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**Nicotinic Acetylcholine Receptors Containing $\alpha 6$ Subunits Contribute to Alcohol
Reward-Related Behaviors**

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Running Head: $\alpha 6$ -containing nAChRs contribute to alcohol reward

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Abstract

Evidence is emerging that neuronal nicotinic acetylcholine receptors (nAChRs) in the mesolimbic dopamine (DA) system are involved in mediating the reinforcing effects of alcohol. Midbrain DA neurons express high levels of $\alpha 6$ subunit-containing nAChRs that modulate DA transmission, implicating their involvement in reward-related behaviors. The present study assessed the role of $\alpha 6$ -containing nAChRs in modulating alcohol reward using transgenic mice expressing mutant, hypersensitive $\alpha 6$ nAChR subunits ($\alpha 6L9'S$ mice). $\alpha 6L9'S$ mice and littermate controls were tested in three well-established models of alcohol reward: 24-hr two-bottle choice drinking, drinking in the dark (DID), and conditioned place preference (CPP). Confocal microscopy and patch-clamp electrophysiology were used to demonstrate the localization and function of hypersensitive $\alpha 6$ subunit-containing nAChRs. Results indicate that female $\alpha 6L9'S$ mice showed significantly higher alcohol intake at low concentrations of alcohol (3% and 6%) in the two-bottle choice procedure. Both male and female $\alpha 6L9'S$ mice drank significantly more in the DID procedure and displayed an alcohol-induced place preference using a low dose of alcohol (0.5 g/kg) that was ineffective in littermate controls. Confocal microscopy showed that $\alpha 6$ subunit-containing nAChRs are selectively expressed on ventral tegmental area (VTA) DAergic, but not GABAergic neurons. Patch-clamp electrophysiology demonstrated that VTA DA neurons of $\alpha 6L9'S$ mice are hypersensitive to ACh. Collectively, these results suggest that $\alpha 6L9'S$ mice are more sensitive to the rewarding effects of alcohol, and suggest that VTA $\alpha 6$ subunit-containing nAChRs modulate alcohol reward. Thus, $\alpha 6$ subunit-containing nAChRs may be a promising therapeutic target for treatment of alcohol use disorders.

Introduction

Alcohol and nicotine are often used together (Grant *et al.*, 2004), possibly because the two drugs interact to influence the rewarding effects of one or both substances (Little, 2000). Nicotine exerts its effects on nicotinic acetylcholine receptors (nAChRs), which are widely expressed in the central nervous system. nAChRs can modulate the excitability of neurons, ultimately affecting the release of neurotransmitters, including acetylcholine (ACh), GABA (McClure-Begley *et al.*, 2009), glutamate (Garduno *et al.*, 2012, Mansvelder & McGehee, 2000), norepinephrine (Azam & McIntosh, 2006), and serotonin (Gotti *et al.*, 2006). Importantly, presynaptic nAChRs located in the mesolimbic reward pathway can strongly modulate dopamine (DA) release (Drenan *et al.*, 2010, Drenan *et al.*, 2008, Grady *et al.*, 2002, Jackson *et al.*, 2009, Salminen *et al.*, 2007).

Alcohol elevates DA levels in the nucleus accumbens (Soderpalm *et al.*, 2009), a brain area involved in the reinforcing properties of alcohol (reviewed by Wise, 1998). Evidence suggests that nAChRs in the ventral tegmental area (VTA) may underlie elevated alcohol-induced accumbal DA levels. Ericson and colleagues (2008) showed that when the nonselective nAChR antagonist mecamylamine was locally administered to the anterior VTA, it blocked alcohol-induced increases in accumbal DA levels, suggesting that nAChRs in the VTA may be involved in modulating alcohol reward. Mecamylamine also reduced alcohol drinking in rodents (Blomqvist *et al.*, 1996, Ericson *et al.*, 1998, Hendrickson *et al.*, 2010) and humans (Chi & De Wit, 2003, Young *et al.*, 2005). The FDA-approved smoking cessation aid varenicline, a partial agonist at $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs (* indicates that other subunits may be present in the pentameric

receptor) (Mihalak *et al.*, 2006), reduced alcohol intake in rodents (e.g., Hendrickson *et al.*, 2010) and in humans (Mckee *et al.*, 2009). However, because both mecamylamine and varenicline are not selective antagonists, it is not clear which nAChR subtypes are involved in alcohol drinking behavior.

The mesolimbic DA system, consisting in part of DA neurons in the VTA and their projections to nucleus accumbens, is crucial to alcohol reward (e.g., Brodie *et al.*, 1999, Gessa *et al.*, 1985). $\alpha 6^*$ nAChRs are enriched in the mesolimbic reward pathway, and previous studies support the claim that $\alpha 6^*$ nAChRs, unlike $\alpha 4$ (non- $\alpha 6$)* nAChRs, are selectively expressed in DA neurons but not in local GABA neurons that inhibit DA cells. Recently, Drenan and colleagues (2008) created mice with mutated $\alpha 6$ nAChR subunits ($\alpha 6L9'S$ mice) in order to selectively amplify the downstream effects of activating $\alpha 6^*$ nAChRs. In $\alpha 6L9'S$ mice, $\alpha 6^*$ nAChRs are markedly hypersensitive to nicotine, showing greater nicotine-stimulated DA release in synaptosome experiments and larger inward currents in response to local application of nicotine to patch-clamped VTA DA neurons in brain slices (Drenan *et al.*, 2008). These results provide evidence that $\alpha 6^*$ nAChRs located on VTA DAergic neurons modulate DA transmission.

Indirect evidence suggests that $\alpha 6^*$ nAChRs may play an important role in modulating alcohol reward-related behaviors. α -conotoxin MII, an antagonist at both $\alpha 3\beta 2^*$ and $\alpha 6^*$ nAChRs, reduced both voluntary drinking (Larsson & Engel, 2004) and operant responding (Lof *et al.*, 2007) for alcohol. However, because α -conotoxin MII is not perfectly selective for $\alpha 6^*$ vs. $\alpha 3^*$ nAChRs, complementary approaches are necessary to confirm the role of specific nAChR subunits in modulating behavior. Mutant mice

with hypersensitive $\alpha 6^*$ nAChRs provide an excellent opportunity to evaluate the specific role of the $\alpha 6$ subunit in alcohol reward.

The purpose of the present study was to assess the involvement of $\alpha 6^*$ nAChRs in modulating alcohol reward by comparing $\alpha 6L9'S$ mice to littermate controls in three well-established models of alcohol reward: 24-hr two-bottle choice drinking, drinking in the dark (DID), and alcohol-induced conditioned place preference (CPP). Confocal microscopy and patch-clamp electrophysiology were used to demonstrate the localization and function of hypersensitive $\alpha 6^*$ nAChRs.

Materials and Methods

Subjects

$\alpha 6L9'S$ bacterial artificial chromosome (BAC) transgenic mice were constructed as previously described (Drenan *et al.*, 2008). Briefly, an ectopic BAC containing a mutant, L9'S allele of the mouse $\alpha 6$ nAChR subunit gene was introduced into fertilized FVB/N embryos and implanted into pseudopregnant Swiss-Webster surrogates. The insertion site within the mouse genome is unknown. Founder animals were back-crossed to C57BL/6 12 to 15 times prior to these studies. Although >90% of the genome is expected to be C57BL/6 following this many rounds of back-crossing, elimination of FVB/N allelic DNA immediately adjacent to the BAC insertion site is not likely. α -conotoxin MII binding experiments demonstrated that $\alpha 6^*$ nAChRs are faithfully expressed in their correct anatomical locations and are not overexpressed in $\alpha 6L9'S$ mice (Drenan *et al.*, 2010, Drenan *et al.*, 2008). Both male and female mice were used in the

current experiment with ages ranging from 64 to 193 days old at the time of experimental procedures. Mice were group housed (unless otherwise indicated) and had free access to food (Rodent Lab Diet 5001, Purina Mills Inc., St. Louis, MO, USA) and water in the home-cage. Ambient temperature in the colony and experimental rooms was maintained at $21\pm 2^{\circ}\text{C}$. Efforts were made to minimize any pain and discomfort to the mice. All experiments were conducted in accordance with the guidelines for care and use of animals provided by the National Institutes of Health (NIH), and all procedures were approved by the Purdue University Institutional Animal Care and Use Committee.

Drugs

Alcohol (95% v/v) was diluted with tap water (drinking studies) or saline (CPP studies) to obtain the desired concentrations (3%, 6%, 10%, 20% v/v). For the place conditioning study, alcohol was administered intraperitoneally (i.p.) in doses of 0.5 and 2.0 g/kg of body weight with injection volumes of 3.15 and 12.6 ml/kg (20% v/v), respectively.

Place conditioning apparatus

The place conditioning apparatus has been utilized in prior place conditioning studies with mice (e.g., Powers *et al.*, 2010). The apparatus consisted of 12 identical Plexiglas boxes (43.2 X 1.6 X 25.4 cm) enclosed in separate ventilated sound- and light-attenuated chambers (76.2 X 50.8 X 20.3 cm). The floor of each box consisted of interchangeable halves with distinct floor textures. One floor texture (the Grid floor) consisted of 4 mm steel rods mounted 3.5 mm apart and the other floor texture (the Hole

floor) was made up of perforated 16 gauge stainless steel with 6.4 mm holes on 9.5 mm staggered centers. Locomotor activity and side position (left or right) for each mouse was continuously monitored in each box with an open field activity frame (SmartFrame Low Density, Lafayette Instrument Co, Lafayette, IN, USA), that contained infrared photobeams along the length and width of each frame (internal frame dimensions: 24.1 X 45.7 cm). Ventilation fans in each box masked outside noise.

Study procedures

24-hr continuous-access alcohol drinking

Mice [n=35; 17 α 6L9'S (9 male/8 female) and 18 non-Tg (10 male/8 female)] were singly housed for 7 days with access to two water-filled tubes (25 ml fitted with metal drinking spouts) in order to habituate them to the experimental environment. On the 8th day, one of the water tubes was replaced with an alcohol tube for each mouse. Alcohol concentration was increased every 4 days: days 1 - 4 (3%), days 5 - 8 (6%), days 9 - 12 (10%), and days 13 - 16 (20%). Every 2 days, mice were weighed, drinking solutions were replaced, and bottle position was alternated to avoid the development of a side preference (Kamens *et al.*, 2010). Evaporation/leakage was estimated from tubes placed on empty cages, and mean volume lost was subtracted from individual drinking values before analysis.

Drinking in the dark (DID)

Drinking in the dark experiments were conducted as previously described (Rhodes *et al.*, 2005). The same mice used in the 24-hr continuous-access alcohol

drinking study remained singly housed for 33 days with normal water bottles. Mice then received one water-filled drinking tube on the home cage for 7 days. On days 8 through 11, body weights were measured and the water tube for each mouse was replaced with a 20% v/v alcohol tube two hours after lights off (lights off at 0945). Mice had access to alcohol for 2 hrs. Amount of alcohol consumed was recorded daily after each 2-hr session.

Alcohol-induced CPP

The place conditioning procedure consisted of 1 pretest, 8 conditioning sessions, and 1 posttest. All phases of the experiment were conducted on consecutive days except that a 48 hr break separated the first four and the second four conditioning sessions. Experimental groups were counterbalanced by genotype, sex, order of exposure to the conditioning stimulus (CS), box enclosure and floor position (i.e., left vs. right side of the box). All animals were transferred on a cart to the procedure room 30 min before the start of experimental procedures each day.

Pretest (Day 1). Initial unconditioned preference for each CS (Grid vs. Hole) was assessed 24 hr prior to the start of the first conditioning trial. Subjects were injected (i.p.) with saline (at a volume equal to a 0.5 or 2.0 g/kg dose of alcohol, depending on the experiment) and placed in the testing box with free access to both floor types for 60 min.

Conditioning Sessions (Days 2-11). Mice underwent a differential place conditioning procedure in which they were randomly assigned to one of two conditioning subgroups within each experimental subgroup. Each conditioning subgroup received 5 minutes of exposure to either a Grid or Hole floor paired with alcohol treatment (4 CS+

conditioning sessions) and the other floor type paired with saline (4 CS– conditioning sessions) on alternating days, for a total of 8 conditioning sessions. The floors and inside of the box were wiped with a damp sponge between each subject.

Separate experiments were run for each dose of alcohol (0.5 and 2.0 g/kg), but all procedures were identical. A total of 32 mice [17 α 6L9'S (8 male/9 female) and 15 non-Tg (4 male/11 female)] were used for the 0.5 g/kg experiment, and 28 mice [17 α 6L9'S (9 male/8 female) and 11 non-Tg (3 male/8 female)] were used for the 2.0 g/kg experiment. The low dose of alcohol (0.5 g/kg) was chosen because we hypothesized that α 6L9'S mice would be more sensitive to alcohol reward, and a 0.5 g/kg dose is below the threshold for producing alcohol-induced CPP in C57BL/6 mice (e.g., Cunningham *et al.*, 1992, Wrobel, 2011). The 2.0 g/kg dose was used as a positive control.

Posttest (Day 12). Mice were tested for CPP 24 hrs after their final conditioning session. As in the pretest, mice had free access to both floors in the same position for 60 min. Mice received an injection of saline (equal volume to a 0.5 or 2.0 g/kg dose of alcohol) directly before being placed in the apparatus to match cues during conditioning.

Brain Slice Preparation for Electrophysiology

Brain slices were prepared as previously described (Engle *et al.*, 2012). α 6L9'S and non-Tg mice were genotyped at 21-28 days after birth. Mice were anesthetized with sodium pentobarbital (100 mg/kg; i.p.) followed by cardiac perfusion with oxygenated (95% O₂/5% CO₂), 4°C NMDG-recovery solution containing (in mM): 93 n-methyl D-glucamine, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na⁺ ascorbate, 2 thiourea, 3 Na⁺ pyruvate, 10 MgSO₄•7H₂O, 0.5 CaCl₂•2H₂O (300-310 mOsm, pH 7.3-

7.4). Brains were removed and retained in 4°C NMDG-recovery solution for 1 min. Coronal slices (250 µm) were cut with a microslicer (DTK-Zero 1; Ted Pella, Redding, CA). Brain slices recovered for 12 min at 33°C in oxygenated NMDG-recovery solution, after which they were held until recording in HEPES holding solution containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na⁺ ascorbate, 2 thiourea, 3 Na⁺ pyruvate, 2 MgSO₄•7H₂O, 2 CaCl₂•2H₂O (300-310 mOsm, pH 7.3-7.4). Coordinates for recordings in SNc/VTA were within the following range: (-3.8 to -2.9 mm from bregma, 4.0 to 4.8 mm from the surface, and 0.0 to 2.0 mm from the midline).

Immunohistochemistry and Confocal Microscopy

Transgenic mice expressing α6* nAChR subunits fused in-frame with green fluorescent protein (GFP) (α6GFP mice; 2 male/2 female) were anesthetized with sodium pentobarbital (100 mg/kg; i.p.) and transcardially perfused with 15 ml of ice-cold PBS followed by 25 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were removed and postfixed for 2 h at 4°C. Coronal sections (50 µm) were cut on a microslicer and collected into PBS. Sections were permeabilized (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 50 mM NaCl, 3 mM MgCl₂, 300mM sucrose) for 1 h at 4°C, blocked (0.1% Triton X-100, 5% donkey serum in TBS) for 1 h at RT, and incubated overnight at 4°C in solutions containing primary antibodies (diluted in 0.1% Triton X-100, 5% donkey serum in TBS). Primary antibodies and final dilutions were as follows: 1:500 rabbit anti-GFP (A11122; Invitrogen), 1:500 sheep anti-tyrosine hydroxylase (TH; AB1542, Millipore), 1:500 mouse anti-GAD67 (MAB5406; Millipore). Sections were washed three times for

10 min each in TBST (0.1% Triton X-100 in TBS) followed by incubation at RT for 1 hr with secondary antibodies (diluted in 0.1% Triton X-100, 5% donkey serum in TBS). Secondary antibodies and final dilutions were as follows: 1:1000 goat anti-rabbit Alexa 488 (A11008; Invitrogen), 1:1000 donkey anti-sheep Alexa 555 (A21436; Invitrogen), 1:1000 donkey anti-mouse Alexa 555 (A31570; Invitrogen). Sections were then washed four times in TBST for 10 min each. All sections were mounted on slides and coverslipped with Vectashield (Vector Laboratories), then imaged with a Nikon (Nikon Instruments) A1 laser-scanning confocal microscope system. Nikon Plan Apo 10X air and 60X oil objectives were used. Alexa 488 was excited with an argon laser at 488 nm, and Alexa 555 was excited with a yellow solid-state laser at 561 nm.

Patch Clamp Electrophysiology

Patch clamp electrophysiology was carried out as previously described (Engle *et al.*, 2012) using tissue collected from adult mice (10 α 6L9'S mice and 5 non-Tg mice). Each recorded cell within a slice was treated as a separate experiment, and each animal yielded between 1 and 6 recorded cells. Patch clamp experiments were not segregated by sex. A single slice was transferred to a 0.8 ml recording chamber (Warner Instruments, RC-27L bath with PH-6D heated platform), and slices were superfused throughout the experiment with standard recording ACSF (1.5–2.0 ml/min) containing (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 24 NaHCO₃, 12.5 glucose, 2 MgSO₄•7H₂O, 2 CaCl₂•2H₂O (300-310 mOsm, pH 7.3-7.4). Cells were visualized with an upright microscope (FN-1; Nikon) using infrared or visible differential interference contrast (DIC) optics. Patch electrodes were constructed from Kwik-Fil borosilicate glass capillary tubes (1B150F-4;

World Precision Instruments, Inc.; Sarasota, FL) using a programmable microelectrode puller (P-97; Sutter Instrument Co.; Novato, CA). The electrodes had tip resistances of 4.5–8.0 M Ω when filled with internal pipette solution (pH adjusted to 7.25 with Tris base, osmolarity adjusted to 290 mOsm with sucrose) containing: 135 mM K⁺ gluconate, 5 mM EGTA, 0.5 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM GTP. Whole-cell recordings were taken at 32°C with an Axopatch 200B amplifier, a 16-bit Digidata 1440A A/D converter, and pCLAMP 10.3 software (all Molecular Devices Axon; Sunnyvale, CA). Data were sampled at 5 kHz and low-pass filtered at 1 kHz. The junction potential between the patch pipette and the bath solution was nulled immediately prior to gigaseal formation. Series resistance was uncompensated.

VTA DAergic neurons were identified as previously described (Drenan et al., 2008). These neurons typically exhibit broad spikes (> 2 msec), slow tonic firing (1 to 6 Hz) in coronal slice preparation, expression of significant I_h currents at hyperpolarized potentials, and significant sag responses in current clamp mode following injection of hyperpolarizing current. To examine the function of somatic nAChRs, acetylcholine was locally applied using a Picospritzer III (General Valve; Fairfield, NJ). Using a high-resolution micromanipulator, (Sutter Instruments; Novato, CA), the pipette tip was moved within 20–40 μ m of the recorded cell 1–2 sec before drug application. ACh-containing ACSF recording solution was then puffed at 10–20 psi for 250 ms. Immediately after the application, the glass pipette was retracted.

Statistical analyses

Behaviors were analyzed using analysis of variance (ANOVA) with the significance level set at $p \leq 0.05$. Between-group factors included genotype and sex. Within-group factors included concentration phase (3%, 6%, 10%, and 20%), day, floor type (Grid or Hole), conditioning session (1-8), trial (4: one CS+ conditioning session and one CS- conditioning session), trial type (CS+ and CS-), block (2-day drinking averages), test (pretest and posttest), time bin (first and last 30 min) or min, where applicable. Only significant interactions including genotype are reported and followed-up with lower-order ANOVAs and *t*-tests, where applicable. Preference test data were averaged into 30 min time bins before analysis since treatment effects on the expression of CPP can change over the course of the 60-min preference test (e.g., Chester *et al.*, 1998, Powers *et al.*, 2010). Pearson correlations between CS+ time and locomotor activity during the posttest were conducted because locomotor activity can affect the expression of CPP (Gremel & Cunningham, 2007). Concentration-response curves for ACh-elicited inward currents in VTA neurons were derived by fitting mean peak current values for each ACh concentration to the Hill equation.

Results

24-hr continuous-access

Alcohol intake. Similar to (Kamens *et al.*, 2010), mice were given access to escalating concentrations of alcohol over a 16-day test period in a two-bottle choice paradigm. Data from non-Tg mice (1 male and 1 female) were excluded from analysis due to restricted fluid flow from the sipper tube, leaving a total of 33 mice included in analyses: 17 $\alpha 6L9'S$ (9 male/8 female) and 16 non-Tg (9 male/7 female). Alcohol intake

(g/kg) was averaged into 2-day blocks to simplify data presentation (Figure 1a). The ANOVA [genotype (2) X sex (2) X concentration phase (4) X block (2: within each concentration)] indicated that females drank more than males [$F(1,29)=23.0$, $p<0.01$] and that intake increased with increasing alcohol concentrations [$F(3,87)=34.9$, $p<0.001$]. A significant four-way interaction [$F(3, 87)=4.7$, $p<0.01$] was also found, and three-way ANOVAs (genotype X sex X block) within each concentration yielded significant three-way interactions at both the 3% [$F(1,29)=4.2$, $p=0.001$] and 6% [$F(1,29)=7.9$, $p=0.01$] concentrations, but not at the 10% or 20%. Follow-up ANOVAs (genotype X sex) at each of the 2 blocks within the 3% and 6% concentrations yielded a main effect of genotype for block 1 [3% concentration; $F(1, 29)=5.1$, $p<0.01$; $\alpha 6L9'S < \text{non-Tg}$] and genotype X sex interactions for blocks 2 [3% concentration; $F(1, 29)=5.4$, $p<0.05$] and 4 [6% concentration; $F(1, 29)=7.0$, $p=0.01$]. The interactions at blocks 2 and 4 resulted from female $\alpha 6L9'S$ mice consuming more alcohol than female non-Tg mice [$F_s(1,13)>11.5$, $p_s<0.01$].

Water intake. Water intake (ml/kg) was averaged into 2-day blocks, as was done for alcohol. The ANOVA [genotype (2) X sex (2) X concentration phase (4) X block (2: within each concentration)] uncovered a significant genotype X concentration phase X block interaction [$F(3,87)=9.8$, $p<0.001$]. Follow-up two-way ANOVAs (genotype X block) within each concentration phase indicated significant genotype X block interactions for the 3% and 6% concentrations [$F_s(1,31)>8.2$, $p_s<0.01$]. One-way ANOVAs comparing genotype at each block within the 3% and 6% concentration phases indicated that non-Tg drank significantly more water than $\alpha 6L9'S$ mice on blocks 2 [3%

concentration; $F(1, 29)=17.7, p<0.001$] and 4 [6% concentration; $F(1, 29)=19.1, p<0.001$], mirroring alcohol intake (data not shown).

Total fluid intake. Total fluid intake (ml/kg: Figure 1b) was averaged into 2-day blocks, as was done for alcohol and water. ANOVA [genotype (2) X sex (2) X concentration phase (4) X block (2: within each concentration)] indicated that females drank more total fluid than males [$F(1,29)=52.3, p<0.001$]. A significant four-way interaction [$F(3,87)=2.7, p<0.05$] was followed-up with three-way ANOVAs within each concentration phase, but these analyses showed no significant effects.

DID

Mice removed from the data set in the 24- hour drinking study (blocked tubes) were included in the DID study, for a total of 35 mice: 17 $\alpha 6L9'S$ (9 male/8 female) and 18 non-Tg (10 male/8 female). A three-way ANOVA [genotype (2) X sex (2) X day (4)] of alcohol intake (g/kg) yielded a main effect of genotype [$F(1, 31)=4.8, p<0.05$], indicating that overall alcohol intake was greater in $\alpha 6L9'S$ mice than non-Tg littermates (Figure 2).

Alcohol-induced CPP

0.5 g/kg

Pretest preference. Analysis of the raw time spent on the Grid and Hole floors [genotype (2) X sex (2) X floor type (2) X time bin (2: first and last 30 min) ANOVA] yielded no effects indicating that mice did not show an unconditioned preference for either floor type.

Pretest activity. Analysis of pretest locomotor activity [genotype (2) X sex (2) X time bin (2: first and last 30 min) ANOVA] indicated that non-Tg mice were more active than $\alpha 6L9'S$ mice [$F(1, 28)=4.4, p<0.05$], that females were more active than males [$F(1, 28)=4.3, p<0.05$], and that activity was lower during the last 30 min compared to the first 30 min [$F(1, 28)=115.8; p<0.01$] (data not shown).

Conditioning activity. Data from one female $\alpha 6L9'S$ mouse was removed from conditioning activity analysis due to equipment malfunction on the second CS+ conditioning trial. Analysis of locomotor activity on CS+ and CS- trials [genotype (2) X sex (2) X trial (4) X trial type (2) ANOVA] indicated a main effect of sex [$F(1, 27)=6.4, p<0.05; \text{females}>\text{males}$] and a genotype X trial interaction [$F(3, 81)=4.0, p=0.01$]. Follow-up analyses of genotype within each trial did not show significance; the interaction was due to a genotype dependent difference in reduced activity across trials.

A genotype X sex X trial type interaction [$F(1, 27)=9.6, p<0.01$] was also found. Follow-up analyses comparing genotype and sex within each trial type showed females were more active than males on both trial types [$F(1,27)>5.7, p<0.05$]. Comparing sex and trial type within each genotype indicated that $\alpha 6L9'S$ females were more active than $\alpha 6L9'S$ males [$F(1,14)=4.4, p=0.05$], and non-Tg mice displayed greater activity on CS+ than on CS- trials [$F(1,13)=4.9, p=0.05$] (Figure 3); $\alpha 6L9'S$ mice did not show a difference in activity levels between CS+ and CS- trial types.

Posttest preference. ANOVAs comparing change in time spent on the CS+ floor from the pretest to the posttest [genotype (2) X sex (2) X test (2) X time bin (2: first and last 30 min)] indicated a significant genotype X sex X test interaction [$F(1,28)=6.4, p<0.05$], but follow-up analyses did not reach significance. There was also a genotype X

test X time bin interaction [$F(1,28)=4.9, p<0.05$]. Two-way ANOVAs (genotype X test) within each time bin indicated a significant two-way interaction in the last 30 min only [$F(1,28)=6.1, p<0.05$]. Analysis of test within each genotype indicated a significant CPP in $\alpha 6L9'S$ mice [$F(1,16)=4.4, p=0.05$], but not in non-Tg mice (Figure 4).

Posttest activity. Analysis of posttest locomotor activity [genotype (2) X sex (2) X time bin (2: first and last 30 min) ANOVA] indicated a genotype X time bin interaction [$F(1,28)=11.8, p<0.01$]. Genotype comparisons within each time bin indicated that non-Tg mice were more active than $\alpha 6L9'S$ mice during the last 30 minutes only [$F(1,30)=13.6, p<0.01$]; there was a trend for the same genotype difference during the first 30 min ($p=0.06$) (data not shown).

Pearson correlations. Correlations of CS+ time and posttest activity during the first and last 30 min indicated no significant relationship. The same analyses run within each genotype also indicated no significant relationships.

2.0 g/kg

Pretest preference. Analysis of the raw time spent on the Grid and Hole floors [genotype (2) X sex (2) X floor type (2) X time bin (2: first and last 30 min) ANOVA] yielded a genotype X sex X time bin interaction [$F(1, 24)=4.7, p<0.05$], but no floor type interaction, indicating that mice did not show an unconditioned preference for either floor type.

Pretest activity. Analysis of pretest locomotor activity [genotype (2) X sex (2) X time bin (2: first and last 30 min) ANOVA] indicated a significant sex X time bin

interaction [$F(1,24)=8.4$, $p<0.01$], due to a greater reduction in locomotor activity in males than females across the test session (data not shown).

Conditioning activity. Analysis of locomotor activity on CS+ and CS- trials [genotype (2) X sex (2) X trial (4) X trial type (2) ANOVA] indicated a significant trial X trial type interaction [$F(3,72)=9.7$, $p<0.01$]. Comparing trial within each trial type indicated a significant reduction in activity across CS- trials only [$F(3,81)=20.5$, $p<0.01$]. Comparing trial type within each trial indicated mice were significantly more active on CS+ days than CS- days for trials 2-4 [$F(1, 27)>20.1$, $ps<0.01$ (Figure 5)].

Posttest preference. ANOVAs comparing change in time spent on the CS+ floor from the pretest to the posttest [genotype (2) X sex (2) X test (2) X time bin (2: first and last 30 min)] indicated a main effect of sex [$F(1,24)=6.3$, $p<0.05$; male>female] and a strong trend for a main effect of test [$F(1,24)=4.0$, $p=0.057$; posttest >pretest] (Figure 6).

Posttest activity. Analysis of posttest locomotor activity [genotype (2) X sex (2) X time bin (2: first and last 30 min) ANOVA] uncovered a main effect of time bin [$F(1,24)=33.6$, $p<0.01$], indicating lower activity during the last than the first 30 min of the posttest (data not shown).

Pearson correlations. Correlations of CS+ time and posttest activity during the first and last 30 min indicated a significant negative relationship during the first 30 min ($r=-0.39$, $n=28$, $p<0.05$). The same analyses run within each genotype indicated significant negative relationships for non-Tg mice only, within both 30 min periods ($rs=-0.75$, $ns=11$, $ps=0.01$).

Immunohistochemistry and Confocal Microscopy

To support the claim that $\alpha 6^*$ nAChRs are selectively expressed in DA neurons but not local GABA neurons, we studied $\alpha 6$ GFP transgenic mice. $\alpha 6$ GFP mice faithfully recapitulate the known, endogenous expression pattern of $\alpha 6^*$ nAChR subunits in the mouse brain (Mackey *et al.*, 2012). We stained ventral midbrain sections in $\alpha 6$ GFP mice with anti-GFP antibodies to localize $\alpha 6^*$ nAChRs, and we co-stained these sections for either tyrosine hydroxylase (TH; a marker of DA neurons) or glutamic acid decarboxylase 67 (GAD-67; a marker of GABAergic neurons).

Confocal microscopy showed the selective expression of $\alpha 6^*$ nAChRs (Figure 7). Analysis of stained sections showed that while $\alpha 6^*$ nAChRs are indeed enriched in DA neurons in VTA and the adjacent substantia nigra pars compacta (SNc) (Figure 7a), GABAergic neurons in ventral midbrain do not express $\alpha 6^*$ nAChRs (Figure 7b). High-resolution confocal images at the border between SNc and SNr show that GFP-tagged $\alpha 6$ nAChR subunits are not localized in GAD67-positive, GABAergic neurons (Figure 7c). Both male and female brains were analyzed. We found no sex differences and no evidence for mis-expression of $\alpha 6$ GFP subunits in brain areas not known to express this subunit (data not shown).

Patch Clamp Electrophysiology

$\alpha 6^*$ nAChRs in $\alpha 6$ L9'S VTA DA neurons may enhance reward signals by being more strongly activated by endogenous ACh levels. To test this idea, we recorded from VTA DA neurons in acutely-prepared brain slices from $\alpha 6$ L9'S and control littermate mice. Following visual identification and the establishment of a stable whole-cell recording, VTA DA neurons were held in voltage clamp mode. A second, drug-filled

micropipette was used to locally deliver ACh to the recorded neuron, resulting in activation of nAChRs (Figure 8a; inset schematic). ACh-evoked currents were generated in response to lower concentrations of ACh in $\alpha 6L9'S$ neurons relative to neurons from control littermate mice (Figure 8a). Peak current amplitudes at each of several ACh concentrations were plotted, and the data were fitted to the Hill equation. In VTA DA neurons, the EC_{50} for ACh was lowered substantially in L9'S neurons compared to responses in control littermate neurons (non-Tg: 208 μM ; $\alpha 6L9'S$: 2 μM ; Figure 8b). A similar EC_{50} trend was obtained from SNc DA neurons (non-Tg: 94 μM ; $\alpha 6L9'S$: 1 μM ; Figure 8c). Together, these results demonstrate that nAChRs in $\alpha 6L9'S$ VTA DA neurons are hypersensitive to ACh. This may partially underlie the increased sensitivity to alcohol reward in $\alpha 6L9'S$ mice.

Discussion

Previous studies have utilized nAChR subunit knockout mice or pharmacological blockade of nAChRs to investigate the relevant nAChR subunits involved in alcohol reward. Here, we studied $\alpha 6L9'S$ mice, which were specifically engineered to amplify and/or isolate functional responses following $\alpha 6^*$ nAChR activation. $\alpha 6GFP$ mice were also used to precisely localize $\alpha 6^*$ nAChRs in individual neurons in the mesolimbic reward system. We found that female $\alpha 6L9'S$ mice drank greater amounts of 3% and 6% alcohol solutions in a two-bottle choice paradigm. $\alpha 6L9'S$ of both sexes drank greater amounts of a 20% alcohol solution in the DID paradigm. Further, $\alpha 6L9'S$ mice exhibited CPP following injection of 0.5 g/kg alcohol, which was insufficient to produce CPP in littermate control mice. Both genotypes showed evidence of CPP in the 2.0 g/kg

experiment, although the main effect did not quite reach statistical significance ($p=0.057$). Our data also showed that $\alpha 6^*$ nAChRs are specifically expressed in midbrain DAergic (but not GABAergic) neurons (Figure 7), and that $\alpha 6^*$ nAChRs in $\alpha 6L9'S$ mice are dramatically more sensitive to endogenous ACh (Figure 8), suggesting that DA reward signals following alcohol exposure are enhanced in $\alpha 6L9'S$ mice. Taken together, these results suggest that genotype, sex and alcohol concentration are interacting to influence sensitivity to the rewarding effects of alcohol.

$\alpha 6^$ nAChRs in alcohol reward-related behaviors*

Interest in studying the modulation of alcohol's rewarding effects by the nicotinic cholinergic system stems from the high co-morbidity between tobacco addiction and alcohol use disorders (AUDs) (Dani & Harris, 2005). Nicotine and/or alcohol interactions at specific nAChR subtypes may explain the co-morbidity. Indeed, particular alleles in either of the $\alpha 5$ - $\alpha 3$ - $\beta 4$ or $\alpha 6$ - $\beta 3$ nAChR gene clusters have been associated with increased risk toward development of nicotine addiction and AUDs in humans (Hoft *et al.*, 2009, Wang *et al.*, 2009), though $\alpha 5^*$ nAChRs do not play a role in alcohol consumption in mice (Santos *et al.*, 2012).

Enhanced alcohol consumption in female $\alpha 6L9'S$ mice in the 24-hr drinking experiment and in both male and female $\alpha 6L9'S$ mice for the DID experiment is consistent with prior studies in which nAChR subtypes have been implicated in alcohol intake and alcohol reward. Mecamylamine, a non-specific nAChR antagonist, reduced voluntary alcohol intake using both 24-hr and limited-access drinking procedures (Blomqvist *et al.*, 1996, Le *et al.*, 2000), and reduced alcohol intake in a binge-drinking paradigm (Hendrickson *et al.*, 2009). Varenicline, an FDA-approved smoking cessation

drug and partial agonist at $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs (Bordia *et al.*, 2012, Mihalak *et al.*, 2006) acts via VTA nAChRs to attenuate alcohol consumption (Hendrickson *et al.*, 2010, Zhao-Shea *et al.*, 2011). Mice lacking $\alpha 4$ nAChR subunits – a genetic alteration that dramatically reduces $\alpha 6^*$ nAChR function (Drenan *et al.*, 2010) – show significantly less alcohol consumption than mice with intact $\alpha 4^*$ nAChR function (Hendrickson *et al.*, 2010). Although mice lacking $\alpha 6$ nAChR subunits ($\alpha 6$ KO mice) demonstrated increased sensitivity to the sedative-hypnotic effects of high alcohol concentrations, they did not show differences in alcohol consumption (Kamens *et al.*, 2012). These results could be due to $\alpha 4\beta 2^*$ nAChR up-regulation, which is seen in $\alpha 6$ KO DA terminals (Champtiaux *et al.*, 2003). Importantly, blockade of $\alpha 6^*$ nAChRs in the VTA with α -conotoxin MII reduced locomotor activity, accumbal DA release, and the rewarding action of alcohol (Larsson *et al.*, 2004). Local VTA infusion of α -conotoxin MII also blocked alcohol-associated conditioned responding (Larsson *et al.*, 2004). These pharmacological and/or genetic loss-of-function studies of $\alpha 4^*$ and $\alpha 6^*$ nAChRs complement the data we present here; prior results in mice with diminished function at $\alpha 4^*$ or $\alpha 6^*$ nAChRs showed reduced alcohol consumption or reward, and in this paper, enhanced $\alpha 6^*$ nAChR function is associated with enhanced sensitivity to the rewarding effects of alcohol.

Intriguingly, we found a sex-dependent genotype difference in alcohol intake at the 3% and 6% concentrations, such that female $\alpha 6L9'S$ mice drank significantly more than female non-Tg mice (Figure 1). These differences in alcohol intake are not related to polydipsia since both genotypes consumed similar amounts of total fluid, which implies the observed differences are specific to alcohol. Future studies comparing intake of other tastants (e.g. saccharine/quinine preference) will be important and allow for

assessment of reward-related genotype differences with other reinforcers. These results suggest a leftward shift in the alcohol reward dose response curve in female $\alpha 6L9'S$ mice. It is interesting to note that while sex-dependent genotype differences were uncovered in the 24-hr drinking paradigm, there were no significant sex-dependent effects in DID or the expression of CPP. $\alpha 6L9'S$ mice, due to the discrete and well-described nature of their genetic alteration, will be useful in future studies to model sex differences in alcohol and/or nicotine reinforcement.

The increased sensitivity to alcohol-induced CPP at the 0.5 g/kg dose in $\alpha 6L9'S$ mice provides further support for the idea that the L9'S mutation produced a leftward shift in the dose-response curve for alcohol reward in these mice (Figure 4). Similarly, Liu and colleagues (2012a) recently reported CPP with a 0.5 g/kg alcohol dose in mice with hypersensitive $\alpha 4^*$ nAChRs (Leu9'Ala) but not in WT mice; no genotype difference was seen with 2.0 g/kg alcohol-induced CPP. Interestingly, $\alpha 6$ subunits are co-expressed with $\alpha 4$ subunits in VTA DAergic neurons (Champtiaux *et al.*, 2003, Liu *et al.*, 2012b), suggesting that $\alpha 6^*$ and $\alpha 4^*$, or possibly $\alpha 6\alpha 4^*$ nAChRs, are involved in modulating alcohol reward-related behavior.

Although we observed no genotype differences in the locomotor-activating effects of alcohol during the CS+ conditioning trials, genotype differences were observed in the absence of alcohol. Non-Tg mice were more active than $\alpha 6L9'S$ mice during both the pretest and posttest of the 0.5 g/kg alcohol study, but not the 2.0 g/kg study. This result is inconsistent with a previous report indicating greater home-cage activity in $\alpha 6L9'S$ than non-Tg mice (Cohen *et al.*, 2012, Drenan *et al.*, 2010, Drenan *et al.*, 2008), but it could be explained by environmental or procedural factors that differ between this and the prior

study. It should be noted that the genetic background of the $\alpha 6L9'S$ mice in both studies was C57BL/6.

Correlations between CS+ time and activity during the posttest yielded a significant negative relationship in non-Tg but not in $\alpha 6L9'S$ mice in the 2.0 g/kg CPP experiment; no correlation was found in the 0.5 g/kg alcohol experiment. Gremel and Cunningham (2007) have previously shown a negative relationship between CPP magnitude and test activity, suggesting that higher levels of activity interfere with the expression of CPP (Gremel & Cunningham, 2007). This negative relationship found in the non-Tg mice (and lack thereof in the $\alpha 6L9'S$ mice) may explain the weaker 2.0 g/kg alcohol-induced CPP in the $\alpha 6L9'S$ mice (Figure 6).

$\alpha 6^$ nAChR neurobiology and the response to alcohol*

Studies of the behavioral and neurobiological action of $\alpha 6^*$ nAChRs are of intense interest for two key reasons: 1) $\alpha 6^*$ nAChRs respond robustly to concentrations of nicotine that are well within the physiological range of nicotine concentrations found in the cerebrospinal fluid of smokers (Salminen *et al.*, 2007), implicating their role in nicotine addiction and as targets for smoking cessation drugs (Quik & McIntosh, 2006), and 2) $\alpha 6^*$ nAChRs are selectively expressed in only a few brain regions (Azam *et al.*, 2002, Champiaux *et al.*, 2002, Drenan *et al.*, 2008, Le Novere *et al.*, 1996), the most important being the mesolimbic and nigrostriatal DA systems. Selective manipulation of nAChR activity in these neurotransmitter systems by $\alpha 6^*$ nAChR agonists, antagonists, or positive allosteric modulators could provide new avenues for development of treatments for Parkinson's disease, smoking cessation, and AUDs (Drenan & Lester, 2012).

Alcohol's action in the brain is complex, likely involving a variety of interactions at specific neurotransmitter receptors, ion channels, and brain circuits in a concentration-dependent manner (Morikawa & Morrisett, 2010). VTA DA neuron activity, specifically, baseline spontaneous firing rate, and the transition from tonic to phasic firing are key neural correlates of alcohol reward (Pfeffer & Samson, 1988, Rassnick *et al.*, 1992). Cholinergic excitation of nAChRs is permissive for activation of DA neuron phasic firing (Mameli-Engvall *et al.*, 2006) and for self administration of several drugs of abuse (Corrigall *et al.*, 1994). Alcohol has well-known effects on GABA_A receptors (Whitten *et al.*, 1996) and NMDA receptors (Lovinger *et al.*, 1989, Maldve *et al.*, 2002), that likely play a role in alcohol addiction. Importantly, several studies support the idea that alcohol may directly potentiate nAChR activity in VTA DA neurons. Co-application of alcohol was sufficient to potentiate peak current amplitude of $\beta 4^*$ nAChRs (Borghese *et al.*, 2002, Covernton & Connolly, 1997), α -bungarotoxin insensitive nAChRs in primary cortical neurons (Aistrup *et al.*, 1999, Marszalec *et al.*, 1999), and $\alpha 4\beta 2$ nAChRs (Zuo *et al.*, 2001, Zuo *et al.*, 2002). Based on these studies and the results we present in this paper, alcohol may also potentiate $\alpha 6^*$ nAChR responses. Future studies will be required to address this hypothesis.

In patch-clamp recordings from VTA DA neurons in acute brain slices, activation of nAChRs with low concentrations of nicotine potentiated an alcohol-induced increase in action potential firing (Clark & Little, 2004). Furthermore, Taylor and colleagues demonstrated that infusion of nicotine into VTA was sufficient to summate with low-concentrations of alcohol to enhance DA release in nucleus accumbens (Tizabi *et al.*, 2002). These results suggest that low-concentrations of alcohol or low-levels of nAChR

activity, which alone may not be sufficient to support changes in behavior, may combine to produce sufficient DA neuron activity to produce the rewarding effects of alcohol. In $\alpha 6L9'S$ mice, stronger levels of ACh-activated $\alpha 6^*$ nAChR activity (Figure 8) may combine with alcohol concentrations that are normally subthreshold (3-6% solutions: Figure 1; 0.5 g/kg alcohol: Figure 4) to produce sufficient stimulation of the mesolimbic DA system to produce alcohol reward. This supposition is strongly supported by recent VTA DA neuron patch clamp experiments with hypersensitive $\alpha 4^*$ nAChR mice, where alcohol was able to summate with bath-applied ACh to increase firing rates specifically via $\alpha 4^*$ nAChRs (Liu *et al.*, 2012a).

Conclusions

The present study is the first to directly demonstrate a role for $\alpha 6^*$ nAChRs in modulating alcohol reward-related behaviors. The data suggest that $\alpha 6L9'S$ mice are more sensitive to alcohol reward, likely a result of hypersensitive $\alpha 6^*$ nAChRs. These results using gain-of-function nAChRs expressed in mice, together with the emerging literature detailing the roles of $\alpha 6^*$ nAChRs in DA neurobiology (Drenan *et al.*, 2010, Drenan *et al.*, 2008, Mackey *et al.*, 2012), suggest that $\alpha 6^*$ nAChRs are a promising therapeutic target for the treatment of AUDs.

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Figure Legends

Figure 1. Data are shown as two-day averages (\pm SEM) of 24-hr two-bottle choice (a) alcohol (g/kg) and (b) total fluid (ml/kg) intake for male (left panels) and female (right panels) α 6L9'S and non-Tg mice. Female α 6L9'S mice drank greater amounts of 3% and 6% alcohol solutions. No differences in total fluid intake were observed. * Indicates a significant difference of genotype ($p < 0.05$); # indicates a significant difference of genotype collapsed across sex ($p < 0.01$).

Figure 2. Data are shown as mean (\pm SEM) alcohol intake (g/kg) collapsed across the 4 days for male and female α 6L9'S and non-Tg mice. α 6L9'S mice showed greater binge-like alcohol drinking (DID) than non-Tg mice. *Indicates a significant main effect of genotype ($p < 0.05$).

Figure 3. Data are shown as mean (\pm SEM) activity counts for α 6L9'S and non-Tg mice during CS+ and CS- trials using a 0.5 g/kg conditioning dose of alcohol. Only activity from trials 1 and 4 are shown to simplify data presentation; trials 2 and 3 showed a similar pattern of activity differences as trial 4. Female α 6L9'S mice were more active than male α 6L9'S mice and non-Tg mice showed significantly greater activity on CS+ trials than CS- trials. * indicates a main effect of trial type ($p < 0.05$; CS+ vs. CS-); # indicates significantly different from male α 6L9'S mice ($p < 0.01$).

Figure 4. Data are shown as mean (\pm SEM) change in time (sec/min) spent on the alcohol-paired floor between posttest and pretest (posttest – pretest difference score) for

$\alpha 6L9'S$ and non-Tg mice, during the first and last 30 min of the preference tests. Note that a bar above the 0 line indicates a preference for the alcohol paired cue, whereas a bar below the 0 line indicates an aversion. Both male and female $\alpha 6L9'S$ mice displayed a significant increase in time spent on the alcohol-paired floor as a result of conditioning with a 0.5 g/kg dose of alcohol, indicating that $\alpha 6L9'S$ mice are more sensitive to the rewarding effects of alcohol. *Indicates main effect of genotype ($p < 0.05$).

Figure 5. Data are shown as mean (\pm SEM) activity counts for $\alpha 6L9'S$ and non-Tg mice during CS+ and CS- trials using a 2.0 g/kg dose of alcohol. All mice showed increased activity on CS+ trials 2-4 compared to CS- trials 2-4. All mice also showed habituation of activity across CS- trials. Only activity from trials 1 and 4 are shown to simplify data presentation. * indicates a main effect of trial type ($p < 0.01$; CS+ vs. CS-); # indicates significantly different from Trial 1 CS- ($p < 0.01$).

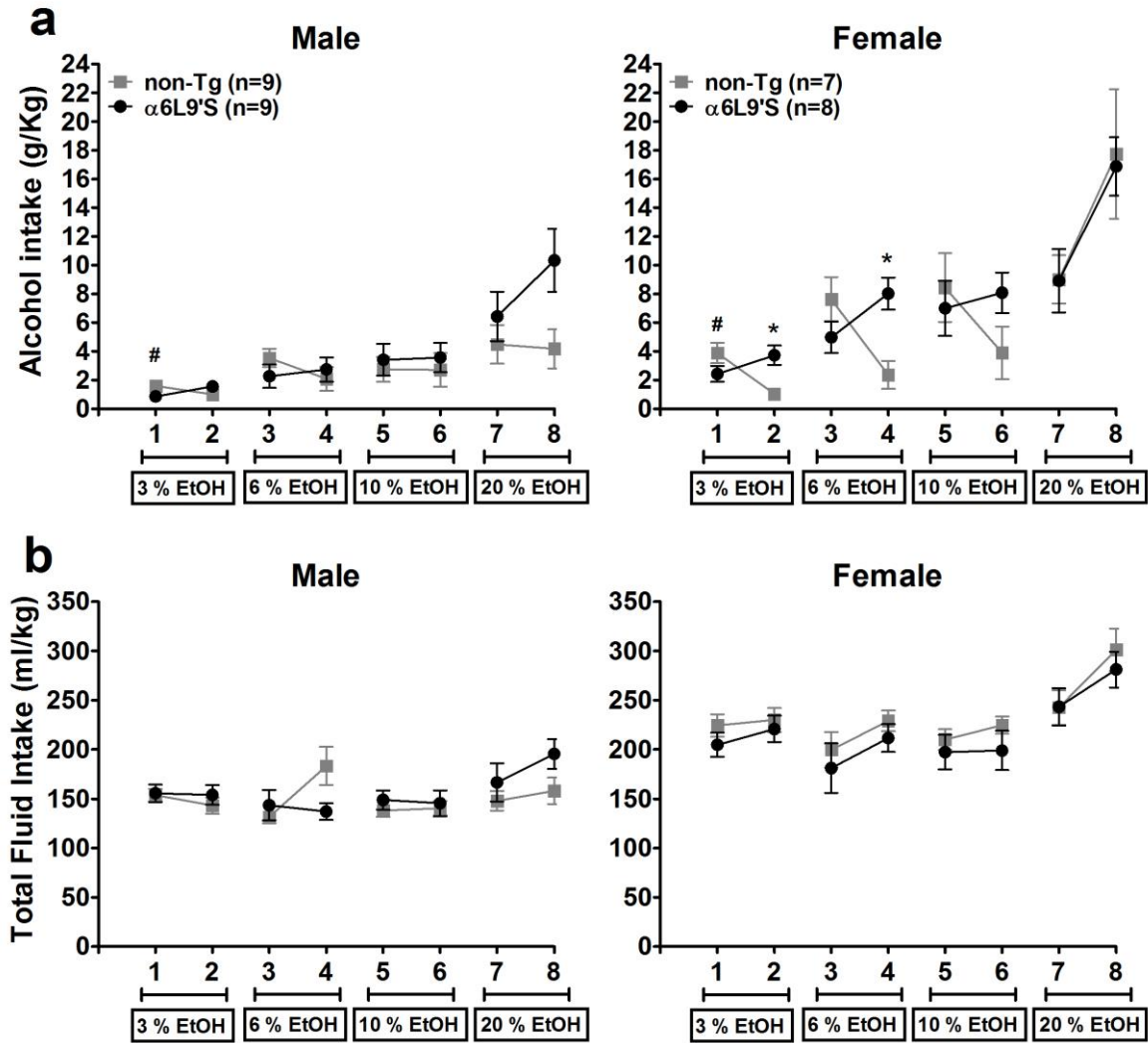
Figure 6. Data are shown as mean (\pm SEM) change in time (sec/min) spent on the alcohol-paired floor between posttest and pretest (posttest – pretest difference score) for $\alpha 6L9'S$ and non-Tg mice, during the first and last 30 min of the preference tests. Note that a bar above the 0 line indicates a preference for the alcohol paired cue. Analysis indicated a main effect of test ($p = 0.057$) very close to significance.

Figure 7. $\alpha 6^*$ nAChRs are selectively expressed in midbrain DA neurons. Ventral midbrain sections from transgenic $\alpha 6GFP$ mice were stained with anti-GFP and anti-TH (a) or anti-GFP and anti-GAD67 (b) antibodies and imaged using confocal microscopy.

High resolution scans at the border between the DA neuron-rich SNc and the GABA neuron-rich SNr (b, white box) were made, showing that $\alpha 6^*$ nAChRs are excluded from GABA neurons (c).

Figure 8. nAChRs in midbrain DA neurons of $\alpha 6L9'S$ mice are hypersensitive to ACh. VTA DA neurons in acutely prepared brain slices from $\alpha 6L9'S$ or non-Tg littermate mice were studied with whole-cell patch clamp recordings. A range of ACh concentrations were directly applied to the recorded DA neuron using a puffer pipette (a, inset), resulting in nAChR-mediated inward currents in $\alpha 6L9'S$ and non-Tg cells (a). Concentration-response curves for ACh-activated inward currents in VTA (b) and SNc (c) DA neurons were generated from the experimental data described in (a).

Figure 1



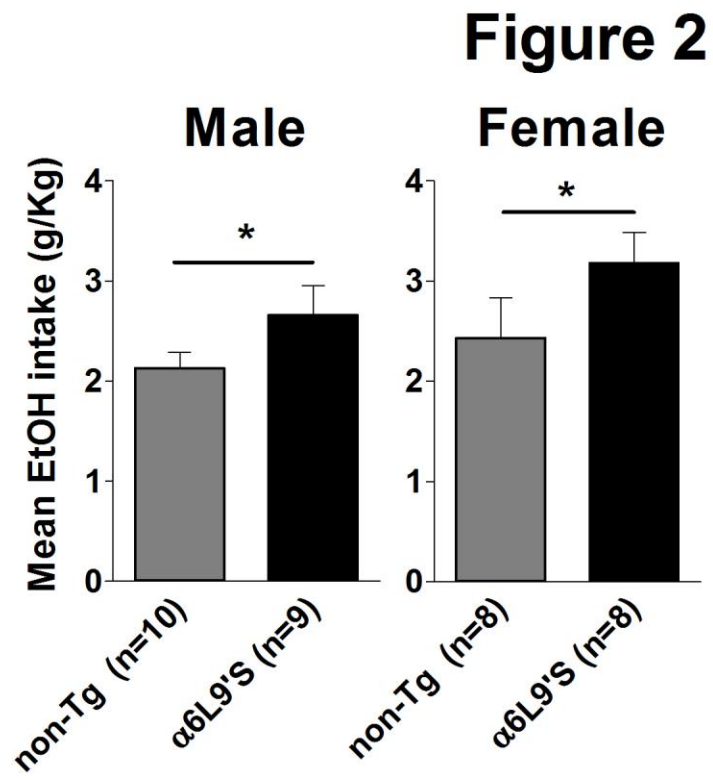


Figure 3

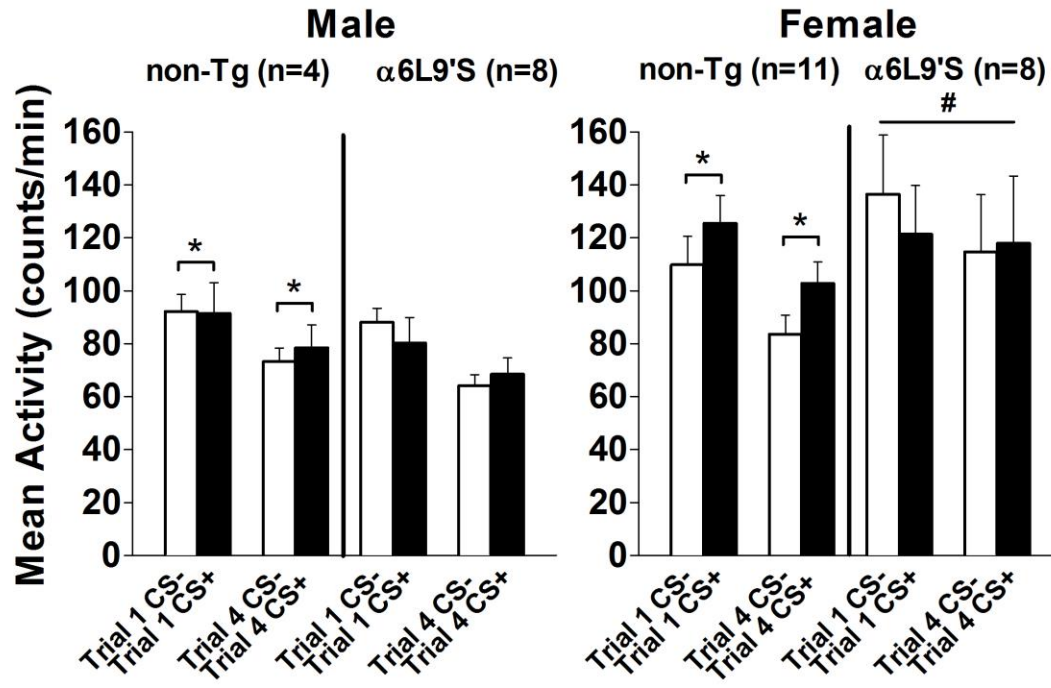


Figure 4

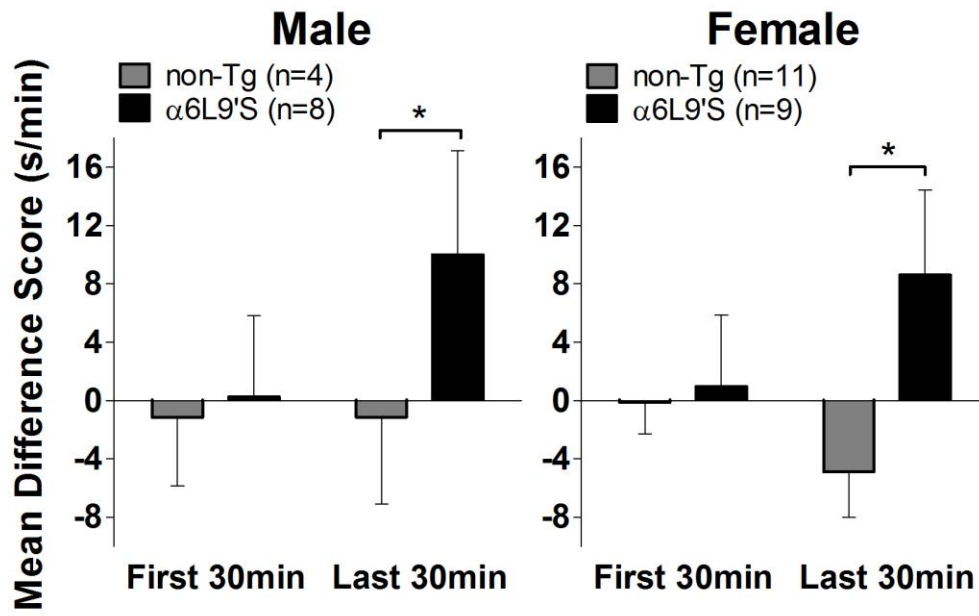


Figure 5

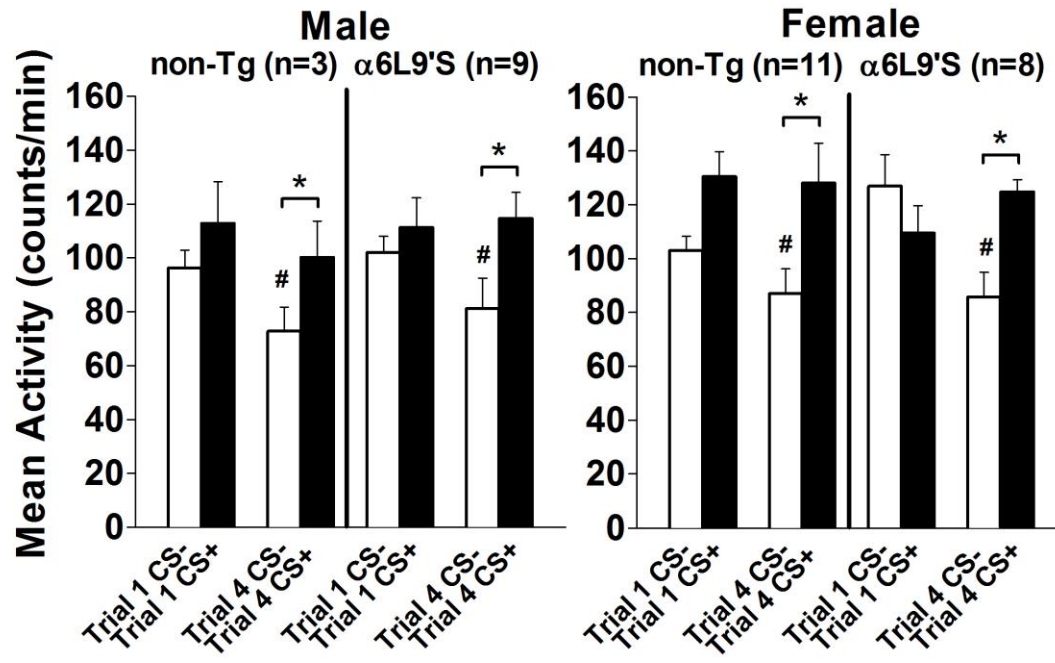


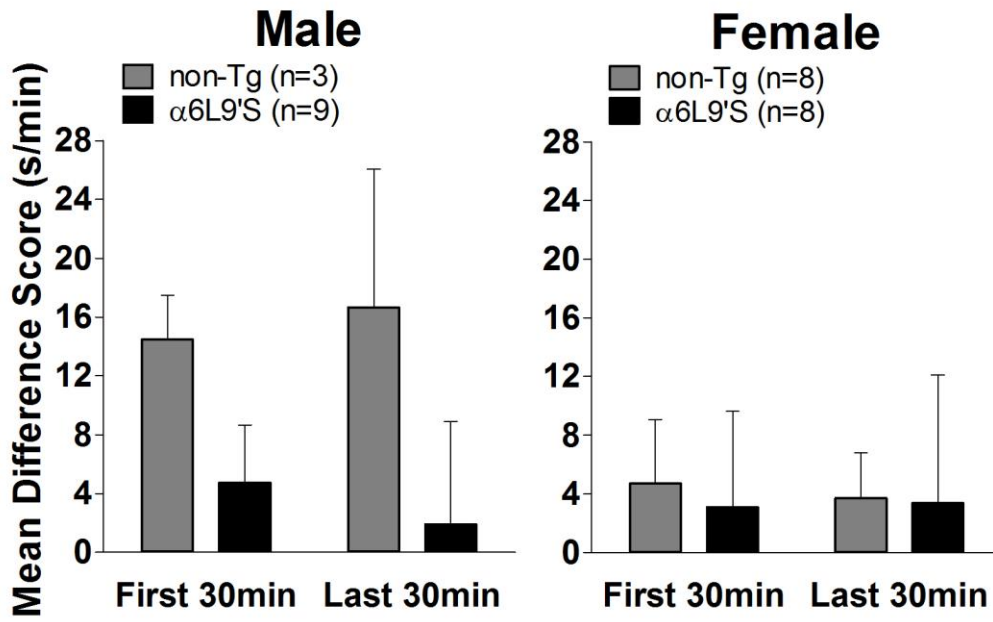
Figure 6

Figure 7

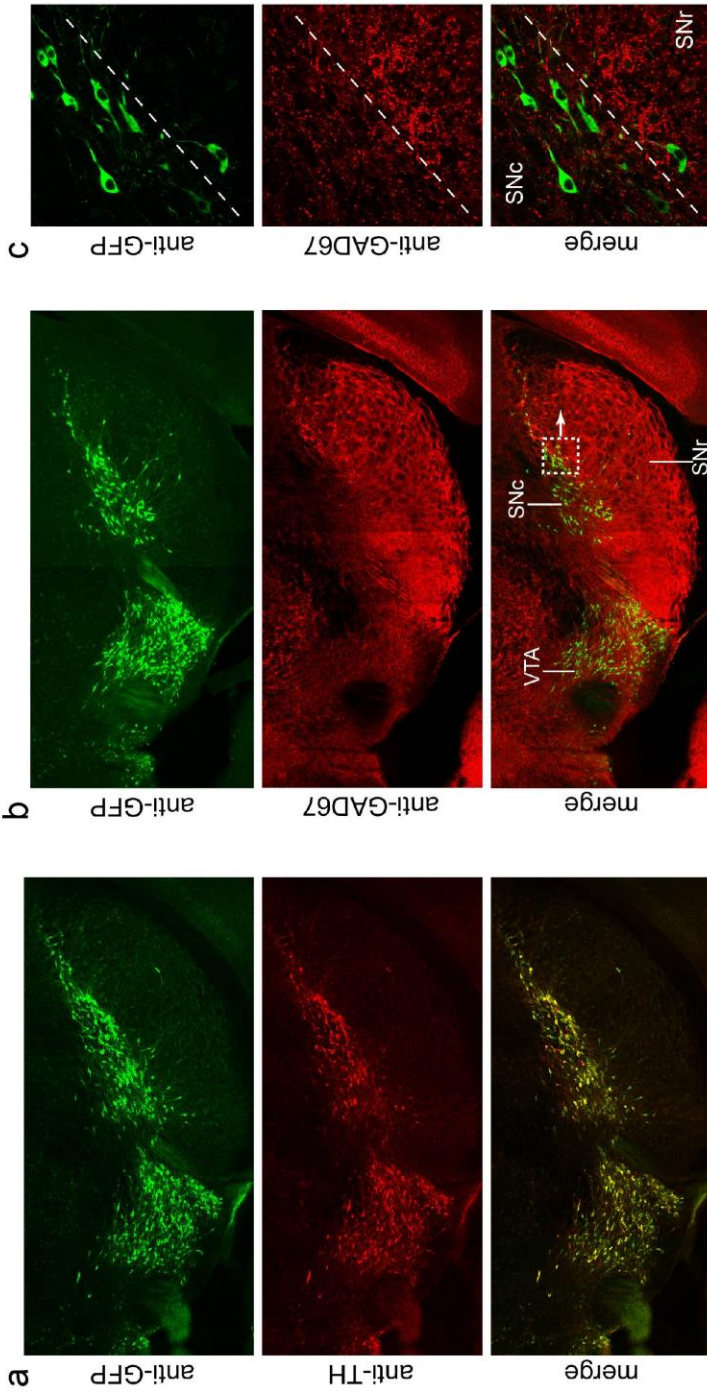


Figure 8

