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Topical hindpaw application of L-menthol decreases responsiveness to heat with biphasic effects on cold sensitivity of rat lumbar dorsal horn neurons

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Abstract

Menthol is used in pharmaceutical applications because of its desired cooling and analgesic properties. The neural mechanism by which topical application of menthol decreases heat pain is not fully understood. We investigated the effects of topical menthol application on lumbar dorsal horn wide dynamic range and nociceptive-specific neuronal responses to noxious heat and cooling of glabrous hindpaw cutaneous receptive fields. Menthol increased thresholds for responses to noxious heat in a concentration-dependent manner. Menthol had a biphasic effect on cold-evoked responses, reducing the threshold (to warmer temperatures) at a low (1%) concentration and increasing threshold and reducing response magnitude at high (10, 40%) concentrations. Menthol had little effect on responses to innocuous or noxious mechanical stimuli, ruling out a local anesthetic action. Application of 40% menthol to the contralateral hindpaw tended to reduce responses to cooling and noxious heat, suggesting a weak heterosegmental inhibitory effect. These results indicate that menthol has an analgesic effect on heat sensitivity of nociceptive dorsal horn neurons, as well as biphasic effects on cold sensitivity, consistent with previous behavioral observations.

Keywords

L-Menthol; heat analgesia; cold hyperalgesia; dorsal horn neurons

Menthol is a commonly used counterirritant extracted from plant oils of the mint family (e.g. *Menta piperita*). Menthol is used in analgesic and antipruritic balms at concentrations ranging from 1–16% (Knight and Draper, 2007). Topical application of L-menthol increases sensitivity to cooling and/or cold pain in human skin (Green, 1992, Wasner et al., 2004, Namer et al., 2005, Hatem et al., 2006, Flühr et al., 2009) and oral cavity (Kalantzis et al., 2007, Albin et al., 2008). Menthol enhances the cooling-evoked responses of primary afferent cold fibers (Schäfer et al., 1986, Swandulla et al., 1986, Swandulla et al., 1987; Wang et al., 1993; Lundy & Contreras, 1995), likely accounting for the perceptual enhancement of cold by menthol. Topical menthol also reduces heat pain in human skin

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(Green, 1985, 1986) and oral cavity (Green, 1986, Albin et al., 2008) and cross desensitizes oral irritancy evoked by the TRPV1 agonist capsaicin (Green and McAuliffe, 2000), and the TRPA1 agonists nicotine (Dessirier et al., 2001) and cinnamaldehyde (Klein et al., 2011a). Effects of menthol on mechanosensitivity are mixed, with reports of anesthesia (Galeotti et al., 2001, Watt et al., 2008) or mechanical allodynia (Binder et al., 2011).

The neural mechanisms by which menthol influences cooling and heat pain sensitivity are incompletely understood. Menthol is known to enhance cooling-evoked responses of dorsal root ganglion (DRG) cells via an action at the cold-sensitive transient receptor potential channel melastatin 8 (TRPM8) (Reid & Flonta, 2002; McKemy et al., 2002, Peier et al., 2002), suggesting a peripheral site for menthol's enhancement of cold sensation. Topical application of menthol to the tongue excited neurons in superficial laminae if trigeminal subnucleus caudalis (Vc), and enhanced their responses to cooling in a dose-dependent manner (Zanotto et al., 2007). Menthol at a high (40%) concentration also inhibited noxious heat-evoked responses (Zanotto et al., 2007). In the present study we wished to further investigate the effects of menthol on lumbar spinal nociceptive neurons, to allow direct comparisons with the effects of topical hindpaw application of menthol on behavioral sensitivity to thermal stimulation in rats (Klein et al., 2010).

Experimental Procedures

Single-unit recording

Experiments were conducted using 54 adult male Sprague-Dawley rats (wt. 302–535 g) under a protocol approved by the UC Davis Institutional Animal Care and Use Committee. Methods were essentially the same as described previously (Merrill et al., 2008, Sawyer et al., 2009a, Sawyer et al., 2009b). Briefly, animals were anesthetized with sodium pentobarbital (induction: 65 mg/kg ip, maintenance: 10 mg/kg iv). Oxygen was delivered via a tracheal cannula. Core body temperature was monitored and maintained by heating pad, and displayed continuously along with the electrocardiogram using a Powerlab interface (AD Instruments, Colorado Springs, CO). A tungsten microelectrode (FHC, Bowdoin, ME; 10 M Ω) was used for extracellular single-unit recording at segments T13 and L1, and action potentials were amplified and connected to a computer through Powerlab (ADInstruments) and Spike 2 (Cambridge Electronic Design, Cambridge, UK) interfaces. When more than one action potential was recorded, they were sorted by spike size and waveform using Spike2 software. Only units that responded to application of cold and/or noxious heat stimuli to the hindpaw were selected for further study.

Thermal, chemical and mechanical stimulation

Thermal stimuli were delivered by a feedback-controlled Peltier device (NTE-2A, Physitemp, Clifton, NJ; 13-mm diameter) attached to a micromanipulator (World Precision Instruments, Sarasota, FL), allowing precise placement of the thermode in contact with the glabrous ventral hindpaw surface. The thermode temperature was controlled by computer to maintain an adapting temperature of 35°C, and to heat to 55°C or to cool to 0°C, with 2 min between the onset of hot and cold stimuli. The thermode-skin interface temperature was measured using a fast thermocouple (IT-18, Physitemp) connected to an electronic thermometer (BAT-12; Physitemp) and displayed continuously. It took approx. 5 sec for the Peltier thermode to heat from 35°C to 55°C, producing a maximum thermode-skin interface temperature that averaged 52.7 °C $\pm$ 0.5 (SEM) and remained above 50°C for a mean of 7.3 $\pm$ 0.5 (SEM) sec. The cooling rate was slower, reducing the thermode-skin interface temperature from 35°C to $\sim$ 0°C over a 55 sec period. A positive thermal response was considered to be an increase of firing 30% above baseline. Only cold- and/or heatresponsive units were investigated further. The mechanosensitive receptive field was mapped using

graded von Frey monofilaments (bending force: 0.68–1258.9 mN) applied in ascending order, followed by gentle blowing, cotton wisp, touching and pinching with a blunt forceps. Cold- and/or heat-sensitive units that additionally exhibited graded responses to increasing force were classified as wide dynamic range (WDR), whereas thermosensitive units that additionally responded only to the largest von Frey hair and pinch were classified as nociceptive specific (NS). Units that did not respond reliably to any mechanical stimulus were classified as mechanically insensitive (MI).

Following unit classification, unit responses to two successive applications of heat and cold stimuli were recorded, and a chemical was then applied to the receptive field by pipette in a volume of 5 μ L. We first applied the vehicle for menthol, followed by application of the noxious heat and cold stimuli three times (noxious heat at 3, 8 and 13 min post-chemical; cold at 5, 10 and 15 min post- chemical application). Mechanical stimuli were retested 2 min after the last cold stimulus. After this, menthol was applied, followed by the identical sequence of thermal and mechanical stimulus application. L-menthol (Givaudan Inc, Cincinnati, OH) was dissolved to a concentration of 1% in a vehicle of 10% ethanol and 1% Tween-80 (Fisher Scientific, Fair Lawn, NJ), and to concentrations of 10% and 40% in a vehicle of 50% ethanol and 5% Tween-80. During chemical application the thermode was lifted away from the hindpaw and then replaced at the same exact location. When re-application of the thermode to the paw surface elicited activity, neuron activity was allowed to return to baseline before proceeding. In some experiments, menthol was applied to the contralateral hindpaw in a volume of 10 μ L, followed by the same sequence of thermal and mechanical stimuli delivered to the ipsilateral paw.

Histology

An electrolytic lesion was made at the end of the recording session through the tungsten microelectrode. The lumbar spinal cord was removed and post-fixed in 10% buffered formalin and cut on a freezing microtome so that spinal cord sections could be examined to identify lesion sites under the light microscope (Sawyer et al., 2009a,b; Merrill et al., 2008).

Data Analysis

The number of thermally-evoked action potentials was summed over 30 sec for heat or 60 sec for cold stimuli, and baseline-corrected by subtracting the number of spontaneous action potentials counted over the same time period prior to the thermal stimulus. A change in firing rate exceeding twice the standard deviation of averaged baseline activity was considered a positive response. The two sets of thermal stimuli delivered prior to chemical application were averaged to represent baseline. Thresholds for heat- and cold-evoked responses were defined as the temperature at which the unit firing rate increased by twice the standard deviation of the immediately preceding baseline firing rate. For mechanical testing, the firing rate 15 sec immediately before each stimulus was summed and subtracted from the firing rate 15 sec after application of the mechanical stimulus. The baseline-corrected number of action potentials elicited by thermal and mechanical stimuli, and temperature thresholds, measured prior to chemical stimulation were compared to the responses post-chemical stimulation by one-way repeated measures ANOVA and Tukey post-hoc comparison tests between time points (SPSS 9.0 software, SPSS, Chicago, IL). Responses between vehicle and menthol treatment groups were also compared using two - way repeated measures ANOVA (SPSS). Error reported is the standard error of the mean (SEM).

Results

Unit classification

Data were collected from a total of 80 lumbar spinal cord units having receptive fields on the glabrous skin of the ipsilateral ventral hindpaw. Fig. 2 shows an example of a unit that responded to both noxious heat and cold stimulation. In 54 experiments only one unit was recorded, while in 5 experiments two units were recorded (one on each side of the spinal cord with >2 hr between recordings; n=10 units) and in 8 experiments two units were recorded simultaneously (n= 16 units) and discriminated post-hoc using the Spike2 software by waveform.

Most unit recording sites were histologically confirmed to be in the superficial laminae of the dorsal horn, while some units were located in deeper laminae (Fig. 1) at an average depth of 535 ± 44 [SEM] μm below the surface. Seventy-one units displayed increasing responsiveness to graded mechanical stimulation and were classified as WDR, 7 units did not respond to innocuous mechanical stimulation but did respond to a 1258.9 mN von Frey and pinch stimulus and were classified as NS, and two cells were classified as MI. The mean baseline firing rate prior to thermal stimulation was 3.9 ± 0.24 impulses/sec across treatment groups, and did not significantly differ over time (data not shown, $F(54, 1377) = 0.72$, $p > 0.05$). The mean baseline firing rate was significantly greater for units responsive to heat and cold compared to those responsive to heat but not cold (4.9 ± 0.4 vs. 2.8 ± 0.2 impulses/sec, $F(1, 158) = 4.28$, $p = 0.04$).

1% menthol

Overall, there were no significant changes in the magnitude of noxious heat-evoked responses following either vehicle or 1% menthol application. This was true for units sensitive to both noxious heat and cooling (n=8), as well as those sensitive to noxious heat but insensitive to cooling (n=7). Although the response magnitude did not change, the mean heat threshold of all 15 heat-sensitive units increased significantly by nearly 3°C at the second and third time points post-menthol (Table 1; $F(3,42) = 3.35$, $p = 0.028$; Tukey post-hoc). Vehicle had no significant effect on heat thresholds (Table 1).

Neither vehicle nor 1% menthol treatment significantly affected the magnitude of responses of cold- and heat-sensitive units to cooling, although the mean cold-evoked responses increased numerically by 26% post-menthol (n=8). The mean threshold of cold-evoked responses decreased significantly (toward warmer temperature) by 4.5°C at the first post-menthol time point (Table 2; $F(3, 21) = 3.24$, $p = 0.043$; Tukey post-hoc). The mean cold threshold was not significantly affected following vehicle. Since some cold-insensitive units might have subliminal cold fiber input that could be sensitized by menthol, we also tested effects of vehicle and menthol on 7 cold-insensitive units. For these units, however, baseline-corrected unit responses during cooling remained near zero following vehicle and 1% menthol, indicating the absence of any cold response.

10% menthol

Following application of 10% L-menthol, heat-evoked responses were numerically reduced in heat- and cold-sensitive units (Fig. 3A) as well as heat-sensitive but cold-insensitive units (Fig. 3B), although there was no significant difference between menthol and vehicle treatment. The mean threshold for heat-evoked responses increased significantly (Table 1; $F(3, 78) = 5.649$, $p = 0.0015$), while vehicle treatment had no effect on threshold.

The mean cold-evoked responses of heat- and cold-sensitive units increased to become significantly higher compared to the pre-menthol response (Fig. 3C, $F(3, 36) = 3.49$,

$p=0.025$). Cold thresholds of these units decreased numerically by $\sim 3^{\circ}\text{C}$, but this was not significantly different from pre-menthol levels or compared to vehicle treatment (Table 2). Vehicle treatment had no effect on cold-evoked responses (Fig. 3C and 3D) or thresholds (Table 2). Interestingly, units that were initially unresponsive to cooling exhibited an increase in cold-evoked firing post-menthol (Fig. 3D). Of these units, 4 of the 14 exhibited a positive response to cooling post-menthol.

40% menthol

Fig. 2 shows an example of a unit's responses to thermal stimuli before and after application of vehicle (Fig. 2A) or 40% menthol (Fig. 2B) to the receptive field on the lateral hindpaw. Whereas vehicle had little effect on heat- and cold-evoked responses, responses to heat and cooling decreased following application of 40% menthol.

Heat-evoked responses decreased following topical application of 40% menthol for the group of units responsive to both heating and cold (Fig. 4A) as well as for the group that responded to heat but not cold (Fig. 4B, $F(3, 27) = 2.99$, $p = 0.037$), with the latter exhibiting a significant change in heat-evoked responses over time. This was accompanied by a significant increase in heat threshold of approx. 5°C (Table 1, $F(3, 66) = 16.41$, $p < 0.001$). Of these latter units, 3 of 10 no longer exhibited a positive response to noxious heat post-menthol. Vehicle treatment had no effect on heat-evoked responses (Fig. 4A and B) or heat threshold (Table 1) over time. Overall, the mean heat threshold for the 40% menthol treatment group was significantly greater compared to vehicle and 1% menthol (Table 1, $F(12, 375) = 2.5$, $p = 0.003$, LSD post-hoc test) but not the 10% menthol group. Additionally, regression analysis revealed a significant correlation between the menthol concentration and change in heat threshold at the first post-menthol time point ($R^2 = 0.27$, $p = 0.002$, Pearson's product-moment correlation).

Following application of 40% menthol, there was a significant decrease in the mean cold-evoked response (Fig. 4C, $F(3, 36) = 3.00$, $p = 0.043$) which returned to pre-application levels by the second post-menthol cold stimulus (Fig. 4C, post #2). Following 40% menthol there was an increase in the mean cold threshold of $\sim 3^{\circ}\text{C}$ that was statistically significant at post-menthol time point #2 (Table 2, $F(3, 36) = 3.8$, $p = 0.019$, Tukey post-hoc test). Eight of the 13 units no longer responded positively to cold following menthol application. Vehicle treatment had no effect on cold-evoked responses (Fig. 4C and D) or cold threshold (Table 2).

Lack of effect of menthol on mechanically-evoked responses

Fifth-eight WDR units exhibited graded responses to a series of calibrated von Frey stimuli, and also light touch and pinch. Neither vehicle nor pretreatment with either 1% ($n = 14$ units), 10% ($n = 23$) or 40% menthol ($n = 21$), had any effect on mechanically-evoked responses, except that 10% menthol resulted in a significant enhancement of the mean touch-evoked response ($p < 0.05$, one-way ANOVA, Tukey post-hoc test).

Contralateral application of 40% menthol

In order to test the counter-irritant effect of menthol, we applied 40% menthol to the hindpaw contralateral to the recorded unit, using the same protocol as for ipsilateral menthol application. Contralateral application of 40% menthol tended to reduce heat-evoked responses of units sensitive to heat and cold ($n = 5$), as well as those sensitive to heat but not cold ($n = 10$). For the latter, the difference between the vehicle vs. 40% menthol group fell just short of statistical significance ($F(3, 27) = 2.4$, $p = 0.08$). Following contralateral application of 40% menthol, there was a maximum mean increase in heat threshold of 2.1°C that was not statistically significant (data not shown). Contralateral application of 40%

menthol also numerically reduce cold-evoked responses, accompanied by an increase in threshold (toward lower temperature) of 2.4°C although neither effect reached statistical significance. For 10 units responsive to heat but not cold, contralateral application of 40% menthol did not result in any appreciable response to cold stimulation.

Discussion

We presently show that topical plantar application of L-menthol increased heat thresholds of nociceptive dorsal horn neurons in a dose-dependent manner. Menthol had biphasic effects on responses to skin cooling, enhancing cold-evoked responses and reducing thresholds at lower (1–10%) concentrations, and decreasing cold-evoked responses with increased threshold at higher concentrations (40%). These data are discussed in relation to the thermosensory effects of topical menthol and possible underlying mechanisms.

The present study focused mainly on spinal WDR neurons. They constitute the majority of neurons contributing to ascending sensory pathways, yet their functional role remains enigmatic (LeBars & Cadden, 2009). Given that WDR neurons typically respond to noxious heat, innocuous or noxious cold, and irritant chemical stimuli, it is assumed that they signal pain. Previous studies support the view that WDR neurons contribute to sensory-discriminative aspects of pain sensation (Mayer et al., 1975; Price & Mayer, 1975; Maixner et al., 1986). The present cold-sensitive units often responded at thresholds within the innocuous cooling range, implying that such innocuous stimuli can activate pain-signaling WDR neurons. A possible sensory correlate is that innocuous cooling (Green & Pope 2003; Green et al. 2008) or application of menthol (Green & Schoen, 2007) to human skin can elicit nociceptive (stinging or burning) sensations.

The present results are largely consistent with previous behavioral studies reporting antinociceptive (Klein et al., 2010) and antihyperalgesic (Proudfoot et al., 2006) effects of topical menthol. Importantly, menthol significantly increased the thresholds of heat-sensitive units in a concentration-dependent manner (Table 1). Neither 1% nor 10% menthol significantly reduced heat-evoked responses (whether or not the neuron responded to cooling), while 40% menthol significantly depressed the heat-evoked responses of cold-insensitive units. The inconsistent effects of menthol might be attributed to the large variability in the magnitude of heat-evoked responses, whereas heat thresholds were more consistent and may provide a more accurate measure of the analgesic action of menthol. The increase in heat thresholds of lumbar dorsal horn neurons by menthol is consistent with our recent report that topical application of menthol-derived cooling compounds significantly inhibited the responses of trigeminal subnucleus caudalis (Vc) neurons to noxious heating of the tongue (Klein et al., 2011b). The high concentration of menthol used in this study is comparable to that used in previous human psychophysical studies (10–40% menthol; Yosipovitch et al., 1996; Hatem et al., 2006; Namer et al., 2005; Wasner et al., 2004) as well as in our prior behavioral study (Klein et al., 2010). The absorption of any chemical into the skin is directly related to its concentration and permeability through the stratum corneum. We thus assume that the greater effect of 40% menthol was due to both higher concentration at intradermal nerve endings as well as recruitment of more nerve endings due to greater diffusion distance in the skin.

Peripheral and/or central mechanisms may contribute to the ability of topical menthol to increase thresholds and reduce the magnitude of dorsal horn neuronal responses to noxious skin heating. Peripherally, a local anesthetic action may be ruled out since even high concentrations of menthol did not significantly affect mechanically-evoked responses of WDR neurons. Although menthol is not known to inhibit TRPV1 in sensory neurons (Macpherson et al., 2006), it has been shown to inhibit TRPA1 in rodents (Macpherson et

al., 2006, Karashima et al., 2007). TRPA1 is co-expressed with TRPV1 (Story et al, 2003, Salas et al., 2009). Thus, menthol might indirectly affect TRPV1 via inhibition of TRPA1. While this might explain the present reduction in heat sensitivity of dorsal horn neurons by 40% menthol, it does not hold for human heat pain, since hTRPA1 activity is not decreased by increasing concentrations of menthol (Xiao et al., 2008). Another possibility is that topical menthol enhances peripheral GABAergic inhibition of nociceptors. Subunits of GABA_A receptors are localized to unmyelinated fibers in glabrous skin, and local intraplantar injection of the GABA_A agonist muscimol reduced formalin-evoked nocifensive behaviors (Carlton et al., 1999). Menthol enhances GABA_A currents (Hall et al., 2004), providing the basis for a potential peripheral antinociceptive effect of menthol.

A central mechanism of heat analgesia by menthol could involve menthol excitation of TRPM8-expressing primary afferent fibers, presumably cold fibers, which would indirectly inhibit heat-sensitive dorsal horn neurons via activation of inhibitory spinal interneurons. Primary afferent fibers expressing TRPM8 terminate in superficial laminae of the spinal dorsal horn (Dhaka et al., 2008) where the majority of heat- and cold-sensitive neurons were presently recorded (Fig. 1). Primary afferent fibers expressing TRPM8 or TRPA1 have partially overlapping projection zones in the spinal cord dorsal horn (Wrigley et al., 2009). Primary afferent fibers expressing TRPM8 terminate near neurons in lamina I that express glutamic acid decarboxylase 65 (Dhaka et al., 2008), a marker for neurons that synthesize the inhibitory neurotransmitter GABA. In whole-cell recordings from identified interneurons in laminae II of mouse spinal cord slices, superfusion with menthol or another TRPM8 agonist, icilin, increased the occurrence of excitatory postsynaptic currents recorded in GFP-labeled GABAergic interneurons (Zheng et al., 2010). These findings suggest that menthol activates TRPM8-expressing sensory afferents, most likely cold fibers, that project to the spinal cord to excite GABAergic inhibitory interneurons in lamina I-II that, in turn, inhibit the heat-evoked responses of dorsal horn neurons within the same segment.

Contralateral topical application of menthol tended to reduce neuronal responses to noxious heat and cooling although the effects failed to achieve statistical significance. This suggests the possibility that topical menthol evokes a weak heterosegmental inhibitory effect on heat-sensitive dorsal horn neurons. Menthol is a topical irritant (Yosipovitch et al., 1996), and other irritant chemicals have been reported to heterotopically suppress pain (Green, 1991). Such a counterirritant effect might explain the mirror-image analgesia that we previously observed behaviorally following topical application of menthol to the rat hindpaw (Klein et al., 2010).

Menthol had biphasic effects on dorsal horn neuronal responses to skin cooling. One percent menthol significantly decreased cold thresholds (toward warmer temperatures; Table 2) and enhanced cold-evoked responses of some neurons although the overall effect was not significant. Ten percent menthol had a biphasic effect, initially reducing and later significantly enhancing the magnitude of cold-evoked responses (Fig. 3C). In addition, some initially cold-insensitive units responded to cooling following application of 10% menthol (Fig. 3D). These results are generally consistent with our prior study of neurons in Vc, which exhibited significantly enhanced responses to cooling of the tongue following topical application of 1% and 10% menthol (Zanotto et al., 2007). Moreover, a menthol-derived cooling compound enhanced responses and reduced thresholds of cold-sensitive Vc neurons (Klein et al., 2011b). In comparing cold-sensitive spinal dorsal horn and Vc neurons, 1% menthol did not significantly enhance the mean cold-evoked response of spinal neurons while it significantly enhanced that of Vc neurons (Zanotto et al., 2007). This difference might be attributed to a lower level of TRPM8 mRNA expression in DRG compared to trigeminal ganglion cells (Vriens et al., 2011).

A high concentration of menthol (40%) significantly decreased the responses of cold-sensitive dorsal horn neurons and increased cold thresholds. This effect was short-lasting (Fig. 4C) and might be due to a peripheral effect of menthol on cold fibers, and/or to a central inhibitory mechanism. Peripherally, TRPM8 expressed in cutaneous nerve endings of cold fibers might become desensitized by the high menthol concentration via several reported mechanisms including a decrease in PIP2 after PLC activation (Liu and Qin, 2005, Rohacs et al., 2005, Daniels et al., 2009), an increase in PKC activation and a subsequent down regulation of TRPM8 (Premkumar et al., 2005, Abe et al., 2006), or calcium-mediated activation of calmodulin (Sarria et al., 2011). Alternatively, TRPA1 has been implicated in transduction of noxious cold from the periphery (Story et al., 2003; Karashima et al., 2009, del Camino et al., 2010), and menthol inhibition of TRPA1 could account for a decrease in response of peripheral fibers to noxious skin cooling. Activation of TRPM8-expressing afferents might also result in central inhibition of cold-sensitive dorsal horn neurons. Presumably, menthol activates cold fibers projecting to the spinal cord, where they excite dorsal horn neurons participating in sensory pathways, as well as GABAergic inhibitory interneurons (see above). Forty percent topical menthol may initially activate a proportionately larger population of spinal inhibitory interneurons, resulting in a significant attenuation in heat- and cold-evoked responses of dorsal horn sensory neurons.

The present data are largely consistent with our previous behavioral study of the effect of topical menthol on heat, cold and mechanical sensitivity in rats (Klein et al., 2010). Topical unilateral application of menthol to the plantar surface resulted in a concentration-dependent heat analgesia and a weaker mirror-image analgesia (Klein et al., 2010). These results are well-matched by the present data, showing that menthol increased the heat thresholds of dorsal horn neurons in a concentration-dependent manner. The mirror-image effect might be due to a weak heterosegmental inhibitory effect of menthol (see above). In addition, menthol had biphasic effects on cold sensitivity of dorsal horn neurons, consistent with the biphasic effects of menthol on behavioral cold sensitivity (Klein et al., 2010). In this latter study, 1% and 10% menthol increased cold avoidance in a thermal preference paradigm, whereas 40% menthol reduced cold avoidance. The enhancement of behavioral cold sensitivity by 10% menthol matches the enhancement of cold-evoked responses of dorsal horn neurons (Fig. 3C). In addition, some initially cold-insensitive dorsal horn neurons responded to cooling after application of 10% menthol (Fig. 3D). This suggests that the responses of previously subliminal cold fibers were sufficiently enhanced by menthol such that they now excited dorsal horn neurons, thus contributing to the increase in sensitivity to skin cooling observed behaviorally. That 1% menthol enhanced behavioral cold sensitivity (Klein et al., 2010), but not neuronal responses to cooling, might be attributed to the use of anesthesia that blunted menthol's effect on neuronal cold sensitivity. The highest (40%) menthol concentration significantly reduced the magnitude and increased the threshold (toward lower temperatures) of responses of dorsal horn neurons to cooling. This is consistent with the behavioral effects of 40% menthol to reduce cold avoidance in the thermal preference test and to increase nocifensive response latency in a cold-plate test (Klein et al., 2010). Finally, menthol generally had little effect on the mechanical sensitivity of dorsal horn neurons, with 10% menthol enhancing responses to touch. Behaviorally, menthol had little effect on mechanical withdrawal thresholds, with a small but significant decrease in mechanical withdrawal threshold at the highest (40%) concentration (Klein et al., 2010). Overall, there is a good correspondence between the modulatory effects of menthol on behavioral and dorsal horn neuronal sensitivity to temperature, supporting the assumption that some thermosensitive dorsal horn neurons likely participate in sensory pathways associated with behavioral thermosensitivity.

Conclusions

Topical application of L-menthol increased thresholds of spinal dorsal horn neurons to plantar heat stimulation, in a concentration dependent manner. Menthol had biphasic effects on responses of dorsal horn neurons to skin cooling, with low concentrations of menthol enhancing responses and lowering thresholds while the high (40%) menthol concentration reduced cold-evoked responses and raised the cold threshold. The modulatory effects of menthol on thermal responses of dorsal horn neurons are consistent with the effects of menthol on temperature sensitivity as assessed in behavioral studies.

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Abbreviations

DRG	dorsal root ganglion
GABA	gamma amino butyric acid
GFP	green fluorescent protein
MI	mechanically insensitive
NS	nociceptive specific
PSTH	peristimulus-time histogram
TRPA1	transient receptor potential ankyrin 1
TRPM8	transient receptor potential melastatin 8
TRPV1	transient receptor potential vanilloid 1
Vc	trigeminal subnucleus caudalis
WDR	wide dynamic range

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Highlights

- Menthol raised thresholds of dorsal horn neuronal responses to noxious heat
- Low-concentration menthol enhanced cold-evoked responses and reduced threshold
- High-concentration menthol reduced cold-evoked responses and increased threshold
- Menthol did not affect mechanosensitivity

Figure 1.
Histologically localized recording sites compiled on L4 spinal cord section (adapted from Paxinos & Watson, 1985).

Figure 2.

Individual example with 40% L-menthol. A: Vehicle. Lower row shows peristimulus time histogram (PSTH, bin width: 1 sec) of unit responses to heating and cooling before and after topical application of vehicle (10% ethanol in 1% Tween, 5 μ L). Gray portions of PSTH indicate activity during heat or cold stimulation. Response at 17 min due to replacement of thermode in contact with hindpaw skin. Upper: traces of thermode-skin interface temperature. Insets show the superficial dorsal recording site (dot) on tracing of spinal section (to left) and the mechanosensitive receptive field (gray) on hindpaw. B: 40% menthol. PSTH of same unit shown in A, before and after topical application of 40% menthol to the receptive field. Note weak response elicited by application of menthol (at arrow). Note response elicited by topical application of menthol (at arrow). Following menthol, heat-evoked responses decreased by 7%, 15% and 17%, and cold-evoked responses decreased by 93%, 52% and 32%, compared to prementhol responses.

Figure 3.

10% L-menthol. A: Units responsive to both cooling and noxious heat ($n = 13$). Graph as in Fig. 3A for vehicle (o) or 10% menthol (■). B: units responsive to noxious heat but not cold ($n = 14$; $p = 0.16$, one-way repeated measures ANOVA). C: Units responsive to both cooling and noxious heat. *: significant difference in responses over time for 10% menthol group ($F(3, 36) = 3.49$, $p = 0.025$, one-way repeated-measures ANOVA, $n = 13$). D: Units responsive to noxious heat but to cold. Following menthol, cooling elicited an increase in firing at time point #2 ($n = 14$).

Figure 4.

40% menthol. A: Units responsive to both cooling and noxious heat ($n = 13$). Graph as in Fig. 3A for vehicle (o) or 40% menthol (■). B: Mean heat-evoked responses of units responsive to noxious heat but not cold ($n = 10$). *: Significant difference over time for 40% menthol group ($p < 0.05$, one-way repeated-measures ANOVA). C: Cold responses of units responsive to both cooling and noxious heat. *: Significant difference over time for 40% menthol group ($p < 0.05$, one-way repeated-measures ANOVA). #: significantly different compared to pre-menthol ($p < 0.05$, Tukey post-hoc test, $n = 13$). D: Cold-evoked responses of units responsive to heat but not initially sensitive to cold ($n = 10$).

Table 1

Heat thresholds. Thresholds are in °C ± SEM.

Treatment	Heat Threshold Pre (°C)	Heat Threshold Post #1 (°C)	Heat Threshold Post #2 (°C)	Heat Threshold Post #3 (°C)
1% Menthol ^{&}	44.5 ± 1.4	46.5 ± 1.2	47.3 ± 1.6 [#]	47.2 ± 1.5 [#]
Vehicle (1%)	44.4 ± 1.2	45.2 ± 1.1	45.7 ± 1.3	45.3 ± 1.4
10% Menthol ^{&}	45.8 ± 0.8	47.8 ± 0.9 [#]	48.3 ± 1.0 [#]	48.2 ± 0.9 [#]
40% Menthol ^{*,&}	46.8 ± 1.2	49.5 ± 1.0 [#]	50.5 ± 1.0 [#]	51.4 ± 1.1 [#]
Vehicle (10,40%)	46.4 ± 1.6	45.5 ± 1.7	47.3 ± 1.7	47.5 ± 1.6

1% menthol and vehicle for 1% menthol: n = 15 for both groups. 10% menthol: n = 27; 40% menthol: n = 23, vehicle (for 10 and 40% menthol): n = 50.

[&] p < 0.05, one-way repeated-measures ANOVA.

[#] p < 0.05, post-hoc Tukey test vs. pre.

^{*} p < 0.05, two-way repeated-measures ANOVA vs. vehicle.

Table 2

Cold thresholds (format as in Table 1).

Treatment	Cold Threshold Pre (°C)	Cold Threshold Post #1 (°C)	Cold Threshold Post #2 (°C)	Cold Threshold Post #3 (°C)
1% Menthol*, &	20.8 ± 2.4	25.3 ± 2.0#	22.9 ± 3.3	22.7 ± 2.4
Vehicle (1%)	23.5 ± 2.3	21.4 ± 2.6	22.7 ± 2.3	21.4 ± 2.6
10% Menthol	16.4 ± 1.1	19.5 ± 1.2	17.9 ± 1.5	18.9 ± 1.4
40% Menthol&	17.0 ± 2.5	14.9 ± 2.6	12.9 ± 2.6#	13.2 ± 2.8
Vehicle (10,40%)	17.5 ± 1.5	17.5 ± 1.4	15.6 ± 1.4	17.5 ± 1.5

1% menthol and vehicle: n=8. 10% menthol: n=13; 40% menthol: n=13; vehicle for 10% and 40% menthol: n=26.