Central Mechanisms of Menthol-Induced Analgesia

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Received May 22, 2012; accepted August 29, 2012

ABSTRACT

Menthol is one of the most commonly used chemicals in our daily life, not only because of its fresh flavor and cooling feeling but also because of its medical benefit. Previous studies have suggested that menthol produces analgesic action in acute and neuropathic pain through peripheral mechanisms. However, the central actions and mechanisms of menthol remain unclear. Here, we report that menthol has direct effects on the spinal cord. Menthol decreased both ipsilateral and contralateral pain hypersensitivity induced by complete Freund’s adjuvant in a dose-dependent manner. Menthol also reduced both first and second phases of formalin-induced spontaneous nocifensive behavior. We then identified the potential central mechanisms underlying the analgesic effect of menthol. In cultured dorsal horn neurons, menthol induced inward and outward currents in a dose-dependent manner. The menthol-activated current was mediated by Cl− and blocked by bicuculline, suggesting that menthol activates γ-aminobutyric acid type A receptors. In addition, menthol blocked voltage-gated sodium channels and voltage-gated calcium channels in a voltage-, state-, and use-dependent manner. Furthermore, menthol reduced repetitive firing and action potential amplitude, decreased neuronal excitability, and blocked spontaneous synaptic transmission of cultured superficial dorsal horn neurons. Liquid chromatography/tandem mass spectrometry analysis of brain menthol levels indicated that menthol was rapidly concentrated in the brain when administered systemically. Our results indicate that menthol produces its central analgesic action on inflammatory pain probably via the blockage of voltage-gated Na+ and Ca2+ channels. These data provide molecular and cellular mechanisms by which menthol decreases neuronal excitability, therefore contributing to menthol-induced central analgesia.

Introduction

Menthol is an organic compound naturally occurring in peppermint oil from mint plants. It is well known for its cooling sensation and is widely used in a number of products including toothpaste, cough drops, mouthwash, chewing gum, and topical analgesics. Menthol has complex peripheral effects. Topical application of low to moderate concentrations of menthol reduces the irritancy of capsaicin, heat hypersensitivity, sprains, and headaches (Green and McAuliffe, 2000; Gentry et al., 2012). The peripheral mechanism of menthol-induced cold hyperalgesia is thought to be induced by activating TRPM8 and TRPA1 channels (Wasner et al., 2008). TRPM8 and TRPA1 have been identified as the cold- and menthol-sensitive channels (McKemy et al., 2002; Peier et al., 2002; Karashima et al., 2007; Bianchi et al., 2012). The peripheral mechanism of menthol-induced cold hyperalgesia is thought to be induced by activating TRPM8 and TRPA1 channels (Gentry et al., 2010; Knowlton et al., 2010). Although the peripheral mechanism of the antinociceptive effect of menthol is not certain, it has been suggested that menthol blocks voltage-gated calcium currents in cultured sensory neurons (Swandulla et al., 1987). A previous study demonstrated that menthol inhibits voltage-gated sodium channel 1.2 currents heterologously expressed in human embryonic kidney 293 cells (Haeseler et al., 2002). A recent report also showed that menthol blocks voltage-gated sodium channels in dorsal root ganglion neurons (Gaudio et al., 2012). Menthol-induced cold sensitivity has been studied extensively. In contrast, only a few reports have directly assessed producing analgesic effects in patients with neuropathic pain (Wasner et al., 2008). TRPM8 and TRPA1 have been identified as the cold- and menthol-sensitive channels (McKemy et al., 2002; Peier et al., 2002; Karashima et al., 2007; Bianchi et al., 2012). The peripheral mechanism of menthol-induced cold hyperalgesia is thought to be induced by activating TRPM8 and TRPA1 channels (Gentry et al., 2010; Knowlton et al., 2010). Although the peripheral mechanism of the antinociceptive effect of menthol is not certain, it has been suggested that menthol blocks voltage-gated calcium currents in cultured sensory neurons (Swandulla et al., 1987). A previous study demonstrated that menthol inhibits voltage-gated sodium channel 1.2 currents heterologously expressed in human embryonic kidney 293 cells (Haeseler et al., 2002). A recent report also showed that menthol blocks voltage-gated sodium channels in dorsal root ganglion neurons (Gaudio et al., 2012). Menthol-induced cold sensitivity has been studied extensively. In contrast, only a few reports have directly assessed

ABBREVIATIONS: TRP, transient receptor potential; CFA, complete Freund’s adjuvant; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; EPSC, excitatory postsynaptic current; GABA, γ-aminobutyric acid; HPLC, high-performance liquid chromatography; Ito, calcium current; I Na, sodium current; I-V, current-voltage; LC/MS/MS, liquid chromatography/tandem mass spectrometry; TTX, tetrodotoxin.

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the analgesic properties of menthol. Systemic menthol reduces acute pain induced by heat and acetic acid (Galeotti et al., 2002). Intrathecal application of menthol decreases mechanical allodynia and thermal hyperalgesia in animals with chronic constriction injury (Proudfoot et al., 2006; Su et al., 2011). The mechanism underlying menthol-induced analgesia is still controversial. It has been suggested that the analgesic effect of menthol is mediated by the activation of TRPM8 in the central terminals of sensory neurons (Proudfoot et al., 2006). However, the role of TRPM8 in mechanical and thermal hypersensitivity has not been established (Colburn et al., 2007; Su et al., 2011). Whether the analgesic effect of menthol is restricted to these particular pain conditions or menthol is a nonselective analgesic for pain hypersensitivity is an open question.

Here, we examined the effect of menthol on pain hypersensitivity induced by inflammation. We demonstrated that menthol attenuates complete Freund’s adjuvant (CFA)-induced primary and secondary pain hypersensitivity and decreases formalin-induced first and second phases of spontaneous nociceptive behavior. We further identified the central mechanisms of menthol-induced analgesia. Our data indicate that menthol directly activates γ-aminobutyric acid type A (GABA\(_A\)) receptors and blocks voltage-gated sodium and calcium channels in dorsal horn neurons. The latter may account for the central spinal mechanisms of menthol-induced analgesic action.

Materials and Methods

All behavioral tests were performed by using 8- to 10-week-old CD-1 male mice purchased from Charles River Laboratories, Inc. (Wilmington, MA). Experiments were done in accordance with the guidelines of the National Institutes of Health (Bethesda, MD) and approved by the Animal Care and Use Committee of Drexel University College of Medicine. Mice were housed in 12-h light/dark cycles. All experiments were performed with the experimenter blind to the drug treatment.

CFA-Induced Mechanical and Thermal Hypersensitivity

CFA-induced inflammatory pain was measured as described previously (Gao et al., 2010). Twenty microliters of 50% CFA was injected subcutaneously into the plantar surface of the right hind paw. Mechanical sensitivity was measured by using a series of von Frey filaments (North Coast Medical, Inc., San Jose, CA) as described previously (Hu et al., 2006). The smallest monofilament that evoked paw withdrawal responses on three of five trials was taken as the mechanical threshold. Thermal sensitivity was measured by using the Hargreaves’ method as described previously (Hu et al., 2006). The smallest monofilament that evoked paw withdrawal responses on three of five trials was taken as the cutoff to prevent potential injury. The latencies were set to approximately 10 s with a maximum of 20 s as the cutoff to prevent potential injury. The latencies were averaged over three trials separated by 15-min intervals.

Formalin-Induced Spontaneous Nociceptive Behavior

The formalin test was performed as described previously (Hu et al., 2006). Mice were habituated for 1 h in a transparent Plexiglas test box (5 × 5 × 10 inches) before any injections. Mice received intraperitoneal injections of 100 mg/kg menthol 15 min before injection with formalin. Twenty microliters of 2% formalin solution was injected subcutaneously into the plantar surface of the right hind paw, and the mouse was returned to the test box immediately. The total time spent in spontaneous nociceptive behavior (licking and lifting of the injected paw) was recorded in 5-min intervals for 1 h.

Cell Culture

Primary cultures of spinal cord superficial dorsal horn neurons were prepared from 2- to 4-day-old CD-1 mouse pups as described previously (Hu et al., 2002). Cultures were used for 1 to 2 days for voltage-gated sodium and calcium current recordings, 3 to 4 days for menthol-induced current recordings, and 5 to 7 days for action potential recordings.

Electrophysiological Recording

Standard whole-cell recordings were made at room temperature by using an EPC 10 amplifier and PatchMaster software (HEKA/Pfalz, Lambrecht, Germany) as described previously (Hu et al., 2007). Electrode resistances were 3 to 5 MΩ with series resistances of 6 to 10 MΩ, which were compensated by >60%. Membrane potentials were corrected for the liquid junction potential. All neurons included in this study had resting membrane potentials ≤ −45 mV, stable input resistance, and leak currents < −80 pA (at −80 mV), which were not subtracted on line.

Records of menthol, Na\(^+\), and Ca\(^{2+}\) currents were performed under voltage-clamp configurations. For menthol-activated current recordings, the membrane voltage was held at −65 mV; the bath solution was Tyrode’s solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM HEPES, and 10 mM glucose. The electrode solution contained 140 mM CsCl (or CsMeSO\(_4\)), 2 mM MgCl\(_2\), 1 mM EGTA, 10 mM HEPES, 3 mM Na\(_2\)ATP, and 0.3 mM Na\(_2\)GTP, pH 7.4. For Na\(^+\) current recordings, the membrane voltage was held at −80 mV, and the extracellular solution contained 105 mM NaCl, 5 mM KCl, 10 mM CsCl, 20 mM tetraethylammonium-Cl, 1 mM MgCl\(_2\), 200 μM CdCl\(_2\), 1 mM CaCl\(_2\), 5 mM HEPES, and 10 mM glucose. The electrode solution contained 140 mM CsMeSO\(_4\), 2 mM MgCl\(_2\), 1 mM EGTA, 10 mM HEPES, 3 mM Na\(_2\)ATP, and 0.3 mM Na\(_2\)GTP, pH 7.4. For Ca\(^{2+}\) current recordings, the membrane voltage was held at −70 mV, the same intracellular and extracellular solutions were used except that CdCl\(_2\) was omitted, and 500 mM tetrodoxin (TTX) and 6 mM BaCl\(_2\) were added in the extracellular solution.

To determine the current-voltage (I-V) relationship, steady-state activation and inactivation, and use-dependent blockade of sodium and calcium channels, the membrane potential was held at −80 mV (I\(_{Na}\)) or −70 mV (I\(_{Ca}\)). For calcium current activation, voltage steps of 250 ms were applied at 10-s intervals in +10-mV increments from −30 to +30 mV. For sodium current activation, a series of voltage steps of 50 ms was applied at 5-s intervals in +10-mV increments from −50 to +60 mV. To determine the voltage dependence of activation of Na\(^+\) or Ca\(^{2+}\) channels, conditioning prepulses ranging from −100 to 10 mV (I\(_{Na}\)) to or 30 mV (I\(_{Ca}\)) were applied in 10-mV increments for 100 ms (I\(_{Na}\)) or 1 s (I\(_{Ca}\)), followed by a step to −20 mV for 25 ms (I\(_{Na}\)) or 10 mV for 200 ms (I\(_{Ca}\)). To determine use-dependent blockade of sodium and calcium channels, a series of 25-ms pulses to −20 mV (I\(_{Na}\)) or 50-ms pulses to 10 mV (I\(_{Ca}\)) were applied at 20 Hz (I\(_{Na}\)) or 10 Hz (I\(_{Ca}\)). For current-clamp recordings and excitatory postsynaptic current (EPSC) recordings, the intracellular solution contained 135 mM KMeSO\(_4\), 5 mM KCl, 2 mM MgCl\(_2\), 1 mM EGTA, 10 mM HEPES, 3 mM Na\(_2\)ATP, and 0.3 mM Na\(_2\)GTP, pH 7.4. The bath solution was Tyrode’s solution.

Determination of the Concentration of Menthol in the Brain

Sample and Calibrator Preparation. Three groups of mice were administered 100 mg/kg i.p. menthol. Whole brain tissues were collected at 5, 30, and 60 min after menthol administration. Each brain sample was weighed, homogenized with two volumes of Milli-Q water by using a dounce homogenizer, and sonicated by using a Sonifier 250 (Branson Ultrasonics Corporation, Danbury, CT). Menthol stock solution was prepared at 25 mg/ml in DMSO. The standard calibrators were prepared in control brain homogenates from
this stock containing 0, 0.006, 0.024, 0.10, 0.39, 1.56, 6.25, and 25 μg/ml menthol.

**Extraction of Menthol and Preparation of Dimethylglycine Ester of Menthol.** The calibrators and brain homogenate samples from mice treated with menthol were mixed with three volumes of hexane containing 100 ng/ml of cyclohexanol (internal standard) by using a vortex. The extracts were then centrifuged at 10,000g for 10 min. Aliquots (300 μl) of the resulting supernatants were mixed with 200 μg of sodium sulfate (anhydrous) for 2 h. The dehydrated hexane extracts (100 μl) were transferred into individual glass vials. Derivatization of menthol with dimethylglycine was made before LC/MS/MS analysis by using a modified method described previously (Jiang et al., 2007). In brief, 30 μl of dimethylglycine hydrochloride (0.5 M) and N,N-dimethylaminopyridine (2 M) in CHCl3, and 30 μl of N-(3-dimethylaminopropyl)N'-ethylcarbodiimide hydrochloride (1 M) were added into the individual samples. The reaction was carried out at 37°C overnight. The resulting solution was evaporated to dryness under nitrogen stream. The residue was reconstituted with 400 μl of 0.1% acetic acid in water/methanol/acetonitrile (100:225:75, v/v/v) and subjected to LC/MS/MS analysis.

**Quantification of Menthol in Brain Tissue Using HPLC Mass Spectrometry.** LC/MS/MS analysis was performed on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems/MD Sciex, Foster City, CA) equipped with a TurbolonSpray source. Chromatographic separation was performed on a Shimazu HPLC system (Shimadzu, Piscataway, NJ) with a Symmetry C18 (3.5 μm, 4.6 × 75 mm) column (Waters, Milford, MA). Mobile phase A was 0.1% acetic acid in water, and mobile phase B was methanol/acetonitrile (75:25, v/v). Mobile phase was delivered at an initial condition of 100% A and a flow rate of 0.5 ml/min with a linear gradient to 20% B in 2 min, then increased to 90% B in 5 min. The column was re-equilibrated with 100% A after washing. Under these conditions, the retention time was 6.3 min for menthol ester and 3.9 min for cyclohexanol ester. The injection volume was 5 μl. The transition is m/z 242 → 104 for menthol ester and m/z 186 → 104 for cyclohexanol ester. The instrument was operated in positive-ion mode, with an ion spray voltage of 4500 V. The curtain gas, ion source temperature, and collision energy were set at 12 psi, 250°C, and 20, respectively.

**Chemicals and Reagents**

DL-Menthol and 6-cyano-7-nitroquinoxaline-2,3-dione were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide as stock solutions. All of these solutions were further diluted to final concentrations in bath solution (for in vitro use). Bicuculline, TTX, and tetraethylammonium chloride were purchased from Sigma-Aldrich and used for derivatization of menthol. All other reagents were HPLC grade and purchased from Sigma-Aldrich.

**Data Analysis**

Off-line evaluation was done by using PatchMaster (HEKA Elektronik) and Origin 8.1 (OriginLab Corp, Northampton, MA). Data were expressed as original traces or means ± S.E.M. For the I-V curves, peak currents at different potentials were normalized to the maximal current (I/Imax). For the activation curves, the peak current was converted to conductance (G) by the formula $G = I/V_m - V_{rev}$, where $V_m$ is the membrane voltage of depolarization pulses and $V_{rev}$ is the reversal potential of the current derived from I-V curves. The voltage dependence of activation and inactivation was fitted with the Boltzmann function: $G/G_{max} = 1/(1 + \exp[(V_{1/2} - V/k)],$ where $G/G_{max}$ is the normalized conductance, $V_{1/2}$ is the potential of half-maximum channel activation, and $k$ is the slope factor. For inactivation, $I/Imax = 1/(1 + \exp[(V_{1/2} - V/k)]$ was used, where $I/Imax$ is the normalized current. For the use-dependent block, inhibition of current amplitudes at each voltage step was calculated as percentage of inhibition $= 100 - 100 \times (I/Imax),$ where $I$ is the value of the current in the presence of menthol, and $I_{max}$ is the value of current in the absence of menthol. Time constants for Ca²⁺ current decay were obtained by using a single exponential function: $y = A \times \exp(-t/T_1),$ where $A$ is the current amplitude, $t$ is time (milliseconds), and $T_1$ is the time constant. For the concentration-dependent curve, the value of the current in the presence of menthol normalized to the current in the absence of menthol was used to calculate the potency of the menthol for blocking the channels. The half-maximal inhibitory concentration (IC50) in the various experimental conditions was determined by fitting the concentration-response curves with the Hill equation: $I/I_0 = 1/(1 + (IC_{50}/C)^n),$ where $I_0$ is the maximum peak current amplitude, $C$ is the menthol concentration, and $n$ is the Hill coefficient. Treatment effects were statistically analyzed with one-way or two-way analysis of variance. Data are presented as mean ± S.E.M. When analysis of variance showed a significant difference, pairwise comparisons between means were tested by the post hoc Bonferroni method. Paired or two-sample $t$ tests were used when comparisons were restricted to two means. Error probabilities of $P < 0.05$ were considered statistically significant. The statistical software Origin 8.1 (OriginLab Corp.) was used to perform fitting of the concentration-response curves and all statistical analyses.

**Results**

**Menthol Reduces CFA-Induced Thermal and Mechanical Hypersensitivity.** To examine the effect of menthol on inflammatory pain, we first tested the effect of menthol in a CFA-induced pain model in mice. Injection of 20 μl of 50% CFA into the hind paw of a mouse produced thermal hyperalgesia in the ipsilateral paw, which recovered completely after 3 weeks (Fig. 1A). However, CFA did not induce thermal hypersensitivity in the contralateral paw. Intraperitoneal administration of menthol (50 and 100 mg/kg) significantly decreased thermal hyperalgesia in a time- and dose-dependent manner with the maximal effect occurring after 30 min (Fig. 1B).

CFA-treated mice fully developed robust mechanical allodynia in the ipsilateral paw 3 h after CFA injection and in the contralateral paw 3 days after CFA injection. CFA-induced mechanical allodynia lasted for 4 weeks (data not shown). Menthol at 100 mg/kg i.p. reduced mechanical allodynia in both ipsilateral and contralateral paws when measured at 96 h after CFA injection (Fig. 1C). To examine its direct effect at the spinal level, menthol was administered intrathecally in 5 μl at a concentration of 50 mM (250 nmol) and produced a marked analgesic effect in both ipsilateral and contralateral paws (Fig. 1D). These results suggest that menthol relieves CFA-induced inflammatory pain and has a direct action in the spinal cord.

**Menthol Reduces Formalin-Induced Spontaneous Nociception.** The formalin test is a useful and reliable model of nociception. It allows for an assessment of analgesic action and provides the ability to distinguish the site of action of analgesics (Hunskaar and Hole, 1987; Shibata et al., 1989). Formalin induces spontaneous biphasic nociceptive behavior. The first phase is caused by acute stimulation of nociceptors. The second phase of nociception is thought to be
involved in central sensitization of dorsal horn neurons (Coderre and Melzack, 1992). Injection of 2% formalin subcutaneously in the hind paw of a mouse resulted in intense spontaneous licking or lifting of the injected paw with a classic biphasic response. A single intraperitoneal injection of 100 mg/kg menthol significantly decreased both the first and second phases of formalin-induced nociception (Fig. 2). This feature of blocking both phases is similar to that observed with centrally acting drugs, such as narcotics, further suggesting that menthol may have a direct central action.

Concentration of Free Menthol in the Brain after a Single Intraperitoneal Administration. To further confirm that menthol has a direct central action, free menthol concentrations in brain tissues were analyzed by LC/MS/MS. Mice received a single intraperitoneal administration of menthol at 100 mg/kg, the same dose used for the assessment of behavioral tests. Whole brain samples were obtained at 5, 30, and 60 min after intraperitoneal menthol injection. Menthol was rapidly absorbed, and a high concentration of menthol (54.6 μg/g) was detected in brain tissue at 5 min after a 100 mg/kg i.p. injection. Its concentration was largely decreased to 12.1 μg/g at 30 min, but remained detectable after 60 min (Fig. 3). This result indicates that menthol has great access to the central nervous system.

**Menthol Activates GABA<sub>A</sub> Receptors in Spinal Dorsal Horn Neurons.** As demonstrated above, menthol has a direct central action and can cross the blood-brain barrier when administered systemically. We were interested in exploring the central mechanisms of menthol-induced analgesia. It has been shown that menthol activates GABA<sub>A</sub> receptors in hippocampal neurons (Zhang et al., 2008). We hypothesized that the central mechanism of menthol-induced analgesic effect may be mediated by the activation of GABA<sub>A</sub> receptors in dorsal horn neurons. To test this hypothesis, we performed patch-clamp recordings in cultured dorsal horn neurons. Bath application of 2 mM menthol induced both inward and outward currents with varying amplitudes in most neurons tested (45/48 neurons). The effects

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**Fig. 1.** Menthol reverses CFA-induced mechanical allodynia and thermal hyperalgesia. A, time course of CFA-induced thermal hyperalgesia (n = 9). *, P < 0.05 compared with baseline. B, time-dependent effect of menthol on CFA-induced thermal hyperalgesia measured 24 h after CFA injection (n = 6–7). *, P < 0.05 compared with vehicle control. C, effects of menthol on CFA-induced mechanical allodynia measured 96 h after CFA injection by intraperitoneal (IP) injection (n = 6–7) or intrathecal (IT) injection (n = 6–8). *, P < 0.05 compared with pre-menthol. Data are presented as mean ± S.E.M.
of menthol were readily reversible upon drug washout (data not shown). We then examined the concentration-response relationships for menthol. Menthol evoked inward currents in a concentration-dependent manner with an EC$_{50}$ value of 1.08 ± 0.04 mM ($n$ = 4; Fig. 4A).

To determine whether menthol-induced current is mediated by the activation of GABA$_{A}$ receptors, we pretreated neurons with bicuculline, a GABA$_{A}$ antagonist. Menthol-induced currents were dramatically attenuated in a concentration-dependent manner (Fig. 4B). To confirm that menthol-induced current was mediated by Cl$^{-}$ channels, we performed voltage-clamp recordings in cultured dorsal horn neurons. Replacement of CsCl in the internal solution with CsMeSO$_{4}$ resulted in a significant shift of the reversal potential (Fig. 4, C and D). These results indicated that the menthol-activated currents were mediated by GABA$_{A}$ channels.

**Menthol Blocks Voltage-Gated Sodium Channels in Dorsal Horn Neurons.** As demonstrated above, the activation of GABA$_{A}$ receptors by menthol required a relatively high concentration. An effective dose (100 mg/kg) of menthol may not be sufficient to reach the concentration required for GABA$_{A}$ activation in the spinal cord. Therefore, other mechanisms may underlie the central analgesic action of menthol. Previous studies indicated that the peripheral mechanism of menthol-induced analgesia is mediated by blocking voltage-gated sodium channels in dorsal root ganglion neurons (Gaudioso et al., 2012). We hypothesized that menthol may block sodium channels in dorsal horn neurons. We performed Na$^{+}$ current recordings with K$^{+}$ and Ca$^{2+}$ currents eliminated pharmacologically (see Materials and Methods). When neurons were held at −80 mV, 10 steps of depolarization from −50 to +60 mV evoked fast activating, fast inactivating voltage-dependent Na$^{+}$ currents, which were completely blocked by 500 nM TTX (data not shown). Sodium currents were activated at approximately −50 mV and reached their peak at approximately −20 mV (Fig. 5, A and B). Bath application of menthol shifted the I-V curve upward and blocked the peak of Na$^{+}$ currents with an IC$_{50}$ of 297.1 ± 64.0 µM and a Hill coefficient of 1.6 ± 0.1 (Fig. 5, B-D). These results show that menthol blocks Na$^{+}$ channels in dorsal horn neurons in a concentration-dependent manner.

**Mechanism of Blocking Na$^{+}$ Channels by Menthol.** To explore the mechanism of menthol’s effect on Na$^{+}$ channels, we first examined a state-dependent block of sodium channels. Neurons were held at −100 or −55 mV and stimulated by a brief test pulse of −20 mV once every 10 s. Bath application of 300 µM menthol blocked approximately 17% of peak Na$^{+}$ current with an IC$_{50}$ of 1936 ± 269 µM when neurons were held at −100 mV (resting state). However, menthol blocked approximately 73% of peak Na$^{+}$ current with an IC$_{50}$ of 89 ± 17 µM when neurons were held at −55 mV (inactivated state) (Fig. 6, A and B). We then studied the possible use-dependent block of sodium channels by menthol. Neurons were depolarized to −20 mV from −80 mV (holding potential). Under this condition, there was a 20 to 30% reduction in sodium currents at the end of stimulation with 20 Hz. However, in the presence of 300 µM menthol there was substantial use-dependent block during 20-Hz stimulation (Fig. 6, C and D). Menthol reduced the peak currents by 44 ± 3% at the first pulse and 64 ± 4% at the 10th pulse. To determine the voltage-dependent block of sodium channels, we tested the effect of menthol on steady-state activation and inactivation. Neurons were stimulated by activation or inactivation protocols as described under Materials and Methods. Bath application of menthol did not alter the activation curve. However, menthol negatively shifted the inactivation curve.

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**Fig. 2.** Menthol reduces formalin-induced nociceptive behavior. A, the time course of nociceptive behavior after subcutaneous formalin (2%; 20 µl) injection into the hind paw after pretreatment with vehicle or 100 mg/kg menthol ($n$ = 8–9). B, total time spent in nociceptive behavior during the first (0–10 min) and second (10–50 min) phases of the formalin response with vehicle or menthol. *, $P < 0.05$ compared with vehicle control.

**Fig. 3.** Menthol concentrations in the brain. Mice were injected with 100 mg/kg i.p. menthol and then sacrificed at 0, 5, 30, or 60 min. Brain menthol concentrations are presented as mean ± S.E.M. ($n$ = 3 mice/group).
Fig. 4. Menthol activates GABA<sub>A</sub> receptors in spinal dorsal horn neurons. A, concentration-response curve of menthol-induced currents (n = 5). Inset, representative recordings of menthol-induced inward currents with four different concentrations at a holding potential of −65 mV. B, representative menthol-induced inward and outward currents recorded in control condition, in the presence of bicuculline (Bicu). C, representative recordings of menthol-induced currents with 140 mM CsCl-based or 120 mM CsMeSO<sub>4</sub> (20 mM CsCl)-based internal solutions. D, I-V relationships of menthol-induced currents recorded with CsCl- or CsMeSO<sub>4</sub>-based internal solutions.

Fig. 5. Menthol blocks voltage-gated sodium channels in cultured spinal dorsal horn neurons. A, representative Na<sup>+</sup> currents evoked by test pulses ranging from −50 to +80 mV for 50 ms from the holding potential of −80 mV before and after application of 300 µM menthol. B, I-V relationships of the Na<sup>+</sup> currents in A (n = 6). The currents measured at the peak were plotted versus test potentials. C, representative sodium currents activated by a voltage step to +20 mV from the holding potential of −80 mV before and after application of 20, 100, 300, and 1000 µM menthol and 5 min after washout of the drug. D, concentration-response curve of menthol-induced inhibition of sodium currents at a holding potential of −80 mV (n = 5–6).
curve by 10 mV without changing the slope factor (Fig. 6E; Table 1). Together, these results suggest that menthol blocks sodium channels in a state-, voltage-, and use-dependent manner.

**Menthol Blocks Voltage-Gated Calcium Channels in Dorsal Horn Neurons.** Menthol has been shown to block calcium channels in dorsal root ganglion neurons (Swandulla et al., 1987). To test whether menthol also blocked voltage-gated Ca\(^{2+}\)/Ba\(^{2+}\) currents in dorsal horn neurons, we performed Ca\(^{2+}\)/Ba\(^{2+}\) current recordings. To increase calcium currents, Ba\(^{2+}\) was used as the major charge carrier. Neurons were depolarized to a series of test pulses: −30 to 60 mV from −70 mV (holding potential). Ca\(^{2+}\)/Ba\(^{2+}\) currents were evoked at approximately 10 mV and reached their maximal amplitude at approximately 10 mV (Fig. 7, A and B), which were resistant to 50 μM NiCl\(_2\) (a known T-type calcium channel blocker) and completely blocked by 200 μM CdCl\(_2\) (data not shown). Bath application of 300 μM menthol shifted the I-V curve upward and blocked the peak of Ca\(^{2+}\)/Ba\(^{2+}\) currents with an IC\(_{50}\) of 124.8 ± 16.0 μM (Fig. 7, B-D). The decay rate of the maximal Ca\(^{2+}\)/Ba\(^{2+}\) current was slow and could be fitted with a single exponential in most neurons (at 10 mV).

### Table 1

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<td>Prementhol</td>
<td>−36.4 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Menthol</td>
<td>−33.8 ± 2.9</td>
</tr>
<tr>
<td>(I_{Ca})</td>
<td>Prementhol</td>
<td>−3.9 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Menthol</td>
<td>1.1 ± 2.1*</td>
</tr>
</tbody>
</table>

\(n\), number of neurons; \(V_{1/2}\), voltage of half-maximal activation or inactivation; \(k\), slope factor.
* \(P < 0.05\) compared with prementhol.
In the presence of menthol, the time constant was significantly decreased from 220 ± 19 to 86 ± 9 ms (n = 6; P < 0.05). These results suggest that menthol blocks Ca\(^{2+}\) channels and facilitates calcium channel inactivation.

**Mechanism of Blocking Ca\(^{2+}\) Channels by Menthol.** To determine the mechanism of the effect of menthol on Ca\(^{2+}\) channels, we also examined the state-dependent block of calcium channels. Neurons were held at −100 or −70 mV and stimulated by a test pulse at 10 mV once every 15 s. Bath application of 300 μM menthol blocked the peak Ca\(^{2+}\) current by 61% at −100 mV or 81% at −70 mV (Fig. 8, A and B), suggesting that menthol may bind to both resting and inactivated Ca\(^{2+}\) channels with preferential binding to inactivated channels. To test the use-dependent block of calcium channels by menthol, neurons were depolarized to 10 mV from −70 mV. Under this condition, a 10-Hz train of 50-ms depolarization pulse produced approximately 50% reduction of the calcium current at the end of the pulse train. Bath application of 150 μM menthol produced a use-dependent block and reduced peak currents by 47% at the first pulse and 78% at the 10th pulse (Fig. 8, C and D). Increased concentrations of menthol did not enhance the use-dependent block (data not shown). To study activation parameters, Ca\(^{2+}\) currents were elicited by applying 250-ms voltage steps ranging from −30 to 30 mV in 10-mV increments from a holding potential of −70 mV. In the presence of 300 μM menthol, a small depolarizing shift in half-activation voltage (V\(_{1/2}\)) by 5.0 mV was observed (Fig. 8E). To determine inactivation parameters, we generated steady-state inactivation curves by using the protocols described under Materials and Methods. Menthol induced a hyperpolarizing shift of the inactivation curve by −8.3 mV (Fig. 8E). Taken together, these results suggest that menthol blocks calcium channels in a voltage-, state-, and use-dependent manner.

**Menthol Reduces Neuronal Excitability and Blocks Synaptic Transmission in Spinal Dorsal Horn Neurons.** To examine the consequence of the block of voltage-gated channels by menthol, we performed current-clamp recordings in cultured dorsal horn neurons. Action potentials were evoked by depolarizing current injections from a holding potential of −65 mV (the Cl\(^−\) reversal potential in our recording conditions). Bath application of 300 or 600 μM menthol dramatically decreased the number of action potentials and slightly reduced the amplitude of action potentials (Fig. 9, A and B), but had no effect on the threshold and first latency (data not shown). Menthol (600 μM) also increased the rheobase (the minimum current required to elicit an action potential) (Fig. 9B). These results indicate that menthol decreases excitability in spinal cord dorsal horn neurons. To test the overall effect of menthol on spontaneous activity in dorsal horn neurons, we recorded spontaneous EPSCs. Neurons were held at −65 mV, and spontaneous EPSCs were recorded with a frequency of 20 to 30 Hz, which were completely blocked by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, a competitive α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid/kainate receptor antagonist (data not shown). It is noteworthy that menthol completely shut down the TTX-sensitive spontaneous EPSCs at concentrations of 100 and 300 μM (Fig. 9C). In contrast, menthol had no effect on miniature EPSCs (in the presence of TTX). This result indicates that menthol blocks spontaneous activity-induced synaptic transmission in dorsal horn neurons.

**Discussion**

Results from the present study reveal that menthol produces an analgesic effect on inflammatory pain. Menthol attenuated CFA-induced thermal and mechanical hypersensitivity, indicating that the analgesic action of menthol is not associated with a specific stimulus. Previous studies and our results suggest that menthol reduces acute pain, neuropathic pain, and inflammatory pain (Swandulla et al., 1987; Gaudioso et al., 2012), indicating that menthol is a nonselective analgesic. Systemic administration of menthol reduced both
ipsilateral and contralateral mechanical allodynia. It is generally accepted that contralateral pain (secondary hyperalgesia) is associated with plastic changes in the central nervous system (Koltzenburg et al., 1999; Filipović et al., 2012). The attenuation of pain by menthol on the contralateral paw may suggest that it acts as a central modulator. The results from the formalin tests demonstrated that menthol decreased both the first and second phases of formalin-induced spontaneous nociceptive responses. It has been suggested that centrally acting drugs such as narcotics inhibit both phases; peripherally acting drugs such as indomethacin and dexamethasone inhibit only the second phase (Hunskaar and Hole, 1987; Shibata et al., 1989). The reduction of both phases may suggest that menthol has a central action. Intrathecal application of menthol had a similar effect on mechanical allodynia, rendering further support for an action of menthol at the spinal level.

The peripheral analgesic action of menthol has been recognized for decades. The peripheral mechanism of menthol is thought to be mediated by blocking voltage-gated calcium and sodium channels (Swandulla et al., 1987; Gaudioso et al., 2012). Other studies have suggested that menthol induces analgesic effect at the spinal level (Proudfoot et al., 2006; Su et al., 2011). Our results provide further evidence that menthol produces a central analgesic action. To explore the central spinal mechanisms underlying this action, we performed a series of electrophysiological experiments in cultured dorsal horn neurons. We found that menthol induced Cl⁻/H⁻ fluxes, which was blocked by bicuculline, a GABA₆ receptor antagonist, indicating that menthol directly activates GABA₆ receptors. This finding is consistent with results from previous studies implicating menthol as a GABA₆ receptor modulator (Watt et al., 2008; Zhang et al., 2008).

We also demonstrated that menthol blocked TTX-sensitive Na⁺ channels in spinal dorsal horn neurons. A higher concentration of menthol (300 μM) slightly blocked Na⁺ channels with an IC₅₀ of approximately 1936 μM at a holding potential of −100 mV, at which point all channels are expected to be in the resting state. This small effect of menthol could be defined as a weak resting channel block. Menthol dramatically decreased Na⁺ currents with an IC₅₀ of 89 μM at −55 mV. The 20-fold increase in blocking potency at a more depolarized holding potential, at which a fraction of the channels are inactivated, suggests that menthol preferen-
Menthol binds to \( \text{Na}^+ \) channels when the channels are inactivated. This finding is further supported by the evidence that menthol induced a hyperpolarizing shift in the steady-state inactivation curve. Our data also showed that menthol-induced inhibition of \( \text{Na}^+ \) currents increases with repetitive depolarization, indicating use-dependent block of \( \text{Na}^+ \) channels. The use-dependent phenotype develops when the interval between pulses is too short to completely recover from inactivation, which represents an accumulation of inactivated channels. The menthol-induced use-dependent block of \( \text{Na}^+ \) channels is most likely caused by the preferential binding of menthol to \( \text{Na}^+ \) channels in the inactivated state. Our findings are consistent with results from a previous study in DRG neurons and DRG neuron-derived F11 cells (Gaudioso et al., 2012). However, menthol blocks \( \text{Na}^+ \) channels in dorsal horn neurons with 2-fold more potency than in DRG neurons (at the same holding potential). This difference could be caused by differences in the subunit composition of \( \text{Na}^+ \) channels between dorsal horn and DRG neurons.

Menthol blocks \( \text{Ca}^{2+} \) channels with a greater potency than \( \text{Na}^+ \) channels. The mechanisms involved in the blocking \( \text{Ca}^{2+} \) channels are similar to those of the blocking \( \text{Na}^+ \) channels. However, menthol accelerated the rate of \( \text{Ca}^{2+} \) current decay during longer test pulses. It produced a more pronounced block of plateau \( \text{Ca}^{2+} \) currents and a weak block of the peak \( \text{Ca}^{2+} \) currents. Menthol-induced acceleration of current decay may result from open channel block, drug-induced inactivation, or both (Lee and Tsien, 1983; Berjukow and Hering, 2001). Our results also showed that menthol blocks resting \( \text{Ca}^{2+} \) channels and shifted the \( \text{Ca}^{2+} \) channel inactivation curves in the hyperpolarization direction without changes in the slope factor, suggesting that menthol may enhance the transition of \( \text{Ca}^{2+} \) channels from the resting to the inactivated state. Furthermore, menthol shifted the steady-state activation of \( \text{Ca}^{2+} \) currents, indicating that menthol may act as a gating inhibitor. The detail mechanisms of menthol-induced calcium channel block remain to be determined. In contrast to a previous report that menthol reduces the
amplitude of low voltage-gated, but not high voltage-gated, calcium currents (Swandulla et al., 1987), we found that menthol reduces the amplitude of high voltage-gated calcium currents. Calcium currents recorded in the present study were activated at higher voltages from the holding potential of −70 mV, at which point most T-type calcium channels are inactivated (Huang, 1989). In addition, these calcium currents were resistant to 50 μM NiCl2, a known T-type calcium channel blocker, further supporting that these currents are mediated by high voltage-gated calcium channels. The discrepancy can be explained by the expression of different subtypes of high voltage-gated calcium channels between dorsal horn and DRG neurons.

As a consequence of the finding that menthol blocked the Na+ channels, we observed that menthol decreased neuronal excitability. Menthol-induced inhibition of repetitive firing is consistent with use-dependent block of Na+ channels. Inhibition of presynaptic Na+ and Ca2+ channels would be expected to reduce neurotransmitter release at the synapse. Indeed, menthol completely blocked the TTX-sensitive spontaneous EPSC but had no effect on the miniature EPSC, which agrees with results from a previous study in monocultured dorsal horn neurons (Tsuzuki et al., 2004). Menthol blocked spontaneous activity-induced synaptic transmission at 100 μM, at which point menthol only inhibits 25% Na+ currents and 40% Ca2+ currents (at −80 mV). The increase in potency could be because a fraction of Na+ and Ca2+ channels are in the inactivated state at −65 mV. The inhibitory effect of menthol increases at more depolarized potentials. In addition, both Na+ and Ca2+ channels are essential for activity-induced synaptic transmission, and menthol hit both Na+ and Ca2+ channels in the same neuron. This double block may account for increasing potency. It is generally accepted that excessive depolarization and abnormal excitability occur in the chronic pain state (Latremoliere and Woolf, 2009). Menthol blocks Na+ and Ca2+ channels with enhanced state-dependent and use-dependent block by preferentially binding to inactivated channels. These features are critical for the suppression of abnormal excitability.

Menthol has been proposed as an activator of TRPM8 (McKemy et al., 2002; Peier et al., 2002) and a modulator of TRPA1 (Karashima et al., 2007; Xiao et al., 2008). To date, there is no report suggesting functional expression of TRPA1 (McKemy et al., 2002; Peier et al., 2002) and a modulator of TRPM8 or TRPA1. GABAA receptors and voltage-gated sodium and calcium channels are the major players in pain signal transmission. However, the activation of GABAA receptors by menthol requires a much higher concentration than that needed for blocking calcium and sodium channels. We observed a GABAA agonist-like sedative effect when menthol was administered at a higher dose (200 mg/kg; data not shown). Therefore, the central mechanisms of the analgesic action of menthol are unlikely to be mediated by blocking GABAA receptors at the doses used for this study. Our LC/MS/MS result shows that menthol has great brain penetration. Menthol with a relative high concentration (12.1 μg/g) at 30 min, but its concentration was largely decreased to 3.1 μg/g at 60 min. This result is consistent with our in vivo data that the efficacy of high-dose menthol (100 mg/kg) is better at 30 min than 60 min. In addition, low-dose menthol (50 mg/kg) significantly reduces CFA-induced thermal hypersensitivity at 30 min, but has no effect at 60 min. The relationship between the brain concentration and behavioral efficacy of menthol further supports the idea that menthol has a central analgesic action. The brain menthol level may also correlate with the concentration required for blocking voltage-gated Na+ and Ca2+ channels.

In summary, our results indicate that menthol produces a central analgesic action by blocking Na+ and Ca2+ channels in dorsal horn neurons, an effect that has also been demonstrated in peripheral neurons (Swandulla et al., 1987; Gaudio et al., 2012). It seems that menthol acts as a nonselective analgesic agent through multiple peripheral and central pain targets, which is probably therapeutically beneficial in treating chronic pain. It may be used as an effective analgesic in chronic pain as well as a topical analgesic.

Acknowledgments

We thank Dr. Seena Ajit, Dr. Alessandro Graziano, and Pamela W. Fried for critically reading and providing valuable comments on the manuscript.

Authorship Contributions

Participated in research design: Pan, Zhao, and Hu.

Conducted experiments: Pan, Tian, Gao, Li, Zhao, and Hu.

Performed data analysis: Pan, Zhao, and Hu.

Wrote or contributed to the writing of the manuscript: Barrett and Hu.

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