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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.

Key words: L-menthol, heat analgesia, cold hyperalgesia, dorsal horn neurons.

INTRODUCTION

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% to 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, 1987; Wang et al., 1993; Lundy and Contreras, 1995), likely accounting for the perceptual enhancement of cold by menthol. Topical menthol also reduces heat pain in human skin (Green, 1985, 1986) and oral cavity (Green, 1986; Albin et al., 2008) and cross desensitizes oral irritancy evoked by the transient receptor potential vanilloid 1 (TRPV1) agonist capsaicin (Green and McAuliffe, 2000), and the transient receptor potential ankyrin 1 (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) (McKemy et al., 2002; Peier et al., 2002; Reid and Flonta, 2002), suggesting a peripheral site for menthol's enhancement of cold sensation. Topical application of menthol to the tongue excited neurons in superficial laminae of 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,b). Briefly, animals were anesthetized with sodium pentobarbital (induction: 65 mg/kg i.p., maintenance: 10 mg/kg i.v.). Oxygen was delivered via a tracheal cannula. Core body temperature was monitored and maintained by heating pad,

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Abbreviations: ANOVA, analysis of variance; DRG, dorsal root ganglion; GABA, gamma amino butyric acid; GFP, green fluorescent protein; MI, mechanically insensitive; NS, nociceptive specific; PSTH, peristimulus-time histogram; SEM, standard error of the mean; 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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and displayed continuously along with the electrocardiogram using a Powerlab interface (AD Instruments, Colorado Springs, CO, USA). A tungsten microelectrode (FHC, Bowdoin, ME, USA; 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 (AD Instruments) 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 Spike 2 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, USA; 13-mm diameter) attached to a micromanipulator (World Precision Instruments, Sarasota, FL, USA), 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 s for the Peltier thermode to heat from 35 to 55 °C, producing a maximum thermode-skin interface temperature that averaged 52.7 °C ± 0.5 (standard error of the mean, SEM) and remained above 50 °C for a mean of 7.3 ± 0.5 (SEM) s. The cooling rate was slower, reducing the thermode-skin interface temperature from 35 °C to ~0 °C over a 55 s period. A positive thermal response was considered to be an increase of firing 30% above baseline. Only cold- and/or heat-responsive 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 μL. 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, USA) was dissolved to a concentration of 1% in a vehicle of 10% ethanol and 1% Tween-80 (Fisher Scientific, Fair Lawn, NJ, USA), 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 μL, 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 (Merrill et al., 2008; Sawyer et al., 2009a,b).

Data analysis

The number of thermally evoked action potentials was summed over 30 s for heat or 60 s 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 s immediately before each stimulus was summed and subtracted from the firing rate 15 s 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 analysis of variance (ANOVA) and Tukey post-hoc comparison tests between time points (SPSS 9.0 software, SPSS, Chicago, IL, USA). Responses between vehicle and menthol treatment groups were also compared using two-way repeated measures ANOVA (SPSS). Error reported is the 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 h 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

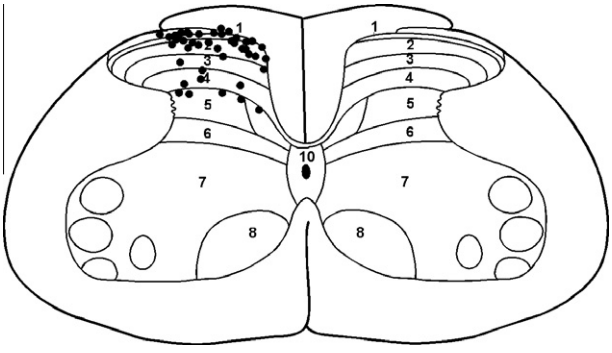

Fig. 1. Histologically localized recording sites compiled on L4 spinal cord section (adapted from Paxinos and Watson, 1998).