Role for Medullary Pain Facilitating Neurons in Secondary Thermal Hyperalgesia

Wendy Kincaid,1 Miranda J. Neubert,1 Mei Xu,1 Chang Jae Kim,2 and Mary M. Heinricher1
1Department of Neurological Surgery, Oregon Health & Science University, Portland, Oregon; and 2Department of Anesthesiology, College of Medicine, Catholic University, St. Paul’s Hospital, Seoul, Korea

Submitted 3 May 2005; accepted in final form 13 September 2005

Kincaid, Wendy, Miranda J. Neubert, Mei Xu, Chang Jae Kim, and Mary M. Heinricher. Role for medullary pain facilitating neurons in secondary thermal hyperalgesia. J Neurophysiol 95: 33–41, 2006. First published September 28, 2005; doi:10.1152/jn.00449.2005. The rostral ventromedial medulla (RVM) has recently received considerable attention in efforts to understand mechanisms of hyperalgesia and persistent pain states. Three classes of neurons can be identified in the RVM based on responses associated with nocifensive reflexes: ON cells, OFF cells, and NEUTRAL cells. There is now direct evidence that ON cells exert a net facilitating effect on spinal nociception and that OFF cells depress nociception. These experiments tested whether the secondary hyperalgesia produced by topical application of mustard oil involves an activation of ON cells in RVM. Firing of a characterized RVM neuron and the latencies of withdrawal reflexes evoked by noxious heat were recorded in lightly anesthetized rats before and after application of mustard oil to the shaved skin of the leg above the knee. Mineral oil was applied as a control. Mustard oil produced a significant increase in ongoing and reflex-related discharge of ON cells, as well as a decrease in the activity of OFF cells. NEUTRAL cell firing was uniformly unchanged after application of mustard oil. The alterations in ON and OFF cell firing were associated with a significant decrease in the latency to withdraw the paw of the treated limb from the heat stimulus, and this hyperalgesia was blocked by microinjection of lidocaine within the RVM. Withdrawals evoked by heating the contralateral hindpaw, forepaw, and tail were unchanged after mustard oil application. These experiments support a pronociceptive role for ON cells and suggest that these neurons contribute to secondary hyperalgesia in inflammation.

INTRODUCTION

In recent years, the attention of investigators interested in pain and pain control has been increasingly focused on understanding mechanisms of hyperalgesia and persistent pain states. The most intense efforts have been directed toward elucidating plasticity in nociceptive transmission pathways, and there is now abundant evidence that both the primary afferent nociceptors and dorsal horn neurons are sensitized in models of hyperalgesia (Koltzenburg 1999; Levine et al. 1993; Ren and Dubner 1999). In comparison, our understanding of supraspinal mechanisms of hyperalgesia is much less advanced. Although an increasing number of functional studies have implicated descending facilitation from the rostral ventromedial medulla (RVM) in hyperalgesia (Heinricher et al. 2003; Porreca et al. 2002; Ren and Dubner 2002), we have comparatively little information concerning the underlying neural basis within the RVM.

Experimental analysis of the RVM mechanisms contributing to enhanced pain states is complicated by two factors. The RVM has long been known to suppress nociception through descending connections to the dorsal horn and to be an important substrate for opioid analgesia (Fields and Basbaum 1978; Mayer and Price 1976). This region is also implicated in a number of functions other than nociceptive modulation, including reproductive behaviors, cardiovascular control, sleep-waking and arousal, thermoregulation, and behavioral suppression (Blessing 2003; Cao et al. 2004; Dampney et al. 2003; Fornal et al. 1985; Grahn and Heller 1989; Morgan and Whitney 2000; Morrison 2001; Murphy et al. 1999; Potas and Dampney 2003; Sugaya et al. 1998). A meaningful analysis of how the RVM contributes to enhanced pain states therefore requires functional identification of the neurons under study, so that mechanisms contributing to nociceptive facilitation can be distinguished from those involved in nociceptive inhibition or other functions.

There is now strong evidence for two populations of nociceptive modulating neurons in the RVM: ON cells and OFF cells (Fields and Heinricher 1985; Fields et al. 2005). ON cells are characterized by a burst of activity associated with nocifensor withdrawal reflexes. Direct, selective activation of ON cells produces hyperalgesia, and a reduction in the threshold at which the ON cell burst is triggered is associated with a decrease in reflex latency (Heinricher and Neubert 2004; McGrath andy et al. 2003; Neubert et al. 2004). However, under basal conditions, ON cell discharge does not regulate nociceptive threshold to any significant degree (Heinricher and McGrath 1998). OFF cells are defined by a pause in firing associated with withdrawal reflexes. Activation of OFF cells results in analgesia, and these neurons are generally thought to exert a net antinociceptive effect (Heinricher and Tortorici 1994; Heinricher et al. 1994; Neubert et al. 2004). RVM neurons that show no nocifensor reflex-related changes in firing are assigned to the NEUTRAL cell category. Although it is not known whether NEUTRAL cells contribute to nociceptive modulation, the response properties of many NEUTRAL cells have been reported to change over the course of hours during prolonged inflammation (Miki et al. 2002). This cell class may therefore be involved in long-term facilitation or inhibition of nociception or in other changes in behavior or physiology during inflammation.

The goal of this study was to identify RVM neurons that could mediate the secondary thermal hyperalgesia produced by...
mustard oil. Topical application of this chemical irritant produces inflammation, activates C-fibers, and sensitizes dorsal horn neurons and spinal reflex circuitry (Pertovaara 1998; Reeh et al. 1986; Schmelz et al. 1996; Woolf and King 1990; Woolf and Wall 1986; Woolf et al. 1994). Mustard oil produces secondary hyperalgesia in humans and awake, behaving animals (Cervero and Laird 1996; Koltzenburg et al. 1992; Mansikka and Pertovaara 1997; Sjölund et al. 1999; Urban et al. 1996). Both the sensitization of dorsal horn neurons and the enhancement of reflexive behaviors after mustard oil are attenuated or blocked by inactivation of the RVM (Mansikka and Pertovaara 1997; Urban et al. 1996, 1999a,b). Functional studies thus show that output from the RVM is required for mustard oil–induced secondary hyperalgesia. However, these studies provide no information about the underlying neural mechanisms within the RVM. These experiments were designed to determine if activation of ON cells and/or NEUTRAL cells is part of a spinal-supraspinal feedback circuit required for secondary thermal hyperalgesia during inflammation produced by mustard oil.

METHODS

Animals, surgical preparation, and nociceptive testing

All experimental procedures followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University. Male Sprague-Dawley rats (Sasco, 250 – 300 g) were anesthetized with pentobarbital sodium (60 mg/kg, ip), and a catheter inserted into an external jugular vein for administration of sodium methohexital. Each rat was placed in a stereotaxic apparatus, a hole was drilled in the skull over the cerebellum, and the dura was removed to allow placement of an electrode in the RVM. Body temperature was maintained at ~37°C by a circulating water pad.

After surgery, the anesthetic level was allowed to lighten until a paw withdrawal reflex (PW) could be elicited by application of noxious heat to the left hindpaw using a feedback-controlled projector lamp focused on the blackened plantar surface of the paw. In a small number of cases, the forepaw or tail was similarly tested with radiant heat. After surgical preparation, the animals were maintained in a lightly anesthetized state using a continuous infusion of methohexital at a rate (15–30 mg/kg/h, iv) that allowed a stable withdrawal latency and prevented any signs of discomfort. The animals did not move spontaneously, nor did they vocalize or produce vigorous or prolonged withdrawal reflexes after noxious pinch. The rate was adjusted for each animal to allow a baseline PW of ~3 s. The protocol was begun after a stabilization period of 30 min, and infusion rate was not altered during the protocol.

Latency of the paw withdrawal or tail flick to heat was used as a measure of noxious responsiveness, as described previously (Barbaro et al. 1989; Neubert et al. 2004). Each trial consisted of a linear increase in temperature at ~1.8°C/s from a holding temperature of 34°C until the reflex occurred or to a maximum of 53°C at 10.6 s. Trials were carried out at 5-min intervals throughout the experiment. The holding temperature obviates any concern that apparent effects on reflex latency could be attributed to changes in skin temperature.

RVM recording and microinjection

A gold- and platinum-plated stainless steel recording microelectrode (FHC, Bowdoinham, ME) was inserted into the RVM for extracellular single unit recording. RVM neurons were classified as previously described (Barbaro et al. 1989; Fields et al. 1983). Spike waveforms were monitored and stored for off-line analysis (Dataview Systems, Thornton, CO) to ensure that the unit under study was unambiguously discriminated throughout the experiment. Spike times were stored with a temporal resolution of 0.1 ms. OFF cells were characterized by an abrupt pause in ongoing activity beginning just before the occurrence of the PW. ON cells were identified by a sudden burst of activity beginning just prior to the occurrence of the PW. Both OFF and ON cells typically responded to noxious pinch, with inhibition and excitation, respectively. NEUTRAL cells were identified by no change in activity associated with PW. NEUTRAL cells did not generally respond to noxious pinch. Because the reflex-related ON cell activation cannot normally be detected when the neuron is already spontaneously active, and because the reflex-related pause can be seen only during active periods, we attempted to test each neuron during both active and silent periods. If a neuron was continuously active during characterization (i.e., it did not exhibit spontaneously occurring silent periods that would allow discrimination of a NEUTRAL cell from a continuously active ON cell), anesthetic depth was temporarily increased to a point at which the withdrawal reflex was lost in an attempt to reduce spontaneous discharge and allow testing for reflex-related activation of the neuron. This approach has been used because ON cell firing typically slows if the anesthetic level is increased in this manner. Anesthetic level was then returned to the level needed to maintain a stable paw withdrawal. Full characterization of a neuron could thus require an hour or more. The behavioral testing protocol was initiated once a well-isolated cell with a robust reflex-related change in activity (or lack of response, in the case of a NEUTRAL cell) was identified.

In a set of experiments without neuronal recording, lidocaine (4%) or saline was microinjected into the RVM in a volume of 500 nl using a glass micropipette (75–100 μm OD) to test whether the RVM was required for secondary hyperalgesia after application of mustard oil in these lightly anesthetized animals.

Protocol and data analysis

We determined the effects of application of mustard oil on withdrawal latency and on the ongoing and reflex-related discharges of RVM neurons. After three baseline PW trials, mustard oil (100%) or mineral oil was applied to the shaved surface of the left or right lateral thigh just above the knee (200 μl saturating a filter paper pledget, ~15 × 12 mm for 2 min). Withdrawal latency and cell activity were monitored for an additional 45 min. Only one protocol was performed in each animal.

Five cell parameters were analyzed. 1) Ongoing activity. Because OFF cells and ON cells often show irregular alternations between periods of silence and activity, cell activity integrated over the 30 s before each withdrawal trial was used as an overall index of ongoing firing. 2) ON cell PW-related burst. Average firing rate in the 3-s period beginning 0.5 s before the PW was recorded for all PW trials. This approach, rather than counting the number of spikes or duration of the reflex-related burst, was necessary because a burst can only be identified in cases in which the neuron is inactive at the time of heat onset. 3) Duration of the OFF cell pause. Duration of the reflex-related pause was determined for those trials that fell at a time when the OFF cell was not already silent at the time of heat onset. 4) Cycling. The proportion of time that a given cell was considered to be in an “active” or “silent” period was defined as described previously (Barbaro et al. 1989). Briefly, an active period was defined as any epoch lasting ≥2 s with a minimum of 1 spike/s, and a silent period as any epoch of ≥2 s without any cell activity. The proportion of time in which each OFF cell was active or silent was calculated for the baseline and posttreatment periods. 5) For NEUTRAL cells, we calculated firing rate (in spikes/s) in the 3-s period beginning 0.5 s before the PW (i.e., analogous to the ON cell burst) as a percentage of ongoing firing (i.e., mean firing rate in spikes/s in the 30-s period before the heat onset).
This allowed us to determine whether NEUTRAL cells developed reflex-related changes in firing after mustard oil application. Data are presented as means ± SE. Wilcoxon’s signed ranks test was used for statistical analysis of cell parameters; ANOVA with repeated measures followed by Dunnett’s test, or t-test for correlated means was used to compare baseline and postinflammation withdrawal latencies. *P < 0.05* was considered statistically significant.

**Histology**

At the conclusion of the experiments, recording sites were marked with an electrolytic lesion and infusion sites by injection of pontamine sky blue dye. Animals were killed with an overdose of methohexital and perfused intracardially with physiological saline followed by 10% formalin. Recording and infusion sites were histologically verified. The RVM was defined as the nucleus raphe magnus and adjacent reticular formation at the level of the facial nucleus. Recording sites were distributed in the RVM as in previous publications from this laboratory (Heinricher and Roychowdhury 1997; Heinricher and Tortorici 1994).

**RESULTS**

**RVM neuron responses to application of mustard oil**

ON cells were potently activated during the application of mustard oil, and a less substantial but significant increase in activity was maintained after the mustard oil was removed (Figs. 1, top trace, and 2). The activation was similar whether the mustard oil was applied to the left (ipsilateral to paw withdrawal testing) or right (contralateral to paw withdrawal testing) hindlimb (Fig. 2). Mineral oil had no effect on the firing of ON cells (Figs. 1, 2nd trace, and 2). The enhanced ongoing activity of ON cells after mustard oil was due primarily to an increase in the proportion of time that the cells were in active phases, whereas firing rates during active periods were increased only slightly (Table 1). However, firing rates during the reflex-related burst were significantly increased (Table 1).

We have previously noted that ON and OFF cells vary in excitability, and that although cells within each class fire in phase, individual neurons are recruited into the active pool at different thresholds (Barbaro et al. 1989). In this study, we noted that mustard oil application frequently recruited ON cells that had had very low or variable excitability during characterization or baseline into the active population. Although we typically did not quantify the activity of these “background” neurons because the electrode position was optimized to record the cell under study, one example of such recruitment in which the second cell was sufficiently well isolated to quantify its activity is shown in Fig. 3. During the baseline period, only the cell in the top trace was active. The neuron in the bottom trace was completely inactive throughout the 15-min baseline period.
in contrast to the activation of ON cells, OFF cell firing was significantly depressed after mustard oil (Figs. 1, 3rd trace, and 2). This decrease in activity occurred irrespective of whether the mustard oil was applied to the hindlimb ipsilateral or contralateral to reflex testing (Fig. 2). The overall decrease in discharge was caused by a decrease in the proportion of time within the active phases, as well as a reduction in firing rates during the active phases (Table 1). The duration of the reflex-related pause was also significantly increased (Table 1).

Neutral cell firing was unaffected by mustard oil (Figs. 1, 4th trace, and 2). Only 1 of the 13 neutral cells challenged with mustard oil showed a change of >25% in ongoing firing rate. In that case, there was a gradual decrement in firing rate that was not temporally linked to application of mustard oil. In addition, no neutral cell exhibited novel reflex-related changes in firing after mustard oil. In baseline, firing rate at the time of PW was 110.3 ± 4.8% of the ongoing firing rate. After mustard oil, this percentage was almost identical, with firing rate at the time of the PW 111.2 ± 7.5% of the ongoing rate (P = 0.34, Wilcoxon’s signed ranks test).

Behavioral responses to application of mustard oil

Mustard oil evoked strong flexion of the entire treated limb throughout the 2-min application period. Animals subsequently displayed frequent “spontaneous” withdrawals and were hyperresponsive to handling of the paw when its position on the heat stimulator was adjusted. Testing for secondary thermal hyperalgesia showed that administration of mustard oil on the left thigh produced a decrease in PW latency to heat applied to the plantar surface of the hindpaw of that limb (Fig. 4). Hyperalgesia was evident at the first test after removal of the mustard oil–soaked pledget and was maintained for the entire 45-min monitoring period. Microinjection of lidocaine, but not saline, in the RVM prevented the decrease in PW latency, showing that the RVM is required for this hyperalgesia (Fig. 5).

The timing of the ON cell burst and OFF cell pause relative to the paw withdrawal was unaffected by mustard oil. The time of the ON cell burst and OFF cell pause relative to the paw withdrawal was unaffected by mustard oil. The time of

<table>
<thead>
<tr>
<th></th>
<th>Pre-Mustard Oil</th>
<th>Post-Mustard Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ON cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of time active</td>
<td>28.4 ± 8.8 %</td>
<td>45.8 ± 9.1 %**</td>
</tr>
<tr>
<td>Firing rate during active periods</td>
<td>5.2 ± 0.6 sp/s</td>
<td>7.2 ± 0.8 sp/s</td>
</tr>
<tr>
<td>Reflex-related firing</td>
<td>8.7 ± 1.4 sp/s</td>
<td>13.5 ± 1.7 sp/s**</td>
</tr>
<tr>
<td>Percentage of PW trials with cell active at heat onset</td>
<td>29.6 %</td>
<td>41.4 %</td>
</tr>
<tr>
<td><strong>OFF cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of time active</td>
<td>92.4 ± 2.0 %</td>
<td>68.7 ± 8.2 %**</td>
</tr>
<tr>
<td>Firing rate during active periods</td>
<td>8.6 ± 1.1 sp/s</td>
<td>6.6 ± 0.9 sp/s**</td>
</tr>
<tr>
<td>Pause duration, s</td>
<td>9.2 ± 2.3 s</td>
<td>13.5 ± 3.2 s*</td>
</tr>
<tr>
<td>Percentage of PW trials with cell silent at heat onset</td>
<td>3.9 %</td>
<td>30.7 %</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Because overall firing of both cell classes was affected similarly when mustard oil was applied to either the left or right hindlimb (ipsilateral and contralateral to reflex testing, respectively), data for both groups were combined. Not surprisingly, the percentage of paw withdrawal trials on which the ON cell under study was active, or the OFF cell silent, closely tracks the proportion of time active/silent for the two cell classes.
the first spike in the reflex-related ON cell burst was determined for all trials on which the burst was well-defined (i.e., the cell was not active at heat onset). The first spike in the burst preceded the paw withdrawal by 0.82 ± 0.19 s at baseline and by 0.77 ± 0.12 s after mustard oil (P > 0.05, t-test for correlated means). The beginning of the OFF cell pause was defined as the final spike before the reflex for all trials on which the cell was active at time of heat onset. The pause preceded the withdrawal by 0.65 ± 0.14 s at baseline and 0.56 ± 0.14 after mustard oil (P > 0.05, t-test for correlated means). The lag from heat onset to onset of the burst or pause was necessarily reduced (because paw withdrawal latency was decreased). The lag from heat onset to the first spike in the ON cell burst was reduced from 2.95 ± 0.37 s at baseline to 2.15 ± 0.09 s after mustard oil (P < 0.05, t-test for correlated means). Similarly, the lag from heat onset to beginning of the OFF cell pause was reduced after mustard oil from 2.10 ± 0.15 s at baseline to 1.40 ± 0.29 s (P < 0.05, t-test for correlated means).

Secondary hyperalgesia was limited to the ipsilateral limb. There was no decrease in PW latency when mustard oil was applied to the contralateral hindlimb, nor was there a change in latency in the forepaw or tail flick withdrawal reflexes after application of mustard oil to the hindlimb (Fig. 6).

**DISCUSSION**

These experiments show that the firing of both ON and OFF cells, but not that of NEUTRAL cells, is significantly altered by application of mustard oil. Mustard oil–evoked changes in ON and OFF cell firing are associated with a decrease in paw withdrawal latency. This secondary hyperalgesia, which is limited to the treated limb, requires the RVM.

**Relationship between changes in activity of ON and OFF cell populations and the PW**

There is now growing evidence that descending modulatory projections from the RVM play an important role in persistent pain states, including those associated with inflammation, nerve injury, and prolonged opioid administration (Heinricher...
and OFF cells a net antinociceptive effect (Fields and Heinricher 2003; Ossipov et al. 2004; Porreca et al. 2002). However, teasing out the specific RVM mechanisms mediating enhanced nociception is complicated by the functional complexity of this region. The RVM is involved in a number of functions other than pain modulation. In addition, the RVM inhibits or facilitates nociception under different conditions, and inflammation recruits descending inhibition in addition to descending facilitation from brain stem systems (Ren and Dubner 2002). In these experiments, we used the ON/OFF/NEUTRAL cell classification originally proposed by Fields et al. (1983), and found a consistent relationship between cell class and the changes in ongoing firing after topical application of mustard oil. As a population, ON cells showed an increase in ongoing firing and OFF cells showed a decrease. NEUTRAL cell firing was unchanged. Mustard oil thus caused a shift in the balance between ON and OFF cell firing, so that ON cells were more likely to be in an active phase and OFF cells in a quiescent phase, at any given time after mustard oil. The likelihood that the heat stimulus would be delivered at a time that the ON cell population was in an active phase and the OFF cell population silent was therefore increased, because the stimulus was delivered to the paw at 5-min intervals irrespective of cell activity. The secondary hyperalgesia produced by mustard oil is therefore consistent with previous demonstrations that withdrawal reflexes occur with a shorter latency if the evoking stimulus is delivered when the ON cells are already firing and OFF cells are inactive (Bederson et al. 1990; Heinricher and Neubert 2004; Heinricher et al. 1989, 2004; Morgan and Fields 1994; Neubert et al. 2004; Ramirez and Vanegas 1989).

These data are correlative but consistent with the accumulating evidence that ON cells exert a net pronociceptive effect and OFF cells a net antinociceptive effect (Fields and Heinricher 1985; Heinricher and Neubert 2004; Heinricher and Tortorici 1994; Heinricher et al. 1994; Neubert et al. 2004). Moreover, inactivation of the RVM with lidocaine prevented secondary hyperalgesia in this experiment. The latter observation supports a causal role for ON cell activation, because ON cells were the only neurons whose activity was increased after mustard oil. However, because lidocaine would presumably block all neurons and fibers of passage through the RVM, we cannot rule out the possibility that ongoing activity in NEUTRAL cells or fibers of passage is the relevant variable.

Secondary hyperalgesia in this experiment was limited to the mustard oil–treated hindlimb. This is consistent with previous studies of reflex circuitry in spinalized as well as intact animals, as well as analyses of secondary hyperalgesia in awake behaving animals (Clarke and Harris 2004; Mansikka and Pertovaara 1997; Mansikka et al. 1996; Pertovaara 1998; Urban et al. 1996; Woolf and King 1990; Woolf and Wall 1986; Woolf et al. 1994). However, this observation is at odds with the generally accepted view that the RVM exerts a global influence over nociceptive inputs from all regions of the body. This idea is based on the whole body analgesia produced by stimulation or morphine microinjection in the RVM (Mayer and Price 1976; Yaksh and Rudy 1978) and on anatomical evidence that the projection from the RVM to the dorsal horn is diffuse, with individual neurons projecting to multiple levels of the spinal cord and even bilaterally (Cho and Basbaum 1989; Huisman et al. 1981; Skagerberg and Björlund 1985). Identified ON and OFF cells have been shown to project to multiple spinal segments (Fields et al. 1995). It would therefore have been predicted that hyperalgesia caused by activation of ON cells and the accompanying decrease in the firing of OFF cells would not be restricted to a single limb. Indeed, Morgan and Fields (1994) previously showed that dipping the foot in hot water activated ON cells and facilitated the withdrawal of the contralateral limb. However, hyperalgesia did not outlast the period of stimulation in their study, and it is possible that we would have seen a contralateral effect had we measured withdrawal latency during the period of mustard oil application.

One possible explanation for the restricted distribution of hyperalgesia may lie in the degree to which ON cells were activated after mustard oil. Activation may have been insufficient to produce behaviorally measurable hyperalgesia in the absence of sensitization at the spinal level. In support of this, the extent to which ON cell firing was increased, although consistent across neurons and statistically significant, was much less than was seen after direct microinjection of neurotransmitters or cholecystokinin in the RVM (Heinricher and Neubert 2004; Neubert et al. 2004). Both of these peptides evoked almost continuous firing in almost all ON cells tested and produced measurable hyperalgesia in the absence of any sensitizing peripheral input. In comparison, the shift in the balance from OFF cell to ON cell populations in this study was much less marked, with ON cells active <50% of the postmustard oil period, on average. This moderate increase in descending facilitation from the RVM would intersect with sensitization of peripheral input and dorsal horn neuron circuitry only at the lumbar level ipsilateral to the mustard oil application. This might explain why behaviorally measurable hyperalgesia is limited to the treated limb.

A second possibility is that descending inhibition recruited in parallel with the descending facilitatory influence from the RVM suppresses excitability in circuits serving other body regions. The mustard oil stimulus could recruit heterotopic inhibition, for example (Le Bars 2002). In addition, descending inhibition is reported to be increased in a time-dependent fashion in a number of acute and persistent pain models and to oppose descending facilitation (Azami et al. 2001; Cervero et al. 1991; Crown et al. 2004; Monhemius et al. 2001; Ren and
Changes in reflex-related firing of ON and OFF cells and the paw withdrawal reflex

It is unlikely that the increased firing rate during the ON cell burst or the increased duration of the OFF cell pause contributed to the shorter paw withdrawal latency. ON and OFF cells do not encode the intensity of the stimulus, but rather the occurrence of the reflex (Fields et al. 1983; Jinks et al. 2004a). Moreover, the ON cell burst and OFF cell pause reflect cell activity concurrent with or after the reflex. Nevertheless, the longer OFF cell pause after mustard oil might be expected to prolong a period of enhanced responsiveness to subsequent stimuli (Ramírez and Vanegas 1989). In addition, it has been suggested that the firing rate of ON cells during the withdrawal-related burst of activity could modulate the magnitude or force of the reflex or might prime responses to subsequent stimuli (Jinks et al. 2004b; Ramírez and Vanegas 1989). However, we measured only reflex latency and not force or magnitude in this study.

RVM neuronal responses to topical application of mustard oil

Mustard oil evoked a pronounced flexion of the entire limb throughout its application. ON cells were strongly activated, and OFF cell discharge was almost completely suppressed during this withdrawal. NEUTRAL cells did not respond during application of mustard oil. These observations show that an equivalent classification would result if either heat- or mustard oil-evoked withdrawals were used to divide RVM neurons into ON, OFF, and NEUTRAL cell classes. In this study, no NEUTRAL cell tested displayed altered ongoing firing or developed reflex-related changes in firing after mustard oil. In contrast, others have reported that most NEUTRAL cells developed reflex-related changes in firing after inflammation of the hindpaw produced by complete Freund’s adjuvant, an inflammatory stimulus with a slower onset and more prolonged time course than mustard oil (Miki et al. 2002). These authors did not report recruitment of inactive cells. The discrepancy between our study and that of Miki et al. (2002) may be caused by technical factors but could also be related to stimulus intensity or to the different time-courses in the two experiments. We limited our protocol to 1 h, because we were confident that a stable anesthetic state would be maintained for that period, whereas Miki et al. (2002) recorded for up to 17 h after the inflammation was induced. It may be that changes in the properties of NEUTRAL cells are responsible for the longer-term plasticity in the pain modulating function of the RVM reported with prolonged inflammation, nerve injury, stress, or opioid administration (Burgess et al. 2002; Heiricher et al. 2003; Hurley and Hammond 2000, 2001; Imbe et al. 2004; Porreca et al. 2001; Ren and Dubner 2002; Xie et al. 2005). Alternatively, NEUTRAL cells may contribute to other behavioral or physiological responses to prolonged inflammation.

Technical issues

The possibility that anesthesia influenced the behavioral hyperalgesia or neuronal responses seen here must be considered. Other investigators using the mustard oil–induced secondary thermal hyperalgesia paradigm have reported that infusion of pentobarbital at a rate sufficient to maintain a lightly anesthetized state interferes with hyperalgesia, particularly at later time points (42–48 min or more after removing the mustard oil, Cleland et al. 1994). However, these authors used the long-acting barbiturate pentobarbital, rather than the shorter-acting methohexital used here, and there was no control for anesthetic depth. In these experiments, PW latency was unchanged in the mineral oil–treated control group, which indicates that the anesthetic level was stable throughout the testing period using our procedures. Moreover, the RVM-dependent change in PW latency that we observed after mustard oil applied proximal to the heat stimulus on the same limb is entirely consistent with previous reports in awake behaving animals (Urban et al. 1996, 1999b). Thus the behavioral measures in this study are unlikely to have been altered by anesthesia as such.

The influence of anesthesia on neuronal firing is also a potential concern. There is no question that the responsiveness of RVM neurons is modulated with anesthetic state and with arousal more generally (Jinks et al. 2004a; Leung and Mason 1995; Oliveras et al. 1991a,b). However, cell activity was unchanged in control animals treated with mineral oil, ruling out changing anesthesia as a possible confound of the effects of mustard oil. Moreover, ON and OFF cells can be identified in unanesthetized animals (Clarke et al. 1994; Leung and Mason 1999), as well as in animals anesthetized with ketamine, halothane, and isoflurane (Clarke et al. 1994; Jinks et al. 2004a; McGarvaughy and Reins 1993; McGarvaughy et al. 1993). Moreover, the many parallels between behavioral results obtained in anesthetized and unanesthetized animals would argue that the fundamental modulatory framework of the RVM is not altered qualitatively under anesthesia (Heiricher and Tortorici 1994; Heiricher et al. 1994, 2001; Meng and Johansen 2004; Meng et al. 2005; Neubert et al. 2004).

Issues of sampling and bias in the recorded population must always be considered in electrophysiological analysis of neuronal populations. Neurons without spontaneous or evoked activity are simply not “seen” by the extracellular electrode, and there is evidence from several brain regions that many or even most neurons are silent. In a recent study of hippocampal pyramidal cells, only a small minority of the pyramidal cells calculated from anatomical data to be within range of an extracellular electrode showed spontaneous activity (Henz et al. 2000). Similarly, Towe and Harding (1970) estimated that the largest 5% of pyramidal tract neurons accounted for ~50% of the sample encountered with an extracellular electrode. The ON and OFF cell populations are known to include neurons with different excitabilities and to fluctuate in excitability over time (Barbaro et al. 1989). Although only neurons with robust reflex-related activity were chosen for quantitative study, we informally noted a number of cases in which a neuron that was silent during baseline or that displayed weak or unreliable
activity in the background before mustard oil application began to respond or to respond more robustly after mustard oil. Although anecdotal, this observation highlights the importance of sampling as an issue in extracellular recording studies. Population methods (e.g., multicontact electrodes or an activity measure such as Fos expression if there were an anatomical marker for ON cells) will be needed to determine the numbers and significance of recruitment of ON cells into the active pool for hyperalgesia.

In summary, these experiments showed that secondary thermal hyperalgesia produced by topical application of mustard oil in lightly anesthetized rats requires the RVM and is associated with activation of the ON cell population and suppression of OFF cell firing. NEUTRAL cells showed no change in ongoing firing and did not develop reflex-related changes in firing after mustard oil. These data show that ON cells are recruited during acute inflammation and suggest that these neurons play a role in secondary hyperalgesia. Further studies using selective inactivation of the ON cell population will be required to test that hypothesis.

**GRANTS**

This study was supported by National Institute of Drug Abuse Grant DA-05608.

**REFERENCES**


Hurley RW and Hammond DL. The analgesic effects of supraspinal 


**Huisman AM, Kuypers HG, and Verburgh CA.** Quantitative differences in collateralization of the descending spinal pathways from red nucleus and other brain stem cell groups in rat as demonstrated with the multiple fluorescent retrograde tracer technique. *Brain Res* 209: 271–286, 1981.


