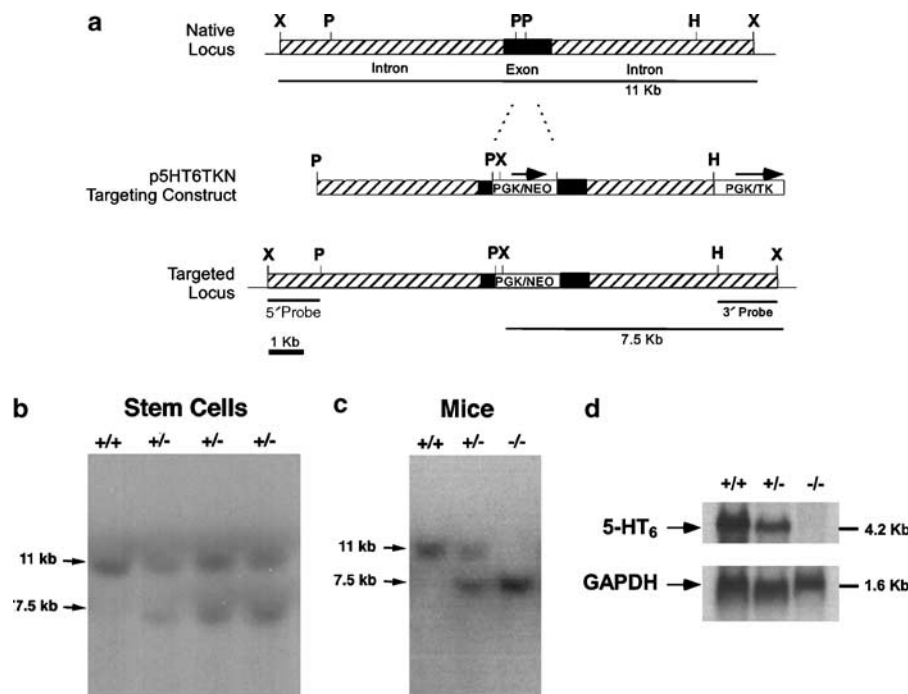


Figure 1 Production of 5-HT₆R null mutant mice. (a) A schematic representation of the native allele, targeting vector, and mutant allele following homologous recombination. The 7.5 and 11 kb fragments obtained from Southern blotting are highlighted. (b) A representative Southern blot of *Xba*I-digested ES cell genomic DNA; the wild-type allele is indicated by an 11 kb fragment, while the mutant allele is indicated by a 7.5 kb fragment. (c) A representative Southern blot of *Xba*I-digested genomic DNA in wild-type (+/+), heterozygous (+/-), and mutant (-/-) mice. (d) A representative Northern blot of whole brain polyA⁺ RNA in wild-type, heterozygous, and mutant mice. X: *Xba*I, P: *Pst*I, H: *Hind*III, PGK: phosphoglycerate kinase, TK: thymidine kinase, NEO: neomycin-resistance cassette.

Northern Blot Analysis

Total RNA was extracted with Trizol reagent (Life Technology) from whole brains. From these total RNAs, poly A⁺ RNA was purified using an oligo dT column as described (Sambrook *et al*, 2001). Nine micrograms of poly A⁺ RNA were run on a formaldehyde gel and transferred to a Duralon-UV membrane (Stratagene), which was then hybridized with a ³²P-radiolabeled DNA probe corresponding to the 300 bp portion of the *htr6* coding region that had been deleted from the targeting vector. Following hybridization, washes and film development, blots were stripped and rehybridized with a GAPDH probe for an internal standard.

Behavioral Testing

Mice were group-housed (4–6 mice per cage) in standard polycarbonate mouse cages (29 × 18.5 × 13 cm) with free access to food and water under a 12 h light/dark cycle. Behavioral assays were performed using male mice between the ages of 3 and 6 months. Unless otherwise indicated, naïve mice were used for each behavioral assay, and cohorts of size *n* = 8 were used for each group. Between subjects, testing apparatus were cleaned with a 0.25% bleach solution, wiped down with water, and then dried. All animals were tested during the light cycle. All experimental conditions were counterbalanced by genotype. Approximately 3 min before each assay, animals were removed from their home cage and placed in a clean holding cage for transfer, except for photobeam activity monitoring, where animals were placed into the test chambers directly from home cages. For all behavioral tests, investigators were blind to genotype and drug treatment.

Open Field

A 4-unit open field was used, consisting of a white Kydex box divided into four separate 50 × 50 × 38 cm chambers, allowing four animals to be tested concurrently. A video camera was mounted directly above the chambers to monitor animals' activity and movement. Four mice were briefly kept in individual holding cages before being simultaneously placed into the open field chambers. Distance traveled was assessed for 30 min with a video tracking system (Poly-track, San Diego Instruments). Assignments to the four chambers were counterbalanced by genotype. Mouse activity within 7 cm of the chamber walls was defined as occurring within the peripheral zone; activity further from the walls was considered to occur within the center zone.

Elevated Zero Maze

A zero maze (34 cm inner diameter, 46 cm outer diameter, on four-braced legs 40 cm off the ground) was used according to the procedures previously described (Heisler *et al*, 1998). Mice were removed from their home cage to a transfer cage, and then placed in the center of one of the closed quadrants. Latency to enter an open quadrant (all four paws), time spent in open quadrants, number of closed-to-open quadrant transitions, and head dips were

live-scored in a blinded fashion during the 6 min test by a remote observer.

Home Cage Activity

Animals were housed individually in rat cages (48 × 27 × 13 cm) with bedding, food and water, under a 12 h light/dark cycle. To assess activity, beam breaks were collected each hour for 3 days with a photobeam activity system (FlexField, San Diego Instruments). Both horizontal locomotor activity (as monitored by a 4 × 8 array of infra-red photobeams) and rearings (as scored by an elevated set of eight infra-red photobeams) were recorded. The animals were placed in the system at noon on day 1.

Prepulse Inhibition

Acoustic startle responses and prepulse inhibition of acoustic startle were evaluated using SR-LAB hardware and software (San Diego Instruments). Mice were placed in clear acrylic testing chambers within the startle chambers. Chambers were not lit during testing. All subjects received a 5 min acclimation period where the only input stimulus was 70 dB background white noise. Following this acclimation, mice were briefly habituated to six 120 dB startle stimuli (0 ms rise time, 40 ms plateau, 0 ms fall time). Startle habituation was followed by prepulse–startle trials. Prepulse stimulus intensities were 4, 8, or 16 dB above the 70 dB background (0 ms rise time, 20 ms plateau, 0 ms fall time), and preceded the 120 dB startle stimulus by 80 ms. During the session, 10 trials each of all three prepulse conditions, as well as control 120 dB startle stimuli presented without a prepulse, and trials of no stimulation (which provide an upper bound on the maximum prepulse inhibition possible) were randomly interspersed and separated by an average of 150 s (range 70–230 s). Peak startle responses were detected and measured by piezoelectric sensors located under the testing chambers.

Morris Water Maze

Three cohorts of 24 animals each (eight per genotype) were trained using a visible platform/hidden platform version of the water maze task. Prior to each training session, mice were removed from their group cages and individually housed in holding cages for the duration of that day's training (approximately 6 h). Investigators were blinded to mouse genotype throughout training and testing. An 8 × 8 cm clear Plexiglas platform with an attached 7.5 × 7.5 × 7.5 cm³ black Plexiglas cube (visible platform) was centered in a randomly selected quadrant of a circular, 92 cm inner diameter polyethylene tank filled with warm water made opaque using white tempura-based paint (DryTemp powder tempura). Multiple objects were placed in the testing room to act as distal cues; these cues remained constant through all experiments. In one training block, a mouse was gently transported from its holding cage and released into the water facing the maze wall. Swimming paths were videotaped and analyzed using a commercially available software package (EthoVision, Noldus Inc.). The trial ended when the mouse mounted the platform with all four paws or if the mouse did not mount the platform after

60 s of swimming. All mice spent 20 s on the platform before returning to the holding cage. Following a 1-min rest interval, the mouse was returned to the maze at a different starting quadrant and the above process repeated until all four quadrants had been tested over four trials. Each mouse underwent two blocks of training per day. On day 3 (block 5), the visible platform was replaced by an 8 × 8 cm clear Plexglas platform submerged 0.5–1 cm below water level (hidden platform). All mice received 5 days (10 blocks) of training to the hidden platform. Once all mice completed the final training block, the hidden platform was removed from the maze, and all mice received a 1-min probe trial starting from the maze location opposite the old platform position. Probe trials for two of the cohorts were performed immediately after the final block of hidden platform testing; the probe trial for the remaining cohort was performed 28 days after the final day of hidden platform testing.

Motor Coordination

Motor coordination was assessed with an Accurotor rotarod (Accuscan Instruments). The rotation rate was accelerated from zero to 30 rpm over 5 min, and continued at 30 rpm for 5 more minutes until the end of one trial. Four animals were tested concurrently in separate 11 cm-wide compartments on a rod approximately 3 cm in diameter and elevated 35 cm. Each animal was assessed over seven trials with 20-min intertrial intervals. In each trial, the latency to fall from the rod was recorded.

Physostigmine-Induced Stretching and Yawning

Wild-type, heterozygous, and homozygous mutant mice were individually transferred to a holding cage where they received an intraperitoneal (i.p.) injection of either saline vehicle or the cholinesterase inhibitor physostigmine at 0.2 and 0.4 mg/kg ($n=8$ per genotype-dosage combination). Yawning, stretch-attend, and locomotor behaviors were continuously scored for 1 h following injection.

Locomotor Response to Amphetamine

Wild-type, heterozygous, and homozygous mutant mice were transferred to rat cages and habituated for 120 min, prior to receiving an i.p. injection of saline on day 1 and amphetamine 10 mg/kg (high dose) on day 2. Additional wild-type and homozygous mutant mice were transferred to rat cages and habituated for 120 min prior to receiving an i.p. injection of saline on day 1, and saline or amphetamine 2 mg/kg (low dose) on day 2. Mice receiving saline on day 2 received amphetamine 2 mg/kg on day 9, and *vice versa*. Cages were positioned on the photobeam activity system; locomotor activity and rearing were recorded for 120 min before and 60 min after i.p. injection. One cohort of 16 mice each (eight wild type, eight mutant) received high-dose amphetamine; two cohorts received low-dose amphetamine.

Ethanol-Induced Ataxia

The Accurotor rotarod apparatus was used as described above with the following modification in procedure. Before initiation of rotation, mice were placed on the stationary

rod facing opposite the direction of rotation, and within 10 s the rod speed was increased to 8 rpm. Each mouse was tested on the rotating rod for a maximum of 100 s. Each mouse was tested in the following sequence: day 1-training session, day 3-saline session 1, day 5-saline session 2, day 8-ethanol 1.5 g/kg, day 12-ethanol 2 g/kg, day 16-ethanol 2.5 g/kg. On the training day, mice were tested for their ability to stay on the rotating rod for more than 100 s for three out of 10 trials with each trial at least 2 min apart. None of the tested mice failed to meet this criterion. On the saline and ethanol test days, mice were first given one test on the rod, followed by i.p. injection of either saline or ethanol and tested 15 min later.

Ethanol-Induced Loss of Righting Reflex (LORR)

Mice were injected with 3.5 g/kg i.p. ethanol and put in a supine position in a plastic V-shaped trough. LORR was confirmed when the animals failed to right themselves and stand on all four paws for three times within a 30-s period. The length of LORR was recorded for each mouse. The test sequence of mice was counterbalanced by genotype.

Locomotor Response to Ethanol

Mice were tested in a photobeam activity system (FlexField, San Diego Instruments) configured to monitor both horizontal locomotor activity and rearings. In each test session, mice were habituated in the activity chamber for 2 h followed by i.p. injection of either saline or 2 g/kg ethanol. After injection, activity monitoring continued for 60 min. The schedule for injection was: day 1-saline, day 2-saline, day 3-ethanol or saline, day 9-saline or ethanol (reversed treatment from day 3).

Ethanol-Induced Hypothermia

At 3 weeks after testing ethanol-induced locomotion, mice were transferred to holding cages 30 min prior to 2 g/kg ethanol administration in a room maintained at ambient temperature. A thermistor probe (TH-5 thermometer, Physitemp) was inserted 1.5 cm into the rectum, and the temperature reading was recorded immediately before and 10, 20, 30, 40, and 60 min after ethanol injection.

Blood Ethanol Concentration

In a time course study, mice were injected with ethanol 3.5 g/kg i.p. and the tail blood was collected 10, 30, and 90 min postinjection. At 270 min postinjection, mice were decapitated and their trunk blood was collected. Whole blood samples (50–100 µl) were immediately placed on ice and centrifuged within 30 min to obtain plasma for further analysis. Plasma ethanol concentration was analyzed in an alcohol oxidation reaction using alcohol dehydrogenase with NAD as a cosubstrate as supplied in a Diagnostics Alcohol Reagent Kit (Sigma). Absorbance at 340 nm of the reaction product was measured with an Ultraspec 1000 spectrophotometer (Pharmacia). An ethanol standard solution (Sigma) was used to standardize the conversion of absorbance to ethanol concentration.

Ethanol Two-Bottle Preference Test

Mice of each genotype were individually housed for 2 weeks. Four control cages (with no mice) were also established at this time to estimate fluid loss from evaporation, dripping, and experimental error. Two bottles (each containing water) were introduced to all cages for 2 days to assess side preferences and basal fluid intake. Bottle spouts had sipper balls to minimize fluid leakage. Ethanol solution was then substituted for water in one bottle; mice first received 3% ethanol for 4 days, followed by 6% ethanol for 4 days, followed by 10% ethanol for 4 days, followed by 20% ethanol for 4 days. Mice had *ad lib* access to both bottles throughout the experiment. Both water and ethanol bottles were weighed daily during this time, and within each cage bottles were switched daily to prevent the development of place preference. On days when ethanol concentrations changed, mice were weighed. To assess ethanol intake over a stable baseline, only data from the last 3 days spent at a given ethanol concentration were further analyzed. Changes in ethanol and water weights from control cages were subtracted from the changes determined in subject cages.

Statistics

Behavioral scores were analyzed for normality using the Shapiro–Wilk's test. One-way or repeated measure ANOVA, followed by Tukey HSD *post hoc* tests, were used to compare the effect of genotype on normally distributed variables. Genotype comparisons of variables that were not normally distributed were analyzed with the Kruskal–Wallis *H* test, followed by Tukey HSD *post hoc* tests. All figures display the mean and standard error of the data to illustrate the central tendency of the variables. For all analyses, significance was assigned at the $p < 0.05$ level. Sigma Stat (SPSS) was the statistical software package used for all analyses.

RESULTS

Generation of 5-HT₆R Null Mutant Mice

Homologous recombinant ES cell clones were produced with an overall frequency of approximately 6% of drug-resistant colonies (Figure 1b). Blastocyst injections of targeted cells yielded chimeric mice that were bred with C57BL/6J females. Germline transmission of the mutation was confirmed by Southern blot analysis (Figure 1c). To assess the abundance of intact 5-HT₆R mRNA within the brains of mutant mice, Northern blot analysis was performed. Blots were probed with a radiolabeled fragment corresponding to the deleted region of the gene. A 4.2 kb band was apparent for wild-type, but not mutant samples; an approximate 50% reduction in the abundance of intact transcript was observed for heterozygotes (Figure 1d). We attempted to confirm loss of protein product via autoradiography using the ligand ¹²⁵I-SB258585 (Roberts *et al*, 2002); however, we were unable to detect specific binding in wild-type brains. This finding is consistent with recent observations indicating that the affinities of ligands used to characterize 5-HT₆Rs in the rat (such as ¹²⁵I-SB258585)

demonstrate reduced affinity for the mouse homolog, and that 5-HT₆R expression levels in the mouse are reduced relative to those in the rat (Hirst *et al*, 2003).

Heterozygote crosses produced wild-type, heterozygous, and homozygous mutant mice in the expected Mendelian ratios, indicating that the mutation does not impair embryonic or postnatal viability. 5-HT₆R mutant mice were healthy and normal in appearance. No phenotypic differences in body weight were noted across cohorts of male mice (Supplementary Figure S-1). No overt phenotypic abnormalities were noted in brain weight, cytoarchitecture, monoamine levels, serotonin, dopamine, and noradrenergic neuron distribution, or striatal dopamine D₁ and D₂ receptor expression (Supplementary Figure S-2).

Absence of Phenotypic Abnormalities of Diverse Baseline Behaviors

Cohorts of wild-type, heterozygous, and 5-HT₆R mutant mice were assessed for behavioral alterations over multiple domains, including exploratory and anxiety-related behaviors, home cage activity, sensorimotor gating, and cognitive function. There were no genotype-related differences in overall exploratory behavior as measured by distance traveled in an open field (Figure 2a), nor were there any differences in open field anxiety-related behaviors as evaluated by time spent in the field center *vs* periphery (Figure 2b). Similarly, there was no evidence of an anxiety-related phenotype in an elevated zero maze (ie no differences in total open quadrant dwell time, number of transitions between open and sheltered maze quadrants, or head dips, Figure 2c). Loss of 5-HT₆R function also appeared to have no effect on circadian modulation of locomotor activity. Wild-type, heterozygous, and mutant mice individually housed for 3 days in photobeam activity systems demonstrated no difference in activity onset or offset timing, or in total activity (measured by photobeam breaks, Figure 2d).

Sensorimotor gating was evaluated by prepulse inhibition of acoustic startle responses. Wild-type, heterozygous, and 5-HT₆R mutant mice appropriately demonstrated increased inhibition of a 120 dB startle stimulus with increasing prepulse magnitudes; however, there were no genotype-related differences in prepulse inhibition (Figure 2e). There were also no phenotypic differences in baseline responses to the 120 dB startle stimulus (data not shown).

To assay for possible changes in cognitive function, we employed a standard Morris water maze protocol to assess place learning. Three separate experiments were performed and demonstrated no phenotypic differences in acquisition of either the visible or hidden platform tasks (Figure 3a). Similarly, no differences were noted in probe trial performance (performed immediately after the last block of hidden platform trials, Figure 3b). To determine if there were differences in consolidation of long-term spatial memories, one probe trial was performed 28 days after the last day of hidden platform training; again, no performance differences were found between wild-type and mutant mice (Figure 3c).

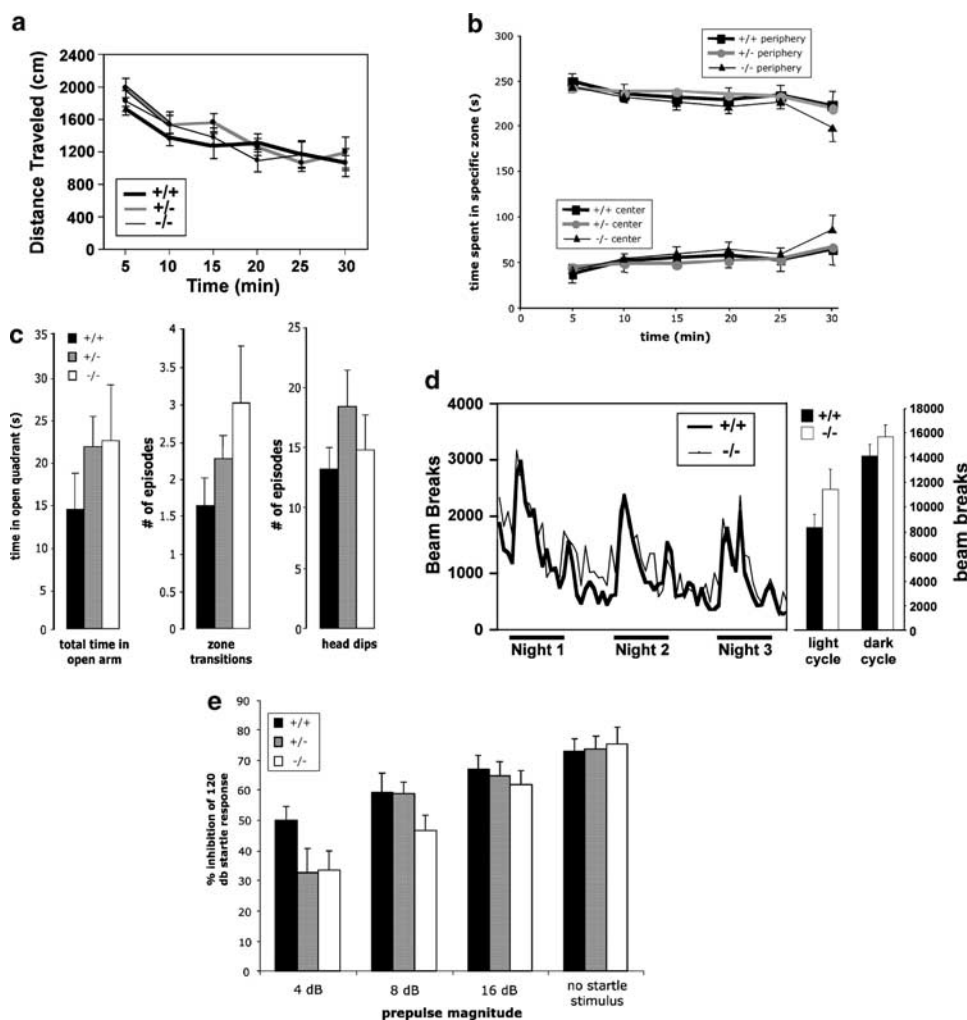


Figure 2 5-HT₆R mutant mice baseline behavioral screening. (a) No phenotypic differences in overall locomotor distance in a novel environment (over 30 min in the open field test, $F_{2,21} = 0.424$, $p = 0.66$). (b) No phenotypic differences in open field measures of anxiety-related behaviors. Top group of lines represents total time spent within 7 cm of open field walls (periphery); bottom group of lines represents total time spent within field center in the same 30 min trials. (c) No phenotypic differences in elevated zero maze measures of anxiety-related behaviors ($F_{2,21} = 0.816$ (total open arm time), 1.755 (zone transitions), 1.027 (head dips), all NS). (d) Similar diurnal patterns of activity in wild-type ($n = 9$) and 5-HT₆R mutant ($n = 10$) mice. Time-of-day had a significant effect on activity, such that animals were more active in the dark phase than in the light phase ($F_{1,17} = 63.46$, $p < 0.001$); however, no phenotypic differences in activity levels or diurnal activity patterns were observed ($F_{1,17} = 2.144$, $p = 0.161$). Right panel depicts cumulative beam breaks over the second and third days (light cycle, $p < 0.14$ Student's t -test) and nights (dark cycle, $p < 0.23$, Student's t -test). (e) No differences noted in prepulse inhibition between wild-type ($n = 15$), heterozygous ($n = 16$), and mutant ($n = 15$) mice receiving prepulses 4, 8, and 16 dB above the baseline 70 dB prior to a 120 dB startle stimulus. The rightmost bars are responses within the startle chamber in the absence of a startle stimulus, and represent the maximum prepulse inhibition that may be possible. Repeated measure ANOVA demonstrated a significant effect of stimulus magnitude across all genotypes ($F_{3,41} = 32.7$, $p < 0.001$), but no significant differences attributable to genotype or a genotype*stimulus interaction. Unless otherwise mentioned, cohort sizes were 8 per genotype, and error bars are ± 1 standard error.

Absence of Phenotypic Abnormalities in Responsiveness to Amphetamine and Physostigmine

Locomotor response to 2 or 10 mg/kg i.p. amphetamine treatment yielded no phenotypic differences (Supplementary Figure S-3). No phenotypic differences in baseline frequency of stretching or yawning behaviors were noted (data not shown). Similarly, no differences in either stretching or yawning behaviors were appreciated between wild-type, heterozygote, or 5-HT₆R mutant mice following treatment with saline vehicle or the cholinesterase inhibitor physostigmine (Supplementary Figure S-4).

5-HT₆ R Mutant Mice have Decreased Sensitivity to the Ataxic and Sedative Effects of Ethanol

In an accelerating rotarod assay, all mice showed improved performance following repeated trials, and no phenotypic differences in motor coordination or motor learning were observed (Figure 4a). However, 5-HT₆R null mutant mice displayed reduced sensitivity to the ataxic effects of a 2 g/kg ethanol injection, remaining on the rotating rod substantially longer than both the wild types and heterozygotes (Figure 4b). A similar trend (that did not reach statistical significance) was noted in mouse rotarod responses after

1.5 g/kg ethanol injection (Supplementary Figure S-5); by contrast, animals of all genotypes were unable to perform the rotarod assay after 2.5 g/kg ethanol injection due to sedation. These findings suggest that 5-HT₆R null mutant mice are less sensitive to the ataxic effects of ethanol. However, since mice in the 2 g/kg experimental group had a single 1.5 g/kg ethanol injection 4 days before testing, we

cannot exclude the possibility that phenotypic differences in ethanol tolerance contributed to these results.

5-HT₆R mutant mice were also more resistant to the sedative effect of ethanol, as tested by LORR. 5-HT₆R mutants regained the righting reflex significantly faster after 3.5 g/kg ethanol injection compared to wild-type animals, with heterozygotes exhibiting an intermediate effect (Figure 4c). Mutant mice also displayed increased initial hyperlocomotor responses to ethanol, with increased locomotion in the first 5 min after 2 g/kg ethanol injection (Figure 4d). By contrast, no phenotypic differences were observed in locomotor responses to saline injections (data not shown).

No differences between wild-type and 5-HT₆R mutant mice were noted in ethanol-induced hypothermia following a single 2 g/kg ethanol injection (Figure 4e). Thus, the 5-HT₆R mutation may dissociate ethanol-induced ataxic and sedative effects from ethanol-induced hypothermic effects. However, more extensive dose-response studies would be required to definitively exclude the possibility that the 5-HT₆R mutation alters hypothermic responses to ethanol. Assays of blood ethanol concentration following a single 3.5 g/kg ethanol injection detected no differences in ethanol metabolism between wild-type and 5-HT₆R mutant mice (Figure 4f).

No Differences in Two-Bottle Ethanol Preference Between Wild-type and 5-HT₆R Mutant Mice

In light of phenotypic differences in acute responses to ethanol, we examined ethanol preference in these mice. Figure 5 depicts the results of a two-bottle preference test, a simple behavioral assay for altered ethanol ingestion between wild-type and 5-HT₆R mutant mice. No differences were noted in either ethanol ingestion (Figure 5a) or ethanol preference ratio (ethanol solution consumed/total fluid intake; Figure 5b) between wild-type and 5-HT₆R mutant mice.

DISCUSSION

We have presented initial analyses of mice bearing a constitutive null mutation of the 5-HT₆ receptor. Although these animals perform normally in a wide variety of standard behavioral assays, they exhibit abnormal responses to ethanol. These mice are less sensitive to the acute ataxic and sedative effects of ethanol as measured in rotarod and loss-of-righting-reflex assays; they also display

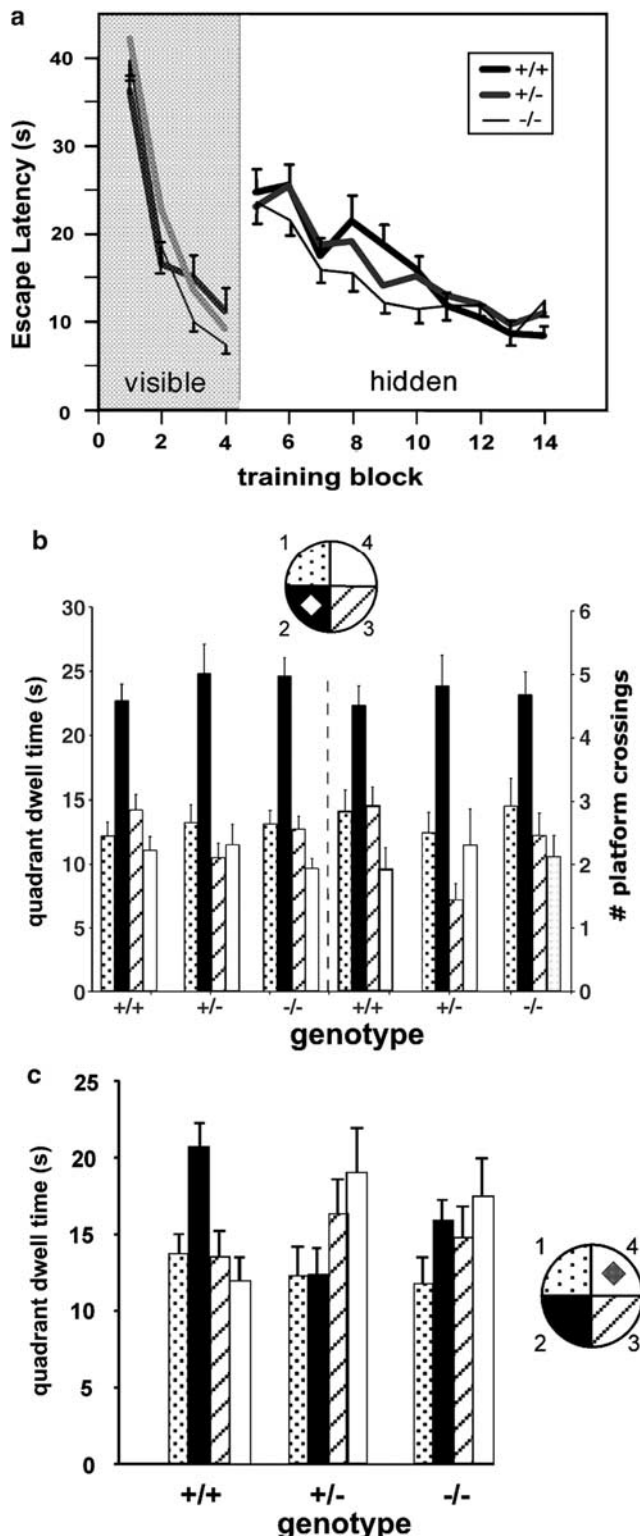


Figure 3 5-HT₆R mutant mice cognitive testing. (a) No phenotypic differences were noted in escape latencies during the visible or hidden platform tasks of the Morris water maze ($n=24$ wild type, 16 heterozygous, 24 mutant, $F_{2,59}=1.417$ for genotype effect (NS), $F_{18,531}=0.211$ for genotype*block interaction (NS)). Mice trained to quadrant 2. (b) No phenotypic differences in probe trial performance (training responses shown in panel a) were noted. Leftmost bars represent quadrant dwell times, rightmost bars represent platform crossings, all mice trained to quadrant 2. (c) No phenotypic differences in 28-day delayed probe trial performance as measured by time spent in trained quadrant (cohorts of eight per genotype, one-way ANOVA, primary factor genotype, $F_{2,19}=2.2378$, $p<0.1341$). Mice trained to quadrant 4. Error bars are ± 1 standard error.

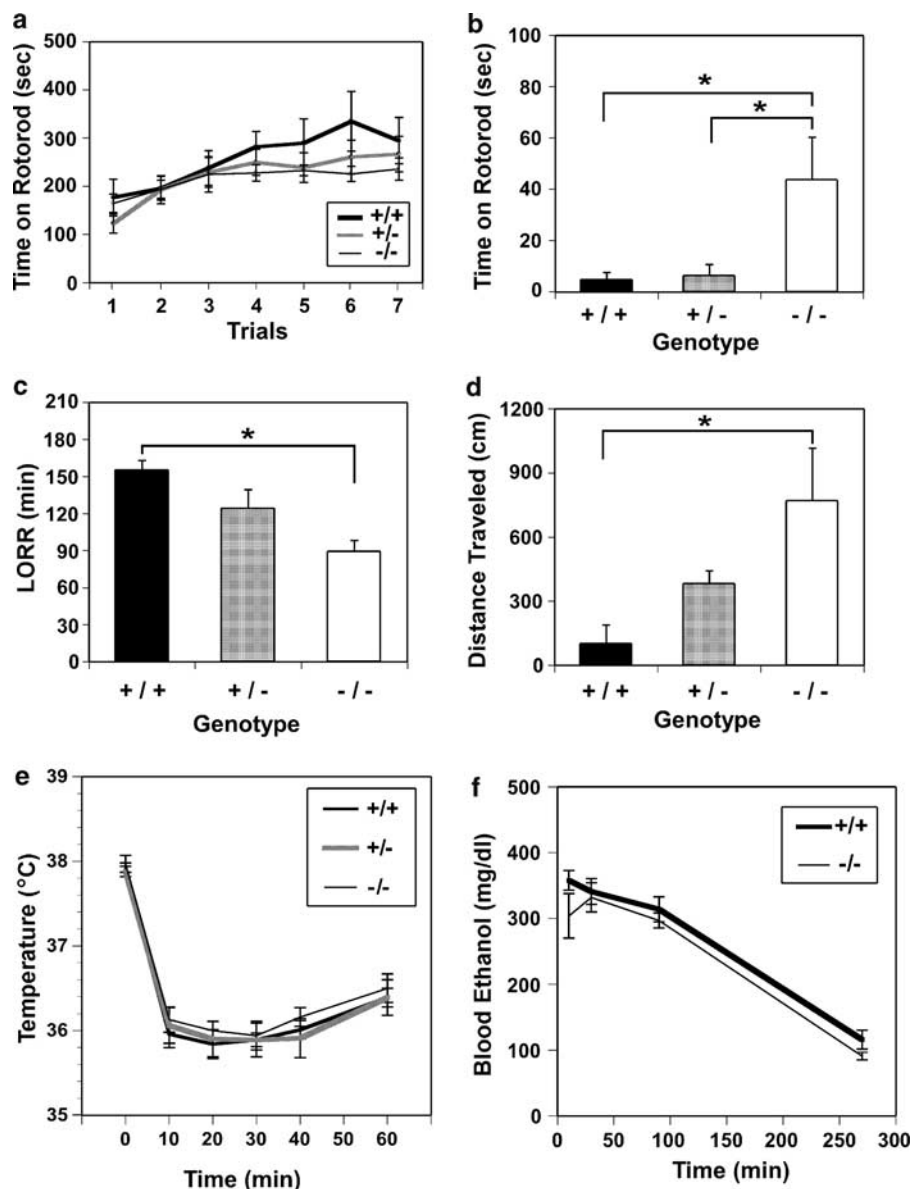


Figure 4 Altered acute ethanol responses in 5-HT₆R mutant mice. (a) No differences in baseline rotarod performance between wild-type, heterozygous, and mutant mice over the seventh training sessions. Performance at the end of the seventh trial session was improved relative to initial performance for all groups ($F_{6,96} = 10.38$, $p < 0.001$ for time, $F_{2,16} = 0.486$, $p = 0.624$ for genotype). (b) Following 2 g/kg ethanol administration, 5-HT₆R mutant mice remained on the rotarod significantly longer than both heterozygote and wild-type mice ($F_{2,23} = 4.753$, $p < 0.05$). (c) Following 3.5 g/kg ethanol administration, 5-HT₆R mutant mice regained their righting reflex more rapidly than either heterozygous or wild-type mice ($F_{2,23} = 8.451$, $p < 0.005$). (d) 5-HT₆R mice show increased sensitivity to ethanol-induced locomotor hyperactivity effects in the initial 5 min after 2 g/kg ethanol ($F_{2,23} = 4.591$, $p < 0.05$) administration. (e) No phenotypic differences in ethanol-induced (2 g/kg) hypothermia ($F_{2,21} = 0.31$, $p = 0.737$) were observed. (f) No differences in blood ethanol concentration ($F_{1,14} = 1.518$, $p = 0.238$) were observed between wild-type and 5-HT₆R mutant mice. Cohort sizes were eight per genotype; error bars are ± 1 standard error. Asterisks denote relationships whose statistical significance were $p < 0.05$.

increased locomotion immediately following ethanol administration. These findings represent the initial indication that 5HT₆Rs contribute to serotonergic influences on ethanol responses.

5-HT₆R-Influenced Behaviors

Initial studies that demonstrated high binding affinities of atypical antipsychotic agents such as clozapine at 5-HT₆Rs (Roth *et al*, 1994) raised the possibility that this receptor influences sensorimotor gating and/or psychotic processes.

Some studies indicated that 5-HT₆R antisense oligonucleotides (Bourson *et al*, 1995; Sleight *et al*, 1996) or 5-HT₆R antagonists (Unsworth and Molinoff, 1994; Sleight *et al*, 1998; Bentley *et al*, 1999; Routledge *et al*, 1999) produced yawning-stretching behaviors that could be blocked by the muscarinic cholinergic antagonist atropine or potentiated by the cholinesterase inhibitor physostigmine. Other studies have implicated 5-HT₆Rs in the modulation of cognitive processes (Russell and Dias, 2002). However, studies of the roles of 5-HT₆Rs in these and other behavioral processes have produced conflicting results.

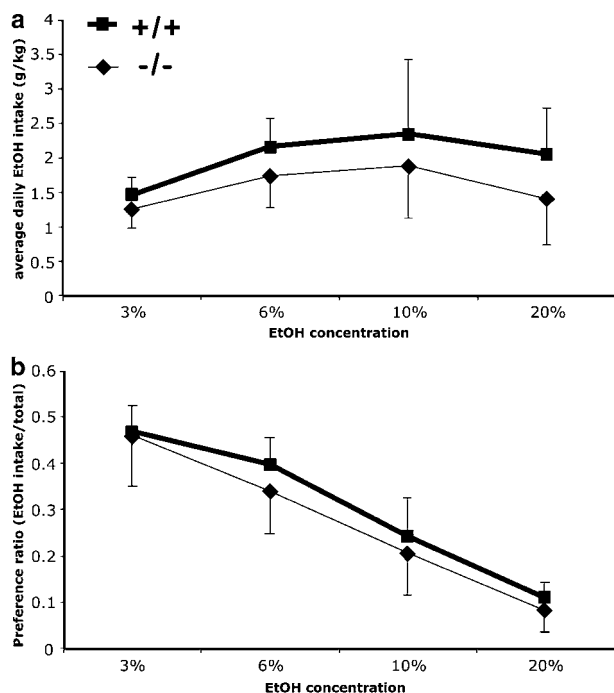


Figure 5 No differences in total ethanol intake (a, $F_{3,15} = 0.47$, $p = 0.503$) or ethanol preference (b, $F_{3,15} = 0.25$, $p = 0.624$) were noted between wild-type and 5-HT₆R mutant mice. Ethanol concentration had a significant effect on ethanol preference in both wild-type and 5-HT₆R mutant mice (b, $F_{3,15} = 8.00$, $p = 0.004$). Cohort sizes were eight per genotype; error bars are ± 1 standard error.

With regard to cognition, rats acutely treated with 5-HT₆R antagonists demonstrated improved spatial memory retention 1 week after Morris maze training (Woolley *et al*, 2001; Stean *et al*, 2002). 5-HT₆R antagonists also enhanced acquisition and reversed a scopolamine-evoked deficit in rat operant autoshaping and passive avoidance tasks (Bos *et al*, 2001; Meneses, 2001; Riemer *et al*, 2003). Pretreatment with a 5-HT₆R antagonist also improved rat performance of an attentional set-shifting task, a rodent analog of the Wisconsin card sorting test, a commonly used assay of cognitive-guided sorting (Hatcher *et al*, 2005). However, other investigators have been unable to demonstrate 5-HT₆R-induced enhancements of cognitive function (Russell and Dias, 2002; Lindner *et al*, 2003). In three different cohorts, we were unable to demonstrate phenotypic differences in either Morris water maze acquisition or probe trial performance.

Similar uncertainty exists regarding the relationship between 5-HT₆Rs and anxiety-related behaviors. In rats, continuous intraventricular infusion of 5-HT₆R antisense oligonucleotides evoked increased anxiety-related behaviors on an elevated plus maze and a social interaction test (Hamon *et al*, 1999; Otano *et al*, 1999). However, similar treatments failed to alter conditioned freezing responses in rats (Yoshioka *et al*, 1998). We have been unable to detect a significant anxiety-related phenotype in 5-HT₆R null mutant mice.

Few studies have investigated how 5-HT₆Rs may modulate responses to substances of abuse. Rats pretreated with the selective 5-HT₆R antagonist SB 258510A demonstrated

enhanced locomotor responses to amphetamine treatment that were accompanied by increased extracellular dopamine efflux in the prefrontal cortex (Frantz *et al*, 2002). However, this effect was not observed in response to cocaine administration (Frantz *et al*, 2002). We were unable to demonstrate differences in locomotor sensitivity to either low- or high-dose amphetamine treatment between wild-type and 5-HT₆R mutant mice. However, a more extensive dose-response study would be required to definitively exclude the possibility that the mutation alters sensitivity to amphetamine at other doses not tested in these animals.

To obtain a more complete behavioral profile of the 5-HT₆R mutant mice, we further surveyed a number of baseline behaviors, including circadian rhythms of locomotor activity, exploration in a novel open field, motor coordination, and sensorimotor gating. We were unable to detect any phenotypic differences in these behaviors, nor in the stretching and yawning behaviors proposed to be influenced by 5-HT₆Rs. Thus, initial behavioral assessment of 5-HT₆R mutant mice demonstrated no significant differences across a wide variety of behavioral domains.

There are several possible explanations for why we were unable to detect differences in behaviors postulated to have 5-HT₆R-mediated influences. 5-HT₆R-evoked phenotypes in yawning-stretching, cognitive performance, and anxiety-related behaviors have not been consistently demonstrated in rats (Woolley *et al*, 2004). Moreover, constitutive deletion of the 5-HT₆R may evoke developmental compensations masking these phenotypes; similarly, changes in behavior resulting from chronic 5-HT₆R deletion may differ from those induced by acute pharmacological or antisense oligonucleotide treatment. Additionally, species differences in 5-HT₆R mRNA expression may contribute to species differences in behaviors sensitive to 5-HT₆R function. Recent evidence indicates that differences between mouse and rat/human 5-HT₆R receptor sequences are associated with differences in their affinities for 5-HT₆R antagonist compounds, while not altering binding affinities for serotonin (Hirst *et al*, 2003).

5-HT₆ Receptors Influence Acute Responses to Ethanol Consumption

Whereas abundant evidence implicates central serotonin systems in responses to ethanol, the relative contributions provided by particular serotonin receptor subtypes remain unclear. The enrichment of 5-HT₆R expression in striatal regions implicated in ethanol responses raised the possibility that these receptors may play a role in these responses. Here, we report that 5-HT₆Rs influence acute responses to ethanol administration.

Following a moderate dose of ethanol, 5-HT₆R mutant mice remained on a rotating rod for a longer duration compared with wild-type mice; similarly, 5-HT₆R mutant mice recovered their righting reflex more rapidly than wild types. These findings suggest that 5-HT₆R mutant mice are less sensitive to the acute ataxic and sedative effects of ethanol. 5-HT₆R mutant mice also displayed increased locomotor activity levels following low-dose ethanol administration. Hyperlocomotion following low-dose ethanol administration is a well-studied phenomenon (Phillips and Shen, 1996); in C57BL/6 mice, this effect is most prominent

in the first 5 min following administration (Crabbe *et al*, 1982). This finding suggests that the mutation enhances locomotor activation and/or reduces behavioral sedation in response to low-dose ethanol. In our experimental design, mice treated with 2 g/kg ethanol in the ataxia test received a single 1.5 g/kg ethanol injection 4 days prior to testing. Therefore, we cannot completely rule out whether ethanol tolerance contributed to our findings. However, 5-HT₆R mutant mice receiving an initial 1.5 g/kg ethanol injection did show a trend, albeit not statistically significant, toward decreased ethanol-induced ataxia. The above findings are also not explained by differences in ethanol clearance.

Since ethanol can induce significant hypothermia, which in turn might influence coordination and locomotion, we specifically tested ethanol-induced hypothermia at the dose (2 g/kg) where these behaviors differed significantly between 5-HT₆R mutant and wild-type mice. However, we did not identify any phenotypic differences in ethanol-induced hypothermia at this dose. This finding does not preclude the possibility that phenotypic differences in ethanol-induced hypothermia may occur at other ethanol doses.

Although no phenotypic differences were observed in a standard two-bottle intake test, several factors preclude us from concluding that 5-HT₆Rs do not influence ethanol self-administration. Ethanol ingestion was less than anticipated for C57BL/6 or C57BL/6-129 mixed genetic background mice (Belknap *et al*, 1993; Mihalek *et al*, 2001; Thiele *et al*, 2000); a floor effect may potentially mask genotypic differences. Furthermore, the mixed genetic background of these mice could obscure the impact of the 5-HT₆R mutation. In addition, differences in circadian patterns of ethanol ingestion (Gill *et al*, 1986) cannot be discerned by a two-bottle preference test. In future work, it will be of interest to test mice congenic for the 5-HT₆R mutation using operant assays that estimate the reinforcing properties of ethanol while eliciting levels of intake that produce pharmacological effects on physiology and behavior (Cunningham *et al*, 2000; Risinger *et al*, 1998; Roberts *et al*, 1999; Samson *et al*, 1998).

Serotonin Systems and Ethanol Responses

Abundant evidence indicates that serotonergic systems influence both ethanol intake and ethanol responses. Human alcoholism syndromes have been subdivided into distinct subcategories associated with alterations in central serotonergic function (Virkkunen and Linnoila, 1996; Higley and Linnoila, 1997) and serotonin transporter promoter polymorphisms (Hallikainen *et al*, 1999). Evidence exists that specific subgroups of alcoholics decrease their ethanol ingestion following selective serotonin reuptake inhibitor (SSRI) therapy (Naranjo and Knoke, 2001; Pettinati, 2001). In animal studies, lines of rats bred for increased ethanol preference demonstrate decreased serotonergic immunoreactivity within the median and dorsal raphe nucleus (Zhou *et al*, 1994) and localized reductions in telencephalon serotonin content and innervation (Murphy *et al*, 1987, 2002; Gongwer *et al*, 1989; Zhou *et al*, 1991; Myers *et al*, 1998; Lucas and McMillen, 2002). Firing rates of dorsal raphe neurons decrease following acute ethanol administration (Pistis *et al*, 1997).

Although our results suggest that 5-HT₆Rs contribute to the serotonergic modulation of ethanol responses, previous studies have also defined roles for other components of serotonergic neurotransmission. Some lines of mice carrying a constitutive 5-HT_{1B} receptor mutation demonstrate increased ethanol consumption compared to wild types (Crabbe *et al*, 1996; Phillips *et al*, 1999; however, see Risinger *et al*, 1999; Bouwknecht *et al*, 2000; Gorwood *et al*, 2002 for conflicting reports). Other serotonin receptors, including 5-HT₃R (Engel *et al*, 1998; Engel and Allan, 1999; Rodd-Henricks *et al*, 2003), 5-HT_{2A}R (West *et al*, 1998; Maurel *et al*, 1999; however, see Wilson *et al*, 2000; Blakley *et al*, 2001 for conflicting reports), and 5-HT_{2C}R (Maurel *et al*, 1999; Tomkins *et al*, 2002) also modulate ethanol ingestion. Finally, multiple lines of evidence demonstrate that SSRIs affect multiple responses to ethanol ingestion, including overall ethanol ingestion, taste aversion, and ethanol discrimination (Gulley *et al*, 1995; Gardell *et al*, 1997; Risinger, 1997; Lamb and Jarbe, 2001).

The precise mechanisms through which loss of 5-HT₆R function leads to increased ethanol resistance are unclear. A large body of literature suggests that the striatum and nucleus accumbens are important loci for the locomotor and behavioral effects of ethanol ingestion (Weiss and Porrino, 2002). Acute ethanol administration evokes serotonin release in the nucleus accumbens (Yoshimoto *et al*, 1992; Yan, 1999). 5-HT₆Rs within the striatum and nucleus accumbens may thus contribute to part of the serotonergic response to ethanol ingestion, and their absence may reduce an aspect of this response.

In summary, we have identified a role for 5HT₆Rs in modulating the ataxic and sedative effects of ethanol in the mouse. This phenotype was not secondary to changes in ethanol pharmacokinetics. Furthermore, particular ethanol-related responses were differentially affected by the mutation, since there were no observed phenotypic effects on ethanol clearance or in ethanol-evoked hypothermia. Our data suggest a specific ethanol phenotype in 5-HT₆R mutant mice, and indicate that 5-HT₆Rs influence behavioral responses to ethanol.

ACKNOWLEDGEMENTS

This research was funded in part by grants from the American Federation for Aging Research (SJB), Department of Veteran's Affairs (HMC), National Alliance for Research on Schizophrenia and Depression (HMC), and Program for Minority Research Training in Psychiatry 5T32MH19126 (HMC). We thank Drs Mark Hamblin and Ruth Kohen for a 5-HT₆ receptor genomic fragment, Dr Roger Pederson for 129-derived embryonic stem cells, and Dr Warren Hirst for use of ¹²⁵I-SB258585. We also thank Jean Danao, Irene Yun, Aileen Sweeney, and Jed Holtzman for their technical skills and support.

REFERENCES

- Agajanian GK, Sanders-Bush E (2002). *Serotonin*. In: Davis KL, Charney D, Coyle J, Nemeroff C (eds). *Neuropsychopharmacology: The Fifth Generation of Progress*. American College of Neuropsychopharmacology: Nashville, TN. pp 15–34.

- Barnes NM, Sharp T (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* 38: 1083–1152.
- Belknap JK, Crabbe JC, Young ER (1993). Voluntary consumption of ethanol in 15 inbred mouse strains. *Psychopharmacology (Berl)* 112: 503–510.
- Bentley JC, Bourson A, Boess FG, Fone KC, Marsden CA, Petit N *et al* (1999). Investigation of stretching behaviour induced by the selective 5-HT₆ receptor antagonist, Ro 04-6790, in rats. *Br J Pharmacol* 126: 1537–1542.
- Blakley GG, Pohorecky LA, Benjamin D (2001). Bidirectional changes in ethanol consumption in rats with site-specific antisense down-regulation of 5-hydroxytryptamine_{2A} receptors in brain. *J Pharmacol Exp Ther* 299: 277–289.
- Boess FG, Riemer C, Bos M, Bentley J, Bourson A, Sleight AJ (1998). The 5-HT₆ receptor selective radioligand [³H] Ro 63-0563 labels 5-hydroxytryptamine receptor binding sites in rat and porcine striatum. *Mol Pharmacol* 54: 577–583.
- Bos M, Sleight AJ, Godel T, Martin JR, Riemer C, Stadler H (2001). 5-HT₆ receptor antagonists: lead optimisation and biological evaluation of *N*-aryl and *N*-heteroaryl 4-amino-benzene sulfonamides. *Eur J Med Chem* 36: 165–178.
- Bourson A, Borroni E, Austin RH, Monsma Jr FJ, Sleight AJ (1995). Determination of the role of the 5-HT₆ receptor in the rat brain: a study using antisense oligonucleotides. *J Pharmacol Exp Ther* 274: 173–180.
- Bouwknicht JA, Hijzen TH, van der Gugten J, Maes RA, Hen R, Olivier B (2000). Ethanol intake is not elevated in male 5-HT_{1B} receptor knockout mice. *Eur J Pharmacol* 403: 95–98.
- Branchek TA, Blackburn TP (2000). 5-HT₆ receptors as emerging targets for drug discovery. *Annu Rev Pharmacol Toxicol* 40: 319–334.
- Crabbe JC, Johnson NA, Gray DK, Kosobud A, Young ER (1982). Biphasic effects of ethanol on open-field activity: sensitivity and tolerance in C57BL/6N and DBA/2N mice. *J Comp Physiol Psychol* 96: 440–451.
- Crabbe JC, Phillips TJ, Feller DJ, Hen R, Wenger CD, Lessov CN *et al* (1996). Elevated alcohol consumption in null mutant mice lacking 5-HT_{1B} serotonin receptors. *Nat Genet* 14: 98–101.
- Cunningham CL, Fidler TL, Hill KG (2000). Animal models of alcohol's motivational effects. *Alcohol Res Health* 24: 85–92.
- Engel SR, Allan AM (1999). 5-HT₃ receptor over-expression enhances ethanol sensitivity in mice. *Psychopharmacology (Berl)* 144: 411–415.
- Engel SR, Lyons CR, Allan AM (1998). 5-HT₃ receptor over-expression decreases ethanol self administration in transgenic mice. *Psychopharmacology (Berl)* 140: 243–248.
- Frantz KJ, Hansson KJ, Stouffer DG, Parsons LH (2002). 5-HT₆ receptor antagonism potentiates the behavioral & neurochemical effects of amphetamine but not cocaine. *Neuropharmacology* 44: 170–180.
- Gardell LR, Whalen CA, Chattopadhyay S, Cavallaro CA, Hubbell CL, Reid LD (1997). Combination of naltrexone and fluoxetine on rats' propensity to take alcoholic beverage. *Alcohol Clin Exp Res* 21: 1435–1439.
- Gérard C, Martres M-P, Lefèvre K, Miquel MC, Vergé D, Lanfumey L *et al* (1997). Immuno-localization of serotonin 5-HT₆ receptor-like material in the rat central nervous system. *Brain Res* 746: 207–219.
- Gill K, France C, Amit Z (1986). Voluntary ethanol consumption in rats: an examination of blood/brain ethanol levels and behavior. *Alcohol Clin Exp Res* 10: 457–462.
- Glatt CE, Snowman AM, Sibley DR, Snyder SH (1995). Clozapine: selective labeling of sites resembling 5HT₆ serotonin receptors may reflect psychoactive profile. *Mol Med* 1: 398–406.
- Gongwer MA, Murphy JM, McBride WJ, Lumeng L, Li TK (1989). Regional brain contents of serotonin, dopamine and their metabolites in the selectively bred high- and low-alcohol drinking lines of rats. *Alcohol* 6: 317–320.
- Gorwood P, Aissi F, Batel P, Ades J, Cohen-Salmon C, Hamon M *et al* (2002). Reappraisal of the serotonin 5-HT_{1B} receptor gene in alcoholism: of mice and men. *Brain Res Bull* 57: 103–107.
- Gulley JM, McNamara C, Barbera TJ, Ritz MC, George FR (1995). Selective serotonin reuptake inhibitors: effects of chronic treatment on ethanol-reinforced behavior in mice. *Alcohol* 12: 177–181.
- Hallikainen T, Saito T, Lachman HM, Volavka J, Pohjalainen T, Ryyanen OP *et al* (1999). Association between low activity serotonin transporter promoter genotype and early onset alcoholism with habitual impulsive violent behavior. *Mol Psychiatry* 4: 385–388.
- Hamon M, Doucet E, Lefevre K, Miquel MC, Lanfumey L, Insausti R *et al* (1999). Antibodies and antisense oligonucleotide for probing the distribution and putative functions of central 5-HT₆ receptors. *Neuropsychopharmacology* 21: 68S–76S.
- Hatcher PD, Brown VJ, Tait DS, Bate S, Overend P, Hagan JJ *et al* (2005). 5-HT₆R antagonists improve performance in an attentional set shifting task in rats. *Psychopharmacol* 181: 253–259.
- Heisler LK, Chu H-M, Brennan TJ, Danao JA, Bajwa P, Parsons LH *et al* (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT_{1A} receptor mutant mice. *Proc Natl Acad Sci USA* 95: 15049–15054.
- Higley JD, Linnoila M (1997). A nonhuman primate model of excessive alcohol intake: personality and neurobiological parallels of type I- and type II-like alcoholism. In: Galanter M (ed). *Recent Developments in Alcoholism, Vol 1*. Plenum Press: New York. pp 191–219.
- Hirst WD, Abrahamsen B, Blaney FE, Calver AR, Aloj L, Price GW *et al* (2003). Differences in the central nervous system distribution and pharmacology of the mouse 5-hydroxytryptamine-6 receptor compared with rat and human receptors investigated by radioligand binding, site-directed mutagenesis, and molecular modeling. *Mol Pharmacol* 64: 1295–1308.
- Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ *et al* (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol Rev* 46: 157–203.
- Kohen R, Metcalf MA, Khan N, Druck T, Huebner K, Lachowicz JE *et al* (1996). Cloning, characterization, and chromosomal localization of a human 5-HT₆ serotonin receptor. *J Neurochem* 66: 47–56.
- Lamb RJ, Jarbe TU (2001). Effects of fluvoxamine on ethanol-reinforced behavior in the rat. *J Pharmacol Exp Ther* 297: 1001–1009.
- Lane H-Y, Lin C-C, Huang C-H, Chang Y-C, Hsu S-K, Chang W-H (2004). Risperidone response and 5-HT₆ receptor gene variance; genetic association analysis with adjustment for nongenetic confounders. *Schizophr Res* 67: 63–70.
- Lindner MD, Hodges Jr DB, Hogan JB, Orie AF, Corsa JA, Barten DM *et al* (2003). An assessment of the effects of serotonin 6 (5-HT₆) receptor antagonists in rodent models of learning. *J Pharmacol Exp Ther* 307: 682–691.
- Lucas LA, McMillen BA (2002). Differences in brain area concentrations of dopamine and serotonin in Myers' High Ethanol Preferring (mHEP) and outbred rats. *J Neural Transm* 109: 279–292.
- Mansour SL, Thomas KR, Capecchi MR (1988). Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* 336: 348–352.
- Maurel S, De Vry J, De Beun R, Schreiber R (1999). 5-HT_{2A} and 5-HT_{2C/5-HT_{1B}} receptors are differentially involved in alcohol preference and consummatory behavior in cAA rats. *Pharmacol Biochem Behav* 62: 89–96.
- Meneses A (2001). Role of 5-HT₆ receptors in memory formation. *Drug News Perspect* 14: 396–400.

- Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL *et al* (2001). GABA_A-receptor δ subunit knockout mice have multiple defects in behavioral responses to ethanol. *Alcohol Clin Exp Res* 25: 1708–1718.
- Monsma Jr FJ, Shen Y, Ward RP, Hamblin MW, Sibley DR (1993). Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol Pharmacol* 43: 320–327.
- Murphy JM, McBride WJ, Lumeng L, Li TK (1987). Contents of monoamines in forebrain regions of alcohol-preferring (P) and -nonpreferring (NP) lines of rats. *Pharmacol Biochem Behav* 26: 389–392.
- Murphy JM, Stewart RB, Bell RL, Badia-Elder NE, Carr LG, McBride WJ *et al* (2002). Phenotypic and genotypic characterization of the Indiana University rat lines selectively bred for high and low alcohol preference. *Behav Genet* 32: 363–388.
- Myers RD, Robinson DE, West MW, Biggs TA, McMillen BA (1998). Genetics of alcoholism: rapid development of a new high-ethanol-preferring (HEP) strain of female and male rats. *Alcohol* 16: 343–357.
- Naranjo CA, Knoke DM (2001). The role of selective serotonin reuptake inhibitors in reducing alcohol consumption. *J Clin Psychiatry* 62(Suppl 20): 18–25.
- Ohmori O, Shinkai T, Hori H, Nakamura J (2001). Novel polymorphism in the 5'-upstream region of the human 5-HT₆ receptor gene and schizophrenia. *Neurosci Lett* 310: 17–20.
- Otano A, Frechilla D, Cobreros A, Cruz-Orive LM, Insausti A, Insausti R *et al* (1999). Anxiogenic-like effects and reduced stereological counting of immunolabelled 5 hydroxytryptamine 6 receptors in rat nucleus accumbens by antisense oligonucleotides. *Neuroscience* 92: 1001–1009.
- Pettinati HM (2001). The use of selective serotonin reuptake inhibitors in treating alcoholic subtypes. *J Clin Psychiatry* 62(Suppl 20): 26–31.
- Phillips TJ, Hen R, Crabbe JC (1999). Complications associated with genetic background effects in research using knockout mice. *Psychopharmacology (Berl)* 147: 5–7.
- Phillips TJ, Shen EH (1996). Neurochemical bases of locomotion and ethanol stimulant effects. *Int Rev Neurobiol* 39: 243–282.
- Pistis M, Muntoni AL, Gessa G, Diana M (1997). Effects of acute, chronic ethanol and withdrawal on dorsal raphe neurons: electrophysiological studies. *Neuroscience* 79: 171–176.
- Riemer C, Borroni E, Levet-Trafit B, Martin JR, Poli S, Porter RH *et al* (2003). Influence of the 5-HT₆ receptor on acetylcholine release in the cortex: pharmacological characterization of 4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine, a potent and selective 5-HT₆ receptor antagonist. *J Med Chem* 46: 1273–1276.
- Risinger FO (1997). Fluoxetine's effects on ethanol's rewarding, aversive and stimulus properties. *Life Sci* 61: PL235–PL242.
- Risinger FO, Brown MM, Doan AM, Oakes RA (1998). Mouse strain differences in oral operant ethanol reinforcement under continuous access conditions. *Alcohol Clin Exp Res* 22: 677–684.
- Risinger FO, Doan AM, Vickrey AC (1999). Oral operant ethanol self-administration in 5-HT_{1b} knockout mice. *Behav Brain Res* 102: 211–215.
- Roberts AJ, Heyser CJ, Koob GF (1999). Operant self-administration of sweetened versus unsweetened ethanol: effects on blood alcohol levels. *Alcohol Clin Exp Res* 23: 1151–1157.
- Roberts JC, Reavill C, East SZ, Harrison PJ, Patel S, Routledge C *et al* (2002). The distribution of 5-HT(6) receptors in rat brain: an autoradiographic binding study using the radiolabelled 5-HT(6) receptor antagonist [¹²⁵I]SB-258585. *Brain Res* 934: 49–57.
- Rodd-Henricks ZA, McKinzie DL, Melendez RI, Berry N, Murphy JM, McBride WJ (2003). Effects of serotonin-3 receptor antagonists on the intracranial self-administration of ethanol within the ventral tegmental area of Wistar rats. *Psychopharmacology (Berl)* 165: 252–259.
- Rogers DC, Hagan JJ (2001). 5-HT₆ receptor antagonists enhance retention of a water maze task in the rat. *Psychopharmacology (Berl)* 158: 114–119.
- Roth BL, Craig SC, Choudhary MS, Uluer A, Monsma Jr FJ, Shen Y *et al* (1994). Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. *J Pharmacol Exp Ther* 268: 1403–1410.
- Routledge C, Price GW, Bromidge SM, Moss SF, Newman G, Riley T *et al* (1999). Characterisation of SB-271046, a potent and selective 5-HT₆ receptor antagonist. *Br J Pharmacol* 127: 21P.
- Ruat M, Traiffort E, Arrang JM, Tardivel-Lacombe J, Diaz J, Leurs R *et al* (1993). A novel rat serotonin (5-HT₆) receptor: molecular cloning, localization and stimulation of cAMP accumulation. *Biochem Biophys Res Commun* 193: 268–276.
- Russell MG, Dias R (2002). Memories are made of this (perhaps): a review of serotonin 5-HT(6) receptor ligands and their biological functions. *Curr Top Med Chem* 2: 643–654.
- Sambrook J, Fritsch EF, Maniatis T (2001). *Molecular Cloning, A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.
- Samson HH, Pfeiffer AO, Tolliver GA (1998). Oral ethanol self-administration in rats: models of alcohol-seeking behavior. *Alcohol Clin Exp Res* 12: 591–598.
- Shinkai T, Ohmori O, Kojima H, Terao T, Suzuki T, Abe K (1999). Association study of the 5-HT₆ receptor gene in schizophrenia. *Am J Med Genet (Neuropsych Genet)* 88: 120–122.
- Sleight AJ, Boess FG, Bos M, Levet-Trafit B, Riemer C, Bourson A (1998). Characterization of Ro 04-6790 and Ro 63-0563: potent and selective antagonists at human and rat 5-HT₆ receptors. *Br J Pharmacol* 124: 556–562.
- Sleight AJ, Monsma Jr FJ, Borroni E, Austin RH, Bourson A (1996). Effects of altered 5-HT₆ expression in the rat: functional studies using antisense oligonucleotides. *Behav Brain Res* 73: 245–248.
- Stein TO, Hirst WD, Thomas DR, Price GW, Rogers D, Riley G *et al* (2002). Pharmacological profile of SB-357134: a potent, selective, brain penetrant, and orally active 5-HT(6) receptor antagonist. *Pharmacol Biochem Behav* 71: 645–654.
- Svartengren J, Axelsson-Lindén P, Edling N, Fhølenhag K, Isacson R, Hillegaart V *et al* (2004). *The Selective Serotonin 5-HT₆ Receptor Antagonist BVT5182 Decreases Food Intake and Body Weight in Both Rats and Mice. Abstract/Viewer/Itinerary Planner*. Society for Neuroscience: Washington, DC. Online.
- Thiele TE, Miura GI, Marsh DJ, Bernstein IL, Palmiter RD (2000). Neurobiological responses to ethanol in mutant mice lacking neuropeptide Y or the Y5 receptor. *Pharmacol Biochem Behav* 67: 683–691.
- Thome J, Retz W, Baader M, Pesold B, Hu M, Cowen M *et al* (2001). Association analysis of HTR₆ and HTR_{2A} polymorphisms in sporadic Alzheimer's disease. *J Neural Transm* 108: 1175–1180.
- Tomkins DM, Joharchi N, Tampakeras M, Martin JR, Wichmann J, Higgins GA (2002). An investigation of the role of 5-HT(2C) receptors in modifying ethanol self-administration behaviour. *Pharmacol Biochem Behav* 71: 735–744.
- Tsai S-J, Chiu H-J, Want Y-C, Hong C-J (1999a). Association study of serotonin-6 receptor variant (C267T) with schizophrenia and aggressive behavior. *Neurosci Lett* 271: 135–137.
- Tsai S-J, Liu H-C, Liu T-Y, Want Y-C, Hong C-J (1999b). Association analysis of the 5-HT₆ receptor polymorphism C267T in Alzheimer's disease. *Neurosci Lett* 267: 139.
- Unsworth CD, Molinoff PB (1994). Characterization of a 5-hydroxytryptamine receptor in mouse neuroblastoma N18TG2 cells. *J Pharmacol Exp Ther* 269: 246–255.
- Virkkunen M, Linnoila M (1996). Serotonin and glucose metabolism in impulsively violent alcoholic offenders. In: Stoff DM,

- Cairns RB (eds). *Aggression and Violence: Genetic, Neurobiological, and Biosocial Perspectives*. Lawrence Erlbaum: Mahwah, NJ, pp 87–100.
- Ward RP, Hamblin MW, Lachowicz JE, Hoffman BJ, Sibley DR, Dorsa DM (1995). Localization of serotonin subtype 6 receptor messenger RNA in the rat brain by *in situ* hybridization histochemistry. *Neuroscience* **64**: 1105–1111.
- Weiss F, Porrino LJ (2002). Behavioral neurobiology of alcohol addiction: recent advances and challenges. *J Neurosci* **22**: 3332–3337.
- West MW, Kalmus G, Myers RD (1998). Limited access to ethanol in genetic drinking rats is suppressed while feeding is enhanced by the mixed 5-HT_{1A} agonist/5-HT_{2A} antagonist FG5938. *Pharmacol Biochem Behav* **60**: 823–828.
- Wilson AW, Costall B, Neill JC (2000). Manipulation of operant responding for an ethanol-paired conditioned stimulus in the rat by pharmacological alteration of the serotonergic system. *J Psychopharmacol* **14**: 340–346.
- Woolley ML, Bentley JC, Sleight AJ, Marsden CA, Fone KC (2001). A role for 5-HT₆ receptors in retention of spatial learning in the Morris water maze. *Neuropharmacology* **41**: 210–219.
- Woolley ML, Marsden CA, Fone KCF (2004). 5-HT₆ receptors. *Curr Drug Targets—CNS Neurol Disorders* **3**: 59–79.
- Yan QS (1999). Extracellular dopamine and serotonin after ethanol monitored with 5-minute microdialysis. *Alcohol* **19**: 1–7.
- Yoshimoto K, McBride WJ, Lumeng L, Li TK (1992). Alcohol stimulates the release of dopamine and serotonin in the nucleus accumbens. *Alcohol* **9**: 17–22.
- Yoshioka M, Matsumoto M, Togashi H, Mori K, Saito H (1998). Central distribution and function of 5-HT₆ receptor subtype in the rat brain. *Life Sciences* **62**: 1473–1477.
- Zhou FC, Bledsoe S, Lumeng L, Li TK (1991). Immunostained serotonergic fibers are decreased in selected brain regions of alcohol-preferring rats. *Alcohol* **8**: 425–431.
- Zhou FC, Pu CF, Murphy J, Lumeng L, Li TK (1994). Serotonergic neurons in the alcohol preferring rats. *Alcohol* **11**: 397–403.

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