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Behavioral Testing of Thermo-Sensitive Transient Receptor Potential (TRP) Channel Agonists Cinnamaldehyde and Menthol on Touch, Temperature and Pain Sensations

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ABSTRACT. It has recently been established that transient receptor potential (TRP) channels play an important role in transducing thermal, mechanical and chemical stimuli for somatic sensation. Several TRP channels exhibit sensitivity to increases or decreases in temperature as well as chemical ligands that elicit similar thermal or painful sensations; these include menthol from mint, mustard oil, cinnamaldehyde, gingerol, capsaicin from chili peppers, camphor, eugenol from cloves, and many others.

Cinnamaldehyde (CA) is a pungent chemical from cinnamon that acts as an agonist of the TRPA1 channel that was originally reported to be activated by cold temperatures below 18°C. CA induces heat hyperalgesia and mechanical allodynia in human skin, and sensitizes responses of spinal and trigeminal dorsal horn neurons to noxious skin heating in rats. TRPA1 is also implicated in cold nociception, however its role in cold pain is more controversial, with discrepant reports that TRPA1 does or does not respond to intense cooling.

Menthol is derived from plants of the mint family and is used in analgesic balms and also in foods and oral hygiene products for its fresh cooling sensation. Menthol enhances cooling by interacting with the cold-sensitive TRPM8 channel, but its effect on pain is less well understood. We have used behavioral methods to investigate if CA and menthol affect sensitivity to thermal (hot and cold), innocuous cold, and mechanical sensitivity in rats.

Unilateral intraplantar injection of CA (5-20%) induced a significant, concentration-dependent reduction in latency for ipsilateral paw withdrawal from a noxious heat stimulus, i.e., heat hyperalgesia. CA also significantly reduced mechanical withdrawal thresholds of the injected paw, i.e., mechanical allodynia. Bilateral intraplantar injections of CA resulted in a signiûcant cold hyperalgesia (cold plate test) and a weak enhancement of innocuous cold avoidance (thermal preference test).

In contrast to CA, menthol dose-dependently increased the latency for noxious heat-evoked withdrawal, i.e. an antinociceptive effect. Menthol did not affect mechanosensation except for a weak allodynic effect at the highest concentration (40%), indicating that it did not exert a local anesthetic effect. Menthol had a biphasic effect on cold avoidance. High concentrations of menthol reduced cold avoidance, i.e. cold hypoalgesia, while low menthol concentrations significantly increased cold avoidance. The highest menthol concentration resulted in cold hypoalgesia (cold plate test), while lower concentrations had no effect.

Overall, our data support the idea that TRPA1 and TRPM8 channels represent promising peripheral targets for pain modulation. *© 2010 Bull. Georg. Natl. Acad. Sci.*

Key words: *cold pain, heat pain, mechanical allodynia, nociception, thermal preference, hyperalgesia, hypoalgesia.*

Introduction

In the past few decades, a tremendous amount of research has been performed in an attempt to elucidate the mechanisms involved in pain perception [1,2]. In this regard, it has recently been established that transient receptor potential (TRP) channels play an important role in transducing thermal, mechanical and chemical stimuli for somatic sensation [3-7]. Several TRP channels exhibit sensitivity to increases or decreases in temperature as well as chemical ligands that elicit similar thermal or painful sensations [8,12]. TRP channels appear to play a mechanosensory or osmosensory role in several musculoskeletal tissues as well [13,14].

Cinnamaldehyde (CA) is a pungent chemical from cinnamon that acts as an agonist of the TRPA1 channel that was originally reported to be activated by cold temperatures below 18°C [15]. CA induces heat hyperalgesia and mechanical allodynia in human skin [16,17,18,19], and sensitizes responses of spinal and trigeminal dorsal horn neurons to noxious skin heating in rats [20,21,22]. TRPA1 is also implicated in cold nociception, however, its role in cold pain is more controversial, with discrepant reports that TRPA1 does [15,23], or does not respond to intense cooling [24].

Knockout mice lacking TRPA1 exhibit normal cold sensitivity [25], or partial [26] or severe [23] deficits in cold pain sensitivity. In humans, topical cutaneous application of CA induces cold hypoalgesia [17] while epilingual CA induces brief cold hyperalgesia [16]. Neither agent affects rat spinal neuronal responses to cooling [20,27]. TRPA1 was originally reported to play a role in mechanotransduction [28]. Mustard oil (allyl isothiocyanate=AITC) induced mechanical allodynia in humans [29]. Intraplantar injection of the TRPA1 agonist 4-hydroxynonenal reduced mechanical paw withdrawal thresholds in mice [30] and blockade of TRPA1 by systemically or locally administered antagonists reversed mechanical hyperalgesia in inflammatory and nerve injury models in mice [31,32].

Menthol is derived from plants of the mint family and is used in analgesic balms and also in foods and oral hygiene products for its fresh cooling sensation [33,34]. Menthol applied to the skin elicits cooling and tingling sensations, and has some anesthetic [35] and *ê*-opioid-mediated antinociceptive properties in the mouse hot-plate test [36]. Menthol enhances cooling by interacting with the cold-sensitive TRPM8 [37,38,39], but its effect on pain is less well understood. TRPM8 is activated by temperatures below 25°C as well as by menthol and other cooling agents [40], and knockout mice lacking TRPM8 exhibit decreased sensitivity to cold surfaces that are normally avoided [8,41,42]. Menthol enhances cooling-evoked gating of TRPM8 transfected in cell lines [43,44,45] and naturally expressed in dorsal root ganglion (DRG) and trigeminal ganglion cells [46,47].

Higher concentrations of menthol enhance cold pain in human skin [17,48,49,50] and oral mucosa [16] and enhance cold avoidance in rats assessed using an operant facial thermal stimulation paradigm [51]. Sensory neurons expressing TRPM8 project to superficial laminae of the spinal cord dorsal horn [52,53] which contain cold-sensitive neurons, that project in the spinothalamic tract [54]. Responses of nociceptive neurons in superficial laminae of trigeminal subnucleus caudalis (Vc) to lingual cooling are enhanced by menthol [55,56].

Based on the studies described above, we hypothesized that intraplantar injection of CA would induce (1) hyperalgesia to noxious heat, (2) cold allodynia and/or cold hyperalgesia, and (3) mechanical allodynia. On the other hand, the apparently opposing effects of menthol on the perception of heat and cold pain prompted the present study. We wished to systematically investigate and compare the modulatory effects of topical menthol on thermal (hot and cold) and mechanical sensitivity in rats using an array of behavioral tests.

Methods

Animals. Adult male Sprague Dawley rats (350-500 g) were singly housed and given rodent chow and water ad libitum. The study protocol was approved by the UC Davis Animal Care and Use Committee.

Application of Chemicals. CA (Sigma-Aldrich, St. Louis MO) at doses of 0.5, 1 or 2 mg/10 µl (i.e., 5%, 10% and 20% or 378.3 mM, 756.7 mM and 1.5 M; in saline + 5% Tween 80, Fisher Scientific, Waltham, MA) or vehicle was injected intraplantar using a 30 gauge needle.

L-Menthol (Givaudan Flavors Corp., Cincinnati, Ohio) dissolved in ethanol and Tween-80 (Fisher Scientific, Fair Lawn, NJ) at concentrations of 0.01%, 0.1%, 1.0%, 10%, or 40% (0.64 mM, 6.4 mM, 64 mM, 640 mM, or 6.4 M, respectively) was topically applied by cotton tip applicator to one or both ventral hindpaws, allowed to dry for 2 min. Vehicles for 0.01%, 0.1%, and 1.0% menthol (10% EtOH + 1% Tween) and 10% or 40% menthol (50% EtOH + 5% Tween) were applied in the same manner separately as controls.

Behavioral testing.

Thermal paw withdrawal (Hargreaves test). Rats were first habituated, over three successive daily sessions, to stand on a glass surface heated to 30ºC +/- 1ºC within a ventilated Plexiglas enclosure. Before formal testing,

baseline latencies for paw withdrawals evoked by radiant thermal stimulation were measured a minimum of three times/ paw, with at least 5 min elapsing between tests of a given paw. A light beam (Plantar Test 390, IITC, Woodland Hills, CA) was focused onto the plantar surface of the hind paw through the glass plate from below, and the latency from onset of the light to brisk withdrawal of the stimulated paw was measured. To prevent potential tissue damage, a cutoff time of 20 sec was imposed if no paw movement occurred. Withdrawal latencies for both the treated and untreated paw were measured at 3, 15, 30, 45, 60 and 120 min post-application of vehicle or CA, or menthol to one hindpaw.

Von Frey mechanical paw withdrawal threshold. Baseline mechanical withdrawal thresholds were assessed using an electronic von Frey filament (1601C, IITC) pressed against the plantar surface of one hindpaw. This device registered the force (g) at the moment that the hind paw was withdrawn away from the filament. Following application of CA or menthol or vehicle, mechanical paw withdrawal thresholds were measured at the same post-application times as noted above for thermal paw withdrawals.

Two-temperature preference test. The apparatus consisted of two adjacent thermoelectric surfaces (each 13.3" x 6.37"; AHP-1200DCP, Teca Thermoelectric, Chicago, IL) that could be independently heated or cooled to a pre-set temperature (-5 to >50ºC) that was maintained within +/-1.0°C. A Plexiglas box enclosed both plates, which were separated by a center partition with an opening in the middle to allow the rat to move freely between the two surfaces. Rats were habituated to the test arena with both plates set at 30°C. They were videotaped from above for 20 min, and the time the animal spent on each plate was recorded. Preference testing was done by setting one plate at 30°C and the other plate at a higher or lower temperature in 5°C increments, using a counterbalanced design. The menthol, or CA or vehicle was topically applied bilaterally as described above. The animal was placed onto one of the plates in a matched block design alternating initial rat position and temperature.

Data analysis. Thermal, mechanical, and cold plate paw withdrawal responses were normalized to baseline averages and subjected to one-way repeated measures analysis of variance (ANOVA) using SPSS 9.0 software (SPSS, Chicago IL) and InStat 3.05 (GraphPad Software, Inc., CA). Multiple comparisons were done post-hoc using Least Significant Difference (LSD) tests. A 95% confidence interval was used for all statistical comparisons and error reported is the standard error of the mean.

Results

CA application.

Thermal Paw Withdrawal Test. CA resulted in a significant, dose-dependent reduction in ispilateral thermal paw withdrawal latency. Fig. 1A shows mean withdrawal latencies of the injected paw *vs*. time relative to injection of vehicle or CA at each concentration tested. There was a dose-dependent reduction in latency, with the 20% CA concentration significantly different from vehicle and 5% CA treatments. The highest dose resulted in a mean reduction to 61.7% of pre-injection baseline by 30 min with partial recovery at 120 min. For the contralateral paw (Fig. 1B), there was an overall significant effect of treatment with the 20% group being significantly different from saline and 5% CA treatments.

Von Frey Paw Withdrawal Test. Mechanically-evoked withdrawal thresholds are plotted *vs.* time for the treated paw in Fig. 1C. At each CA concentration thresholds were significantly different from vehicle, but not from each other, indicating that a maximal reduction in withdrawal threshold (to 44.4% of baseline) was achieved at the lowest (5%) concentration of CA. Mean withdrawal thresholds for the contralateral paw (Fig. 1D) were not significantly affected at any CA concentration.

Menthol application.

Thermal Paw Withdrawal Test. The hindpaw receiving topical menthol exhibited a concentration-dependent increase in withdrawal latency (Fig. 2A). The 40%, 10% and 1% menthol treatment groups were significantly different from vehicles, and the 40% group was significantly different from all other concentrations (Fig. 2A, *: $p<0.01$, repeated-measures ANOVA). The 10% menthol group was not significantly different from 1% menthol ($p=0.07$), and the 0.1% and 0.01% groups did not differ significantly from vehicle. There was an apparent mirror image effect, in that for the contralateral hindpaw, the 40% menthol group was significantly different from all other concentrations (*: $p<0.01$) which were not significantly different from vehicle (Fig. 2B). Fig. 2C and D show data for the vehicle controls. For the treated hindpaw, there was a significant difference between vehicle groups (*: $p<0.01$), with 50% ethanol treatment resulting in a significant reduction in thresholds (Fig. 3C; *: $p<0.01$). For the contralateral hindpaw, there was no significant difference between ethanol concentrations, both of which were ineffective (Fig. 2D).

Von Frey Paw Withdrawal Test. For the ipsilateral (treated) hindpaw, the 0.1-10% menthol groups were not significantly different from vehicle (Fig. 3A). Only the 40% menthol group was significantly different from all

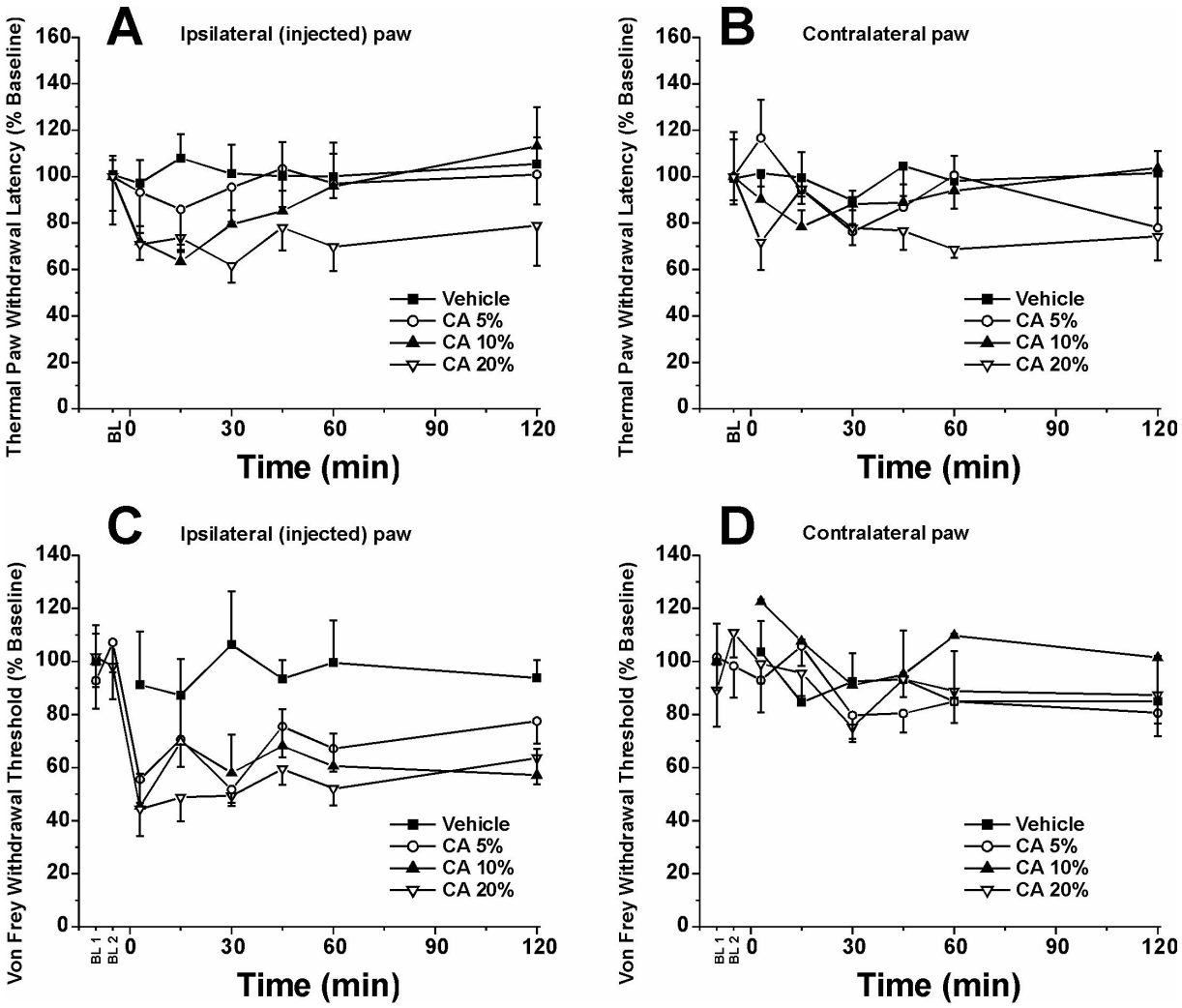

Fig. 1. A: Normalized mean thermal paw withdrawal latency vs. time following ipsilateral intraplantar injection of vehicle (control) or CA at each indicated concentration. There was a significant effect of CA concentration with the 20% CA group significantly different from vehicle and 5% CA groups ($p<0.05$ for both, n=8). BL: pre-injection baseline. B: As in A for paw contralateral to CA injection. There was a significant effect of CA concentration with the 20% group significantly different from vehicle and 5% groups ($p<0.05$ for both). C: As in A for von Frey mechanically-evoked withdrawal of the injected paw. There was a significant effect of CA concentration, with 5, 10 and 20% CA all different from vehicle ($p< 0.05$) but not from each other. D: As in C for contralateral paw. There was no significant effect of CA concentration ($p>0.5$).

other groups (*: $p<0.05$, repeated measures ANOVA) indicating allodynia. For the contralateral hindpaw, none of the menthol concentration groups were significantly different from the vehicles (Fig. 3B). Fig. 3C and D show data with vehicle controls. There was no significant difference between vehicle groups for the ipsilateral or contralateral hindpaws.

Two-temperature Preference Test. Naïve animals significantly avoided temperatures below 30°C and above 35°C (Fig. 4A). Untreated (naïve) rats exhibited no preference for either surface when they were both set at 30°C, indicating an absence of positional preference (Fig. 4A). Naïve rats did not show a preference for 35°C *vs.* 30°C, indicating that their preferred temperature lies within this range, or possibly 1-2°C higher or lower since we only tested temperature differentials of 5°C. When one of the plates was set to a temperature of 25°C and lower, or 40°C and higher, there was a temperature-dependent decrease in the percent time spent on the colder or hotter plate which was significantly different compared to time spent on the 30°C plate (Fig. 4A). Fig. 4B plots the mean number of times rats crossed between the two plates. The maximum number of crossings was observed when both plates were set at 30°C, and decreased when the non-neutral plate was set at higher or lower temperatures (Fig. 4B). The greatest decline in the number of crossings, as well as time spent on the non-neutral plate, was observed for the largest temperature differences, i.e. 0°C and 50°C *vs.* 30°C (Fig. 4A, B).

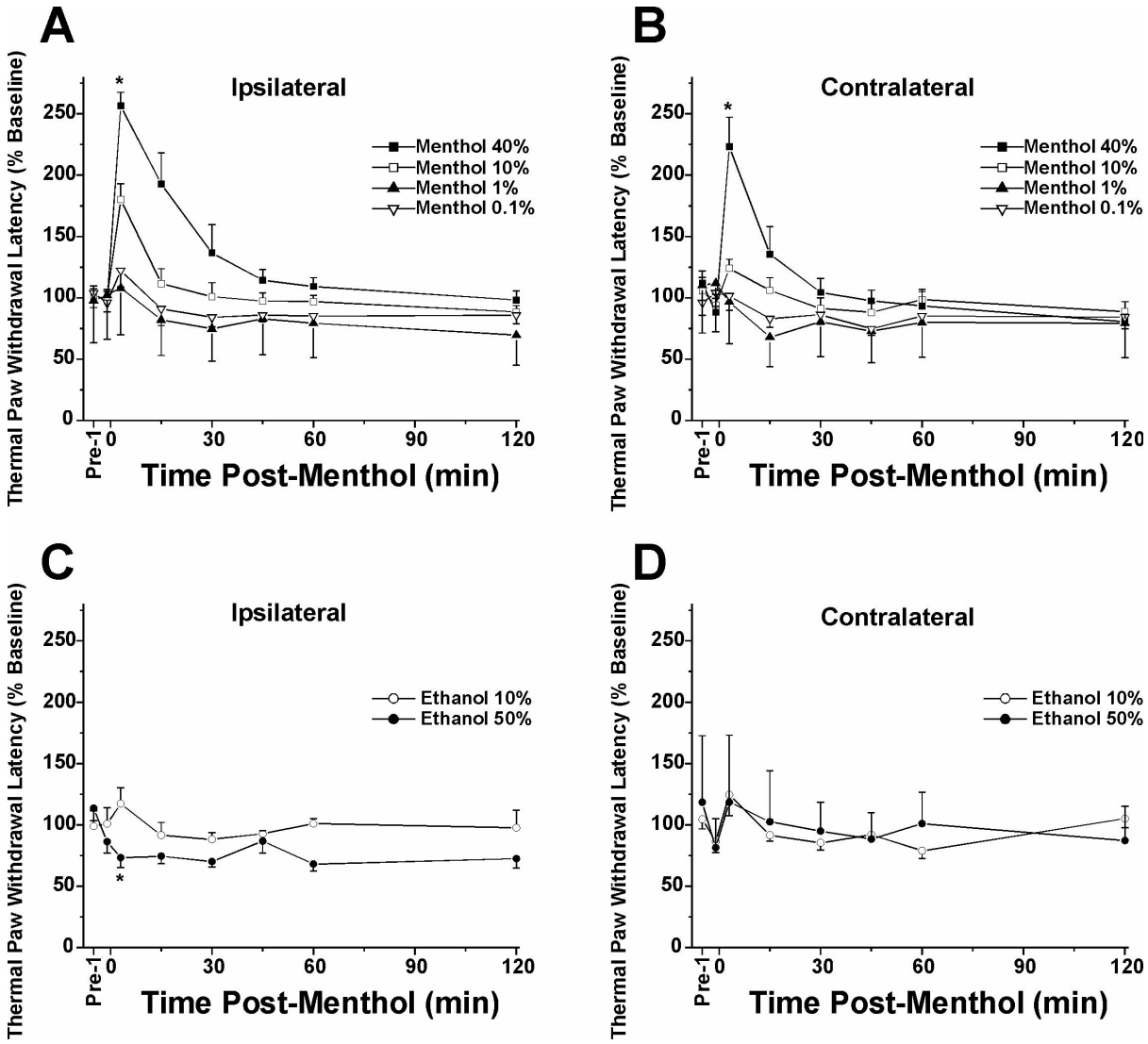

Fig. 2. A: Thermal paw withdrawal latency (Hargreaves) test: A: ipsilateral hindpaw. The hindpaw receiving topical menthol exhibited a concentration-dependent increase in withdrawal latency (analgesia). Groups of animals tested at concentrations of 40%, 10% and 1% menthol were significantly different from vehicles. Forty percent menthol was different from all other concentrations (*$p<0.01$, repeated-measures ANOVA), while 10% menthol was not different from 1% menthol ($p=0.07$). Data for 0.01% menthol are similar to 0.1% menthol group and omitted for clarity (n=8/group). B: Contralateral hindpaw. There was a weak mirror-image effect. The 40% menthol group was significantly different from all other concentrations (*$p<0.01$), which were not different from vehicle (0.01% menthol omitted). C: Vehicle controls: ipsilateral hindpaw. There was a significant difference between groups (*$p<0.01$). D: Vehicle controls: contralateral hindpaw. There was no significant difference between ethanol concentrations, both of which were ineffective.

For formal testing using menthol, one plate was set at 30°C and the other at 15°C or 20°C in a counterbalanced design. These temperature differentials were chosen because the degree of avoidance of both temperatures (20-30%; see Fig. 4A) was intermediate compared to warmer or colder temperatures, thus allowing for menthol-induced shifts in either direction (i.e., avoiding floor or ceiling effects). Menthol had a biphasic effect on temperature preference. In the 15°C *vs.* 30°C preference test (Fig. 5A), treatment with high menthol concentrations (10% and 40%; vertically-striped and open bars) resulted in rats spending a significantly ($p<0.05$) lower proportion of time on the 30ºC plate compared to vehicle controls (diagonally-hatched and dark gray bars), indicating cold hyposensitivity. At lower concentrations (0.01%-1%; horizontally-striped, light gray and stippled bars in Fig. 5A), rats spent significantly more time on the 30ºC plate ($p<0.05$), indicating cold hypersensitivity. At the lowest menthol concentration (0.01%) there was a significant decline in the number of plate crossings (9.7 ± 1.3) when compared to naïve or vehicle treated animals (16.7 ± 2.3 and 15.4 ±1.8,

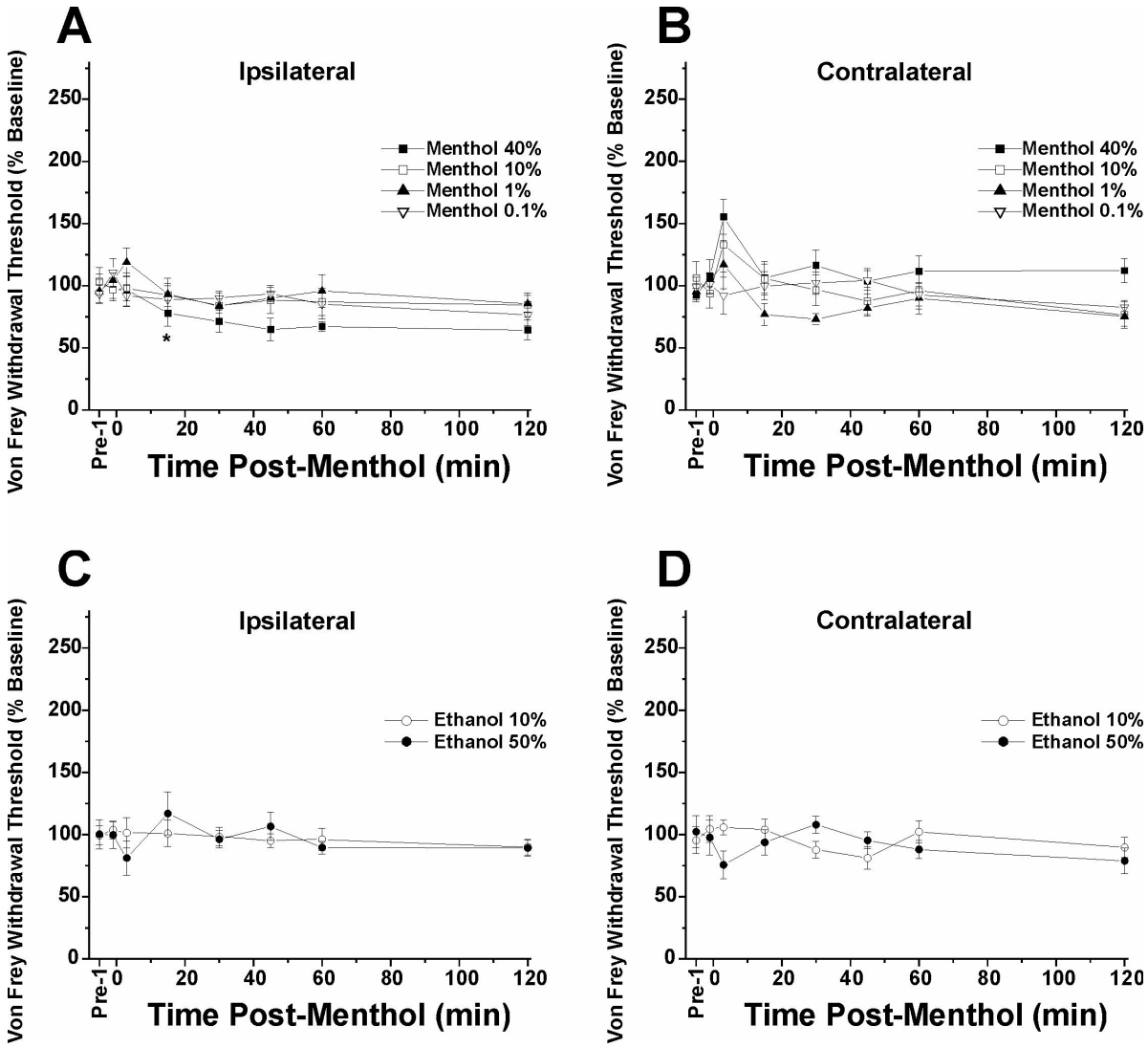

Fig. 3. A: von Frey paw withdrawal threshold: ipsilateral hindpaw. The 0.1-10% menthol groups were not significantly different from vehicle (10% ethanol). Only the 40% menthol group was significantly different from all other groups (*$p<0.05$, repeated measures ANOVA) indicating allodynia. Data for 0.01% menthol are similar to 0.1% menthol treated groups and omitted for clarity (n=8/group). B: Contralateral hindpaw. None of the menthol concentration groups were significantly different from the vehicles (0.01% menthol omitted). C: Vehicle controls: ipsilateral hindpaw. There was no significant difference between 10% +1% Tween-80 and 50% ethanol+5% Tween-80 vehicle groups. D: Vehicle controls: contralateral hindpaw. No significant difference between ethanol concentrations.

respectively, $p<0.05$ for both). There were no significant differences among control groups, with the 10% ethanol, 50% ethanol, and untreated naïve groups exhibiting preferences for the 30ºC plate of 79.9 ± 6.6%, 77 ± 7.0%, and 80.3 ± 6.0%, respectively.

In the 20ºC *vs.* 30ºC preference test (Fig. 5B), a similar biphasic effect was noted, with the highest menthol concentration (40%) resulting in significantly less time, and the lowest concentration (0.01%) resulting in significantly more time, spent on the 30ºC plate. At the lowest concentration (0.01%) of menthol, there was also a significant decline in the number of plate crossings (8.25± 0.8) when compared to naïve or vehicle treated animals (16.7 ± 2.3 and 15.4 ±1.8, respectively, $p<0.05$). At the highest menthol concentration tested (40%) there was a significant increase in the number of plate crossings compared to naïve or vehicle treated animals (18.4 ±1.8 *vs.* 12.2 ± 1.3 and 11.6 ±1.5, respectively, $p<0.05$). There were no significant differences among control groups, with the 10% ethanol, 50% ethanol, and untreated naïve groups exhibiting preferences for the 30ºC plate of 78.1 ± 8.4%, 76.1 ± 7.9%, and 71.0 ± 9.0%, respectively.

Discussion

The present data provide a comprehensive view of

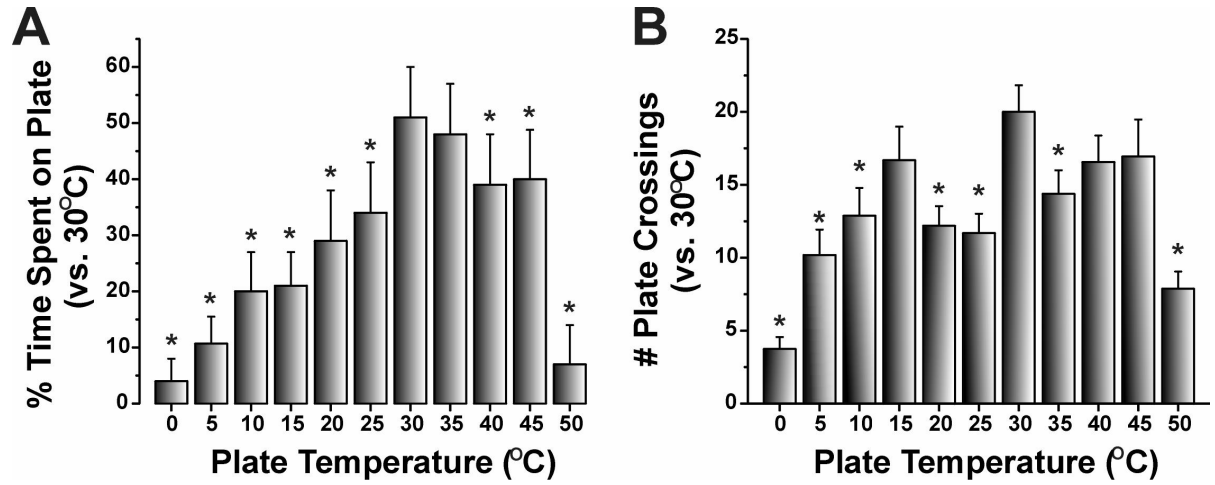

Fig. 4. Two-temperature preference test. Rats were placed on one of two adjacent thermoelectric plates whose temperatures could be set independently. One plate was set at 30°C and the other at a warmer or colder temperature in 5?C increments in a counterbalanced design. The rat was free to move from one surface to the other through an opening in a vertical barrier between the two plates. A: The graph plots the mean percentage of time naive rats spent on the warmer or colder plate relative to the thermoneutral (30°C) plate over a 20 min period. Rats significantly avoided temperatures <30?C and >35?C. *p<0.05, paired t-test. B: As in A for mean number of crossings between the thermoneutral (30?C) and warmer/ cooler plate over a 20 min period. Na?ve animals, *p<0.05, unpaired t-test, n=16/group for 5-45?C; n=8/group for 0?C and 50?C.

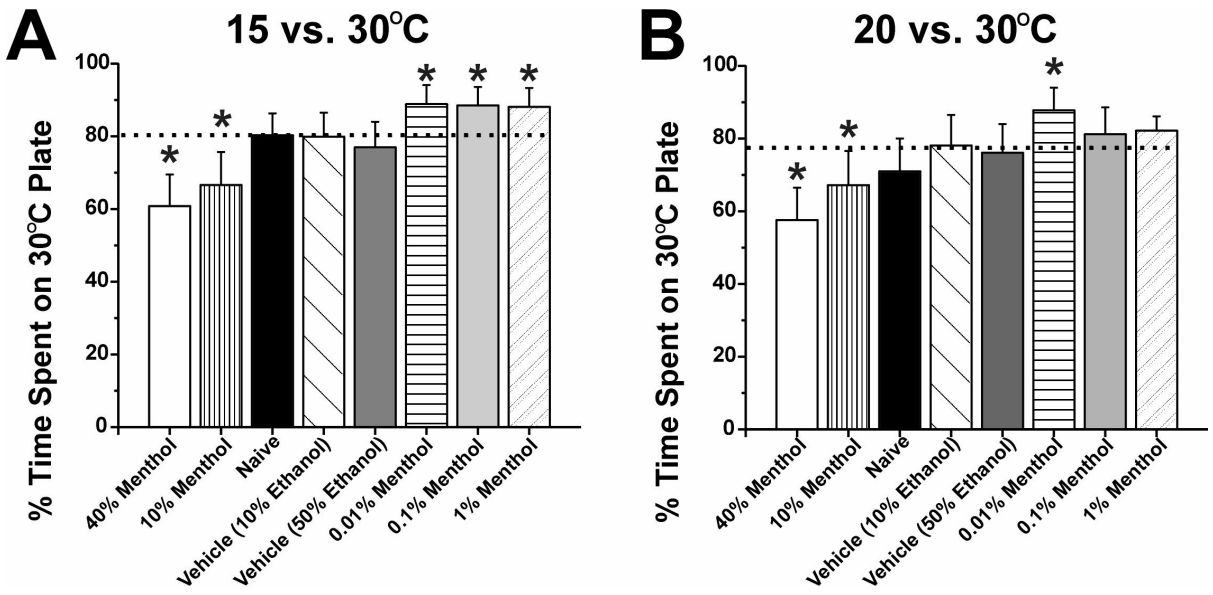

Fig. 5. Biphasic effects of menthol on thermal preference. A: Graph plots % time rat stood on 30 vs. 15°C plate. Horizontal dashed line indicates that naive and vehicle-treated rats avoided the colder plate ~80% of the time as a reference. At high (40%, 10%; open and vertically-striped bars) menthol concentrations rats spent significantly less time on the 30°C plate compared to vehicle controls (diagonally-hatched and dark gray bars) (*p<0.05, n=16/group), indicating cold hyposensitivity. At lower concentrations (0.01% and 1%; horizontally-striped, light gray and stippled bars), rats spent significantly more time on the 30?C plate (*p<0.05, n=16/group), indicating cold hypersensitivity. B: As in A for 20°C vs. 30°C preference test. Note significant cold hyposensitivity with 40% menthol and significant hypersensitivity at the lowest menthol concentration of 0.01%, (*p<0.05, n=16/group).

effects of intraplantar CA on thermal (hot and cold) and mechanical sensitivity. CA induced a dose-dependent heat hyperalgesia lasting >2 hr at the highest dose, mechanical allodynia, and cold hyperalgesia.

CA enhancement of heat sensitivity is consistent with previous studies. Topical application of CA (795 mM) to human forearm skin evoked burning pain and heat hyperalgesia [17]. Epilingual CA (16 mM) produced brief heat hyperalgesia [16]. CA enhanced responses of spinal [20] and Vc neurons to noxious heat [22]. These and the present findings are consistent with a role for TRPA1 in heat pain and heat hyperalgesia.

The dose-dependent increase in magnitude and duration of heat hyperalgesia induced by CA was similar

to that induced by intraplantar capsaicin (1-30 μg dose range) in rats using the same method [57]. Since TRPA1 is co-expressed in sensory neurons that express TRPV1 [15,58], heat hyperalgesia induced by CA might involve its activation of intradermal nociceptor nerve endings to engage an intracellular mechanism leading to enhanced heat sensitivity of TRPV1. Alternatively, CA may cause intradermal release of inflammatory mediators that lower the heat threshold of TRPV1 [59,60]. CA at the highest concentration may have also triggered central sensitization, leading to the observed reduction in withdrawal latency for the contralateral paw (Fig. 1B). Consistent with this, topical application of AITC (TRPA1 agonist) to the lateral hind limb significantly reduced the tail flick latency in rats in a manner dependent on the integrity of the rostral ventromedial medulla [61].

In humans, CA on forearm skin induced cold *hypoalgesia* [17] whereas epilingual CA or AITC briefly enhanced cold pain [16]. Lingual application of CA significantly enhanced cold-evoked responses of superficial Vc neurons in rats [22] but did not affect responses of lumbar spinal wide dynamic range (WDR)-type neurons to skin cooling [20]. These discrepancies regarding the effects of CA on cold pain perception and neuronal responses may partly involve the route of delivery and accessibility of CA to intradermal nociceptors. In our preliminary investigation [62] and in the present study, intradermal injection of CA allowed for a direct access to nociceptive nerve endings to result in significant cold hyperalgesia and enhancement of cold avoidance.

The prolonged enhancement of mechanosensitivity (i.e. allodynia) following CA (Fig. 1C) is consistent with previous studies showing a prolonged decrease in mechanical withdrawal threshold in mice following intraplantar injection of a TRPA1 agonist [30] and with allodynia induced in human skin by topical application of AITC [29]. A role for TRPA1 in mechanical allodynia is further supported by reports that TRPA1 antagonists attenuated inflammation- or nerve injury-induced decreases in mechanical paw withdrawal thresholds in mice [31,32] and decrease mechanically evoked responses in C fibers in mice [63]. However, these behavioral data are inconsistent with our electrophysiological data showing that neither CA nor AITC had any significant effect on the mechanical sensitivity of spinal WDR neurons [20]. Similarly, only 1 of 9 subjects experienced mechanical allodynia following application of 10% CA to forearm skin [17]. The mismatch between our behavioral observation of a CA-induced increase in mechanosensitivity and lack of CA effect on neuronal mechano-sensitivity [20] may involve the route of administration as noted earlier.

Our study has shown that menthol increased paw withdrawal latencies to noxious heat in a concentration-dependent manner, indicating an antinociceptive effect. The highest menthol concentration also significantly increased cold plate latencies, consistent with antinociception. However, lower menthol concentrations did not significantly affect nocifensive latencies in the cold plate test, indicating that menthol more effectively suppresses heat compared to cold pain. These effects are unlikely to be explained by a local anesthetic effect, since the highest menthol concentration increased mechanosensitivity (allodynia). Menthol had a biphasic effect on innocuous cold sensitivity, with high menthol concentrations reducing and low menthol concentrations enhancing avoidance of cooler surfaces.

Topical balms and other over-the-counter products for pain relief often contain menthol concentrations of 5% to 15% or even higher. The present data indicate that topical application of menthol in this concentration range is antinociceptive for heat pain, and also for cold pain at the highest menthol concentration. These findings are consistent with previous studies showing menthol suppression of heat pain [16,64-66] and capsaicin irritancy [67]. The mechanism of menthol's antinociceptive effect is not certain although many menthol-sensitive DRG [47] and Vc neurons [56] respond to capsaicin and other noxious stimuli and innocuous cooling can elicit nociceptive sensations [68]. Topical paw application of menthol has also been reported to reverse behavioral reflex sensitization to noxious heat and mechanical stimulation in the rat chronic constriction injury model of neuropathic pain [69]. These data suggest that menthol-sensitive primary afferent fibers can inhibit nociceptive pathways. A peripheral mechanism could involve menthol inhibition of nociceptors, possibly by blocking TRPA1 expressed in nociceptive nerve endings [70,71]. Another mechanism is menthol activation of cold receptors that centrally inhibit spinal nociceptive neurons. A third possibility is that menthol engages supra-segmental or supra-spinal circuits to result in descending inhibition of spinal nociceptive neurons.

Topical application of menthol elicits oral irritation [72-74] and cutaneous cold pain [75,76] and directly excites many cold-sensitive nociceptive Vc neurons [22,56]. Unilateral menthol induced a weaker mirror-image antinociceptive effect in the contralateral hindpaw (Fig. 2B), suggesting the involvement of hetero-segmentally-organized antinociceptive circuits that exerted a depressant effect on nociceptive neurons bilaterally. This

would be akin to counter-irritation, which was demonstrated in a human psychophysical study in which chemically-evoked irritation on one arm was suppressed by a stronger irritant stimulus delivered to the opposite side [77].

There was a small but significant reduction in paw withdrawal latency following the 50% ethanol vehicle (Fig. 2C) that might be attributed to ethanol sensitization of TRPV1 expressed in nociceptors responsive to noxious heating [78]. This effect might have slightly reduced the analgesic effect of high menthol concentrations (10% and 40%) that were dissolved in 50% ethanol.

The present data revealed a biphasic effect of menthol on innocuous cold sensitivity, with high menthol concentrations reducing and low menthol concentrations enhancing the avoidance of cold temperatures. We tested preference for a thermo-neutral (30ºC) surface *vs.* 15ºC and 20ºC surfaces, since the latter cold temperatures are avoided about 70-80% of the time by naïve rats (Fig. 5). Rats receiving high (10% and 40%) menthol concentrations avoided the 15ºC and 20ºC surfaces to a significantly lesser degree, indicating indifference to the colder surface that might reflect cold hypoalgesia. They also exhibited a high number of plate crossings, comparable to naïve animals tested with both plates set at 30ºC. We reasoned that when animals initially stood on the colder plate and perceived it to be aversive, they tended to subsequently avoid it thus resulting a fewer plate crossings. However, while there was a systematic decline in time spent on surfaces having progressively colder or hotter temperatures (Fig. 5A), the relationship of plate crossings to temperature difference was more variable (Fig. 5B) suggesting that plate crossings are a less sensitive measure of cold or heat aversion. The indifference to cold temperatures might be attributed to a peripheral desensitization of TRPM8 by high menthol concentrations [79] or a central inhibitory effect, as described above.

In contrast, low concentrations of menthol (0.01%-1%) significantly increased avoidance of the 15ºC and 20ºC surfaces and reduced the number of plate crossings. These results might reflect cold allodynia, or they may indicate an increase in sensitivity to innocuous cold that is aversive to the animal but not actually painful. It is interesting that the decrease in cold sensitivity observed in TRPM8 knockout mice appears to disappear below 10°C [8], a temperature that is often reported to be painful. This would be consistent with a role for TRPM8 in innocuous cold sensation but not pain. In any event, the increase in cold or cold pain sensitivity may involve menthol enhancement of thermal gating of TRPM8 expressed in cold receptors and/or nociceptors.

The opposing effects of 40% menthol in human (cold hyperalgesia) *vs.* rat (cold hyposensitivity) might be explained by allometric scaling and/or differences in dermal diffusion. We presently applied menthol to both ventral hindpaws, which constitutes a substantially larger percentage of the overall body surface area of a rat, as compared to the restricted region of volar forearm skin treated with menthol in the human studies [17,48,50,76]. Moreover, dermal absorption of chemicals, which is a function of the total area of application and concentration [80-83], is greater in the rat *vs.* human [84]. For example, permeation of L-menthol through the stratum corneum was four times higher in the hairless rat compared to human skin [85]. We speculate that the relative area of chemical stimulation and species differences in skin permeation explain why several-fold lower concentrations of menthol induced cold hypersensitivity in rats compared to the human studies.

The mechanism of cold transduction has been elusive, and despite the discovery of thermoTRPs it remains a complex issue [86-88]. The behavioral effects of topical menthol application seen in this study are consistent with effects on TRPM8. An important role for TRPM8 becomes apparent when this channel is missing. TRPM8 null mice exhibit a deficit in cold avoidance and lower incidence of cold-sensitive afferent fibers [8,41,42]. However, the authors cannot rule out the possibility that menthol may interact with other channels expressed in sensory neurons [71,89,90]. Recent observations suggest that cold sensation likely involves multiple channels, including potassium channels [88,91,92], in transducing and modulating the transmission of temperature information [66,88,93,94].

Conclusions

The thermosensitive TRP channels represent an important set of new targets for the development of analgesic drugs. Moreover, future biophysical studies of these fascinating ion channels will reveal the molecular mechanisms that have evolved to detect changes in environmental temperature, a function critical to the survival of all life forms. Overall, our data support the idea that TRPA1 and TRPM8 channels represent promising peripheral targets for pain modulation.

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ადამიანისა და ცხოველთა ფიზიოლოგია

თერმომგრძნობიარე გარდამავალ რეცეპტორულ პოტენციალთა არხების აგონისტების, დარიჩინის ალდეჰიდისა და მენთოლის მოქმედების ეფექტები შეხების, ტემპერატურისა და ტკივილის მგრძნობელობაზე

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(წარმოდგენილია აკადემიის წევრის ვ. ოკუჯავას მიერ)

არცთუ დიდი ხნის წინ აღმოჩენილ იქნა, რომ გარდამავალ რეცეპტორულ პოტენციალთა (გრპ-TRP) არხები მნიშვნელოვან როლს ასრულებენ თერმული, მექანიკური და ტკივილის შეგრძნებების აღმოცენებაში. ზოგიერთი TRP არხი ავლენს მგრძნობელობას მაღალი ან დაბალი ტემპერატურის მიმართ, ისევე როგორც რიგი ქიმიური ლიგანდების მიმართ, რომლებიც აღძრავენ თერმულ ან ტკივილის შეგრძნებას. მათ მიეკუთვნება მენთოლი, რომელსაც შეიცავს პიტნა, დარიჩინის ალდეჰიდი, მდოგვის ზეთი, მიხაკის ზეთი, კაპსაიცინი (შეიცავს ცხარე წიწაკა) და სხვა.

დარიჩინის ალდეჰიდი (ДА) წარმოადგენს ცხარე, პიკანტურ ქიმიურ ნივთიერებას, რომელიც მოქმედებს როგორც TRPA1 არხის აგონისტი. ეს რეცეპტორული არხი ასევე აქტივდება სიცივის მოქმედებით, 18°C-ზე დაბალი ტემპერატურით. ადამიანის კანზე ზემოქმედებისას და იწვევს სითბური ტკივილისადმი მგრძნობელობის გაზრდას (ჰიპერალგეზია) და მის მოქმედებამდე ნეიტრალურ მექანიკურ გამღიზიანებელზე ტკივილის შეგრძნებას (მექანიკური ალოდინია). TRPA1 არხი აგრეთვე ჩართული უნდა იყოს გაციებით გამოწვეულ ტკივილის აღქმაში (ნოციცეფცია), მაგრამ მისი როლი ამ პროცესში ნათლად გამოკვეთილი არ არის.

მენთოლი მიიღება პიტნის ოჯახის მცენარეების ფოთლებიდან და მსგავსად ДА-სა გამოიყენება კვებისა და ფარმაცევტულ მრეწველობაში. იგი ასევე სასარგებლო უნდა იყოს ტკივილის გასაყუჩებლად. მენთოლი ზრდის სიცივის შეგრძნებას სიცივისადმი მგრძნობიარე TRPM8 არხთან ურთიერთქმედებისას, მაგრამ მისი მოქმედება ტკივილთან დაკავშირებით ჯერ კიდევ დაუდგენელია. ჩვენ გამოვიყენეთ ქცევითი მეთოდები, რათა შეგვესწავლა დარიჩინის ალდეჰიდისა და მენთოლის მოქმედების თავისებურებანი თერმულ (სითბო და სიცივე), არამტკივნეულ სიცივესა და მექანიკურ მგრძნობელობაზე ვირთაგვებში.

მიღებულმა შედეგებმა გვიჩვენეს, რომ ДА-ის (5-20%) უკანა თათში ტერფქვეშა უნილატერალური ინიექცია იწვევს იპსილატერალური თათის მოცილების რეფლექსის ფარული პერიოდის სარწმუნო, დოზა-დამოკიდებულ შემცირებას სითბური ტკივილის სტიმულებზე, ანუ ჰიპერალგეზიას. იგი ასევე ამცირებს თათის მექანიკური გაღიზიანების ზღურბლს, ე.ი. იწვევს მექანიკურ ალოდინიას. ДА-ის ბილატერალურმა ინფექციამ გამოავლინა ჰიპერალგეზია სიცივეზე (ცივი ფირფიტის ტესტი) და არამტკივნეულ სიცივეზე განრიდება (თერმული უპირატესობის ტესტი).

ДА-სგან განსხვავებით, მენთოლი დოზა-დამოკიდებულად ზრდის თათის მოცილების რეფლექსის ლატენტობას სითბურ ტკივილზე, ე.ი. გააჩნია ანტინოციცეპტური ეფექტი. ჩვენი მონაცემებით იგი არ უნდა მოქმედებდეს მექანორეცეპტორებზე, თუმცა მაღალ კონცენტრაციაზე (40%) გააჩნია სუსტი ალოდინიური ეფექტი, რაც მიუთითებს მის მცირე ლოკალურ ანესთეზიურ უნარზე. სიცივისადმი განრიდების ტესტმა

გამოავლინა მენთოლის ბიფაზური მოქმედება. მაღალ კონცენტრაციაზე იგი ამცირებს სიცოცხლე განრიდებას, მაშინ როცა დაბალ კონცენტრაციაზე სარწმუნოდ ზრდის ამ უკანასკნელ ეფექტს.

მიღებული შედეგები ადასტურებენ მოსაზრებას, რომ TRPA1 და TRPM8 არხები წარმოადგენენ იმედის მომცემ პერიფერიულ სამიზნეებს ტკივილის მოდულაციისთვის ახალი ტიპის ანალგეზიური პრეპარატების შექმნის თვალსაზრისით.

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