Pain-facilitating medullary neurons contribute to opioid-induced respiratory depression

Abbreviated title: Separating analgesia from respiratory depression

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ABSTRACT

Respiratory depression is a therapy-limiting side-effect of opioid analgesics, yet our understanding of the brain circuits mediating this potentially lethal outcome remains incomplete. Here we studied the contribution of the rostral ventromedial medulla (RVM), a region long implicated in pain modulation and homeostatic regulation, to opioid-induced respiratory depression. Microinjection of the µ-opioid agonist DAMGO in the RVM of lightly anesthetized rats produced both analgesia and respiratory depression, showing that neurons in this region can modulate breathing. Blocking opioid action in the RVM by microinjecting the opioid antagonist naltrexone reversed the analgesic and respiratory effects of systemically administered morphine, showing that this region plays a role in both the analgesic and respiratory depressant properties of systemically administered morphine. The distribution of neurons directly inhibited by RVM opioid microinjection was determined using a fluorescent opioid peptide, dermorphin-Alexa594, and found to be concentrated in and around the RVM. The non-opioid analgesic improgan, like DAMGO, produced antinociception but, unlike DAMGO, stimulated breathing when microinjected into the RVM. Concurrent recording of RVM neurons during improgan microinjection showed that this agent activated RVM ON-cells, OFF-cells and NEUTRAL-cells. Since opioids are known to activate OFF-cells but suppress ON-cell firing, the differential respiratory response to these two analgesic drugs is best explained by their opposing effects on the activity of RVM ON-cells. These findings show that pain relief can be separated pharmacologically from respiratory depression, and identify RVM OFF-cells as important central targets for continued development of potent analgesics with fewer side-effects.

Keywords: rostral ventromedial medulla, analgesia, improgan, pain-modulation, rat
INTRODUCTION

While opioids remain the most powerful tool available for treating moderate to severe pain, their utility is limited by side-effects, especially potentially lethal respiratory depression. Given this risk and the low therapeutic index for many opioids, clinicians often under-treat pain (Nickerson and Attaran 2012; Webster et al. 2011). Despite the clinical and social significance of opioid-induced respiratory depression, the underlying neural mechanisms and circuits are still not fully understood.

In contrast to respiratory depression, the analgesic actions of opioids have been studied intensely, and we now know that these agents produce pain relief by engaging an endogenous brainstem pain modulatory system. This system is the driving force behind the natural suppression or enhancement of pain in different behavioral states (Fields 2004). Its output influences pain behavior via projections from the rostral ventromedial medulla (RVM) to dorsal horn nociceptive circuits. Inactivation or lesion of the RVM can interfere with the analgesic effects of systemically administered opioids, and µ-opioid agonists applied directly in the RVM produce a potent analgesia (Fields et al. 2006; Proudfit 1980; 1981).

Two classes of RVM neurons, the “ON-cells” and “OFF-cells,” respond to opioids (Fields et al. 2006; Heinricher et al. 2009). ON-cells facilitate nociception, and these neurons are defined by activation during nociceptive withdrawal behaviors. Conversely, OFF-cells suppress nociception, and this cell class is defined by a withdrawal-related pause in activity. Drugs that prevent the OFF-cell pause produce behavioral antinociception, independent of whether ON-cell activity is changed (Heinricher and Ingram 2008; Heinricher et al. 2010b; Neubert et al. 2004a). µ-Opioids, for instance, given systemically or locally in the RVM, produce continuous OFF-cell firing while inhibiting ON-cell activity. Whether these changes in OFF-cell and ON-cell activity
collectively or separately relate to other effects of opioids, including respiratory depression, is not yet known.

The constituent regions of the RVM, including portions of raphe magnus, raphe pallidus, and raphe obscurus at the level of the facial nucleus, have also been tied to other regulatory functions, including thermogenesis and cardiovascular control (Cao et al. 2004; Lovick 1997; Nakamura and Morrison 2007). Although these areas have not been strongly implicated in opioid-mediated respiratory depression, they have been linked to respiratory modulation (Dias et al. 2012; Dias et al. 2007; Hellman et al. 2007; Hellman et al. 2009; Madden and Morrison 2005; Menuet et al. 2011; Rice et al. 2009; Taylor et al. 2006; Verner et al. 2004). Nevertheless, the neuronal and physiological overlap of these homeostatic functions with pain modulation is not well-understood, in part due to the lack of mechanistic studies that include both parameters.

Here we show that the RVM contributes to opioid-induced respiratory depression at doses that simultaneously produce behavioral analgesia. In this same brain region, the non-opioid analgesic improgan also relieves pain, yet stimulates respiration. This functional separation reflects independent actions of the two distinct populations of opioid-sensitive RVM neurons, the ON-cells and the OFF-cells. Thus, while these results demonstrate an overlap of opioid-induced respiratory depression and analgesia within a common brainstem region, they also show promise for dissociating these two effects pharmacologically, at the level of functionally distinct neuronal populations.
MATERIAL AND METHODS

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Surgical Preparation and Anesthesia

Deep surgical anesthesia was induced in male Sprague-Dawley rats (250-350 g, Charles River) using 4% isoflurane in humidified O₂ at 1.25 l/min, and placed in a stereotaxic apparatus. For surgical preparation (no more than 20 min), the isoflurane concentration was reduced to 3% and a small craniotomy performed to allow placement of a recording electrode and/or glass microinjection pipette in the RVM. Animals were placed on a circulating warm-water pad to support body temperature.

After surgical preparation the isoflurane concentration was reduced from 3% to 1.5% over 30 minutes (-0.5% every 10 minutes), and then further adjusted in increments of 0.25% until a tail flick reflex was evoked (see Nociceptive Testing below) without other signs of discomfort. This concentration was maintained for at least 30 min prior to the initiation of the experimental protocol. Isoflurane concentration and gas flow rate were fixed for the duration of the protocol.

Nociceptive Testing

Nociceptive thresholds were measured using tail flick (TF) latency. Using a feedback-controlled radiant heat source, the ventral side of the animal's tail was maintained at 34 °C between trials and then heated at a constant rate of 1.7 °C/s until a tail movement was detected or the cut-off temperature of 53 °C was reached. A motion transducer detected movement of the tail. Three locations, at 2, 4, and 6 cm from the tip of the tail, were tested in rotation to avoid
sensitization and tissue damage. The holding temperature allowed us to rule out the possibility
that any changes in latency could be attributed to changes in skin temperature. TF latency was
defined as the difference in time between the point at which the tail surface temperature reached
36 °C and the occurrence of the reflex. This protocol, with trials at 5 min intervals, produces a
stable measure of nociceptive responsiveness over several hours (Martenson et al. 2005). The
baseline threshold was the average withdrawal latency of three trials taken immediately prior to
the drug injection. To aid in comparisons of drug effect among groups, TF latency is sometimes
expressed as percent of maximum possible effect: \(\% \text{ MPE} = \frac{\text{post-drug latency – baseline latency}}{\text{cut-off latency – baseline latency}}\).

Respiration, Heart Rate, and Rectal Temperature

Breathing was monitored by using two different noninvasive methods, both of which provide
accurate measurements of breathing rate and relative tidal volume as compared to whole-body
plethysmography (Cleary et al. 2012). Initial experiments used accelerometry-based
plethysmography (Devonshire et al. 2009), where an accelerometer was attached to the chest
wall of the animal to detect movements associated with breathing. In later experiments,
respiration was monitored using ventilation pressure-transduction (Cleary et al. 2012), which
measures small changes in pressure just outside the animal’s nose resulting from inhalation and
exhalation. The respiratory signals were amplified, filtered, and recorded for off-line analysis
(Spike2; CED, Cambridge, UK). Respiratory rate was determined by averaging the inter-breath
interval over the 60-s period before each TF. Relative respiratory amplitude was determined by
expressing peak-to-peak amplitude, a correlate of tidal volume (Cleary et al. 2012), as a
percentage of the pre-drug baseline. Heart rate was derived from the electrocardiogram. Body
temperature was measured using a rectal thermometer (TH-5; Physitemp, Clifton, NJ).
For the improgan microinjection experiments (see Experimental Protocols below), a gold-and platinum-plated stainless-steel recording microelectrode (Microprobe, Gaithersburg, MD) was attached to the microinjection pipette so that the tips were separated by no more than several hundred microns (Heinricher et al. 2010a; Heinricher et al. 2010b; Heinricher et al. 1994; Neubert et al. 2004b). The assembly was lowered to the RVM using anatomical landmarks. Cell recordings made before, during and after the improgan microinjection were stored for later off-line analysis to ensure accurate discrimination throughout the recording. Prior to the start of the recording protocol, each neuron was unambiguously classified as an ON-, OFF, or NEUTRAL-cell using standard criteria (Fields et al. 1983). ON- and OFF-cells are defined by a sudden activation or cessation in firing rate, respectively, beginning just prior to a nociceptive reflex such as the TF response. NEUTRAL-cells show no change in firing rate correlated with the occurrence of a nociceptive reflex. Spontaneous firing was determined by measuring the firing rate in a 30-s period immediately prior to each tail flick trial (5-min intervals). Reflex-related changes in firing were determined in a 3-s period beginning 0.5 s prior to the tail flick response.

Experimental Protocols

In the first set of experiments, the contribution of neurons in the RVM to the antinociceptive and respiratory depressant actions of systemically administered morphine was determined by microinjection of the opioid antagonist naltrexone in the RVM. Tail flick trials were initiated at 5-min intervals throughout the protocol. Following a 15-min baseline period, morphine (0.66 mg/kg, i.v.) was given. Ten minutes later, naltrexone (3 µg/200 nl) or artificial CSF (aCSF, 200 nl) was microinjected into the RVM over a period of approx. 5 min, or into surrounding regions
as off-site controls. Naloxone (0.27 mg/kg, i.v.) was given systemically at the end of the experiment to show that morphine effects were receptor-mediated and reversible.

In the second set of experiments, we examined the effects of direct RVM microinjection of DAMGO ([D-Ala², N-Me-Phe⁴, Gly-ol]-enkephalin, 200 pmol/200 nl) or the non-opioid analgesic improgan (15 or 30 nmol, Hough et al. 2000) on nociception, respiratory parameters, heart rate, and body temperature. In the improgan experiments, activity of an RVM neuron was also recorded as described above. A 15-min baseline was established followed by microinjection of drug or vehicle (injected over five to ten minutes, beginning immediately after the last baseline TF). TF, respiratory measurements, heart rate, rectal temperature, and cell activity (in the improgan experiments) were recorded for the next hour.

In the third set of experiments, we microinjected either the GABAₐ receptor antagonist bicuculline (22 pmol/200 nl) to disinhibit RVM neurons, or the GABAₐ receptor agonist muscimol (18 pmol/200 nl) to suppress activity of RVM neurons (Heinricher and Tortorici 1994; Martenson et al. 2009). The protocol in this third set was identical to that for DAMGO and improgan, with TF, respiration, heart rate and rectal temperature recorded before and after microinjection of bicuculline or muscimol. However, cell data were not recorded in this set of experiments.

Verification of microinjection and recording sites

Microinjection locations and recording sites were marked by either fluorescent beads (FluoSpheres, Invitrogen, Eugene, OR) injected with the drug or by an electrolytic lesion created after the experimental protocol. Animals were overdosed with isoflurane and then transcardially perfused with physiological saline followed by 10% formalin. Brains were removed and stored
overnight in 10% formalin. The brainstem was sectioned at 60 µm on a cryostat and mounted for microscopic examination.

Identification of opioid-sensitive brainstem neurons

To identify neurons in the region of the RVM that contain post-synaptic µ-opioid receptors and that could thus drive RVM opioid-induced changes in nociception and respiration, we microinjected a peptide µ-opioid agonist, dermorphin, that was fluorescently labeled using Alexa Fluor 594 (Arttamangkul et al. 2000; Arttamangkul et al. 2006). Dermorphin-A594 was dissolved in either 3% DMSO in saline (6 pmol/200 nl injections) or 30% DMSO in saline (66 pmol/200 nl).

For injection of dermorphin-A594 into the RVM, animals were initially anesthetized using 5% isoflurane for placement of a jugular catheter, and the anesthetic then switched from inhaled isoflurane to intravenous methohexital (30 – 60 mg /hr). After achieving a stable baseline for at least 25 minutes, dermorphin-A594 was injected into the RVM. In some experiments, 45 minutes prior to the injection of dermorphin-A594, an injection of the irreversible mu-opioid antagonist, β-funaltrexamine (β-FNA, 300 nl, 6 nmol, Tocris Bioscience), was injected into the RVM. Heart rate, respiratory rate, and rectal temperature were measured as described above. Nociceptive threshold was measured by placing a Peltier device on the left hindpaw, slowly increasing temperature from 35 to 53 °C, and noting the temperate at which a withdrawal was initiated. EMG recordings from the left calf were used to determine the beginning of the withdrawal. Antinociception is expressed as percent of maximum possible effect (%MPE). These experiments allowed a comparison of the analgesic efficacy and respiratory and autonomic depressive effects of dermorphin-A594 with those of DAMGO and improgan.
Physiological and nociceptive parameters were monitored before and after injection of dermorphin-A594. Sixty minutes after injection, animals were overdosed with methohexital and perfused transcardially with solutions of physiological saline and of 10% formalin. The brains were removed, fixed overnight in 10% formalin, and sectioned at 60 µm using a cryostat. Sections were mounted on glass slides with permount, visualized on an Olympus BX51 fluorescent microscope (Olympus, Center Valley, PA), and photographed using a Microfire A/R camera attachment (Optronics, Inc., Goleta, CA). For each brain, an experimenter blinded to the treatment conditions photographed eight representative brainstem sections between -1.08 and -3.96 mm (relative to the interaural line), with the same intensity and exposure for each photograph.

Mean fluorescence for each section was quantified using the open-source image processing package Fiji (http://www.fiji.sc). Fluorescence was measured in the RVM, a midline area roughly 2 mm in width and 1 mm in height directly dorsal to the pyramidal tracts at the level of the facial nucleus. Background intensity for each section was also measured and then subtracted from the overall fluorescence.

Statistical analysis

All data are represented as mean ± SEM. Drug effects on TF latency, hindpaw withdrawal threshold, respiratory rate, heart rate, and rectal temperature were determined using one- or two-way ANOVA, with post-hoc comparisons used where indicated. Differences in mean RVM fluorescence between treatment groups and the effects of dermorphin-A594 relative to baseline were analyzed using unpaired and paired t-tests, respectively. Respiratory amplitude was analyzed using a Friedman’s analysis of variance by rank. RVM neurons exhibit a wide range of basal firing rates. A within-cell analysis approach was therefore used in which cell firing data
post-treatment were compared to baseline using a Wilcoxon's signed ranks test for matched
samples. Analyses were performed using GraphPad Prism or Statview. $P < 0.05$ was considered
statistically significant.
RESULTS

The RVM contributes to antinociceptive and respiratory-depressant actions of systemically administered morphine.

The RVM is defined functionally, as the area where low-current electrical stimulation produces behavioral antinociception, and includes the nucleus raphe magnus and adjacent reticular formation at the level of the facial nucleus (Fields and Heinricher 1985). We first determined whether this region is required for respiratory depressant actions of systemically administered morphine, as well as for analgesia. Respiratory parameters (rate and amplitude) were measured in parallel with the TF response evoked by noxious radiant heat. The latter is an index of nociception widely employed in awake behaving animals that can also be used in lightly anesthetized subjects (Fields and Heinricher 1985).

As shown in Figure 1, systemically administered morphine produced both potent analgesia and a significant decrease in respiratory rate (ANOVA, $p < 0.05$ compared to baseline for all groups). Both effects were reversed by focal application of the opioid antagonist naltrexone in the RVM, but not by aCSF vehicle. Naltrexone microinjections in areas immediately surrounding the RVM (dorsal, rostral, and caudal) were ineffective (Figure 1, naltrexone placement control group). Subsequent systemic administration of naloxone, a highly lipophilic, short-acting opioid antagonist, reversed antinociception and respiratory depression in RVM-vehicle and placement control groups, showing that both effects were opioid receptor-mediated and reversible. These data demonstrate that opioid receptors in the RVM contribute to respiratory depression as well as to antinociception produced by systemically administered morphine.
Distribution of neurons in the RVM driving opioid-induced changes in respiration, heart rate, and pain threshold

We next determined the distribution of neurons in the RVM and surrounding areas that could be the direct target of µ-opioid agents. By microinjecting the µ-opioid agonist dermorphin labeled with an Alexa Fluor 594 fluorophore (dermorphin-A594), we could identify individual cells in the RVM and surrounding regions that bind the agonist and internalize the µ-opioid receptor. These labeled neurons are potential drivers for the physiological and behavioral effects produced by opioid microinjections into the RVM.

We first determined that dermorphin-A594, like DAMGO, could produce antinociception and alter breathing when microinjected in the RVM. The higher dose of dermorphin-A594 (66 pmol/200 nl) produced significant effects on heat-evoked withdrawal (%MPE: 64.6 ± 18.4, n = 5, p < 0.05), respiratory rate (-16.2 ± 3.6 breaths/min, p < 0.05), heart rate (-23.0 ± 7.9 beats/min, p < 0.05), and body temperature (-0.28 ± 0.10 °C, p < 0.05), consistent with results from microinjections of DAMGO into the RVM (see next section).

To identify the minimal distribution of neurons that could produce behavioral effects, we used the lowest dose of dermorphin-A594 (6 pmol/200 nl) that consistently produced measurable, albeit small, antinociception (%MPE: 9.5 ± 3.5, n = 4, p < 0.05) and then mapped the distribution of fluorescently labeled neurons. With this lower dose, respiratory rate was significantly decreased (-10.8 ± 3.0 breaths/min, p < 0.05), although there were no changes in heart rate (-25.0 ± 11.0 beats/min, p > 0.05) or body temperature (-0.21 ± 0.09 °C, p > 0.05).

Many neurons with strong A594 fluorescence were visible in the area immediately surrounding the injection site (Figure 2), including numerous cells in the nucleus raphe magnus, nucleus raphe pallidus, raphe obscurus, and reticularis gigantocellularis pars alpha. Distinctly fluorescent
single neurons were visible as far as 1 mm rostral and caudal to the injection site. Some larger
neurons were also visible in the area dorsal to the injection site (nucleus reticularis
gigantocellularis), predominantly in the sections containing the injection site or the track of the
injector.

In control experiments with this lower dose of dermorphin, injecting the irreversible mu-
opioid antagonist beta-funaltrexamine (beta-FNA) 45 minutes prior to dermorphin-A594 injection significantly attenuated mean fluorescent labeling in the RVM (dermorphin-A594: 6.4 ± 1.1 arbitrary units averaged across all rostro-caudal levels, n = 4; beta-FNA pretreatment: 1.8 ± 0.63, n = 4 ; p < 0.05 by unpaired t-test, Figure 2f).

The RVM supports opioid-induced respiratory depression

To compare the analgesic and respiratory effects of direct local RVM administration of the
mu-opioid agonist DAMGO with those of the non-opioid analgesic improgan (Hough et al. 2000),
we recorded nociception, respiration, heart rate, and body temperature simultaneously before and
after microinjection of the two agents. We also recorded RVM neuronal activity in the improgan
experiments, but not in the DAMGO experiments, since the effect of local DAMGO injection on
activity of RVM neurons has been defined previously (Heinricher et al. 1994).

Microinjections of DAMGO or improgan in the RVM at sites shown in Figure 3 produced
can nonicceptive effects of dermorphin and DAMGO were seen at 10-20 and
35-45 min post-injection, respectively, consistent with the known time-courses of these agents.

Injections of improgan in areas surrounding the RVM, mostly dorsal and rostral (see Figure 3)
resulted in a small, but statistically significant, increase in TF latency (1.8 ± 0.5 s, n = 23,
$ p < 0.01$).
Although both DAMGO and improgan produced antinociception when microinjected into the RVM, only DAMGO produced a significant respiratory depression, decreasing both respiratory rate and amplitude (Figure 4). In marked contrast, improgan in the RVM stimulated both respiratory rate and amplitude. The peak effects of improgan and DAMGO were seen at 10-20 and 35-45 min post-injection, respectively, for both rate and amplitude. Vehicle injection had no effect on respiration, and injections of improgan in areas surrounding the RVM produced only a modest increase in respiration (4.8 ± 0.18 breaths/min, \( p < 0.05 \)). These data demonstrate that the analgesic actions of drugs in the RVM are not inextricably linked to respiratory depression.

**Effects of DAMGO and improgan on heart rate and body temperature are also distinct**

DAMGO microinjection resulted in a decrease in heart rate while improgan induced a substantial increase (Figure 4). Peak effects on heart rate were evident at 10-20 min and 35-45 min post-injection with improgan and DAMGO, respectively. A small but statistically significant decrease in heart rate (6.0 ± 2.0 beats/min) was seen in vehicle-treated controls over the course of the experiment. Injections of improgan in areas surrounding the RVM produced a statistically significant increase in heart rate (25 ± 6.6 beats/min, \( p < 0.01 \)).

Like heart rate, body temperature was also differentially affected by DAMGO and improgan. DAMGO microinjection decreased, whereas improgan increased, body temperature (Figure 4). The peak effects of improgan and DAMGO on temperature were seen at 35-45 min post-injection. The delayed time-course for improgan in this case presumably reflects the kinetics of whole-body temperature change. Injections of improgan in areas surrounding the RVM produced a small but statistically significant increase in body temperature (0.1 ± 0.03 °C, \( p < 0.01 \)).
Thus, like respiratory depression, reduced autonomic output following manipulations of the RVM can also be dissociated from analgesia.

Changes in RVM neuronal activity from DAMGO and improgan administration

From a pain-modulating perspective, all neurons recorded in the RVM can be assigned to one of three mutually exclusive classes: OFF-cells (defined by nociceptive reflex-related inhibition of activity), ON-cells (characterized by nociceptive reflex-related activation), and NEUTRAL-cells (unresponsive to noxious stimuli, Fields et al. 2006). Both OFF-cells and ON-cells function as pain-modulating neurons, respectively suppressing and facilitating spinal nociceptive processing. The effects of \( \mu \)-opioids on the firing of these RVM cell classes have been well documented. Local or systemically administered \( \mu \)-opioid receptor agonists, including morphine and DAMGO, indirectly activate OFF-cells through presynaptic disinhibition, suppress ON-cell firing through direct inhibition, and do not alter NEUTRAL-cell firing (Fields et al. 2006; Heinricher and Ingram 2008). The effects of locally administered improgan on the different RVM cell classes have not been studied. We therefore recorded the activity of physiologically identified neurons within the RVM during the improgan injections described above (Figure 4).

As expected from previous work, these neurons exhibited a wide range of basal firing rates. Improgan activated the pain-inhibiting OFF-cells in the RVM. Ongoing firing of these neurons was increased substantially (Figure 5). Further, improgan prevented the characteristic inhibition of OFF-cell firing during noxious stimulation \( (p = 0.03, n = 7, \text{Wilcoxon’s signed rank test compared to baseline, data not shown}) \). Improgan activation of OFF-cells thus mimics the net opioid effect of increasing the firing of these neurons (Heinricher et al. 1994). However, unlike opioids, improgan also strongly activated both ON-cells and NEUTRAL-cells (Figure 5).
The differential effects of RVM DAMGO and improgan on respiration and autonomic parameters is therefore best explained by changes in the firing of the ON-cells, since only this cell class responds differentially to the two drugs.

Functional effects of stimulating or blocking all RVM neurons

To corroborate the behavioral and physiological effects of RVM DAMGO and improgan, we examined the effects of non-selective excitation or inhibition of all RVM neurons on nociception, respiration, and autonomic parameters. The goal of these experiments was to contrast effects of selective manipulations of ON- and OFF-cells using opioids with non-selective activation or inhibition to confirm the contributing role of these two cell classes to analgesia, heart rate, thermogenesis, and respiratory control.

To non-selectively excite RVM neurons, we microinjected the GABA_A receptor antagonist bicuculline into the RVM. Like improgan, bicuculline activates both ON- and OFF-cell classes (Heinricher and Tortorici 1994). The physiological response to bicuculline generally mimicked the response to improgan rather than DAMGO, with antinociception accompanied by increases in respiratory rate, heart rate, and body temperature (Figure 6). These data verify the above finding with improgan that concurrent activation of ON- and OFF-cells in RVM stimulates respiration at the same time that it produces analgesia.

To confirm that suppression of activity of a subset of RVM neurons was relevant to opioid-induced respiratory depression, we blocked activity of all RVM neurons by microinjecting the GABA_A receptor agonist muscimol (Martenson et al. 2009). Breathing, heart rate, and body temperature were all significantly reduced following RVM blockade, although nociceptive threshold was not altered (Figure 6). Inhibiting all RVM neurons thus reproduces the respiratory
depressant actions of DAMGO, and further, points to a role for this region in maintenance of basal respiratory function.

**DISCUSSION**

These experiments show that the RVM, a region long implicated in pain modulation and homeostatic regulation, contributes to both the analgesic and respiratory depressant properties of $\mu$-opioids. To determine whether RVM mechanisms of antinociception can be separated from those mediating respiratory depression, we compared the behavioral, physiological and neuronal effects of DAMGO with those of improgan, a non-opioid analgesic (Table 1). While both drugs produced analgesia when microinjected into the RVM, DAMGO produced respiratory depression, whereas improgan stimulated breathing. Locally applied DAMGO, like systemically administered morphine, is known to activate OFF-cells and suppress ON-cell firing (Heinricher et al. 1994). Here, local improgan activated both ON- and OFF-cells. Thus, while OFF-cells show the same response to both DAMGO and improgan, the two drugs have opposing effects on ON-cells. The differential respiratory response to these two analgesic drugs in the RVM is therefore most readily explained by their opposing effects on the activity of ON-cells. By contrast, the common analgesic response to the agents is accounted for by their ability to activate OFF-cells.

**Neural basis for analgesia and respiratory depression mediated by the RVM**

While histochemical and anatomical approaches to the study of RVM neurons are as yet incomplete, their physiological classification is comprehensive. That is, by definition, every RVM neuron recorded can be identified as an ON-, OFF-, or NEUTRAL cell. These three cell classes have been identified in barbiturate-, ketamine- and isoflurane-anesthetized rats as well as in decerebrate-unanesthetized and awake animals (Clarke et al. 1994; Heinricher et al. 2010b;
ON-cells facilitate nociception, and local or systemically administered μ-opioids suppress ON-cell activity. OFF-cells suppress nociception, and opioids increase OFF-cell firing through disinhibition. Sustained OFF-cell activity mediates the analgesic action of morphine and other μ-opioids. The NEUTRAL-cells do not respond to μ-opioid agonists, whether given systemically or locally (Fields et al. 2006; Heinricher and Ingram 2008). Therefore, one or both of the two opioid-sensitive cell classes, the ON-cells and OFF-cells, must mediate the physiological and behavioral effects of μ-opioids in the RVM, including respiratory depression and analgesia.

To better understand how μ-opioids act in the RVM to depress respiration, we compared the effects of opioids with those of improgan, a non-opioid analgesic. This compound does not cross the blood-brain barrier, but when administered intracerebroventricularly, it acts at an unknown receptor site to stimulate descending antinociception through RVM OFF-cell activation (Heinricher et al. 2010b; Nalwalk et al. 2004), a finding consistent with the present results. The surprising observation in the current study was that improgan, applied directly in the RVM, produced a powerful respiratory stimulation in parallel with analgesia, allowing us to investigate the cellular basis for the differential influence on respiratory control and nociception. Locally administered improgan activated not only OFF-cells, mimicking the effect of μ-opioids on these neurons, but also ON-cells, an effect opposite to that of μ-opioids. Although NEUTRAL-cell firing was also increased by local improgan, these neurons do not respond to opioids (Barbaro et al. 1989), which argues against a role for this cell class in opioid-induced respiratory modulation via the RVM. These data therefore confirm the already substantial evidence that the OFF-cells are the analgesic output from the RVM (Fields et al. 2006; Heinricher and Ingram 2008), but more important, suggest that RVM effects on respiration are mediated by ON-cells. A role for
ON-cells in opioid-induced respiratory depression was unexpected but fits well with established interactions between pain and respiration. For instance, acute noxious stimuli, which activate ON-cells, have long been recognized to attenuate opioid-induced respiratory depression (Borgbjerg et al. 1996; Kamei et al. 2011; McQuay 1988). Should OFF-cells play any role in modulating respiration or autonomic parameters, that influence is masked by the overriding effect of the ON-cells.

Dissociation of analgesia from respiratory depression at the level of the RVM

Since OFF-cells appear to mediate analgesia but not respiratory depression, our data imply that further separation of respiratory depression from analgesia is possible, based on both neural substrate and pharmacology. µ-Opioid activation of OFF-cells is indirect, through a presynaptic mechanism, whereas inhibition of ON-cells is a direct postsynaptic effect (Heinricher and Ingram 2008; Heinricher et al. 1992; Pan et al. 1990). Because the pre- and post-synaptic actions of µ-opioids invoke distinct second-messengers and channels (Heinricher and Ingram 2008), presynaptic mechanisms could be critical targets for “pure” opioid-like analgesia. Focusing on OFF-cell-selective pathways, including the presynaptic µ-opioid receptors and downstream molecules, therefore has the potential to provide potent pain relief without the risk of respiratory depression. Indeed, cannabinoids, like opioids, act in the RVM to produce analgesia, but do not produce significant respiratory depression. This disparity between opioid and cannabinoid actions could be explained by the fact that cannabinoids do not have direct post-synaptic inhibitory actions on RVM ON-cells (Meng et al. 1998; Vaughan et al. 1999).

The RVM has the potential to modulate respiration through several pathways. Raphe magnus and raphe obscurus both send direct projections to the phrenic motor nucleus (Holtman et al. 1986; Holtman et al. 1984; Hosogai et al. 1998), and stimulation of either raphe magnus or...
pallidus influences activity of phrenic motoneurons (Lalley 1986; Millhorn 1986). Alternatively, the RVM has numerous afferent and efferent connections within the brainstem, and could modulate relays at various stages of the chemosensory pathways or contribute to chemosensory-evoked activations (Guyenet et al. 2010; Huckstepp and Dale 2011; Nattie 2011; Pattinson et al. 2009). For example, medullary raphe regions are recognized to modulate chemosensory function of the retrotrapezoid nucleus (Depuy et al. 2011; Dias et al. 2008; Hilaire et al. 2010; Mulkey et al. 2007; Viemari and Tryba 2009).

Distribution of opioid-inhibited neurons in the RVM and surrounding brainstem

Due to technical challenges with the use of MOR1 antibodies in the medullary core, the distribution of neurons with postsynaptic µ-opioid receptors in the RVM and surrounding brainstem regions has not been defined precisely, and attempts to quantify or visually identify RVM neurons that express the µ-opioid receptor have met with limited success. We found that the fluorescent µ-opioid dermorphin-A594 microinjected into the RVM labeled somata of neurons that bound and internalized this ligand. This approach holds significant promise for labeling functional receptors where immunohistochemical techniques are not optimal. In addition, it gives a more direct measure of the spread of the injected drug than traditional dye approaches or calculations of injectate volumes.

Labeled neurons were found primarily in raphe magnus and nucleus reticularis gigantocellularis pars α, but were also concentrated in raphe pallidus. Neurons in the area of raphe magnus and reticularis gigantocellularis pars α that exhibit inhibitory responses to µ-opioid agonists have been found without exception to be ON-cells (Barbaro et al. 1989). Whether opioid-sensitive neurons in raphe pallidus also exhibit the physiological properties of ON-cells has not been investigated systematically. Raphe pallidus is strongly implicated in homeostatic
regulation, especially control of body temperature (Cao and Morrison 2003; Madden and Morrison 2005; Morrison 2011). However, raphe pallidus has significant anatomical and functional overlap with more dorsal aspects of the RVM, and neurons from throughout the RVM project to the intermediolateral cell column (IML, Berner et al. 1999; Henry and Calaresu 1974; Loewy 1981; Morrison 2011). Functional projections to the IML from the medullary raphe raise core temperature by engaging multiple mechanisms of thermogenesis, including brown-adipose tissue activation, vasoconstriction, and fusimotor activity (Blessing and Nalivaiko 2001; McAllen et al. 2010; Nakamura et al. 2004). Control of thermogenesis by opioid-sensitive ON-cells fits with previous observations that DAMGO injected into the RVM attenuates stimulus-evoked increases in activity of brown adipose tissue (Nason and Mason 2006).

Some labeled neurons were also found immediately rostral and caudal to the RVM, at the level of the superior olive and in the area of raphe obscurus dorsal to the inferior olive. Opioid-sensitive cell populations rostral and caudal to the RVM have also not been characterized, but neurons with respiration-related activity have been identified in the medial medulla immediately caudal to the RVM (Lindsey et al. 1994; Pilowsky et al. 1995). The observation that an opioid microinjected in the RVM can directly influence neurons beyond the conventional boundaries of this region raises the possibility that opioid-induced analgesia and respiratory depression are mediated not by RVM OFF- and ON-cells but by opioid-responsive neurons in surrounding regions (Depuy et al. 2011; Zhang et al. 2007). However, it seems unlikely that these areas were the primary target of the injected analgesic drugs, since local application of an opioid antagonist in areas surrounding the RVM did not prevent the analgesic or respiratory-depressant effects of systemically administered morphine. Further, it has been shown that microinjections of DAMGO caudal and lateral to the RVM, at the level of raphe obscurus, do not activate OFF-cells
or produce behavioral antinociception (Heinricher et al. 1994). Nevertheless, it is doubtful that a clear functional boundary can be drawn between the RVM and adjacent reticular areas, and there is likely to be significant anatomical overlap in the distributions of neurons important in pain modulation, respiration, and autonomic function (Kerman 2008; Lovick 1997; Rathner et al. 2001; Strack et al. 1989).

Integration of pain modulation and homeostatic regulation in the RVM

Control of respiration occurs through the cooperative actions of a network of brain regions, with contributions from the cerebral cortex, hypothalamus, and multiple sites in the brainstem (Dean and Nattie 2010; Feldman et al. 2003; Guyenet 2008; Guyenet et al. 2010; Horn and Waldrop 1998; Nattie and Li 2009). While the outputs of these areas may converge before reaching respiratory motor neurons, no single brain site is responsible for all aspects of breathing. Thus, just as systemically administered opioids modulate nociception through synergistic spinal and supraspinal actions (Bodnar 2000; Budai and Fields 1998; Hirakawa et al. 1999; Yaksh and Rudy 1978), these agents likely depress respiration through concurrent actions in multiple brain areas, including rostral ventrolateral medulla, pre-Bötzinger complex, nucleus ambiguus, and cerebral cortex (Gray et al. 1999; Hassen et al. 1983; Lalley 2006; Miyawaki et al. 2002; Montandon et al. 2011; Pattinson et al. 2009; Stucke et al. 2008; Zhang et al. 2007).

The present findings reinforce the idea of a distributed opioid influence on respiration by showing that activation of opioid receptors in the RVM, a well-known pain-modulating region, can also significantly depress breathing. While these data show that the RVM contributes to decreases in respiration at clinically relevant analgesic doses, higher, potentially lethal doses almost certainly have multiple targets, including direct effects on respiratory premotor neurons (Lalley 2003; Mustapic et al. 2010; Stucke et al. 2008).
While the contribution of RVM ON-cells to opioid-induced respiratory depression is novel, the finding is not out of line with a long-standing view of this region as important for coordinating physiological and behavioral aspects of defense in response to both internal and external challenges to homeostasis (Bandler and Shipley 1994; Lovick 1997). The neuronal basis of this coordination of function deserves further study. Whether a single neuron can modulate nociception, respiration and autonomic parameters in parallel, or if defined cell populations or subpopulations separately regulate each of these functions is a long-standing question that is yet to be resolved (Brazier and Hobson 1980).

**Conclusion**

Given the multiple functions integrated within the RVM, it has been argued that separating opioid-mediated analgesia from side-effects would be impossible (Mason 2011). While the present data show that respiration, body temperature, and heart rate can be modulated by altering the activity of opioid-sensitive neurons in the RVM, the effects on all three homeostatic parameters are separable from pain inhibition.

An important clinical and scientific goal is to develop drugs that effectively relieve pain without producing respiratory depression. Our findings demonstrate a common central site of opioid action for respiratory depression and analgesia, but also show promise for further dissociation of these effects pharmacologically at the level of functionally distinct neuronal populations within the RVM.
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Author Contributions

RSP and DRC performed the experiments, including surgical preparation, single-cell recording, and physiological measurements. RSP and MMH analyzed the data. DRC designed and built the respiratory monitor. JMN contributed to the methods used with impropagan treatment. MMH, DRC, and LBH generated the hypotheses, and contributed significantly to the writing of the manuscript.
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Table Legend

Summary of the responses of RVM neurons and the associated changes in tail flick latency, respiratory rate, heart rate, and temperature to local application of vehicle, DAMGO, improgan, bicuculline, and muscimol in the RVM. Activation of OFF-cells is coupled to hypoalgesia, whereas changes in respiratory rate and autonomic parameters are linked to drug effects on the ON-cells.
Figure Legends

Figure 1  Respiratory depression and antinociception produced by systemically administered morphine are blocked by an opioid-receptor antagonist in the RVM. Animals underwent baseline testing and were given morphine systemically (MOR). At the point labeled RVM, naltrexone or aCSF was microinjected into the RVM. Injections that missed the RVM are shown as placement controls. All animals then received naloxone systemically, to verify the reversibility of any effect. Respiration was quantified and tail flick trials were initiated at 5-min intervals throughout the protocol. (6-9 animals/group, no difference among groups in baseline, *p < 0.05, **p < 0.01, ***p < 0.001 compared to aCSF using ANOVA followed by a Bonferroni post-hoc test) (Significant effect of MOR on breathing and tail flick relative to baseline not marked for clarity, repeated-measures ANOVA, p < 0.05 compared to baseline for all groups).

Figure 2  Dermorphin-A594 labeling of single neurons in and around the RVM. a. Images were taken from a ventral area of sections representing the RVM and rostrally and caudally adjacent brainstem. b. Representative image showing the distribution of fluorescent cells at 1.92 caudal to the interaural line after a 200 nl microinjection of dermorphin-A594. c. View of individual RVM neurons with dermorphin-A594 labeling. d. Representative sections from same animal as in b showing the distribution of fluorescent neurons at different rostral/caudal levels. Distance from interaural line is given. e. Labeling for dermorphin-A594 from representative animal pretreated with beta-FNA. f. Pre-treatment with beta-FNA significantly attenuated
fluorescence from dermorphin-A594 microinjection. 4 animals/group. Schematics showing the extent of the RVM, including raphe pallidus, can be found in Figure 3. Figure 3 Locations of improgan and DAMGO microinjection sites in and around the RVM. There were 36 microinjections of improgan inside, and 23 outside, the RVM. Ten DAMGO microinjections were inside the RVM. Distances from lambda are indicated adjacent to each section. The RVM encompasses the ventromedial medulla at the level of the facial nucleus, ventral to a line drawn across the dorsal aspect of the facial nucleus and medial to the lateral edges of the pyramidal tracts.

Figure 4 Effects of improgan and DAMGO microinjections into the RVM on TF latency, respiratory rate, respiratory amplitude, heart rate, and body temperature. Improgan and DAMGO were injected over a period of 5 to 10 min immediately following a 15-min baseline (BL, average of three trials).

There were no differences among groups in any of these parameters in baseline (one-way between-groups ANOVA, 10 – 36 animals/group). *p < 0.05, **p < 0.01, ***p < 0.001 compared to pre-injection baseline using repeated-measures ANOVA followed by Dunnett’s test (respiratory rate, heart rate, body temperature) or Friedman’s analysis of variance by ranks followed by a Dunn’s test (respiratory amplitude).

Figure 5 All RVM neuronal classes are activated following local application of the non-opioid analgesic improgan. a. Ratemeter records showing firing rate (in spikes/s) of a typical OFF-cell, ON-cell and NEUTRAL-cell before and after local microinjection of improgan during the period indicated below the trace. Triangles indicate TF trials, with closed triangles indicating that the animal responded to the heat, open triangles...
that there was no response prior to the cut-off time.

b. Group data confirm that all three RVM cell classes exhibit an increase in firing rate following improgan, but not vehicle, microinjection. 6-8 cells/group, \( *p < 0.05 \) compared to pre-injection baseline, Wilcoxon’s signed-ranks test.

Figure 6 Effects of RVM improgan and DAMGO, compared to bicuculline and muscimol. TF latency (expressed as percent maximum possible effect, %MPE), change in respiratory rate, change in heart rate, and change in body temperature. Each data set was analyzed using an ANOVA followed by a Dunnett’s test for comparison to aCSF vehicle control. 9 – 36 animals/group, **\( p < 0.01 \), ***\( p < 0.001 \) compared to aCSF group.
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