

Menthol decreases oral nicotine aversion in C57BL/6 mice through a TRPM8-dependent mechanism

Lu Fan,¹ Shrilatha Balakrishna,¹ Sairam V Jabba,² Pamela E Bonner,² Seth R Taylor,¹ Marina R Picciotto,¹ Sven-Eric Jordt^{1,2}

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¹Department of Psychiatry, Yale Tobacco Center of Regulatory Science (TCORS), Yale School of Medicine, New Haven, Connecticut, USA

²Department of Anesthesiology, Duke University School of Medicine, Durham, North Carolina, USA

Correspondence to

Dr Sven-Eric Jordt, Department of Anesthesiology, Duke University School of Medicine, Box 3094 MS27, Durham, NC 27710-3094, USA; sven.jordt@duke.edu

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ABSTRACT

Background Nicotine is a major oral irritant in smokeless tobacco products and has an aversive taste. Mentholated smokeless tobacco products are highly popular, suggesting that menthol increases their palatability and may facilitate initiation of product use. While menthol is known to reduce respiratory irritation by tobacco smoke irritants, it is not known whether this activity extends to oral nicotine and its aversive effects.

Study design The two-bottle choice drinking assay was used to characterise aversion and preference in C57BL/6 mice to a range of menthol concentrations (10–200 µg/mL). Then, effects of menthol on oral nicotine aversion were determined. Responses were compared with those in mice deficient in the cold/menthol receptor, TRPM8, expressed in trigeminal sensory neurons innervating the oral cavity.

Results Mice showed aversion to menthol concentrations of 100 µg/mL and above. When presented with a highly aversive concentration of nicotine (200 µg/mL), mice preferred solutions with 50 or 100 µg/mL menthol added over nicotine alone. In contrast to wild-type mice, *Trpm8*–/– showed a strong aversion to mentholated (100 µg/mL) nicotine (200 µg/mL) and preferred nicotine alone. *Trpm8*–/– mice show aversion to lower concentrations of menthol than wild-type mice.

Conclusions Oral menthol can reduce the aversive effects of oral nicotine and, at higher concentrations, acts as an irritant by itself. Menthol's effects in relation to nicotine require TRPM8, the cool temperature sensing ion channel that activates analgesic and counterirritant mechanisms. These mechanisms may underlie preference for menthol-containing smokeless tobacco products and may facilitate initiation of product use.

INTRODUCTION

Smokeless tobacco products (snuff, chewing tobacco, snus and dissolvables) containing menthol are highly popular.^{1–2} Menthol adds a fresh, cooling and minty taste, sensations that are reminiscent of other tobacco products such as menthol cigarettes and of confectionary products such as chewing gum and mint lozenges. Menthol may increase the palatability of smokeless products that contain tobacco ingredients with aversive flavours, thereby facilitating initiation of product use.³

Nicotine levels in smokeless tobacco products are sufficient to be perceived as irritating by humans, eliciting burning and tingling sensations in the mouth.^{4–5} Nicotine also has an aversively bitter taste.⁵ In recent studies in mice, we demonstrated that menthol inhibits the respiratory irritation response to cigarette smoke irritants such as

acrolein and to cigarette smoke itself.^{6–7} These mice had never been exposed to cigarette smoke before, mimicking initiating users. Higher levels of cotinine were detected in the mice inhaling mentholated cigarette smoke than in the mice inhaling non-mentholated smoke, implying that menthol increases nicotine uptake.⁷ These effects were observed at menthol levels present in smoke of commercial menthol cigarettes.⁷ Together these data suggested that menthol may facilitate smoking initiation by reducing aversion to noxious smoke.^{6–8}

It is currently controversial whether menthol's counterirritant activity extends to oral nicotine and its aversive effects. Most studies examining the effects of menthol in the context of tobacco use were performed in chronic smokers and provide no information about menthol's behavioural effects during initiation of tobacco use. Menthol, at high levels, can also be perceived as irritating.^{4–5} A human psychophysical study demonstrated that menthol applied to the tongue at irritating concentrations caused desensitisation to nicotine later applied to the same location.⁴ Another human study using mentholated nicotine gums observed that menthol reduced pain and irritation elicited by nicotine, but only transiently, and some test participants perceived the high menthol concentrations in this study as irritating.⁵

Pain and irritation are initiated when noxious chemicals activate peripheral sensory nerves.⁹ Smoke irritants such as acrolein activate the sensory irritant receptor, TRPA1, in nerve endings in the mucosa of the throat and nasal passages.¹⁰ Oral nicotine activates nicotinic acetylcholine receptors (nAChRs) in sensory nerves innervating the tongue and oral cavity.^{11–12} Nicotine also activates TRPA1, albeit higher concentrations are required than for nAChRs.¹³ The bitter taste associated with nicotine is likely mediated by bitter taste receptors in oral taste buds that signal to gustatory nerves.^{11–14}

Menthol also has complex interactions with sensory nerves and their receptors. The cooling effect of menthol is mediated by the cold/menthol receptor, TRPM8, expressed in a subset of neurons sensitive to cool temperature.^{15–16} Activation of these neurons by menthol is thought to underlie menthol's analgesic and counterirritant effects. Indeed, menthol failed to inhibit smoke-induced respiratory irritation when mice were treated with a TRPM8 inhibitor before exposure. The irritant effects of menthol at higher concentration are likely mediated by TRPA1.^{17–18}

Mice are obligate nasal breathers, and access for inhaled air to the oral cavity is normally blocked. Thus, oral and respiratory sensing are separated,

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allowing selective study of oral effects. Here we used the two-bottle choice paradigm in mice to investigate the effects of menthol on oral aversion to nicotine. The role of TRPM8 in these effects was investigated by comparing responses of wild-type mice with responses of TRPM8-deficient mice.

METHODS

Animals

Male C57BL/6 mice (adults aged 11 weeks) purchased from Charles River Laboratories were maintained in a AAALAC-certified facility in a temperature and humidity controlled room at a 12-hour light–dark cycle with unlimited access to food and water. *Trpm8*^{−/−} mice were a gift from David Julius (University of California, San Francisco) and backcrossed into C57BL/6 background using marker-assisted backcrossing (Charles River Laboratories, Wilmington, Massachusetts, USA). All experimental protocols were approved by the Institutional Animal Care and Use Committees of Duke University and Yale University. No adverse events were noted during the course of the described experiments.

Two-bottle choice assay

Mice were housed individually in Med Associates behavioural chambers with water supply from drinking tubes manufactured from 25 mL serological pipette tubes with 0.2 mL graduations and stainless steel sipper tubes (Ancare, Part Number, OT99.5, with steel collars at a 90° angle). Tubes were placed in each cage at an equal distance from food. Locations of the tubes were switched every 24 hours to control for side preferences. Each tube contained about 20 mL of liquid. The tubes were calibrated for fluid loss (leakage and evaporation) by placing them in empty cages for 4 days. Average fluid loss per 24 hours per two tubes was 0.4 mL. Mice were acclimated to the two-tube system for 2–3 days with water only for 24 hours/day. To determine preference or aversion, choice solutions were provided overnight from 18:00 to 10:00. This period was chosen since mice are nocturnal, and the animal could remain close to their regular light–dark cycle. The rest of the day mice received tubes

filled with plain water and drank *ad libitum*. Control groups were provided with water only throughout the experiment. Liquid consumption from each tube was measured by comparing weights of the drinking tubes before and after the choice drinking periods. Investigators tasked with filling and swapping tubes were different from investigators reading fluid consumption. The latter were blinded to the tube contents.

Menthol-flavoured solutions

L(−) menthol was used in all studies, the minty and cooling menthol isomer added to tobacco products.¹⁹ Menthol solutions (10–200 µg/mL) were prepared by dissolving L(−)-menthol (Acros Organics) in distilled deionised H₂O (ddH₂O). Nicotine solutions (200 µg/mL) were prepared fresh daily by dissolving nicotine base (Sigma) in dd H₂O or 10–200 µg/mL menthol solutions. Drinking tubes with nicotine solutions were protected from light by covering with aluminium foil.

Statistical analysis

For flavour preference study, data were averaged across the 4 days. One-way ANOVA was used to compare daily averaged liquid consumption among groups, and Tukey post hoc was used to compare between individual groups. In addition, a two-way ANOVA was performed to determine if daily liquid consumption differed from day to day within individual groups due to changes in tolerance over the period of experiment. Paired t-test was used to compare consumed liquid volume between the two choices within each group (**p*<0.05; ***p*<0.01; ****p*<0.0005; *****p*<0.0001).

RESULTS

Aversion to high concentrations of menthol in wild-type C57BL/6 mice

Groups of male adult C57BL/6 mice were given the choice between water and water containing different concentrations of menthol (10–200 µg/mL) overnight, with water *ad libitum*

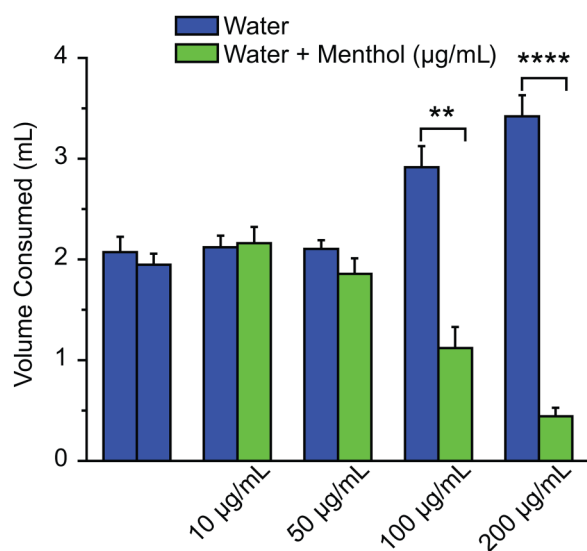


Figure 1 Effects of menthol on oral water consumption in mice in the two-bottle test. Consumption volumes of adult male C57BL/6 mice given the choice between water and menthol-containing water (1–100 µg/mL), averaged over four nightly testing periods. Bars represent mean±SEM. ***p*<0.01; *****p*<0.0001; *n*=5 per group.

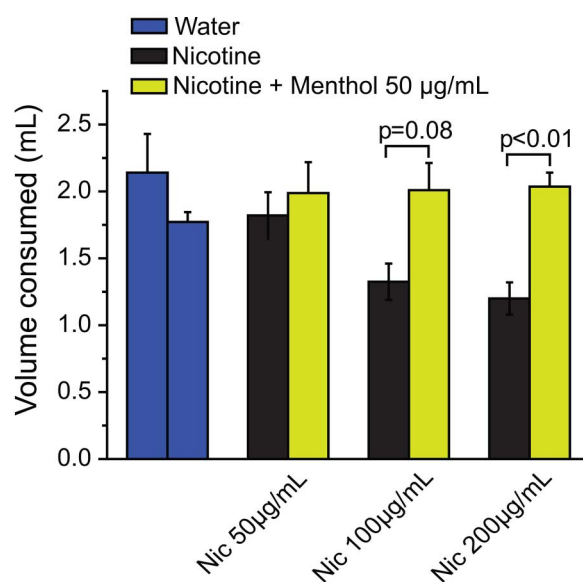
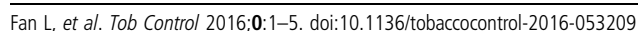


Figure 2 Effects of menthol on oral nicotine aversion in mice in the two-bottle test. Mice were given the choice between non-mentholated and mentholated (50 µg/mL) nicotine solutions, with nicotine contents of 50, 100 or 200 µg/mL. Water only served as control (blue). Bars show average consumption during four consecutive nightly choice periods. Error bars show SEM. *n*=5–7 for each group.

Figure 3 Consumption volumes of wild-type and *Trpm8*^{−/−} mice in the two-bottle test with choice between nicotine only and mentholated nicotine. (A) Consumption volumes averaged over four nights of WT and *Trpm8*^{−/−} mice. Mice were given choice of bottles filled with the same nicotine solution (200 μg/mL, black) or given the choice between nicotine (200 μg/mL, black) or nicotine (200 mg/mL) plus menthol (100 μg/mL, yellow). Error bars represent SEM. **p*<0.05; ***p*<0.01; *n*=5–8 for each group. Nightly consumption volumes during four nights of (B) the same WT mice and (C) the same *Trpm8*^{−/−} mice. Two-way ANOVA analysis was performed to examine for potential tolerance. WT, wild-type.



The above studies demonstrate that menthol has aversive effects at higher concentrations. To examine the effects of menthol on nicotine consumption, we therefore chose non-aversive or weakly aversive concentrations of menthol (50 or 100 $\mu\text{g/mL}$) and tested responses of mice given the choice between mentholated and non-mentholated nicotine solutions. A range of nicotine concentrations with known aversive effects was chosen (50, 100 and 200 $\mu\text{g/mL}$).²⁰ When offered 200 $\mu\text{g/mL}$ nicotine, mice clearly preferred the mentholated (50 $\mu\text{g/mL}$) nicotine solution over nicotine alone (figure 2).

Wild-type mice also preferred nicotine solution (200 $\mu\text{g}/\text{mL}$) containing 100 $\mu\text{g}/\text{mL}$ menthol (figure 3A). When mice deficient in the cold/menthol receptor, TRPM8, were given the same choice, the preference was reversed (figure 3A). *Trpm8* $^{-/-}$ mice clearly preferred the non-mentholated nicotine solution and avoided the mentholated solution. Preference for mentholated nicotine solution was evident on each of the 4 days of testing in wild-type mice (figure 3B). *Trpm8* $^{-/-}$ mice, however, preferred non-mentholated nicotine solution on all 4 days (figure 3C). Also, a two-way ANOVA was performed to determine if daily consumption of nicotine only solution versus mentholated nicotine differed from day to day within individual groups due to potential development of tolerance over the experimental period. Neither in wild-type nor in *Trpm8* $^{-/-}$ mice there was a change in tolerance for the solution they showed aversion against.

Intrigued by the observation of strong aversion to mentholated nicotine solution in *Trpm8*^{−/−} mice we examined whether aversion to menthol or nicotine alone differs between wild-type and *Trpm8*^{−/−} mice. The dose–response relationship for menthol aversion showed a clear shift to lower menthol concentrations in *Trpm8*^{−/−} mice, with a 50 $\mu\text{g/mL}$ solution clearly producing aversion (figure 4A, B). Wild-type and *Trpm8*^{−/−} mice displayed robust aversion to nicotine at 100 and 200 $\mu\text{g/mL}$ (figure 4C, D).

The present study clearly demonstrates that menthol can diminish the oral aversion of mice to nicotine. Mice display aversion to higher menthol concentrations, but non-aversive or mildly aversive concentrations of menthol do not produce more aversion when combined with nicotine. Instead, mentholated nicotine solutions are favoured.

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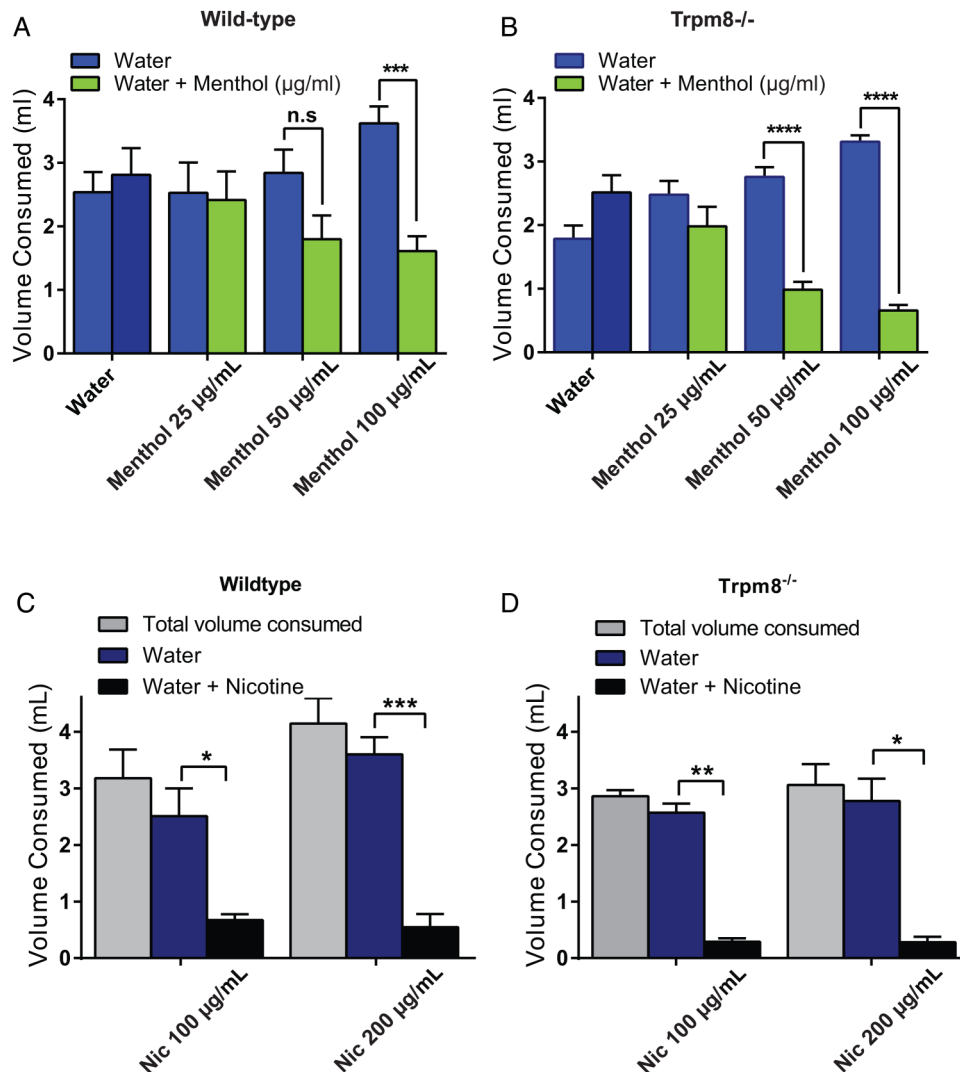


Figure 4 Comparison of menthol and nicotine sensitivity of wild-type and *Trpm8*^{-/-} mice in the two-bottle test. (A) Consumption volumes averaged over four nights of wild-type mice given the choice of water only (blue) or water plus menthol (25, 50 and 100 µg/mL, green). Error bars represent SEM. ****p*<0.001; *n*=3 per group. (B) Consumption volumes averaged over four nights of *Trpm8*^{-/-} mice given the choice of water only (blue) or water plus menthol (25, 50 and 100 µg/mL, green). Error bars represent SEM. *****p*<0.0001; *n*=4 per group. (C) Consumption volumes averaged over four nights of wild-type mice given the choice of water only (blue) or water plus nicotine (100 or 200 µg/mL, black). *n*=5 per group. (D) Consumption volumes averaged over four nights of *Trpm8*^{-/-} mice given the choice of water only (blue) or water plus nicotine (100 or 200 µg/mL, black). *n*=4 per group.

0.07–0.14 mg/mL (445–890 µM) for pouches with higher menthol contents (see online supplementary table 1). Similarly, menthol concentration in the saliva would be 0.075–0.15 mg/mL (477–954 µM) for larger pouches with lower menthol contents and 0.13–0.26 mg/mL (827 µM–1.66 mM) for higher menthol-containing pouches (see online supplementary table 1). All these concentrations are sufficient to stimulate, and potentially saturate, TRPM8 and TRPA1 activity. The menthol concentrations diminishing nicotine aversion in mice in the present study, 50 µg/mL=320 µM and 100 µg/mL=640 µM, are within the range of the estimated saliva concentrations and will strongly activate TRPM8 and TRPA1.

Nicotine contents vary between 2 and 10 mg per pouch, depending on product.²² If released completely, average concentrations in saliva would exceed 50 µg/mL, potentially reaching 500 µg/mL. The nicotine concentrations used here were 50–200 µg/mL (0.3–1.23 mM), well within this range. At these concentrations, peripheral nicotine activates nAChRs in nociceptor sensory neurons innervating the oral cavity. Nicotine may also

activate TRPA1 at these concentrations, however, reported dose–response data are inconsistent and depend on the experimental model or preparation.¹³ Activation of these targets will elicit irritation and pain and contribute to oral nicotine aversion. Nicotine also tastes bitter, a sensation mediated by bitter taste receptors expressed in the taste buds of the tongue.

In the current study, TRPM8, the cold/menthol receptor, was proven to be essential for menthol's effects on oral nicotine aversion. In contrast to wild-type mice in which menthol diminished nicotine aversion, the same combination of menthol (100 µg/mL) with nicotine (200 µg/mL) was avoided by TRPM8-deficient mice that strongly favoured plain nicotine solution. It is likely that TRPM8-expressing trigeminal sensory neurons innervating the oral cavity and tongue activate inhibitory circuits in the trigeminal nucleus that dampen input from nicotine-activated nociceptors, thereby diminishing oral irritation and pain. Menthol is also an irritant, activating irritant receptor TRPA1 in nociceptors. It is likely that menthol-activated TRPM8-expressing neurons also dampen menthol's irritant

effects, similar to its effect on nicotine. In TRPM8-deficient mice, this diminishing effect is gone; menthol's irritancy is unmasked and adds to the irritancy of nicotine instead. This effect likely explains why *Trpm8*^{-/-} mice avoided mentholated nicotine solution and preferred plain nicotine solution.

The present study demonstrates that menthol, at concentrations similar to those estimated for saliva of smokeless tobacco product users, has strong receptor-mediated pharmacological effects on the observed behavioural aversion to nicotine. The mice used in our study had not been exposed to nicotine before, resembling initiating smokeless tobacco product users. Menthol's effect on nicotine aversion did not change over the 4 days of our experiment, suggesting that menthol's effects do not desensitize in the first days of the initiation period.

Humans and rodents express TRPM8 ion channels in the trigeminal neurons innervating the oral cavity. The amino acid sequences of human and mouse TRPM8 are 94% identical (NCBI Blast algorithm), with identity especially high within the transmembrane moiety binding to menthol and conducting ions. This degree of identity is in the same range, or higher, than for most human and mouse nAChRs ($\alpha 2$: 85% identical, $\beta 4$: 94% identical). This high degree of species similarity extends to the dose–response relationship with menthol that are also almost identical, with EC_{50} s for human TRPM8 = $3.7 \pm 0.1 \mu\text{M}$ and for mouse TRPM8 = $4.1 \pm 1.3 \mu\text{M}$.^{24, 25} On the basis of these similarities, it can be assumed that menthol has similar effects in humans, diminishing nicotine's irritant effects in the oral cavity at the menthol concentration used here.²⁴ This effect may facilitate initiation of smokeless tobacco product use and increase the population of users developing and maintaining nicotine addiction.

What this paper adds

- ▶ Menthol is a popular flavour among users of oral smokeless tobacco products such as snuff and snus. It remains unclear whether oral menthol masks the aversive sensory effects of nicotine and other tobacco constituents and thereby facilitates initiation.
- ▶ This paper demonstrates that menthol reduces oral aversion to nicotine in mice, in the concentration range released by smokeless tobacco products.
- ▶ The oral counterirritant effects of menthol are absent in mice deficient in TRPM8, the cold/menthol receptor expressed in sensory nerves, demonstrating that menthol has pharmacologically potent and receptor-mediated effects.
- ▶ Regulation of menthol levels in smokeless product may be a means to modulate smokeless product use initiation and preferences.

Correction notice This article has been corrected since it was published Online First. In the Abstract, '50% or 100 mg/mL' has been corrected to '50 or 100 mg/mL' in the sentence 'When presented with a highly aversive concentration of nicotine (200 mg/mL), mice preferred solutions with 50 or 100 mg/mL menthol added...'.

Contributors S-EJ and MRP conceived of the study. LF, SB, SVJ, PEB and SRT performed experiments. LF, SB, SVJ, MRP and S-EJ contributed to data analysis; interpretation of data; writing of the manuscript and review, revision and approval of the final article. S-EJ is guarantor of the study.

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Competing interests None declared.

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Data sharing statement Data are available for download on request from the Duke Department of Anesthesiology's primary data server.

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