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Reduced Oral Ethanol Avoidance in Mice Lacking Transient Receptor Potential Channel Vanilloid Receptor 1

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Abstract

Ethanol is a known oral trigeminal stimulant and recent data indicate that these effects are mediated in part by transient receptor potential channel vanilloid receptor 1 (TRPV1). The importance of this receptor in orally mediated ethanol avoidance is presently unknown. Here, we compared orosensory responding to ethanol in TRPV1-deficient and wild type mice in a brief-access paradigm that assesses orosensory influences by measuring immediate licking responses to small stimulus volumes. TRPV1^{-/-} and control mice were tested with six concentrations of ethanol (3, 5, 10, 15, 25, 40%), capsaicin (0.003, 0.01, 0.03, 0.1, 0.3, 1 mM), sucrose (0.003, 0.01, 0.03, 0.1, 0.3, 1 M), and quinine (0.01, 0.03, 0.1, 0.3, 1, 3 mM) and psychophysical concentration-response functions were generated for each genotype and stimulus. TRPV1 knockouts displayed reduced oral avoidance responses to ethanol regardless of concentration, insensitivity to capsaicin, and little to no difference in sweet or bitter taste responding relative to wild type mice. These data indicate that the TRPV1 channel plays a role in orosensory-mediated ethanol avoidance, but that other receptor mechanisms likely also contribute to aversive oral responses to alcohol.

Keywords

TRPV1 receptor; Knockout mice; Ethanol; Trigeminal; Sensory behavior

Introduction

Ethanol is a complex chemosensory stimulus that activates the gustatory system via its sweet and bitter taste components (Blizard 2007; Di Lorenzo et al. 1986; Hellekant et al. 1997; Kiefer and Lawrence 1988; Kiefer and Mahadevan 1993; Kiefer et al. 1990; Lemon et al. 2004; Scinska et al. 2000), the olfactory system (Kiefer and Morrow 1991; Mattes and DiMeglio 2001), and oral somatosensory (i.e., trigeminal) circuits responsible for the detection and processing of chemical irritation (Carstens et al. 1998; Danilova and Hellekant 2002; Green 1987). The ability of ethanol to stimulate neural pathways involved in trigeminal processing is well-established across species. Oral application of ethanol to the tongue activates peripheral lingual afferent fibers of the trigeminal nerve in the cat (Hellekant 1965), rat (Simon and Sostman 1991) and non-human primate (Danilova and Hellekant 2002), and produces a concentration-dependent increase in activity of central neurons in the rodent brain stem trigeminal subnucleus caudalis (Carstens et al. 1998). Stimulation of such substrates presumably underlies the burning and irritant sensations to

oral alcohol reported in human psychophysical studies, particularly at higher concentrations (Diamant et al. 1963; Green 1987, 1988, 1990; Wilson et al. 1973).

Recent findings indicate that ethanol's trigeminal stimulant effects may be mediated in part by its interaction with the transient receptor potential channel vanilloid receptor 1 (TRPV1; Trevisani et al. 2002, 2004). TRPV1 is a non-selective cation channel prevalent in peripheral nociceptive sensory neurons where it mediates detection and transduction of noxious chemical and thermal stimuli, including capsaicin and other vanilloids, acidic pH and intense heat ($>43^{\circ}\text{C}$; Caterina et al. 1997; Szallasi et al. 2007, for review). TRPV1 is known to be expressed in primary sensory neurons from trigeminal ganglia (Caterina et al. 1997), which innervate the head and oral cavity and transmit somatosensory information centrally to the brain stem trigeminal nucleus (Arvidsson and Gobel 1981; Jacquin et al. 1983; Marfurt 1981; Takemura et al. 1991). Ethanol evokes concentration-dependent increases in activity of cultured trigeminal ganglion neurons, as well as TRPV1-expressing HEK cells, which are inhibited by the competitive TRPV1 receptor antagonist capsazepine (Trevisani et al. 2002). Additionally, ethanol potentiates electrophysiological responses of the TRPV1 channel to a variety of agonists, including capsaicin, protons, the endo-cannabinoid anandamide and heat (Trevisani et al. 2002). Ethanol-induced peripheral inflammatory responses have also been shown to be dependent on TRPV1 receptor activation (Trevisani et al. 2002, 2004). Immunohistochemical studies have demonstrated heavy localization of TRPV1 receptors on sensory fibers that innervate the oral epithelium (Ishida et al. 2002; Kido et al. 2003), tissue which ethanol is able to rapidly penetrate (Mistretta 1971).

Despite the known capacity of ethanol to stimulate orosensory trigeminal pathways and its exclusive oral route of self-administration in humans, no studies have directly manipulated trigeminal mechanisms to examine their role in alcohol ingestive responses. Previous data indicate that other orosensory components of alcohol, specifically its ability to stimulate neural circuits involved in sweet taste processing (Hellekant et al. 1997; Lemon et al. 2004), are importantly involved in its consumption. For example, independent genetic deletion of several proteins critical for sweet taste reception and transduction (T1r3, α -gustducin and TRPM5) results in substantial reductions in alcohol preference in mice (Blednov et al. 2008; Brasser et al. 2006). Less is known about how ethanol's oral trigeminal stimulus properties are involved in sensory responding to the drug or regulation of alcohol intake.

The goal of the present study was to directly examine the contribution of the TRPV1 receptor to oral ethanol acceptance/avoidance by measuring orosensory responses to alcohol in mice lacking the TRPV1 channel compared to C57BL/6J wild type controls. A brief-access stimulus exposure paradigm designed to assess orosensory-mediated responding was employed to generate psychophysical functions for ethanol and prototypic sweet (sucrose), bitter (quinine) and trigeminal (capsaicin) stimuli in each genotype. It was hypothesized that inhibition of ethanol-induced trigeminal activation via deletion of TRPV1 would directly reduce orosensory alcohol avoidance, suggesting a mechanistic role for this receptor in behavioral oral ethanol sensitivity.

Materials and methods

Animals

Thirty-two naive adult male and female TRPV1 knockout (B6.129X1-*Trpv1*^{tm1Jul/J}) and age-matched C57BL/6J (B6) wild type mice ($n = 16/\text{genotype}$; $n = 8/\text{sex/genotype}$) obtained from Jackson Laboratories (Bar Harbor, ME) were used. Mice were 14 weeks of age at the start of the experiment. Knockout mice were generated by targeting of the *Trpv1* gene in JM1 embryonic stem cells from the 129X1/SvJ mouse strain, as described by Julius and colleagues (Caterina et al. 2000). Germline chimeras were crossed to C57BL/6 females and

the resulting heterozygous males backcrossed to C57BL/6 females for ten generations (Caterina et al. 2000; JAX[®]Mice Database). The TRPV1^{-/-} mutant strain was maintained at Jackson via a homozygous mating strategy, precluding the availability of littermate controls (which control for potential maternal/environmental influences on behavior), and thus the B6 background strain served as the appropriate control (JAX[®]Mice Database). Mean body weights at the start of the experiment were 23.56 g (± 0.81 SE) and 23.33 g (± 1.52 SE) for knockout and control mice, respectively. Animals were housed individually in standard shoebox cages (29.5 $\times$ 18.5 $\times$ 13 cm) in a vivarium that maintained a 12-h light/dark cycle and an ambient temperature of approximately 23°C. All training and testing occurred during the light phase of the cycle. Food and water were available ad libitum except for water restriction conditions noted below. All procedures were approved by the Institutional Animal Care and Use Committee at San Diego State University.

Apparatus

Training and testing were conducted in a Davis MS-160 lickometer apparatus (DiLog Instruments, Tallahassee, FL). This device allows for automated within-session presentation of multiple stimulus solutions to an animal in the form of individual sampling trials of short duration (e.g., 5–10 s) during which immediate lick responses are monitored (see Smith 2001). Mice gained access to a stainless steel drinking spout on each trial through a small aperture in the front wall of a 30 $\times$ 14.5 $\times$ 15 cm testing chamber, with availability of the spout determined by the opening and closing of a motorized shutter. Delivery of a given stimulus solution was determined by the positioning of a motorized table/block apparatus just outside of the chamber that could accommodate up to 16 different stimulus tubes. Lick activity was detected via a high-frequency AC contact circuit and all data collection (i.e., lick counts), as well as presentation and timing of all stimuli, were controlled precisely via computer and associated software.

Training

Mice initially were given 4 days of training with water as the only available stimulus in order to familiarize them with the apparatus and to train them to lick the spout to receive fluid. During the training phase, an overnight water restriction schedule was in effect in order to motivate performance on the task, with mice receiving their sole daily fluid intake in the apparatus. If any animal fell below 80% of its baseline body weight on any training day, 1 h of supplemental water access was additionally given after its training session to facilitate maintenance of body weight. On the first two training days, subjects were given a 30 min period of continuous access to water through a single sipper tube that began when the animal took its first lick. On the last 2 days of training, mice were allowed access to water during 40 5-s trials separated by 10-s inter-presentation intervals to familiarize them with the brief-access trial procedure.

Stimulus testing

Each mouse was subsequently tested during separate weeks for short-term lick responses to the following four stimuli: ethanol (3, 5, 10, 15, 25 and 40%), sucrose (0.003, 0.01, 0.03, 0.1, 0.3 and 1 M), quinine HCl (0.01, 0.03, 0.1, 0.3, 1 and 3 mM), and capsaicin (0.003, 0.01, 0.03, 0.1, 0.3 and 1 mM). Each stimulus was tested over five consecutive daily sessions, and stimuli were presented in serial order (ethanol, sucrose, quinine, capsaicin) across weeks with a 1 week break interposed between testing of different stimuli. Within each test session, mice were presented with all six concentrations of a given stimulus and a vehicle control (see below) during discrete, brief-access exposure trials (10-s trials: ethanol, sucrose and capsaicin; 5-s trials: quinine). Solutions were presented randomly within blocks of seven trials (six blocks total), such that animals were allowed to sample each stimulus concentration and the vehicle control once/block and six times during a given test session

(30 trial replications total at each concentration over the five test days for each stimulus). Upon opening of the shutter on each presentation, 30 s was allowed for trial initiation and the trial duration began with the animal's first lick on the sipper tube. If a mouse failed to initiate sampling during the 30-s period, the shutter closed and the table was automatically repositioned for the next trial. All presentations were separated by 10-s inter-presentation intervals during which the shutter remained closed. Test sessions were approximately 20–30 min in length. Brief-access testing has been used extensively to measure orosensory responsiveness in rodents (Boughter et al. 2002; Davis 1973; Dotson and Spector 2004; Glendinning et al. 2002, 2005; Smith et al. 1992). The specific trial durations for sucrose and quinine reported here were based on those previously shown to produce reliable concentration-response functions for these stimuli in brief-access tests (Boughter et al. 2002, 2005; Brasser et al. 2005; Spector et al. 1996). Longer trial durations for ethanol and sucrose prevent truncating the expression of any appetite responses to these stimuli, and longer trial durations for capsaicin were employed to allow for the delayed response latency to this stimulus on the tongue (Okuni 1977).

The overnight water restriction conditions present during training remained in effect during the 5 days of testing for each stimulus with the exception that mice were given 1 h of supplemental water access following ethanol and sucrose test sessions. Prior work in our laboratory has shown this fluid access schedule to maintain an appropriate level of motivation and to generate reliable psychophysical functions for stimuli that possess a preferred component. Although ethanol additionally possesses aversive orosensory properties, supplemental water administration during ethanol testing similar to that employed for sucrose helps support the observation of any appetitive responding to ethanol (e.g., to its sweet taste properties), as these responses may become more pronounced under manipulations which reduce its trigeminal component. During testing of solely non-preferred stimuli (quinine, capsaicin), supplemental water administration was withheld to promote sampling of these stimuli. Water was available *ad libitum* immediately following the last (i.e., Day 5) test session for each stimulus and during the weeks intervening stimulus testing.

Stimuli

All solutions were prepared fresh prior to testing using reagent grade chemicals (Sigma–Aldrich, St. Louis, MO) and were presented at room temperature. Ethanol, sucrose and quinine HCl were dissolved in deionized water and capsaicin in a vehicle of 1.5% ethanol/1.5% Tween 80 in deionized water. Stimulus concentrations were chosen to encompass a full behavioral orosensory response range based on previous studies (e.g., Spector et al. 1996; St John et al. 1994) and preliminary testing.

Statistical analysis

For each individual mouse, the mean number of licks to each stimulus concentration and to the vehicle control was calculated across all trials sampled over the five test days for each stimulus. Nonsampled trials (i.e., those with zero licks) were excluded from the data analysis such that only valid trials for which mice were attending to the tube and had initiated sampling were evaluated. In order to standardize responses to ethanol, quinine HCl and capsaicin to responses to vehicle (which serves to control for individual differences in licking behavior that are non-orosensory in nature), stimulus/vehicle lick ratios for each mouse were determined by dividing the mean number of licks to each stimulus concentration by the mean number of licks to vehicle. A stimulus/vehicle lick ratio of 1.0 indicates equal responding to a given stimulus concentration relative to vehicle, with ratios approaching zero representing increased levels of lick suppression. To standardize responses to sucrose, a purely preferred stimulus that produces concentration-dependent increases in

licking behavior, standardized lick ratios for each mouse were determined by dividing the mean number of licks to each sucrose concentration by the maximum potential number of licks that animal could generate in a 10 s trial (see Glendinning et al. 2002, 2005). This standardization measure is appropriate for assessing responses to highly preferred stimuli because it avoids the high variability associated with stimulus/vehicle lick ratios for stimuli that evoke large increases in licking relative to vehicle, while controlling for any individual or strain differences in local lick rate, similar to the stimulus/vehicle lick ratio (Dotson and Spector 2004; Glendinning et al. 2002, 2005). Maximum potential number of licks/trial was calculated by multiplying by a factor of ten the subject's local lick rate (licks/s) based on its first 2 days of sipper tube training (local lick rate = $1/\text{mean interlick interval (s)}$; ILIs < 50 ms and > 200 ms filtered when calculating mean ILI). A standardized lick ratio near zero indicates minimal licking to a given sucrose concentration, with a ratio of 1.0 representing maximal responding. Grubb's test was used to detect any outlying individual lick ratio values among subjects of the same genotype and sex at each concentration of each stimulus tested.

For each stimulus, lick ratio data were subsequently analyzed using $2 (\text{genotype}) \times 2 (\text{sex}) \times 6 (\text{concentration})$ mixed analyses of variance (ANOVAs), with genotype and sex as between-subject factors and concentration as a within-subject factor. To assess any strain differences in responding for water/vehicle during training and testing, mean number of licks to water during sipper tube and brief-access training, as well as mean number of licks to vehicle during testing of each stimulus, were additionally analyzed using one-way ANOVAs with genotype as a factor. New-man-Keuls test was used to assess specific differences accompanying significant main effects or interactions from the overall ANOVAs. Alpha level for post hoc comparisons was 0.05. In cases where overall analysis of the lick ratio data yielded a significant main effect of concentration or interaction of concentration with other factors, one-sample *t*-tests were used to determine whether the stimulus/vehicle lick ratios for each stimulus concentration were significantly above or below 1.0 (i.e., the indifference point) and matched *t*-tests were used to determine whether the standardized lick ratios for each sucrose concentration were significantly different from those for water. A Bonferroni correction was employed for these analyses to adjust the alpha level for the use of multiple comparisons on each data set (i.e., $\alpha = 0.05/\text{number of comparisons}$).

Results

Training

TRPV1 knockout and B6 control mice did not differ in mean number of licks to water during initial sipper tube training (NS effect of genotype: $F(1, 30) = 0.55, P = 0.46$). Mean number of licks to water/session averaged across the two sipper tube training sessions was $813.94 (\pm 79.00 \text{ SE})$ for controls and $733.19 (\pm 74.76 \text{ SE})$ for knockouts. Local lick rates (licks/s) calculated from the mean ILI data during sipper tube training also did not differ for controls and knockouts (control: $8.39 (\pm 0.05 \text{ SE})$ and knockout: $8.43 (\pm 0.08 \text{ SE})$; NS effect of genotype: $F(1, 30) = 0.17, P = 0.68$). Genotypes showed similar mean total lick responses to water during brief-access trial training (NS effect of genotype: $F(1, 30) = 0.18, P = 0.68$), with mean number of licks/session averaged across the two brief-access training sessions of $884.78 (\pm 46.08 \text{ SE})$ and $855.38 (\pm 52.06 \text{ SE})$ for controls and knockouts, respectively. Mean licks/trial during brief-access training also did not differ between genotypes (control: $30.33 (\pm 1.04 \text{ SE})$ and knockout: $31.16 (\pm 1.13 \text{ SE})$; NS effect of genotype: $F(1, 30) = 0.30, P = 0.59$). The mean estimated volume of water consumed/session during sipper tube and brief-access training sessions, respectively, was $0.890 \text{ ml} (\pm 0.062 \text{ SE})$ and $1.001 \text{ ml} (\pm 0.039 \text{ SE})$. These estimates were calculated by multiplying the average total lick measurements for each mouse during sipper tube and brief-access training

by previously reported mean volume/lick intake of water in mice of 1.15 μ l under experimental parameters highly similar to those in the current study (including the use of 2.7 mm orifice tubes; Dotson and Spector 2005).

Stimulus testing

Ethanol—TRPV1 deficient mice displayed significantly higher lick ratios for ethanol than B6 control mice regardless of concentration (main effect of genotype: $F(1, 28) = 14.68$, $P < 0.001$, Fig. 1a). Both knockouts and controls nevertheless suppressed their lick responses to ethanol with increasing concentration, reflecting the presence of a concentration-dependent aversion in both genotypes (main effect of concentration: $F(5, 140) = 112.29$, $P < 0.001$; genotype $\times$ concentration interaction NS, Fig. 1b). Post hoc analyses of the concentration effect indicated no difference in responding to 3 and 5% ethanol, and then a significant decline in all lick ratios for successive concentrations above 5% (i.e., 5–10, 10–15, 15–25, 25–40%; P 's ≤ 0.001). No other effects from the overall ANOVA were significant. Lick ratios for ethanol were significantly below 1.0 (i.e., indifference) at concentrations $\geq 10\%$ (all t 's ≥ 6.6 , P 's < 0.001), but did not differ from 1.0 at 3 and 5% (both t 's < 0.95).

Mean number of licks to vehicle during ethanol testing did not significantly differ between B6 controls and TRPV1 knockout mice (NS effect of genotype: $F(1, 30) = 3.31$, $P = 0.08$). Mean licks/trial to the water vehicle was 37.62 (± 1.45 SE) and 32.96 (± 2.11 SE) for controls and knockouts, respectively.

Capsaicin—TRPV1 null mice were indifferent to capsaicin across concentration, whereas B6 control mice displayed a concentration-dependent avoidance (main effect of genotype: $F(1, 19) = 45.84$, $P < 0.001$; main effect of concentration: $F(5, 95) = 4.15$, $P < 0.01$; genotype $\times$ concentration interaction: $F(5, 95) = 4.92$, $P < 0.001$, Fig. 2). Across-genotype comparisons indicated suppressed lick ratios in wild type mice compared to knockouts at the three highest concentrations (0.1, 0.3 and 1 mM; P 's < 0.05). Lick ratios did not differ across concentration among TRPV1 knockouts, while B6 mice exhibited equivalent levels of responding to 0.003, 0.01, and 0.03 mM, and then a significant decline in responding at 0.1, 0.3 and 1 mM capsaicin relative to all lower concentrations (P 's < 0.01). No other effects from the overall ANOVA were significant. Lick ratios for capsaicin in B6 controls were significantly below 1.0 at concentrations ≥ 0.1 mM (all t 's ≥ 8.9 , P 's < 0.001) but not at lower concentrations (all t 's ≤ 2.4), and lick responses to capsaicin among knockouts did not vary significantly from the indifference point at any concentration (all t 's ≤ 3.1). Eight control subjects for which a full set of ratio data could not be calculated due to failure to respond to one or more capsaicin concentrations were excluded from the analysis. These exclusions resulted from an overall suppressive effect of capsaicin exposure on sampling in several control subjects, an effect not present in knockouts. One additional control subject was excluded because its lick ratio values at 0.003, 0.01 and 1 mM were significant outliers ($P < 0.05$) relative to all other subjects of the same genotype and sex (Grubb's test). No other individual ratio values for capsaicin met criteria for an outlier according to this test.

Mean number of licks to vehicle during capsaicin testing was significantly lower in B6 controls relative to TRPV1 knockouts (effect of genotype: $F(1, 21) = 101.80$, $P < 0.001$). Mean licks/trial to vehicle was 6.76 (± 1.08 SE) in controls and 45.19 (± 2.44 SE) in knockouts, further reflective of a robust suppressive effect of capsaicin exposure on overall responding in control mice. Mean lick values to capsaicin across concentration in control mice were: 0.003 mM—4.01 (± 0.92 SE), 0.01 mM—4.04 (± 1.42 SE), 0.03 mM—4.28 (± 1.18 SE), 0.1 mM—2.47 (± 0.60 SE), 0.3 mM—1.66 (± 0.31 SE), and 1 mM—1.63 (± 0.27 SE).

Sucrose—Regardless of genotype, all animals displayed greater avidity for higher sucrose concentrations relative to lower concentrations (main effect of concentration: $F(5, 140) = 112.37$, $P < 0.001$, Fig. 3). Post hoc analyses of the concentration effect indicated a significant increase in standardized lick ratios for sucrose for all concentrations above 0.01 M (i.e., 0.01–0.03, 0.03–0.1, 0.1–0.3, 0.3–1 M; P 's < 0.05), but no difference in SLRs between 0.003 and 0.01 M. B6 controls had higher lick ratios for sucrose than TRPV1 knockouts (main effect of genotype: $F(1, 28) = 6.30$, $P < 0.05$) and males also displayed significantly elevated lick ratios compared to females (main effect of sex: $F(1, 28) = 8.81$, $P < 0.01$). Both genotype and sex effects, however, were driven primarily by reduced appetitive responding for sucrose in knockout females (borderline genotype $\times$ sex interaction: $F(1, 28) = 3.95$, $P = 0.06$), as controls of both sexes and knockout males exhibited similar overall mean SLRs for sucrose. No other effects from the overall ANOVA were significant. Standardized lick ratios for sucrose were significantly greater than those for water at concentrations ≥ 0.03 M (all t 's ≥ 6.2 , P 's < 0.001), but did not differ from those for water at 0.003 and 0.01 M (both t 's ≤ 2.7).

Mean number of licks to vehicle during sucrose testing did not differ between B6 controls and TRPV1 knockout mice (NS effect of genotype: $F(1, 30) = 0.35$, $P = 0.56$). Mean licks/trial to the water vehicle was $24.24 (\pm 3.34 \text{ SE})$ and $22.03 (\pm 1.71 \text{ SE})$ for controls and knockouts, respectively.

Quinine—Both TRPV1 deficient and B6 control mice showed a similar concentration-dependent avoidance of quinine, with an orderly decrease in lick responses with each successive increase in concentration (main effect of concentration: $F(5, 140) = 621.15$, $P < 0.001$, post hoc comparisons: 0.01–0.03, 0.03–0.1, 0.1–0.3, 0.3–1, 1–3 mM; P 's < 0.001 , Fig. 4). The overall analysis additionally yielded a significant genotype $\times$ sex interaction ($F(1, 28) = 4.28$, $P < 0.05$) and three-way interaction of genotype $\times$ concentration $\times$ sex ($F(5, 140) = 4.55$, $P < 0.001$). Follow-up comparisons on the interactions, however, failed to reveal a significant difference in quinine lick ratios between TRPV1 knockouts and controls at any concentration for either males or females (all P 's NS). The source of the three-way interaction appeared to be due to a tendency for reduced aversion to intermediate concentrations of quinine in knockout females compared to knockout males. No other effects from the overall ANOVA were significant. Lick ratios for quinine in control mice of both sexes and knockout males were significantly below 1.0 at concentrations ≥ 0.1 mM (all t 's ≥ 4.2 , P 's ≤ 0.004) and in knockout females were below the indifference point at concentrations ≥ 0.3 mM (all t 's ≥ 9.0 , P 's < 0.001). Quinine lick ratios at 0.01 and 0.03 mM did not significantly differ from 1.0 for any group (all t 's ≤ 2.6).

Mean number of licks to vehicle during quinine testing did not differ between B6 controls and TRPV1 knockout mice (NS effect of genotype: $F(1, 30) = 0.14$, $P = 0.71$). Mean licks/trial to the water vehicle was $34.32 (\pm 0.70 \text{ SE})$ and $34.73 (\pm 0.85 \text{ SE})$ for controls and knockouts, respectively.

Discussion

The present study demonstrates that genetic deletion of the TRPV1 receptor in mice results in a reduction, but not an absence, of aversive orosensory responses to alcohol, indicating a contributing role for this receptor in orally mediated ethanol avoidance. These data are in line with prior *in vitro* evidence that ethanol interacts with this receptor on primary sensory neurons of the trigeminal system (Trevisani et al. 2002) and anatomical evidence for pronounced expression of this receptor on sensory fibers that innervate the oral cavity (Ishida et al. 2002; Kido et al. 2003). The findings presented here suggest that activation of

TRPV1 receptors orally by ethanol partially mediates aversive sensory responses to this stimulus on a functional level.

The attenuated aversion to alcohol observed among TRPV1 null mice in the current study was not concentration-dependent, with mutant mice displaying a fairly uniform decreased orosensory aversion to alcohol across a broad concentration range from 3 to 40%. That TRPV1 receptors are sensitive to ethanol at low concentrations is consistent with the ability of ethanol concentrations as low as 0.3–3% to activate isolated rodent trigeminal ganglion neurons, an effect which is inhibited by the TRPV1 antagonist capsazepine (Trevisani et al. 2002). Primary trigeminal afferents in primates have also been reported to respond to orally applied ethanol at concentrations as low as 0.7 M (~4%; Danilova and Hellekant 2002). Ethanol's trigeminal stimulant effects, however, have been shown to increase as a function of concentration in both peripheral trigeminal nerve fibers (Danilova and Hellekant 2002) and central neurons in the brain stem trigeminal subnucleus caudalis (>15%; Carstens et al. 1998), consistent with psychophysical ratings in humans of greater burning and irritant sensations with increasing alcohol concentration (Diamant et al. 1963; Green 1987, 1988; Wilson et al. 1973). The present findings of a uniform reduction in ethanol avoidance in TRPV1 deficient mice independent of concentration would suggest that mechanisms other than TRPV1 may be responsible for concentration-dependent increases in trigeminal activity with rising ethanol concentration. Central trigeminal neurons in caudalis receive convergent input from at least three identified oral somatosensory receptors, including TRPV1, TRPA1 and TRPM8 (Zanotto et al. 2007). It is therefore possible that ethanol stimulates other receptors on oral trigeminal afferents or acts via a more non-specific mechanism to primarily produce its trigeminal stimulant effects at high concentrations.

The maintenance of some avoidance to alcohol in TRPV1 knockouts further indicates that mechanisms other than TRPV1 contribute to aversive oral behavioral responses to alcohol. As mentioned above, it is likely that ethanol's trigeminal properties are not solely mediated via its interaction with TRPV1, consistent with evidence that a proportion of the neuronal response to ethanol in trigeminal ganglion neurons is insensitive to the TRPV1 blocker capsazepine (Trevisani et al. 2002). In primates, ethanol also has been shown to stimulate a subset of peripheral trigeminal nerve fibers that are not responsive to capsaicin (Danilova and Hellekant 2002), a potent TRPV1 agonist (Caterina et al. 1997), suggesting that ethanol can activate the trigeminal system via additional, TRPV1-independent mechanisms. Apart from its trigeminal properties, there is also evidence that ethanol may possess a bitter gustatory component, as conditioned taste aversions to alcohol cross-generalize to mixtures of sucrose and quinine in rats (Kiefer and Lawrence 1988; Kiefer and Mahadevan 1993) and to either tastant alone in C57BL/6J mice (Blizard 2007). Thus, the concentration-dependent aversion to alcohol maintained in TRPV1 deficient mice in the current study may involve additional trigeminal and/or gustatory mechanisms.

It is important to emphasize that the avoidance curves for ethanol observed in C57BL mice in brief-access testing differ from those observed in long-term two-bottle intake tests, where this strain typically displays preference for ethanol at concentrations $\leq 15\%$, with peak preference around 10% (Bachmanov et al. 1996; Brasser et al. 2006; Blednov et al. 2008). These differences may result from postingestive influences on responding in long-term intake tests, but also from water restriction conditions inherent in brief-access tests to motivate short-term sampling in mice, which tend to elevate water responses relative to non-deprived conditions typically employed in long-term tests. While water restriction did not prevent the detection of appetitive responding for sucrose (a purely preferred stimulus that generates high levels of responding compared to water), for complex stimuli such as ethanol that possess both appetitive and aversive components, expression of the aversive properties may be more readily apparent under such conditions. While these methodological factors

make it somewhat more difficult to evaluate ethanol's appetitive orosensory properties in short-term tests, these conditions facilitate evaluation of differences in ethanol avoidance responses between TRPV1 knockouts and their C57BL background strain.

Distinct from the reduction in orosensory ethanol avoidance observed among TRPV1 deficient mice, deletion of the receptor fully abolished behavioral orosensory sensitivity to capsaicin, with null mice displaying a complete lack of aversion to capsaicin at any concentration tested. These data are the first to index orosensory-mediated responding to capsaicin in TRPV1 mutant mice across concentration, and concur with initial findings that TRPV1 knockout mice show no decrease in consumption of a 0.125% saccharin solution when tainted with 0.1 mM capsaicin (Caterina et al. 2000). The present data therefore strongly reinforce the obligatory role of the TRPV1 receptor in mediating oral behavioral sensitivity to this trigeminal stimulus. Absence of the TRPV1 channel had little effect on orosensory responding for sucrose or quinine, prototypic sweet and bitter tastants, with slightly lower sucrose responses among knockout females, and no consistent differences between genotypes in lick responses to quinine. Oral quinine stimulation has not been shown to evoke activity in central trigeminal neurons in vivo (Simons et al. 2003; but see Liu and Simon 1998). Although the current study did not measure differences in salt responses between genotypes, prior work has demonstrated that TRPV1 deficient mice display reduced avoidance of high salt concentrations (Ruiz et al. 2006), which are known to stimulate central trigeminal pathways (Carstens et al. 1998; Sudo et al. 2002) and elicit irritant sensations in humans (Gilmore and Green 1993; Green and Gelhard 1989). Collectively, these data indicate that while the TRPV1 receptor is necessary for trigeminal sensitivity to capsaicin, it also plays a non-exclusive role in mediating responses to other trigeminal stimulants as well, including ethanol and NaCl.

One issue that should be considered when evaluating differences between the TRPV1 knockout and B6 control strain in the present study is that the knockout mice were generated using an embryonic stem cell line from the 129 strain (Caterina et al. 2000), and thus retain a fragment of 129 donor genes flanking the ablated TRPV1 locus which could have potentially contributed to the differences observed between knockout and B6 mice in addition to the influence of the target gene (Bolivar et al. 2001; Crusio 2004). While this possibility cannot be entirely ruled out, previous data have demonstrated no difference in capsaicin preference between 129 and B6 mouse strains (Bachmanov et al. 1996), in contrast to the large difference in capsaicin sensitivity between TRPV1 knockout and B6 mice reported here. Similarly, 129 mice are known to display reduced ethanol preference relative to the B6 strain (Bachmanov et al. 1996), an effect opposite to the elevated ethanol responses among knockouts compared to B6 mice in the current study. Genome-wide mapping of quantitative trait loci influencing ethanol preference and intake in F₂ hybrids between the B6 and 129 strains has also not revealed polymorphisms affecting these phenotypes on chromosome 11 (Bachmanov et al. 2002), which contains the 129 donor fragment in TRPV1 knockouts (Caterina et al. 2000). Thus, the presently observed differences in ethanol and capsaicin responding between TRPV1 knockouts and controls would not be clearly predicted from the presence of 129 donor genetic material surrounding the null mutation in the knockout strain. By contrast, there is direct electrophysiological and pharmacological evidence for interaction of both capsaicin and ethanol with the TRPV1 receptor (Caterina et al. 1997; Trevisani et al. 2002, 2004). It is possible that the slightly lower sucrose responses among knockout females in the current study could be influenced by residual 129 genes in the knockout strain, as 129 mice are known to display reduced sucrose preference relative to B6 mice (Bachmanov et al. 1996); however, genetic polymorphisms between the B6 and 129 strains on chromosome 11 in the area of the TRPV1 gene that affect sucrose ingestive responses have yet to be identified. It should be emphasized that additional research is necessary to determine if the present findings

obtained in one inbred mouse strain (B6) generalize to other strains, as factors contributing to ethanol avoidance responses may differ across strains.

The brief-access psychophysical assay employed in the present study was specifically chosen to assess the role of the TRPV1 channel in initial orosensory responsiveness to ethanol. While the impact of altered aversive sensory responses to ethanol mediated via the TRPV1 receptor and/or other trigeminal or gustatory substrates on long-term ethanol intake remains to be fully examined, a recent study reported elevated preference and consumption of alcohol in TRPV1 null mice relative to wild type controls in a standard two-bottle choice paradigm (Blednov and Harris 2007). The present data suggest that higher ethanol intake among TRPV1 knockouts in such long-term tests may be mediated in part by a reduction in the aversive sensory characteristics of alcohol. Manipulation of alcohol's appetitive orosensory component, specifically inhibition of its sweet taste properties, has previously been shown to substantially reduce ethanol consumption and preference in long-term tests (Blednov et al. 2008; Brasser et al. 2006), emphasizing the importance of orally derived reinforcement in alcohol intake. Identifying the specific mechanisms that mediate oral ethanol avoidance and preference is critical to understanding how the sensory processing of this drug ultimately influences its ingestion.

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Fig. 1.

a Stimulus/vehicle lick ratios for ethanol in TRPV1 knockout (TRPV1^{-/-}) and C57BL/6J (B6) wild type control mice averaged across concentration. TRPV1 null mice displayed reduced oral avoidance of alcohol relative to wild type mice (main effect of genotype: $F(1, 28) = 14.68$, $***P < 0.001$). **b** Stimulus/vehicle lick ratios for ethanol in TRPV1 knockouts and B6 controls as a function of concentration. Regardless of genotype, lick responses to ethanol declined from 5 to 40% (main effect of concentration: $F(5, 140) = 112.29$, $P < 0.001$; post hoc comparisons, P 's ≤ 0.001). Lick ratio = mean licks to stimulus/mean licks to deionized water. Mean ($\pm$ SE) lick ratio data are shown. $n = 16$ /genotype

Fig. 2.

Stimulus/vehicle lick ratios for capsaicin in TRPV1 knockout (TRPV1^{-/-}) and wild type mice as a function of concentration. TRPV1 null mice were indifferent to capsaicin across concentration, whereas B6 mice displayed suppressed lick ratios at 0.1, 0.3, and 1 mM capsaicin relative to all lower concentrations (genotype $\times$ concentration interaction: $F(5, 95) = 4.92$, $P < 0.001$; post hoc comparisons, P 's < 0.01). Post hoc comparisons between genotypes indicated lower lick ratios to capsaicin in B6 mice compared to knockouts at the three highest concentrations (0.1, 0.3, and 1 mM; $*P$'s < 0.05). Lick ratio = mean licks to stimulus/mean licks to vehicle. Mean ($\pm$ SE) lick ratio data are shown. $n = 7$ for wild type and $n = 16$ for knockout mice

Fig. 3.

Standardized lick ratios for sucrose in TRPV1 null (TRPV1^{-/-}) and wild type control mice as a function of concentration and gender. Higher lick ratios for sucrose were observed in males compared to females (main effect of sex: $F(1, 28) = 8.81$, $P < 0.01$) and in B6 controls compared to TRPV1 knockouts (main effect of genotype: $F(1, 28) = 6.30$, $P < 0.05$), with both effects mediated by a trend for reduced sucrose responses in knockout females (borderline genotype $\times$ sex interaction: $F(1, 28) = 3.95$, $P = 0.06$). Regardless of genotype and gender, lick ratios for sucrose increased with concentration from 0.01 to 1 M (main effect of concentration: $F(5, 140) = 112.37$, $P < 0.001$; post hoc comparisons, $P < 0.05$). Standardized lick ratio = mean licks to stimulus/maximum potential licks per trial. Mean ($\pm$ SE) lick ratio data are shown. $n = 16$ /genotype

Fig. 4. Stimulus/vehicle lick ratios for quinine in TRPV1 null (TRPV1^{-/-}) and B6 wild type mice as a function of concentration and gender. Lick responding for quinine decreased with concentration from 0.01 to 1 mM (main effect of concentration: $F(5, 140) = 621.15$, $P < 0.001$, all comparisons $P < 0.001$). Post hoc comparisons on a three-way interaction of genotype $\times$ concentration $\times$ sex ($F(5, 140) = 4.55$, $P < 0.001$) did not reveal a significant difference in quinine lick ratios between TRPV1 knockouts and B6 controls at any concentration for either gender (all P 's NS). Lick ratio = mean licks to stimulus/mean licks to deionized water. Mean ($\pm$ SE) lick ratio data are shown. $n = 16$ /genotype