



Contents lists available at ScienceDirect

European Journal of Pain

journal homepage: [www.EuropeanJournalPain.com](http://www.EuropeanJournalPain.com)

## Role of RVM neurons in capsaicin-evoked visceral nociception and referred hyperalgesia

Raul Sanoja<sup>a,b,\*</sup>, Victor Tortorici<sup>a</sup>, Carlos Fernandez<sup>a</sup>, Theodore J. Price<sup>b,1</sup>, Fernando Cervero<sup>b</sup>

<sup>a</sup>Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 20632, Caracas 1020A, Venezuela

<sup>b</sup>Anesthesia Research Unit (Faculty of Medicine), Faculty of Dentistry and McGill Center for Research on Pain McGill University, Montreal, Quebec, Canada

### ARTICLE INFO

#### Article history:

Received 10 December 2008

Received in revised form 7 April 2009

Accepted 7 April 2009

Available online xxx

#### Keywords:

Capsaicin

Descending facilitation

Hyperalgesia

AP5

NMDA

### ABSTRACT

Most forms of visceral pain generate intense referred hyperalgesia but the mechanisms of this enhanced visceral hypersensitivity are not known. The on-cells of the rostral ventromedial medulla (RVM) play an important role in descending nociceptive facilitation and can be sensitized to somatic mechanical stimulation following peripheral nerve injury or hindpaw inflammation. Here we have tested the hypothesis that visceral noxious stimulation sensitizes RVM ON-like cells, thus promoting an enhanced descending facilitation that can lead to referred visceral hyperalgesia. Intracolonic capsaicin instillation (ICI) was applied to rats in order to create a hyperalgesic state dependent on noxious visceral stimulation. This instillation produced acute pain-related behaviors and prolonged referred hyperalgesia that were prevented by the RVM microinjection of AP5, an NMDA selective antagonist. In electrophysiological experiments, ON-like RVM neurons showed ongoing spontaneous activity following ICI that lasted for ~ 20 min and an enhanced responsiveness to von Frey and heat stimulation of the hindpaw and to colorectal distention (CRD) that lasted for at least 50 min post capsaicin administration. Moreover, ON-like cells acquired a novel response to CRD and responded to heat stimulation in the innocuous range. OFF-like neurons responded to capsaicin administration with a brief (<5 min) inhibition of activity followed by an enhanced inhibition to noxious stimulation and a novel inhibition to innocuous stimulation (CRD and heat) at early time points (10 min post capsaicin). These results support the hypothesis that noxious visceral stimulation may cause referred hypersensitivity by promoting long-lasting sensitization of RVM ON-like cells.

© 2009 European Federation of Chapters of the International Association for the Study of Pain. Published by Elsevier Ltd. All rights reserved.

Cite this article as: Raul Sanoja, Victor Tortorici, Carlos Fernandez, Theodore J. Price, Fernando Cervero, Role of RVM neurons in capsaicin-evoked visceral nociception and referred hyperalgesia. *Eur J Pain* xxx (2009) xxx–xxx [doi:10.1016/j.ejpain.2009.04.006]

Full text available online on ScienceDirect<sup>®</sup>, [www.sciencedirect.com](http://www.sciencedirect.com).

### 1. Introduction

In humans, referred hyperalgesia to somatic areas is an important symptom of visceral irritation or inflammation (Giamberardino, 1999; Giamberardino, 2000; Vergnolle, 2008). Peripheral mechanisms of nociceptor sensitization are well documented (Farmer and Aziz, 2008; Gasbarrini et al., 2008) but the central mechanisms that cause referred hyperalgesia, as a result of this peripheral sensitization, are not completely understood. The

rostral ventromedial medulla (RVM) contains a network of neurons that have well identified nociceptive-response profiles associated with pain modulation (Fields et al., 1983; Brink and Mason, 2004; Fields, 2004). Recent electrophysiological studies have shown that after peripheral nerve injury the RVM putative pain facilitating neurons, called ON cells, become sensitized and that level of sensitization is functionally linked to hyperalgesic responses in superficial tissues (Carlson et al., 2007; Goncalves et al., 2007). The RVM's role in nerve injury-induced hyperalgesia is further supported by anatomical lesions of its descending projections (Ossipov et al., 2000), by the pharmacological manipulation of the RVM output (Chen et al., 2004; Xie et al., 2005), and by the selective ablation of RVM pain facilitating neurons (Porreca et al., 2001; Burgess et al., 2002).

Several studies also suggest a crucial role for the RVM in the behavioral and physiological responses to noxious visceral

\* Corresponding author. Present address: University of Arizona, Department of Pharmacology, 1501 N Campbell Ave., Bldg. 221, RM 660, Tucson AZ 85724, USA. Tel.: +520 626 4286.

E-mail address: [sanoja@email.arizona.edu](mailto:sanoja@email.arizona.edu) (R. Sanoja).

<sup>1</sup> Present address: University of Arizona, Department of Pharmacology, 1501 N Campbell Ave., Bldg. 221, RM 660, Tucson AZ 85724, USA.

stimulation. In an urinary bladder distention model, the electrical stimulation of the RVM produced an intensity-dependent inhibition of the visceromotor response evoked by bladder distension that was dependent on endogenous opioidergic systems (Randich et al., 2008). In addition, during colon-rectal distention (CRD), the electrical stimulation of the RVM produced biphasic effects (inhibition with high stimulating currents and facilitation with low currents) (Zhuo and Gebhart, 2002). Also, chemical intracolonic irritants produce visceral hyperalgesia in rodents (Laird et al., 2001) and this response can be attenuated in a dose-dependent manner by the RVM microinjection of APV, a selective NMDA receptor antagonist (Coutinho et al., 1998). Based on these observations, we have tested the hypothesis that RVM neurons can be sensitized by the activation of visceral nociceptors and can thus contribute to the enhanced sensitivity observed in somatic regions during visceral pain.

## 2. Methods

All experimental procedures were carried out on male Sprague–Dawley rats (240–300 g). Behavioral experiments were done at the *Instituto Venezolano de Investigaciones Científicas*, IVIC (Venezuela) and electrophysiology experiments were done at McGill University (Canada). Animals used at IVIC were born and bred inside of the Institution and the ones used in McGill University were purchased from Charles Rivers (Boucherville, Canada) and kept in the McGill Animal Holding Facility until the day of the experiment. All animal experiments were approved by IVIC's Bioethical Committee for Animal Research and the McGill University Institutional Review Board and followed guidelines established by the International Association for the Study of Pain and the Society for Neuroscience.

### 2.1. Behavioral tests

Before testing, the animals were habituated to the testing area for 30 min in a plexiglass box (12.5 × 18 × 15 cm) with a wire mesh floor.

#### 2.1.1. Intracolonic capsaicin instillation (ICI)

We used the test originally developed for mice (Laird et al., 2001) and adapted for use in rats. To proceed with the intracolonic injection we first applied petroleum jelly (Vaseline) in the perianal area to avoid the stimulation of somatic areas by contact with the irritant, then 200 µl of capsaicin (0.1% w/vol., Tocris, MO, USA) or 0.9% saline (same volume) was administered by introducing a transparent cannula (Plastic catheter, 1.02 mm OD, Portex, Herts, UK) 7 cm long into the colon via the anus. Right after the instillation each rat was placed in the plexiglass box. The spontaneous behavior to this stimulus was observed and counted directly for 20 min. Postures defined as abdominal nociceptive-related behaviors were: hunching, hump-backed position, abdominal retractions, licking of the abdomen, stretching the abdomen, and squashing the lower abdomen against the wire mesh (Wesselmann et al., 1998; Laird et al., 2001).

#### 2.1.2. Mechanical stimulation of the abdomen

The frequency of withdrawal responses to the application of a single von Frey filament to the abdomen was used as a test of referred mechanical hyperalgesia. A calibrated von Frey filament with the force of 45 g was applied 10 times to the abdomen and the number of positive withdrawal responses noted. The filament was applied for ~2 s with an inter stimulus interval of 5–10 s. Care was taken not to stimulate the same point twice in succession to avoid learning or sensitization.

### 2.2. Electrophysiology recordings

Rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.). Rectal temperature was kept at ~37 °C with a feedback controlled electric blanket. One catheter was placed in the left carotid artery for continuous arterial blood pressure recordings and another in the left jugular vein for injection of anesthetic. The trachea was cannulated to allow continuous end-tidal CO<sub>2</sub> recordings as well as to provide humidified air. The level of anesthesia was maintained (pentobarbital sodium 10–12 mg/kg/h) such that there were no reflex motor responses on application of noxious stimuli and corneal reflexes were also absent. Pupillary constriction was also monitored and used as an indicator of adequate anesthesia. Thereafter, each rat was placed in a stereotaxic apparatus, a hole was drilled in the skull over the cerebellum, and the dura mater was removed to allow placement of an electrode in the RVM. Typically, we waited more than one hour between the exposure of the cerebellum and the start of recordings, in order to reach the same anesthesia level in all animals used in these experiments.

Intracolonic capsaicin produces a long-lasting hyperalgesic state characterized by strong nociceptive-related abdominal and somatic behaviors. This response does not allow the use of lightly anesthetized rats in the electrophysiology studies to conduct classical ON- and OFF-cells recordings correlated with tail flick reactions (Fields et al., 1983). Instead, we verified in each experiment the stereotaxic coordinates (Paxino and Watson, 1998) to ensure that the tip of the electrode was within RVM boundaries and, in addition, each neuron was characterized for baseline nociceptive properties. For this reason we refer to these neurons as ON-like and OFF-like cells rather than ON- and OFF-cells, respectively. Non-reflex withdrawal-dependent classification of RVM ON- and OFF-cells has been used previously using the classical ON- and OFF-cell nomenclature (Pertovaara et al., 2001; Ansah et al., 2008; Pacharinsak et al., 2008); however, we have chosen to use the ON- and OFF-like cell terminology to avoid any undue confusion over reflex withdrawal classification.

#### 2.2.1. Recording techniques

Recordings were made with tungsten microelectrodes (9–12 MΩ, FHC Inc., ME, USA). The electrical activity of the neurons was amplified, filtered, displayed on an oscilloscope, digitized by a computer interface (CED 1401, Cambridge Electronic Design, Cambridge, UK), and analyzed with a computer running Spike2 software (CED). All data were stored for off-line analysis. One ON-like or OFF-like neuron was recorded per animal during each experiment. NEUTRAL-like cells were analyzed only off-line in those experiments where data was captured from these cells with an ON- or an OFF-like cell.

#### 2.2.2. Characterization of the neurons

An RVM nociceptive neuron was chosen for further study if its activity changed because of noxious pinch, stimulation of the tail with a small toothless forceps or radiant noxious heat stimulation of the right hindpaw. Pinch stimulation was applied for 5 s. Radiant heat (ramps of 12 s duration raising the temperature at ~2 °C/s from 30 °C to a maximum of 55 °C) was delivered with a light bulb source with feedback control. These tests were used to confirm that a given RVM neuron showed ON- or OFF-like activity. RVM neurons were classified as OFF-like cells if they had an abrupt inhibition in ongoing activity during the application of a noxious stimulus. ON-like cells were identified by a sudden burst of activity with application of a noxious stimulus. NEUTRAL-like cells were recognized because they did not change their activity in response to noxious stimulation. Cells were only used for analysis if they showed identical responses to heat and pinch stimulation.

In those experiments where the goal was to study the response properties of the neurons after injury, extra innocuous and noxious tests were performed in different body segments. Hindpaw mechanical responsiveness was ascertained by stimulation of the left hindpaw with a calibrated 100 g von Frey filament applied for 5 s. For colonic stimulation, a calibrated colorectal distention (CRD) device was used consisting of a small balloon (latex, 2 cm) secured onto a feeding cannula (18 Ga., Harvard Instruments, MA, USA) placed inside the colon (7 cm from anal orifice) and inflated at an innocuous pressure (up to 15 mm Hg) and to a noxious pressure (up to 80 mm Hg) for 10 s each. The balloon was always fully withdrawn 5 s after noxious pressure was applied. Between innocuous and noxious stimulation trials the pressure was released to wait until neurons recovered their previously ongoing activity.

### 2.2.3. Electrophysiology experimental design

Two experimental designs using different groups of animals were performed:

Spontaneous activity of RVM nociceptive neurons after capsaicin application:

After neuron characterization, the rat received either intracolonic capsaicin or saline instillation, following the same procedure as in the behavioral experiments. The spontaneous activity of the neuron was then monitored for more than an hour. No other stimulation was performed on these animals.

Evoked activity in RVM nociceptive neurons after capsaicin application:

The same experiments were performed in a new group of animals as described before but the von Frey, CRD and heat stimuli were applied 10, 30 and 50 min after the capsaicin application while the activity of the neuron was monitored for up to 2 h.

### 2.2.4. Electrophysiology experimental analysis

Neuronal firing was measured as spikes/s. Baseline (BL) rates were assessed as the mean ongoing activity (spontaneous activity) one minute before starting the neuron characterization and within an interval of capture equal to 30 s prior to any stimulation. Spontaneous activity during the capsaicin application and every 10 min thereafter, corresponds to RVM neuron average activity for the time period with a bin size of 30 s. Neuronal firing elicited by noxious mechanical stimuli or innocuous and noxious CRD was recorded during the time of stimulus application. Firing evoked by radiant heat was recorded in the innocuous range (40–43 °C) and in the noxious range (52–55 °C). Spikes per second for evoked

responses are reported as the number of evoked spikes per second with background activity subtracted.

### 2.3. Microinjection into RVM

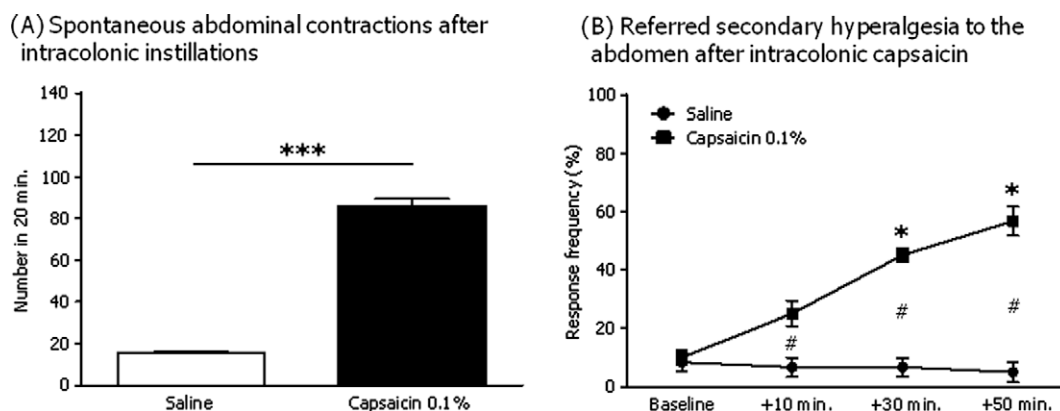
Under thiopental anesthesia (60 mg/kg) a stainless steel 25-gauge guide cannula was stereotaxically (Paxino and Watson, 1998) placed at 2 mm dorsal to the nucleus raphe magnus and anchored to the cranium with stainless steel screws and dental cement. During the following week, the rats were observed and tested to ascertain that they showed no evidence of somatosensory damage. After the habituation period inside the plexiglas box and baseline mechanical testing, a 27-gauge microinjection cannula was introduced through the guide cannula to reach the RVM. This microinjection cannula was previously filled and connected to a Hamilton syringe with polyethylene tubing. Either D-AP5 (2 nmol in 0.2  $\mu$ l saline; Tocris, MO, USA) or 0.2  $\mu$ l saline alone were microinjected into the RVM over a 60 s period. The microinjection cannula was left in place for another 60 s. Rats received only one microinjection, and the experimenter was unaware of its content. Five minutes after the microinjection, capsaicin was instilled in the colon and visceral pain-related behaviors were observed and referred, mechanical hyperalgesia testing was done 30 min after (at the peak of visceral hyperalgesia, see Section 4).

### 2.4. Statistics

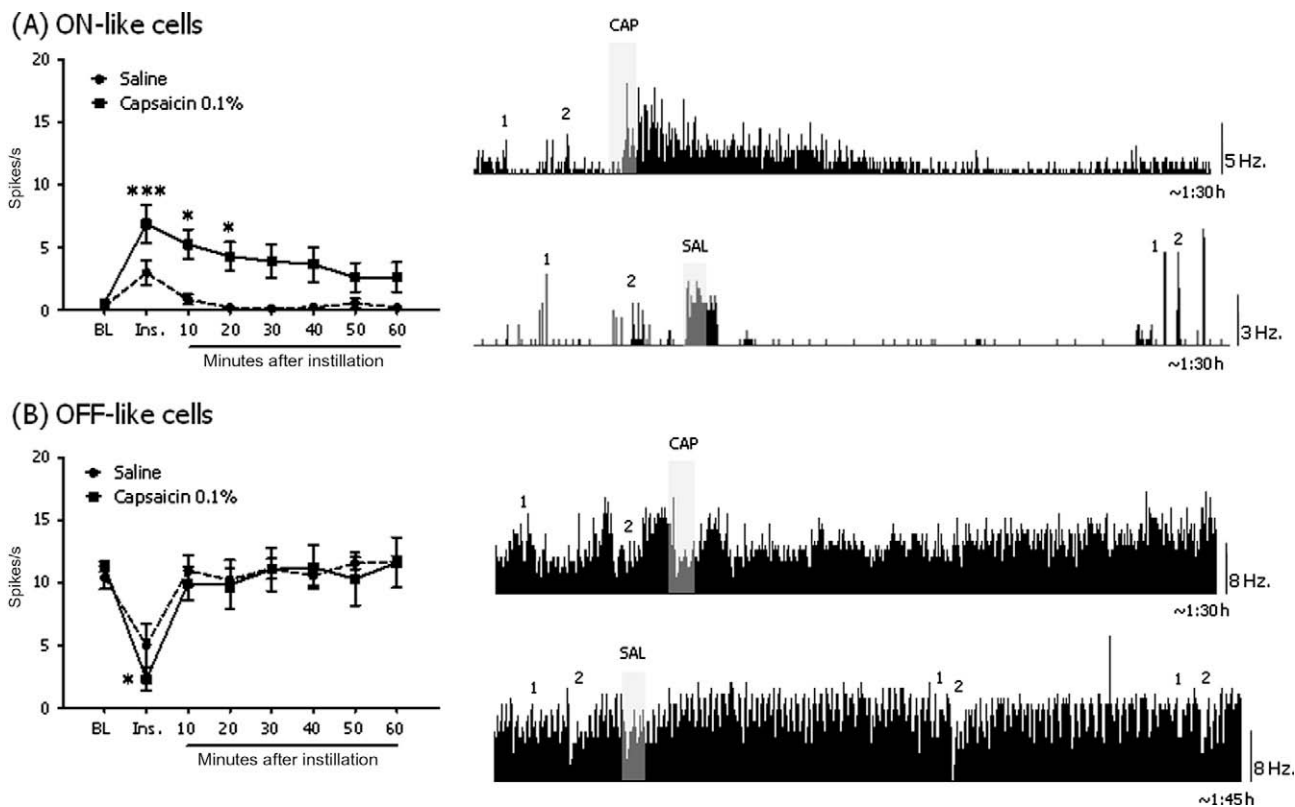
Data was processed using Prism 5 for Windows (GraphPad Software Inc., CA, USA). Results are expressed as mean  $\pm$  S.E.M. Unpaired *t* test was used to compare two groups (Figs. 1 and 6) and Wilcoxon rank test was used to analyze the changes after intracolonic instillation in same group over time (Fig. 1B). In experiments where repeated measures were done Friedman's test with Dunn's post-hoc analysis was used (Fig. 2). In experiments where stimulations were applied repeated measures ANOVA was done with Bonferroni post-hoc analysis (Fig. 3–5).

### 2.5. Histology

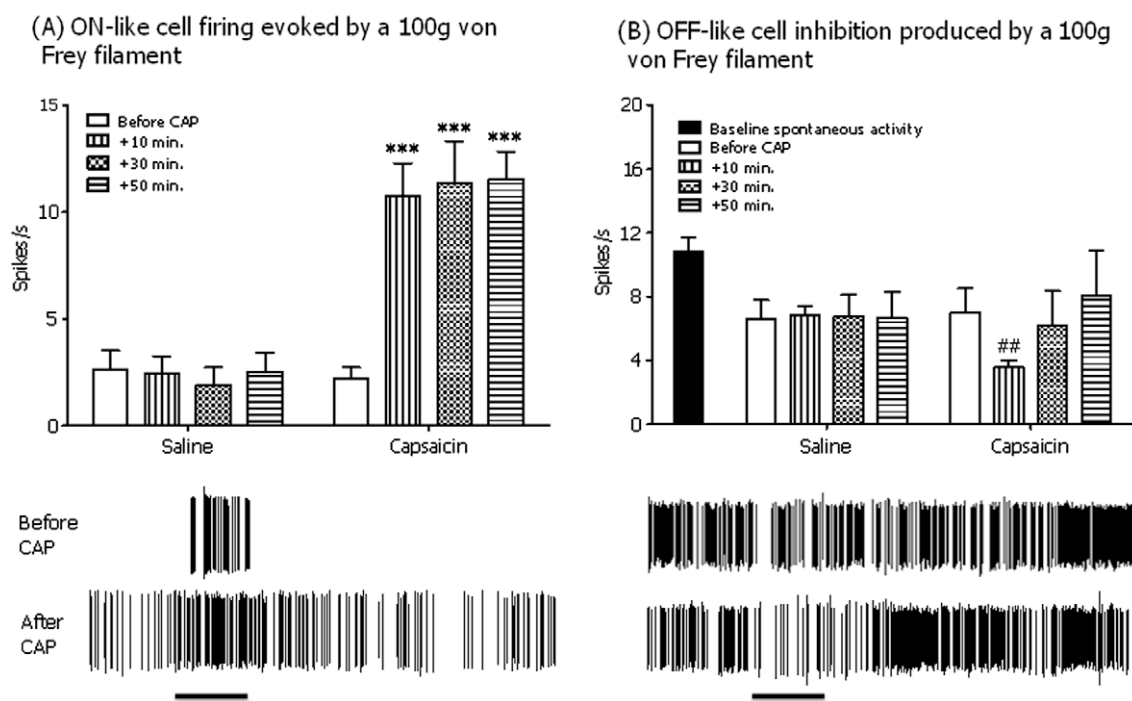
Neuronal recording sites were marked electrolytically. Animals were killed at the end of the experiment with an overdose of Pentothal, and the brain was excised and fixed in 10% formalin. The lesions were identified in 50  $\mu$ m transverse sections with reference to a stereotaxic atlas (Paxino and Watson, 1998).



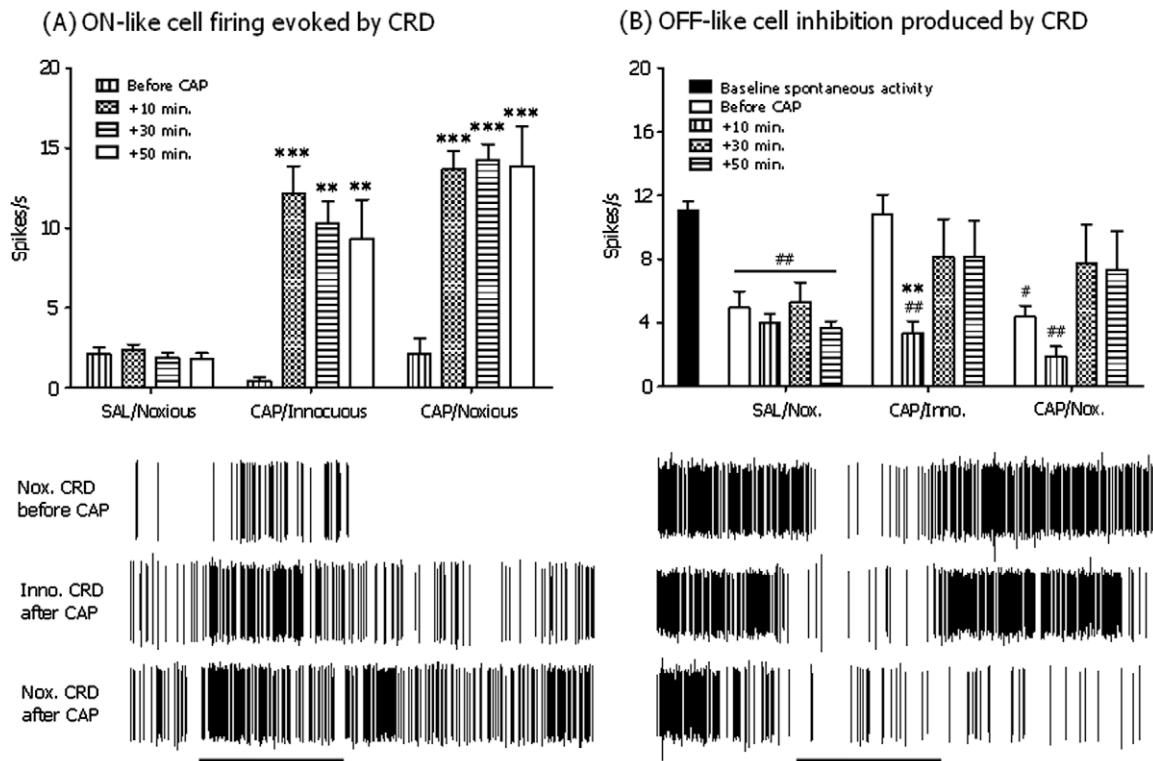
**Fig. 1.** Behavioral responses after intracolonic instillation of capsaicin in rats. (A) Spontaneous abdominal contractions after intracolonic instillations of capsaicin ( $n = 6$ ) or saline ( $n = 6$ ). (B) Referred secondary hyperalgesia to the abdomen after intracolonic instillation of capsaicin. Positive withdrawal response frequencies during abdominal stimulation with a 45 g von Frey filament before (baseline) and after intracolonic capsaicin ( $n = 6$ ) or saline ( $n = 6$ ). \* $p < 0.05$ , \*\*\* $p < 0.0001$  vs. baseline; # $p < 0.05$ , saline vs. capsaicin.



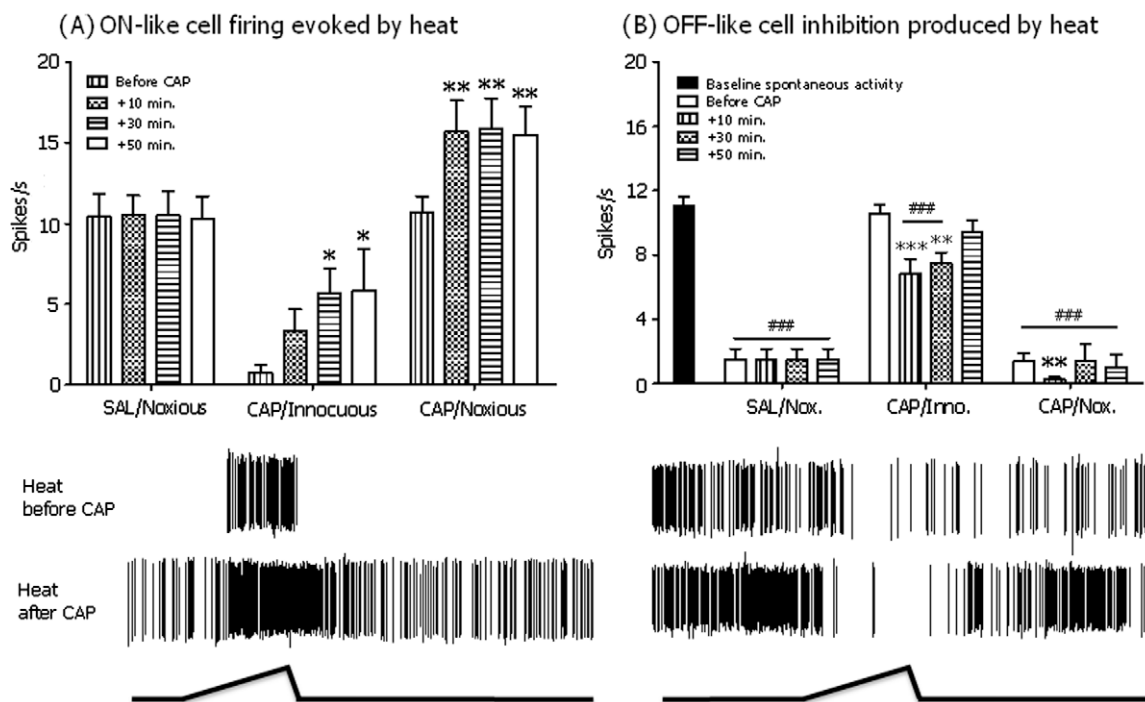
**Fig. 2.** Activity of RVM cells evoked by intracolonic capsaicin. Left, (A) ON-like cell activity evoked by saline ( $n = 6$ ) or capsaicin ( $n = 8$ ). (B) OFF-like cell activity evoked by saline ( $n = 6$ ) or capsaicin ( $n = 8$ ). Right, ratemeter examples of a typical experiment are shown next to the matching graph. Shadow rectangles represent the instillation moment. Applications of noxious tail pinch and radiant heat to the paw are, respectively, indicated by 1 and 2. Bin size = 1 s.  $^*p < 0.05$ ;  $^{***}p < 0.001$ .



**Fig. 3.** Responses of RVM neurons to von Frey filament stimulation. (A) ON- and (B) OFF-like cells before and after intracolonic instillation in rats. In B, baseline spontaneous activity is shown to compare with inhibition of activity evoked by each stimulation. ON-like cells (CAP  $n = 6$ , SAL  $n = 4$ ) and OFF-like cells (CAP  $n = 6$ , SAL  $n = 4$ ).  $^{***}p < 0.001$  vs. before capsaicin (CAP) and  $^{##}p < 0.01$  vs. baseline spontaneous activity. The lower panels show sample activity captured for 40 s (before, during and after each stimulus was applied). The duration of the stimulus is indicated by the bar.



**Fig. 4.** Responses of RVM neurons to colon-rectal distention (CRD). (A) ON- and (B) OFF-like cells before and after intracolonic capsaicin in rats. The upper panel shows mean activity of the RVM cells. In B, baseline spontaneous activity is shown to compare with inhibition of activity evoked by each stimulation. ON-like cells (CAP  $n = 6$ , SAL  $n = 4$ ) and OFF-like cells (CAP  $n = 6$ , SAL  $n = 4$ ).  $^{*}p < 0.01$  and  $^{***}p < 0.001$  vs. before capsaicin (CAP).  $^{#}p < 0.05$  and  $^{##}p < 0.01$  vs. baseline spontaneous activity. Lower panel shows sample activity captured for 40 s (before, during and after each stimulus was applied). The duration of the stimulus is indicated by the bar.



**Fig. 5.** Responses of RVM neurons to radiant heat applied to the right hindpaw. (A) ON- and (B) OFF-like cells before and after intracolonic capsaicin in rats. The upper panel shows mean activity of the RVM cells. In B, baseline spontaneous activity is shown to compare with inhibition of activity evoked after each stimulation. ON-like cells (CAP  $n = 6$ , SAL  $n = 4$ ) and OFF-like cells (CAP  $n = 6$ , SAL  $n = 4$ ).  $^{*}p < 0.05$ ,  $^{**}p < 0.01$  and  $^{***}p < 0.001$  vs. before capsaicin (CAP).  $^{###}p < 0.01$  vs. baseline spontaneous activity. Lower panel shows sample activity captured for 40 s (before, during and after each stimulus was applied). Length of the stimulus is indicated by a ramp (30–55 °C in 12 s).

### 3. Results

#### 3.1. Behavioral reaction to capsaicin

Intracolonic instillation of capsaicin (Fig. 1A) evoked a significant increase ( $p = 0.0022$  vs. saline instillation) in visceral pain-related behaviors. These behaviors were mainly characterized by abdominal contractions. Some abdominal contractions were also seen with intracolonic instillation of saline. These were of a shorter duration, fewer in frequency and were related to the volume injected and colonic distention; a similar phenomenon has been reported in mice (Laird et al., 2001). Intracolonic capsaicin induced abdominal contractions that lasted for 15 min and the dose of capsaicin used did not generate freezing behavior or catalepsy as has been reported in mice (Laird et al., 2001; Sanoja and Cervero, 2005).

Intracolonic capsaicin produced a referred, secondary mechanical hyperalgesia to the abdomen (Fig. 1B). Baseline values were 10% response frequency. In intracolonic capsaicin treated rats, the response frequency to mechanical stimulation was significantly higher than in saline treated animals by 30 min post instillation ( $p = 0.0310$  vs. baseline) and this hyperalgesia lasted at least 50 min ( $p = 0.0345$  vs. baseline).

The following electrophysiology experiments were designed to assess the firing properties of putative RVM pain modulatory neurons during the evolution of intracolonic capsaicin-induced pain-related responses and referred hyperalgesia.

#### 3.2. Physiological reactions to capsaicin

Baseline values for both mean blood pressure (BP) and heart rate (HR) were not significantly different during neuron characterization (CAP vs SAL, BP:  $119.47 \pm 2.51$  vs.  $121.17 \pm 2.82$  mmHg and HR:  $358.42 \pm 4.85$  vs.  $361 \pm 10.29$  bpm) between different rats groups. Rats BP dropped 9.39% right after instillation of capsaicin into the colon, an effect seen for approximately 1 min with the concomitant heart rate increase compensation. This transient vascular change did not affect neuron discharges. We concluded that vascular parameters were only lightly influenced by the dose of capsaicin used and the depth of anesthesia employed.

#### 3.3. Spontaneous neuronal output following intracolonic capsaicin

Intracolonic capsaicin evoked alterations in firing in 70% of the population recorded. In this series of experiments, after the application of capsaicin or saline no further noxious stimulations were made to the animal because the goal was to study spontaneous changes in firing patterns after capsaicin or saline instillation into the colon. Under the level of anesthesia used, most of the ON-like cells chosen had an initial ongoing activity of  $0.5 \pm 0.25$  spike/s before the capsaicin application (Fig. 2A). OFF-like cells included were those with a regular ongoing activity  $11 \pm 0.40$  spike/s (Fig. 2B); under this level of anesthesia spontaneous inhibition in these types of cells were unusual but with noxious stimulation they showed a decrease in their ongoing activity. NEUTRAL-like cells (integral analysis was done off-line), which were differentiated from ON- or OFF-like cells because of their firing properties during characterization (Mason, 1997; Leung and Mason, 1998), did not respond to any noxious stimulation applied to the animals or to the capsaicin challenge. Their mean firing frequency was  $11 \pm 11$  spikes/s.

As expected, intracolonic capsaicin produced a sustainable increase in firing of ON-like cells with long-lasting discharges that were significantly above baseline for 20 min, with a peak activity of  $6.84 \pm 1.47$  spike/s (Fig. 2A). This ON-like cell activity corresponded with the spontaneous pain-related behaviors seen in the conscious animals. Spontaneous discharges continued in these

ON-like neurons following intracolonic capsaicin after the initial 20 min of analysis and, in some cases, the enhanced activity continued for the whole recording period. A temporary but transient increase in maximal discharges of ON-like cells after saline instillation ( $2.97 \pm 1.02$  spikes/s) was also noted. The duration of this change in ON-like cell activity paralleled behavioral changes observed with saline.

OFF-like cell activity after intracolonic capsaicin was not an equivalent mirror-like effect to what was found in ON-like cells (Fig. 2B). Intracolonic capsaicin produced an incomplete inhibition of the OFF-like cell activity of 80%. This inhibition was transient and 10 min after capsaicin instillation into the colon OFF-like cell activity returned to baseline. Intracolonic saline also induced a transient 50% decrease in OFF-like cells firing.

#### 3.4. Neural activity of RVM modulatory neurons evoked by noxious stimulation before and after intracolonic capsaicin

In a different group of rats we performed recordings of ON- and OFF-like cells before and after intracolonic capsaicin or intracolonic saline and compared their baseline (BL) firing activity elicited by noxious stimulation with those elicited at 10, 30 and 50 min after capsaicin. These experiments were then followed for an additional hour after the last stimulation to examine the possibility of response adaptations. Off-line analysis of NEUTRAL-like cells did not show any relation between their activity and noxious mechanical or thermal stimulation, before or after the hyperalgesic state was induced with capsaicin.

In this new series of experiments, all the of neurons that responded to von Frey, CRD and heat during characterization also responded to intracolonic capsaicin indicating that this subpopulation of ON- and OFF-like cells were activated by noxious skin stimulation and by noxious colonic stimulation. Mean levels of activity of ON-like cells prior to stimulation were  $1.5 \pm 0.75$  spikes/s and those of the OFF-like cells were  $11 \pm 0.80$  spikes/s. NEUTRAL-like cell activity was not modified after intracolonic capsaicin by innocuous or noxious activity.

During neuron characterization (prior to intracolonic instillation of capsaicin or saline), von-Frey stimulation in the middle of left hindpaw increased ON-like cell firing rates to  $2.40 \pm 0.20$  spikes/s (Fig. 3A) and reduced OFF-like cell firing rates to  $6.75 \pm 0.51$  spikes/s (Fig. 3B). ON-like cell activity after intracolonic capsaicin instillation was increased 4-fold during von Frey stimulation; a significant increase that was observed during the entire recording period (Fig. 3A, up to 50 min post capsaicin instillation. ANOVA: 4,29  $F = 23.92$ ). The enhanced responsiveness of ON-like cells paralleled the observed behavioral referred hyperalgesia (Fig. 1B). On the other hand, the OFF-like cell activity was reduced significantly in the intracolonic capsaicin group but only during the first 10 min (to  $3.65 \pm 0.42$  spike/s. ANOVA: 4,29  $F = 4.675$ ) as no significant effects were observed at the later time points.

Innocuous CRD (15 mm Hg) before any intracolonic instillation did not change the firing rate of ON- or OFF-like cells, but noxious CRD (80 mm Hg) produced an increment in firing up to  $2.15 \pm 0.92$  spikes/s in ON-like cells and a reduction in firing to  $4.50 \pm 1.00$  spikes/s in OFF-like cells (Fig. 4). In saline treated animals noxious CRD stimulation did not change ON-like cell activity over time (Fig. 4A. ANOVA: 4,19  $F = 1.7$ ) and innocuous stimulation had no effect on ON-like cell activity. After intracolonic capsaicin, CRD produced a 5-fold increase in the activity of ON-like cells which remained throughout the 50 min recording period (Fig. 4A. Innocuous capsaicin ANOVA: 4,29  $F = 12.27$ , and noxious capsaicin ANOVA: 4,29  $F = 23.92$ ). Interestingly, this effect was seen in ON-like cell activity after capsaicin for both innocuous and noxious CRD stimulation (Fig. 4A). OFF-like cells showed inhibition in their activity in response to noxious CRD stimulation at all time points after

intracolonic saline instillation but this change in activity was not altered over time (Fig. 4B. ANOVA: 4,19  $F = 1.616$ ). In saline treated animals OFF-like cells never showed a decrease in activity in response to the innocuous CRD (data not shown). In capsaicin treated animals the OFF-like cell inhibition was enhanced at 10 min post instillation in response to noxious CRD and OFF-like cells acquired a novel inhibition in their firing in response to innocuous CRD at this time point (Fig. 4B. ANOVA: 4,29  $F = 4.858$ ). All OFF-like cell inhibitions were absent in response to noxious CRD stimulation at the 30 and 50 min time points (Fig. 4B. ANOVA: 4,29  $F = 2.977$ ).

Prior to intracolonic instillation, thermal stimulation increased ON-like firing activity up to  $10.50 \pm 1.10$  spikes/s and caused an inhibition in OFF-like cell to  $1.45 \pm 0.50$  spikes/s (Fig. 5). Responses in ON- and OFF-like cells were never observed below  $43^\circ\text{C}$  prior to intracolonic capsaicin or in intracolonic saline treated animals. In animals treated with saline, ON-like cell firing remained unchanged in response to noxious heat (Fig. 5A, ANOVA: 4,19  $F = 66.96$ ) as was the OFF-like cell inhibition (Fig. 5B, ANOVA: 4,19  $F = 6.591$ ). After intracolonic capsaicin, ON-like cell activity was increased 1.5-fold in the noxious heat range ( $> 43^\circ\text{C}$ , ANOVA: 3,23  $F = 6.984$ ) at all time points and ON-like cell discharges were observed in the innocuous heat range ( $< 43^\circ\text{C}$ , ANOVA: 3,23  $F = 3.456$ ) starting at the 30 min time point and continuing to the 50 min time point (Fig. 5A). In the case of OFF-like cells, the inhibition was greater at the 10 min time point after intracolonic capsaicin instillation in the noxious range (Fig. 5B. ANOVA: 4,29  $F = 36.23$ ). Moreover, OFF-like cells displayed a novel inhibition in their discharge in the innocuous temperature range at 10 and 30 min post capsaicin instillation (Fig. 5B. ANOVA: 4,29  $F = 6.032$ ).

### 3.5. Behavioral reaction to capsaicin after RVM-NMDA receptor blockade

Our previous behavioral experiments showed that intracolonic instillation of capsaicin-evoked visceral pain-related behaviors and a referred hyperalgesia to the abdomen. This same treatment also enhanced ON-like cell spontaneous and evoked activity. The following series of experiments were designed to link changes in RVM neuron activity with nociceptive behaviors and referred hyperalgesic responses evoked by irritation of the colon.

Animals which received saline into the RVM 5 min prior to intracolonic capsaicin showed visceral pain-related behaviors (Fig. 6A) and these behaviors were comparable with those seen in rats which did not have any RVM manipulation and also were instilled with capsaicin (Fig. 1A). Microinjection of the NMDA receptor antagonist, AP5, into the RVM 5 min prior to intracolonic

capsaicin attenuated capsaicin-evoked visceral pain-related behaviors ( $p < 0.0001$ , SAL vs. AP5). Preemptive treatment with AP5 also blocked referred secondary hyperalgesia to the abdomen (Fig. 6B) measured 50 min after intracolonic instillation of capsaicin.

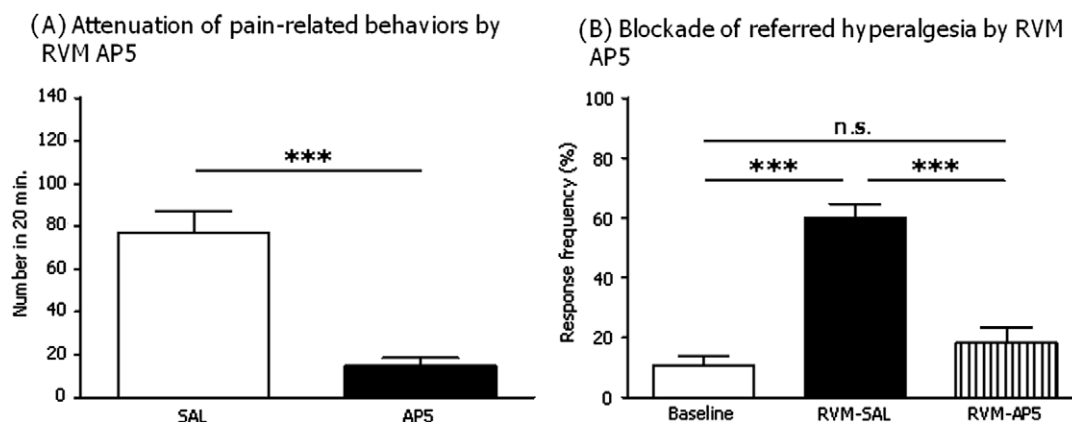
## 4. Discussion

Our results indicate that RVM ON-like cells are sensitized by capsaicin instillation into the colon and that these cells are involved in the spontaneous pain caused by noxious visceral stimulation as well as in the referred hyperalgesic state that persists after colonic irritation.

We have shown that intracolonic capsaicin instillation in the rat creates pain-related behaviors and a long-lasting referred abdominal hyperalgesia. The results of our study show that capsaicin instillation into the colon stimulates ongoing activity in ON-like RVM neurons that parallels the duration of pain-related behaviors and causes enhanced ON-like cell activity when these cells are activated by noxious somatic or visceral stimulation. ON-like RVM neurons also acquire novel responses to innocuous stimulation (CRD or heat) following intracolonic capsaicin instillation. Moreover, we have also shown that the NMDA antagonist AP5, which is known to block RVM ON cell activity (Xu et al., 2006), is also able to attenuate visceral hyperalgesia and block the referred secondary hyperalgesia to the abdomen provoked by intracolonic capsaicin instillation.

These findings suggest that the development of somatic and visceral hyperalgesia and allodynia in this model is paralleled by an increase in the responsiveness of RVM ON-like cells, which supports the hypothesis that RVM ON cells are involved not only in the nociceptive behaviors evoked by visceral stimulation but also in the referred hyperalgesia resulting from noxious stimulation of visceral organs.

This notion is in line with the results of studies in neuropathic rats where ON cells also increased their firing rate in response to noxious stimulation following peripheral nerve injury (Carlson et al., 2007; Goncalves et al., 2007) and with studies involving mustard oil evoked paw hyperalgesia, where some ON-cells increased their firing rates and others that were inactive before mustard oil application became responsive (Kincaid et al., 2006). On the other hand, pharmacological blockade of descending facilitation from the RVM is also capable of inhibiting referred allodynia in visceral pain models (Vera-Portocarrero et al., 2006a) as well as peripheral nerve injury-induced allodynia in the spinal nerve ligation (Wei and Pertovaara, 1999a,b; Burgess et al., 2002; Vera-



**Fig. 6.** Behavioral responses after microinjection of AP5 or saline into the RVM followed by intracolonic capsaicin in rats. (A) AP5 (2 nmol in 0.2  $\mu\text{l}$ ,  $n = 6$ ) or SAL (0.2  $\mu\text{l}$ ,  $n = 6$ ) microinjections into RVM were done 5 min before intracolonic instillations of capsaicin 0.1% and pain-related behaviors are shown. (B) Positive withdrawal response frequencies during abdominal stimulation with a 45 g von Frey filament before RVM microinjections (baseline) and 50 min after intracolonic capsaicin. Same animals as in (A). \*\*\*  $p < 0.01$ .

Portocarrero et al., 2006b) or the enhanced responses of wide-dynamic-range neurons in the chronic constriction injury (CCI) model (Sanoja et al., 2008).

Activation of ON cells in the RVM is mediated by NMDA receptors as demonstrated by the fact that NMDA antagonists significantly decrease ON cell firing without changes in OFF or NEUTRAL cell firing patterns (Heinricher and McGaraughy, 1998). Blockade of this receptor in the RVM with MK-801 or kynurenic acid, two different NMDA antagonists, also attenuates behavioral pain responses in neuropathic rats (Wei and Pertovaara, 1999b; Sanoja et al., 2008). In our study we have shown that selective blockade of ON-like cells in RVM with AP5 is also capable of inhibiting primary and secondary visceral hyperalgesia. Taken together, these findings indicate that increased RVM-dependent descending facilitation is likely to accompany hyperalgesia and/or allodynia in a variety of chronic pain conditions, including referred visceral hyperalgesia.

Ten minutes after ICI of capsaicin we observed an enhanced OFF-like cell inhibition evoked by mechanical (CRD and von Frey) or noxious heat stimulation. Interestingly, in the case of innocuous stimulation with CRD or heat, OFF-like cells acquired a novel inhibitory response that was still present 30 min after ICI. It has been reported that, after peripheral nerve injury, OFF-cell inhibition in the RVM is increased and OFF-cells acquire novel response properties to lower force von Frey stimulation that match the lowered reflex withdrawal thresholds (Carlson et al., 2007). As noted above, we did not observe similar changes in response to CRD stimulation aside from those at early time points post ICI of capsaicin. On the other hand, the attenuation of the OFF-like cell inhibition that we observed may be expected to cause a net increase in descending facilitation further exacerbating visceral hyperalgesia or spontaneous pain following colonic irritation. These differences in OFF-cell responses highlight the differences in RVM neuronal responses to visceral versus somatic stimulation in the presence of injury or inflammation. Indeed, it has been suggested that different subpopulations of ON- and OFF-cells can be identified in the RVM depending on whether or not they receive somatic only or both somatic and visceral inputs (Brink and Mason, 2003, 2004).

Dorsal horn neurons that receive inputs from afferents that innervate the colon have convergent inputs from somatic regions (Olivar et al., 2000). Colonic irritation with capsaicin would be expected to sensitize these neurons in such a manner that their convergent inputs might also be sensitized. The alterations observed in the response properties of ON-like neurons in the RVM may reflect increased activity in these neurons that drive, in turn, an increase in ON-like cell responses in the RVM. On the other hand, the lack of parallel changes in OFF-like cell activity for ongoing firing in the 30 min period following capsaicin instillation, and in response to noxious mechanical stimulation at later time points, may indicate that higher brain centers (Guan et al., 2003; Ren and Dubner, 2007) may drive the changes in RVM output caused by colonic irritation. For instance, it has been reported that intraplantar injection of capsaicin causes a rapid increase in RVM 5-HT levels (Smith et al., 2006) and that intraplantar capsaicin-evoked hyperalgesia is blocked by NK-1 receptor antagonist injection into the RVM while NK-1 antagonists have no effect in normal animals (Pacharinsak et al., 2008).

Our results do not agree with previous reports by Brink and Mason (Brink and Mason, 2003; Brink et al., 2006). These authors, using tail or paw noxious heat stimulation to classify ON and OFF cells, found no relationship between the neuronal responses to CRD and to somatic noxious stimulation. In their experiments nearly equal numbers of ON cells were excited, inhibited or unaffected by CRD. A similar type of response was observed for neutral cells while almost half of the OFF cells were excited and smaller numbers were inhibited or unaffected by colon stimulation. Based

on these results the authors suggested that the responses to CRD and to heat noxious stimulation must be related in a way that would be obscured by the cell classification system they used. Therefore they compared the change in the number of spikes evoked during the total length of the CRD (20 s) and heat stimuli (10 s) regardless of whether or not those changes met their criteria for a response. This minimizes the contribution of a reflex related response and challenges the traditional criteria used to characterize RVM cells. It remains to be demonstrated that the disparity of responses to CRD and to somatic noxious heat reported by Brink and colleagues reflects functional subclasses of RVM cells. Their hypothesis implies that ON cells excited by CRD facilitate responses to CRD itself, which in turn augments excitation of OFF cells that then will act to suppress somatic stimulation (Brink et al., 2006). Our results showed responses to visceral and somatic stimulation that were always in the same direction, in agreement with previous reports that used i.p. injections of bradykinin (Guilbaud et al., 1980), and in line with the referred somatic allodynia observed behaviorally after intracolonic capsaicin.

Due to the long-lasting hyperalgesic state induced by intracolonic capsaicin we decided not to use lightly anesthetized rats in the electrophysiology studies which prevented the use of a traditional ON- and OFF-cell characterization based on the tail flick reflex. However, we believe that the responses of the RVM cells following intracolonic capsaicin and the behavioral responses after RVM-NMDA receptor blocking create an adequate level of evidence to assume that our cells reflect the typical RVM activity associated with the descending control of nociception and promote a good scenario to explain the referred hyperalgesic responses evoked by the colon irritation. Our results suggest that RVM responses to somatic noxious stimulation can be used to predict the results evoked by other modalities of stimulation applied in different areas of the experimental animals which not only expands but also reinforces the hypothesis of an endogenous pain modulatory system.

In conclusion, we have shown that intracolonic capsaicin in the rat causes pain-related behaviors that correlate with sustained enhanced activity in RVM ON-like cells and with more transient changes in OFF-like cells. Moreover, intracolonic capsaicin injection evokes behavioral referred hyperalgesia in the rat and an increased ON-like cell response to noxious stimulation of the colon and these behavioral manifestations can be blocked by NMDA receptor antagonist microinjection into the RVM. These findings link functional changes in RVM ON-like neural activity with behavioral endpoints related to referred, somatic hyperalgesia after colonic irritation. These results indicate that painful stimulation of the viscera is capable of altering the output of the RVM such that putative descending facilitatory neurons increase their basal and evoked firing rates contributing to spontaneous pain as well as primary and referred hyperalgesia after visceral irritation.

## References

- Ansah OB, Goncalves L, Almeida A, Pertovaara A. Enhanced pronociception by amygdaloid group I metabotropic glutamate receptors in nerve-injured animals. *Exp Neurol* 2008;216:66–74.
- Brink TS, Hellman KM, Lambert AM, Mason P. Raphe magnus neurons help protect reactions to visceral pain from interruption by cutaneous pain. *J Neurophysiol* 2006;96:3423–32.
- Brink TS, Mason P. Raphe magnus neurons respond to noxious colorectal distension. *J Neurophysiol* 2003;89:2506–15.
- Brink TS, Mason P. Role for raphe magnus neuronal responses in the behavioral reactions to colorectal distension. *J Neurophysiol* 2004;92:2302–11.
- Burgess SE, Gardell LR, Ossipov MH, Malan Jr TP, Vanderah TW, Lai J, et al. Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. *J Neurosci* 2002;22:5129–36.
- Carlson JD, Maire JJ, Martenson ME, Heinricher MM. Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury. *J Neurosci* 2007;27:13222–31.
- Chen Q, King T, Vanderah TW, Ossipov MH, Malan Jr TP, Lai J, et al. Differential blockade of nerve injury-induced thermal and tactile hypersensitivity by

- systemically administered brain-penetrating and peripherally restricted local anesthetics. *J Pain* 2004;5:281–9.
- Coutinho SV, Urban MO, Gebhart GF. Role of glutamate receptors and nitric oxide in the rostral ventromedial medulla in visceral hyperalgesia. *Pain* 1998;78:59–69.
- Farmer AD, Aziz Q. Recent advances in chronic visceral pain. *Curr Opin Support Palliat Care* 2008;2:116–21.
- Fields H. State-dependent opioid control of pain. *Nat Rev Neurosci* 2004;5:565–75.
- Fields HL, Bry J, Hentall I, Zorman G. The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. *J Neurosci* 1983;3:2545–52.
- Gasbarrini G, Montalto M, Santoro L, Curigliano V, D'Onofrio F, Gallo A, et al. Intestine: organ or apparatus? *Dig Dis* 2008;26:92–5.
- Giamberardino MA. Recent and forgotten aspects of visceral pain. *Eur J Pain* 1999;3:77–92.
- Giamberardino MA. Visceral hyperalgesia. In: Devor M, Rowbotham MC, Wiesenfeld-Hallin Z, editors. *Proceedings of the 9th world congress on pain*. Seattle: IASP Press; 2000. p. 500–23.
- Goncalves L, Almeida A, Pertovaara A. Pronociceptive changes in response properties of rostral ventromedial medullary neurons in a rat model of peripheral neuropathy. *Eur J Neurosci* 2007;26:2188–95.
- Guan Y, Guo W, Zou S-P, Dubner R, Ren K. Inflammation-induced upregulation of AMPA receptor subunit expression in brain stem pain modulatory circuitry. *Pain* 2003;104:401–13.
- Guilbaud G, Peschanski M, Gautron M, Binder D. Responses of neurons of the nucleus raphe magnus to noxious stimuli. *Neurosci Lett* 1980;17:149–54.
- Heinricher MM, McGaraughty S. Analysis of excitatory amino acid transmission within the rostral ventromedial medulla: implications for circuitry. *Pain* 1998;75:247–55.
- Kincaid W, Neubert MJ, Xu M, Kim CJ, Heinricher MM. Role for medullary pain facilitating neurons in secondary thermal hyperalgesia. *J Neurophysiol* 2006;95:33–41.
- Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F. A new model of visceral pain and referred hyperalgesia in the mouse. *Pain* 2001;92:335–42.
- Leung CG, Mason P. Physiological survey of medullary raphe and magnocellular reticular neurons in the anesthetized rat. *J Neurophysiol* 1998;80:1630–46.
- Mason P. Physiological identification of pontomedullary serotonergic neurons in the rat. *J Neurophysiol* 1997;77:1087–98.
- Olivar T, Cervero F, Laird JM. Responses of rat spinal neurones to natural and electrical stimulation of colonic afferents: effect of inflammation. *Brain Res* 2000;866:168–77.
- Ossipov MH, Hong Sun T, Malan Jr P, Lai J, Porreca F. Mediation of spinal nerve injury induced tactile allodynia by descending facilitatory pathways in the dorsolateral funiculus in rats. *Neurosci Lett* 2000;290:129–32.
- Pacharinsak C, Khasabov SG, Beitz AJ, Simone DA. NK-1 receptors in the rostral ventromedial medulla contribute to hyperalgesia produced by intraplantar injection of capsaicin. *Pain* 2008;30:34–46.
- Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. San Diego: Academic Press; 1998.
- Pertovaara A, Keski-Vakkuri U, Kalmari J, Wei H, Panula P. Response properties of neurons in the rostral ventromedial medulla of neuropathic rats: attempted modulation of responses by [1DMe]NPYF, a neuropeptide FF analogue. *Neuroscience* 2001;105:457–68.
- Porreca F, Burgess SE, Gardell LR, Vanderah TW, Malan Jr TP, Ossipov MH, et al. Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu-opioid receptor. *J Neurosci* 2001;21:5281–8.
- Randich A, Mebane H, DeBerry JJ, Ness TJ. Rostral ventral medulla modulation of the visceromotor reflex evoked by urinary bladder distension in female rats. *J Pain* 2008;9:920–6.
- Ren K, Dubner R. Pain facilitation and activity-dependent plasticity in pain modulatory circuitry: role of BDNF-TrkB signaling and NMDA receptors. *Mol Neurobiol* 2007;35:224–35.
- Sanoja R, Cervero F. Estrogen-dependent abdominal hyperalgesia induced by ovariectomy in adult mice: a model of functional abdominal pain. *Pain* 2005;118:243–53.
- Sanoja R, Vanegas H, Tortorici V. Critical role of the rostral ventromedial medulla in early spinal events leading to chronic constriction injury neuropathy in rats. *J Pain* 2008;9:532–42.
- Smith VA, Beyer CE, Brandt MR. Neurochemical changes in the RVM associated with peripheral inflammatory pain stimuli. *Brain Res* 2006;1095:65–72.
- Vera-Portocarrero LP, Xie JY, Kowal J, Ossipov MH, King T, Porreca F. Descending facilitation from the rostral ventromedial medulla maintains visceral pain in rats with experimental pancreatitis. *Gastroenterology* 2006a;130:2155–64.
- Vera-Portocarrero LP, Zhang ET, Ossipov MH, Xie JY, King T, Lai J, et al. Descending facilitation from the rostral ventromedial medulla maintains nerve injury-induced central sensitization. *Neuroscience* 2006b;140:1311–20.
- Vergnolle N. Postinflammatory visceral sensitivity and pain mechanisms. *Neurogastroenterol Motil* 2008;20(Suppl. 1):73–80.
- Wei H, Pertovaara A. Influence of preemptive treatment with MK-801, an N-methyl-D-aspartate receptor antagonist, on development of neuropathic symptoms induced by spinal nerve ligation in the rat. *Anesthesiology* 1999a;91:313–6.
- Wei H, Pertovaara A. MK-801, an NMDA receptor antagonist, in the rostral ventromedial medulla attenuates development of neuropathic symptoms in the rat. *Neuroreport* 1999b;10:2933–7.
- Wesselmann U, Czakanski PP, Affaitati G, Giamberardino MA. Uterine inflammation as a noxious visceral stimulus: behavioral characterization in the rat. *Neuroscience Letters* 1998;246:73–6.
- Xie JY, Herman DS, Stiller CO, Gardell LR, Ossipov MH, Lai J, et al. Cholecystokinin in the rostral ventromedial medulla mediates opioid-induced hyperalgesia and antinociceptive tolerance. *J Neurosci* 2005;25:409–16.
- Xu M, Kim CJ, Neubert MJ, Heinricher MM. NMDA receptor-mediated activation of medullary pro-nociceptive neurons is required for secondary thermal hyperalgesia. *Pain* 2006;127:253–62.
- Zhuo M, Gebhart GF. Facilitation and attenuation of a visceral nociceptive reflex from the rostral ventral medulla in the rat. *Gastroenterology* 2002;122:1007–19.