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Physiological properties of pain-modulating neurons in rostral ventromedial medulla in female rats, and responses to opioid administration

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ABSTRACT

Functional pain disorders disproportionately impact females, but most pain research in animals has been conducted in males. While there are anatomical and pharmacological sexual dimorphisms in brainstem pain-modulation circuits, the physiology of pain-modulating neurons that comprise a major functional output, the rostral ventromedial medulla (RVM), has not been explored in female animals. The goal of this study was to identify and characterize the activity of RVM cells in female, compared to male, rats. ON- and OFF-cells were identified within the RVM in females, with firing properties comparable to those described in males. In addition, both ON- and OFF-cells exhibited a sensitized response to somatic stimuli in females subjected to persistent inflammation, and both ON- and OFF-cells responded to systemically administered morphine at a dose sufficient to produce behavioral antinociception. These data demonstrate that the ON-/OFF-cell framework originally defined in males is also present in females, and that as in males, these neurons are recruited in females in persistent inflammation and by systemically administered morphine. Importantly, this work establishes a foundation for the use of female animals in studies of RVM and descending control.

Introduction

Chronic pain disorders disproportionately impact females, and while studies in healthy humans indicate that there are likely few sex differences in basal pain threshold, males and females may experience pain differently (Fillingim et al., 2009; Mogil, 2012; Racine et al., 2012a). One factor that could contribute to sex differences in pain experience is sexual dimorphisms in brainstem pain-modulation circuits. The rostral ventromedial medulla (RVM) is a major functional output of the best-studied pain-modulating circuit. The RVM has been well-characterized anatomically, physiologically, pharmacologically, and functionally in male animals. Although there is some evidence for anatomical and pharmacological sexual dimorphism in brainstem pain-modulating circuits (Bobeck et al., 2009; Boyer et al., 1998; Loyd & Murphy, 2006; 2009; Tershner et al., 2000), the physiology of pain-modulating neurons in females has been almost entirely unexplored.

A large body of evidence based almost exclusively on findings in males indicates that the RVM modulates nociceptive transmission through projections to the spinal and trigeminal dorsal horns. Two classes of neurons, termed "ON-cells" and "OFF-cells", have been identified physiologically in males: activity of ON-cells increases, whereas activity of OFF-cells ceases prior to behavioral responses evoked by noxious stimuli (Fields et al., 1983a). These two cell classes respectively amplify and suppress nociceptive transmission. A shift in the balance between ON- and OFF-cell population output can therefore produce enhanced or diminished nociception and pain behaviors (Heinricher & Fields, 2013; Heinricher et al., 2009). RVM receives information via sensory pathways, including noxious somatic input, forming a recurrent circuit (Chen & Heinricher, 2019a; Chen & Heinricher, 2019b). Input from higher structures to RVM forms a circuit through which cognitive

Abbreviation: RVM, rostral ventromedial medulla; PAG, periaqueductal gray; CFA, complete Freund's adjuvant.

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and emotional factors can influence pain (Heinricher & Fields, 2013).

Given the evidence for anatomical and pharmacological differences in this brainstem pain-modulating circuit between males and females, it is surprising that few studies have considered the physiological properties of pain-modulating neurons in females (Craft et al., 2004; Rojas-Piloni et al., 1998). Both human and animal literature show that there is a similar organization in periaqueductal gray (PAG) to RVM connectivity in males and females (Kong et al., 2010; Loyd et al., 2007; Loyd & Murphy, 2006). However, some sexual dimorphisms in PAG-RVM circuitry have been identified. There is a greater number of PAG-RVM output neurons in female than male rats, but at the same time, a smaller percentage of this population is activated in females during inflammation or following systemic morphine administration (Loyd et al., 2007; Loyd & Murphy, 2006). There are also differences in opioid effects in both PAG and RVM, with most authors reporting lesser potency or efficacy of localized application of mu-opioid agonists in females (Bernal et al., 2007; Bobeck et al., 2009; Boyer et al., 1998; Loyd et al., 2007; Loyd & Murphy, 2006; 2009; Loyd et al., 2008; Tershner et al., 2000). The purpose of the present study was to identify and fully characterize the activity of RVM cells in female compared to male animals.

We first compared RVM neuronal activity in naïve males and females to determine whether there are any basal differences in physiological properties. Second, since women report higher prevalence of chronic pain than men (Fillingim et al., 2009; Mogil, 2012; Racine et al., 2012a), we extended these studies of RVM properties to a model of persistent, localized inflammation (injection of Complete Freund's Adjuvant into the plantar surface of one hindpaw). Finally, since the analgesic actions of opioids are reported to depend on sex (Mogil, 2020; Nasser & Afify, 2019), we also determined the responses of RVM neurons to systemically administered morphine.

Materials and methods

All experiments followed the guidelines of the National Institutes of Health and the Committee for Research and Ethical Issues of the International Association for the Study of Pain, and were approved by the Institutional Animal Care and Use Committee at the Oregon Health & Science University. Male and female Sprague Dawley rats from Charles River were used in all experiments, weighing <380 and 260 g, respectively, at time of recording. Animals were acclimated for at least 12 days in the vivarium before testing.

Surgical preparation and anesthesia for physiological characterization of RVM neurons

Rats were housed in 12 h light/12 h dark cycles, and experiments were performed during the light phase. Following previously described methods (Cleary & Heinricher, 2013; Martenson et al., 2016), animals were anesthetized (4% isoflurane) and a catheter placed in the external jugular vein for subsequent infusion of methohexital. Animals were then transferred to a stereotaxic apparatus and kept deeply anesthetized. A small craniotomy was drilled at the midline approximately 2 mm posterior to the lambda suture to gain access to RVM. After surgery, anesthesia was adjusted so that the animal withdrew its hindpaw to noxious heat exposure but did not display spontaneous movement. Animals were maintained at this stable anesthetic plane for the duration of the experiment by infusion of methohexital at a constant rate. Heart rate and body temperature were also monitored. There was no significant difference in heart rate or body temperature between males and females (HR: $t_{36} = 0.76$, $p = 0.46$, Temp: $t_{36} = 0.63$, $p = 0.53$). Experimental protocol was initiated once the methohexital flow rate was not adjusted for a minimum of 20–30 min. Males required a higher anesthetic rate compared to females (t_{35}=2.84, $p = 0.0076$, males: 60.89 ± 1.09 mg/kg/h, females: 56.18 ± 1.19 mg/kg/h) to achieve a similar anesthetic plane (Merkel & Eger, 1963).

Inflammation

Persistent inflammation was induced in a subset of female animals prior to experiments. Rats were briefly anesthetized with isoflurane (4%, 4–5 min) and CFA (0.1 ml) was injected subcutaneously into the plantar surface of the right hindpaw. Rats were returned to their home cage for 3 to 6 days to model persistent inflammation, since inflammation peaks at this time (Ren, 1999; Ren & Dubner, 1999). There was no significant difference in anesthetic dose required to maintain CFA-treated females at an anesthetic depth similar to that employed for naïve females ($t_{41} = 0.74$, $p = 0.46$, F CFA: 57.8 ± 1.82 mg/kg/h, F naïve: 56.18 ± 1.19 mg/kg/h). There was also no effect of treatment on heart rate or body temperature (HR: $t_{41} = 1.42$, $p = 0.16$, Temp: $t_{41} = 0.84$, $p = 0.41$).

Characterization of RVM neurons under basal conditions and in persistent inflammation

All testing was performed in low ambient light conditions (<5 lx). A gold- and platinum-plated stainless-steel microelectrode was placed in the RVM to record cell activity. Signals were amplified and band-pass filtered (Neurolog, Digitimer) then transmitted to a computer for real-time spike detection and monitoring using Spike2 (CED, Cambridge, UK). EMG activity, heart rate, and paw heat-stimulus temperature were also recorded using Spike2. Identified neurons were classified as ON-, OFF-, or NEUTRAL-cells based on changes in firing rate associated with nocifensive withdrawal (Cleary & Heinricher, 2013; Fields et al., 1983a; Martenson et al., 2016). ON-cells are defined by a burst in activity beginning just prior to withdrawal from a noxious stimulus. OFF-cells stop firing just prior to withdrawal.

After isolating and identifying a cell as an ON- or OFF-cell, one heat trial was performed on each hindpaw approximately 4 min apart (some trials were delayed in order to capture an ON-cell in a quiet state or an OFF-cell in an active state). Noxious heat was applied by lightly resting a Peltier device (Yale Instruments, New Haven, CT) on the plantar surface of the paw. Paw surface temperature was held at 35 °C before heat onset, and temperature then increased at a rate of approximately 1.5 °C to a maximum of 53 °C. To avoid damage to the paw, the Peltier device was removed upon limb movement, determined using EMG. von Frey fibers (4, 15, 26, 60, and 100 g) were applied to the webbing between the toes. Each fiber was applied three times to each paw, in ascending order, for 8 s. Three interdigital testing sites were alternated, with a minimum of 30 s between each trial. Longer inter-trial intervals (up to 5 min) were sometimes necessary to capture an ON-cell in a quiet state or an OFF-cell in an active state. Paw withdrawal was monitored visually as well as with EMG. In experiments using CFA, inflammation was confirmed visually in CFA-treated animals and paws were measured with calibrated calipers applied at the widest point across the dorsal-plantar surface. The treated hindpaw was significantly larger than those of untreated females ($t_{35} = 17.84$, $p < 0.0001$, CFA: 7.97 ± 0.16, Naïve: 4.46 ± 0.089). In experiments using systemic morphine administration, a thermal stimulus (cut-off temperature of 53 °C, 12 s) was also used.

Response of characterized RVM neurons to opioid administration

Surgical preparation was as above. Opioids were administered systemically via either a second jugular catheter (n = 10) or intraperitoneal injection (n = 25). After isolating and identifying a cell, one heat trial was performed every 5 min as described above. After a minimum of 3 trials to establish baseline cell and behavioral response, morphine sulfate was given in increments of 0.5 mg/kg every 10 min until there was no behavioral response on two of three successive heat trials (12-s cut-off). Naloxone (1 mg/kg i.v. or i.p.) was then administered, and ongoing firing and paw withdrawal-related changes in activity were recorded for a minimum of three trials. The average dose required to produce analgesia in these experiments in female animals was 1.86 mg/kg, which falls within the range of doses that are sufficient to suppress

noxious evoked reflexes in lightly anesthetized male animals (Barbaro et al., 1986; Heinricher et al., 1999; Heinricher et al., 2001a).

Histology

At the end of each experiment, the recording site was marked with an electrolytic lesion. Animals were euthanized by methohexital overdose and perfused transcardially with saline and 10% formalin. Brains were removed, and the lesion site reconstructed. The RVM was defined as the nucleus raphe magnus and adjacent reticular formation medial to the lateral boundary of the pyramids at the level of the facial nucleus. For characterization of RVM physiology, a total of 21 cells from 17 males, 26 cells from 21 naive females, and 25 cells from 22 CFA-treated females were recorded (1–2 cells per animal, although only one protocol was performed in each animal, two identifiable cells were isolated in some experiments). In experiments focused on opioid responses, a total of 45 cells was recorded from 35 females (1–2 cells per animal). Cells were distributed throughout RVM in both males and females (Fig. 1).

Data processing and analysis

At the conclusion of each experiment, action potential waveforms were individually examined to verify correct waveform sorting. Thermal-evoked paw withdrawal latency was defined as the average time from heat onset till paw withdrawal based on EMG activity. Mechanical withdrawal thresholds for each paw were determined based on the minimum force at which a withdrawal was observed in at least two out of three trials.

Ongoing activity was defined as the average firing rate during the two 30-s periods prior to each heat trial. Evoked firing for ON-cells was defined as the total number of spikes in the longest burst during heat, or as the total number of spikes in all bursts initiated during mechanical stimulation. A "burst" was defined as the first action potential after stimulus onset until the last action potential that preceded a 2-s quiet period. However, if an ON-cell was already active prior to heat stimulus onset, then the number of action potentials in the 3-s period around the paw withdrawal was used as the evoked response. Similarly, if an ON-cell was active prior to application of the von Frey fiber, the number of action potentials during the 8-s stimulation was considered the evoked response. Peak firing rate during stimulation was also determined for ON-cells. The stimulus-evoked pause exhibited by OFF-cells was quantified as the percent suppression. In heat trials, this was the firing rate in the 3-s period around the paw withdrawal relative to the firing rate 10-s prior to heat onset. For mechanical stimulation trials, this was the firing rate in the 8-s during mechanical stimulation relative to that in the 8-s period prior to mechanical stimulation. The longest pause duration during stimulation was also determined. A "pause" was defined as the time period between one spike that was preceded within 2 s by another action potential and terminated when two action potentials occurred within 2 s. Cell response threshold was also determined by finding the force required to elicit a minimum 50% change in cell in activity in at least two out of three trials.

Behavioral and cellular data from naïve animals were averaged between the left and right paw for subsequent data analysis. Behavioral data and reflex-related cell parameters were compared between naïve males and females using unpaired *t*-tests, and between the contralateral and ipsilateral paw of CFA-treated females using paired *t*-tests. For tests with von Frey fiber stimulation, data from naïve males and females were compared using 2-factor ANOVA with repeated measures on force. Data from CFA-treated females was analyzed using a 2-factor ANOVA with paw and force as within-subject factors.

In a separate set of experiments looking at effects of morphine administration on activity of RVM neurons in females, three time periods were defined for the purpose of analysis. The "baseline" was defined as the three heat trials prior to the first dose of morphine, the "morphine" period as the final three trials prior to naloxone (two of three consecutive trials with no withdrawal within the 12-s cut-off, as described above), and the "naloxone" period was the three trials after naloxone administration that resulted in at least two paw withdrawals. Ongoing activity was defined as the average firing rate during three 30-s periods prior to the heat trial in each time period. Evoked firing for ON-cells was defined as the total number of spikes in the longest burst during

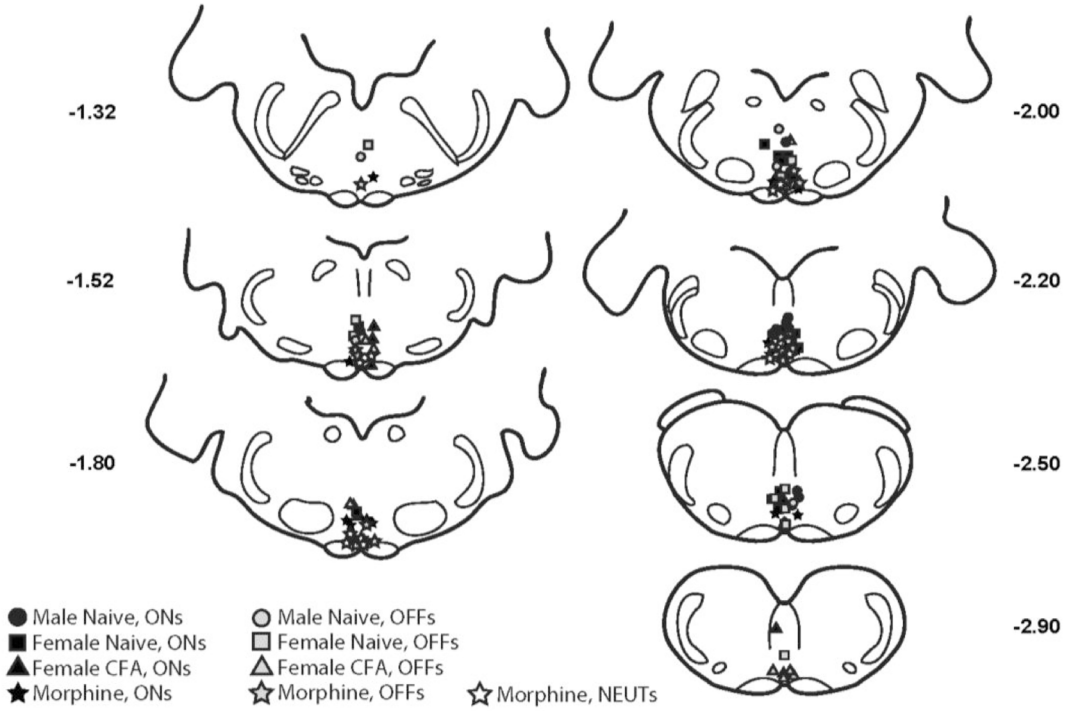

Fig. 1. Histologically verified recording locations within the RVM. Recording sites were distributed between −1.32 and −2.90 mm (relative to the interaural line). The majority of cells were distributed between −1.52 and −2.50 mm caudal to the interaural line.

heat. In the morphine time period when the paw-withdrawal was completely lost, cell activity around the average paw-withdrawal temperature at baseline + 0.5 °C was collected to define stimulus-related cell activity. Behavioral and cellular data obtained in the baseline period were compared with the averages of the three post-morphine trials and the three post-naloxone trials using repeated-measures ANOVA. Quantitative data are presented as mean ± SEM, unless otherwise specified. Parameters with highly skewed distributions were log-transformed for analysis, and back-transformed data presented as geometric mean ± 95% confidence intervals.

Results

No differences in RVM cell ongoing firing and noxious somatic stimulus-related responses in male and female animals

The first set of experiments compared the firing properties of RVM OFF- and ON-cells in female and male animals. Examples of the reflex-related changes in firing of an OFF-cell and ON-cell recorded from a female animal during heat-evoked withdrawal are shown in Fig. 2. Quantification of reflex-related changes in activity is shown in Fig. 3. There was no difference in heat-evoked OFF-cell suppression and pause duration (Fig. 3a,b) or ON-cell total evoked spikes and peak-firing rate (Fig. 3c,d) between the sexes. There was no significant difference in heat-evoked withdrawal latency (Fig. 3e). Comparison of ongoing firing rates (Fig. 4) similarly demonstrated no significant differences between males and females.

We then compared OFF- and ON-cell responses during stimulation with von Frey fibers at forces ranging from 4 to 100 g. In naive female and male animals, OFF- and ON-cells responded to forces in the frankly noxious range (60 and 100 g) that were sufficient to evoke a withdrawal reflex in either sex (Fig. 5a-d). As with heat stimulation, there was no difference between the sexes in cell responses or behavioral threshold (Fig. 5e).

Persistent inflammation following CFA injection produces mechanical but not thermal hyperalgesia in female animals

We next characterized RVM cell responses during persistent inflammation in females. Animals were treated with an injection of CFA in the right hindpaw 3 to 6 days prior to recording. We found that local administration of CFA produced mechanical hyperalgesia in the treated paw (Fig. 6a) in female animals, with a statistically significant decrease in threshold when tested 3–6 d after CFA injection. This decrease was substantial in that stimulation of the inflamed paw even with an innocuous force (≤26 g) evoked a withdrawal response in 81.8% of the

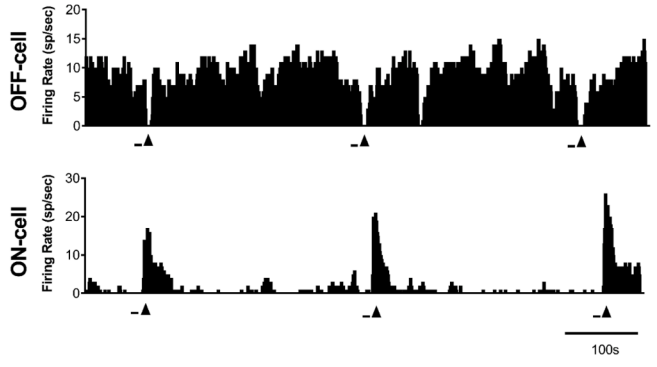

Fig. 2. Representative OFF- and ON-cell responses associated with heat-evoked withdrawal in female animals. Ratemeter records (1 s bins) show cell firing rate, with heat onset (black bars) and paw withdrawal (black triangles) shown below each trace. The OFF-cell firing ceased at the time of paw withdrawal, while the ON-cell responded with a burst of activity.

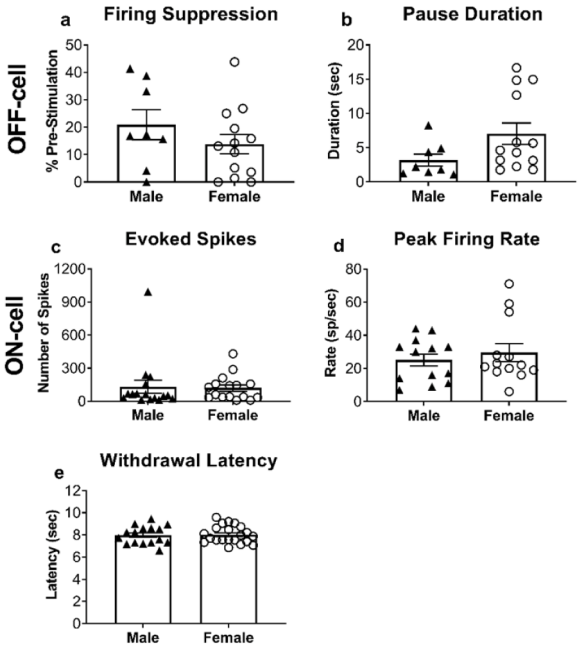

Fig. 3. Heat-evoked reflex-related responses in naïve males and females and paw withdrawal latencies. There was no significant effect of sex on any cell parameter. **a**. OFF cell suppression ($t_{19} = 1.15$, $p = 0.27$, n = 8 M, 13F). **b**. OFF cell pause duration ($t_{19} = 1.84$, $p = 0.082$, n = 8 M, 13F). **c**. ON-cell evoked spikes in burst ($t_{24} = 0.21$, $p = 0.84$, n = 13 M, 13F). **d**. ON-cell peak firing rate ($t_{24} = 0.69$, $p = 0.50$, n = 13 M, 13F). **e**. There was also no significant difference in thermal withdrawal latency between males and females ($t_{35} = 0.16$, $p = 0.88$, n = 16 M, 21F).

animals tested, whereas this was never seen with stimulation of the contralateral paw. Females did not exhibit thermal hyperalgesia at 3–6 d post-injection (Fig. 6b), with no difference in heat-evoked withdrawal latency between the inflamed and contralateral paw. These data are consistent with prior work in males (Cleary & Heinricher, 2013).

Evoked responses of RVM neurons in female animals with persistent inflammation

Stimulus-response functions for the OFF- and ON-cell responses evoked by von Frey fiber stimulation in females with persistent inflammation are shown in Fig. 7. The OFF-cell pause (cell suppression and pause duration, Fig. 7a,b) and ON-cell burst (total evoked spikes and peak firing, Fig. 7c,d) for stimulation of the inflamed and contralateral paw were compared. OFF- and ON-cells developed both increased responses to noxious (60–100 g) stimulation of the inflamed paw compared to the control paw, and novel responses to innocuous stimulation (≤26 g) of the inflamed paw (Fig. 7a,b,d,e). Thresholds were lowered for stimulation of the inflamed paw, but not the contralateral paw (Fig. 7c,f). The responses of RVM cells are thus consistent with the mechanical hypersensitivity seen in these animals.

Opioid response of RVM neurons in female animals

In a third set of experiments, we determined the response of RVM ON-, OFF-, and NEUTRAL-cells to systemic administration of morphine in female animals. NEUTRAL-cells were defined by an absence of response during noxious-evoked withdrawal. Fig. 8 shows firing of an OFF-, ON-, and NEUTRAL-cell in baseline, after systemic administration of morphine sufficient to inhibit heat-evoked withdrawal, and following reversal of the morphine effect with naloxone. In baseline, the OFF-cell

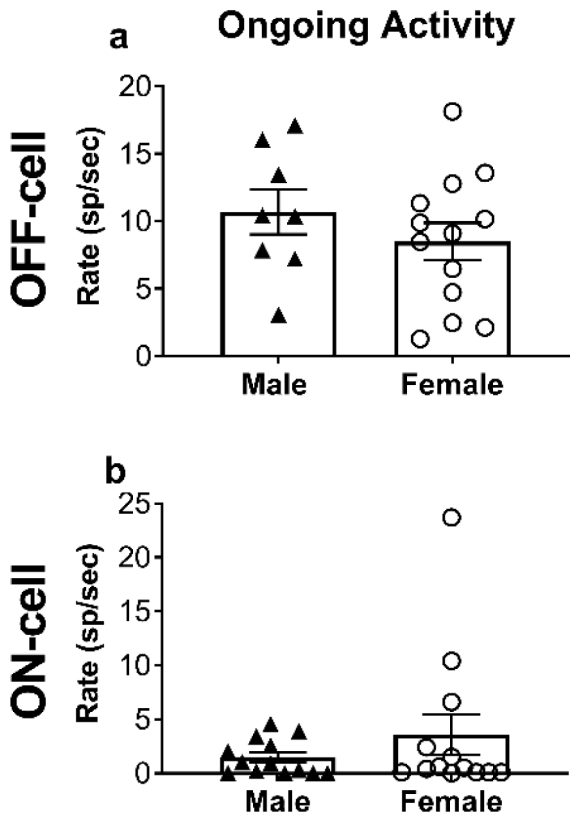

Fig. 4. Ongoing firing of ON- and OFF-cells. **a**. There was no significant difference in OFF-cell ongoing firing rate between male and female animals ($t_{19} = 1.0$, $p = 0.33$, n = 8 M, 13F). **b**. There was no significant difference in ON-cell ongoing firing rate between male and female animals ($t_{24} = 1.09$, $p = 0.29$, n = 13 M, 13F).

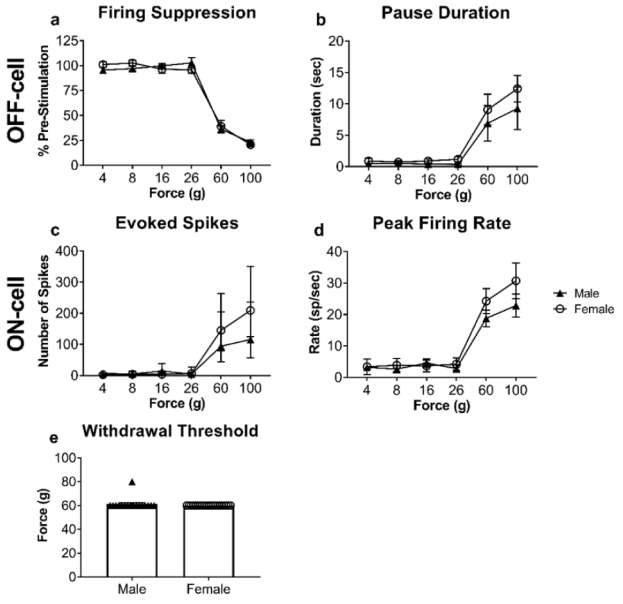

Fig. 5. Mechanically evoked cell response and withdrawal in naïve males and females. For all cell parameters, there was no significant effect of sex, although there was a significant effect of force. **a**. OFF-cell suppression (Sex: $F_{1,19} = 0.0057$, $p = 0.94$; Force: $F_{5,95} = 220$, $p < 0.0001$; Interaction: $F_{5,95} = 1.14$, $p = 0.34$; n = 8 M, 13F). **b**. OFF-cell pause duration (Sex: $F_{1,19} = 0.85$, $p = 0.37$; Force: $F_{5,95} = 21.47$, $p < 0.0001$; Interaction: $F_{5,95} = 0.35$, $p = 0.88$; n = 8 M, 13F). **c**. Evoked spikes in ON-cell burst (Sex: $F_{1,24} = 0.023$, $p = 0.88$; Force: $F_{5,120} = 76.21$, $p < 0.0001$; Interaction $F_{5,120} = 2.38$, p = 0.042; n = 13 M, 13F; data are displayed as geometric mean +/- 95% CI). **d**. ON-cell peak firing rate (Sex: $F_{1,24} = 0.65$, $p = 0.43$; Force: $F_{5,120} = 71.87$, $p < 0.0001$; Interaction: $F_{5,120} = 1.77$, $p = 0.12$; n = 13 M, 13F). e. There was no significant difference in mechanical withdrawal threshold between males and females ($t_{36} = 1.12$, $p = 0.27$, n = 17 M, 21F).

exhibits the defining "pause" in activity at the time of the paw withdrawal, and the ON-cell exhibits a substantial increase in firing rate. NEUTRAL-cell firing is unchanged. After morphine, the paw withdrawal itself is eliminated. The OFF-cell becomes continuously active and during application of heat to the paw, and ON-cell firing is almost completely suppressed, with no burst of activity during the heat stimulus. NEUTRAL-cell firing continues as in baseline. These effects were reversed by systemic naloxone administration.

Group data are shown in Fig. 9. Overall, there was a statistically significant increase in the ongoing firing of OFF-cells and decrease in that of ON-cells. Two of fifteen OFF-cells studied, both with very low ongoing activity prior to morphine, became inactive after morphine, and two of fifteen ON-cells showed an overall increase in activity. The OFF-cell pause and ON-cell burst during noxious heat application were significantly depressed, however, one ON-cell failed to show a suppression of activity during noxious heat. NEUTRAL-cells exhibited ongoing activity at 19.4 spike/s on average, ranging from 11 to 36 spikes/s for individual neurons in the present sample. The firing rate was unchanged following morphine administration. These observations are consistent with the effects of systemically administered morphine on the activity of RVM cells in males (Barbaro et al., 1986; Fields et al., 1983b).

Discussion

The primary goal of this study was to identify and characterize pain-modulating neurons in the RVM in females. Since RVM is a major output of brainstem pain-modulatory circuitry that can amplify or suppress pain-transmission by actions at the dorsal horn (Heinricher & Fields, 2013; Heinricher et al., 2009), sex-related differences in the activity or organization of this system could in principle predispose females to develop chronic pain conditions. As in prior work in males, we were able to identify ON-, OFF- and NEUTRAL-cells in the RVM in females, with reflex-related activation of ON-cells and suppression of OFF-cell firing. Firing properties in females were comparable to those in males recorded in parallel experiments. In addition, both ON- and OFF-cells exhibited a "sensitized" response to somatic stimuli in females subjected to persistent inflammation, with lowered thresholds and enhanced responses to suprathreshold stimuli. Finally, both ON- and OFF-cells responded to systemically administered morphine at a dose sufficient to produce behavioral antinociception. Thus, the physiological properties of RVM neurons in females, sensitization in a persistent inflammatory state, and response to systemically administered morphine are entirely consistent with what is known of these neurons in males. Overall, these findings validate the defining features of RVM cells by extending them to females.

We first considered ongoing activity levels and noxious-evoked responses of RVM cells in naïve animals. There was no significant difference between the two sexes in cell firing parameters, showing that RVM cells in females have similar response properties to those in males under basal conditions. Thus, despite some anatomical and pharmacological differences in pain-modulation circuitry upstream of RVM and in RVM itself (Bobeck et al., 2009; Boyer et al., 1998; Loyd & Murphy, 2006; 2009; Tershner et al., 2000), the properties of RVM neurons are comparable in males and females under basal conditions. Moreover, since the RVM contributes to basal nociceptive "tone" (Heinricher et al., 1989), this observation of similar output from RVM in males and females

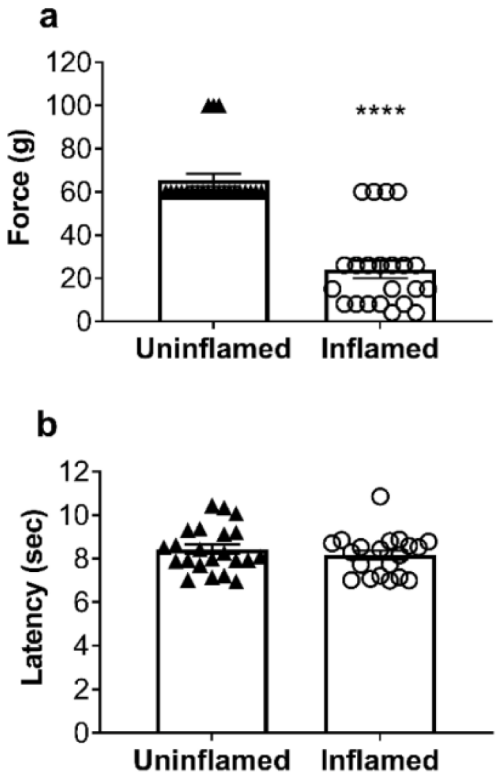

Fig. 6. Mechanical but not thermal hypersensitivity in females with persistent inflammation. **a**. There was a significant difference between paws for mechanically-evoked paw withdrawal threshold (paired *t*-test, $t_{21} = 11.61$, $p < 0.0001$, n = 22). **b**. No significant difference between paws for heat-evoked paw withdrawal latency (paired *t*-test, $t_{19} = 0.95$, $p = 0.35$, n = 20).

is consistent with our own observation of no difference between males and females in thermal or mechanical nociception, and more generally, the lack of evidence for a robust sex difference in basal nociceptive

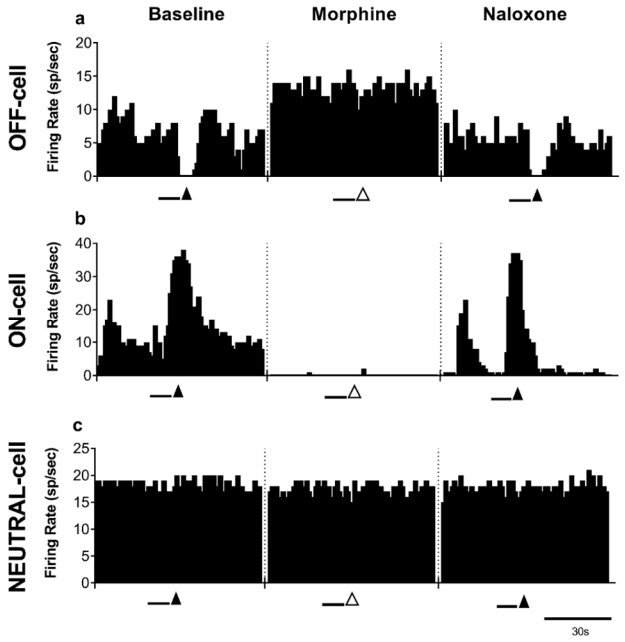

Fig. 8. Representative RVM cell response to systemic morphine administration in female animals. Ratemeter records (1 s bins) show the effect of systemic morphine administration on the activity of an **a**. OFF-cell, **b**. ON-cell, and **c**. NEUTRAL-cell. Heat onset (black bars) prior to morphine administration and after naloxone administration resulted in paw withdrawal (black triangles). Analgesic doses of morphine resulted in a loss of paw withdrawal (open triangles).

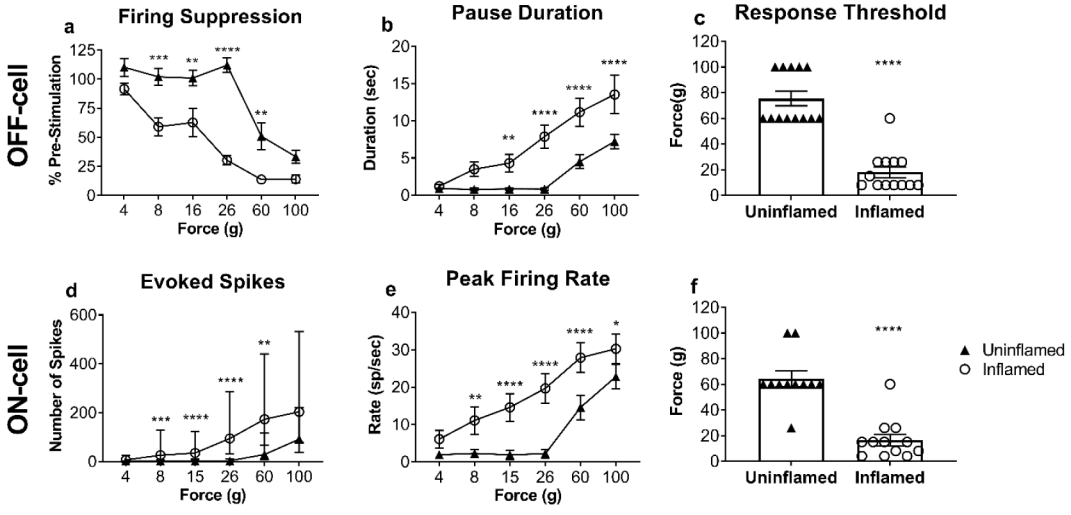

Fig. 7. Shift in cell stimulus–response curve for mechanical stimulation of CFA treated paw. For all cell parameters, there was a significant effect of force, paw, and force × paw interaction. **a**. OFF-cell suppression: force ($F_{5,60} = 47.28$, $p < 0.0001$), paw ($F_{1,12} = 51.71$, $p = 0.00012$), force × paw ($F_{5,60} = 5.58$, $p = 0.0003$), n = 13 cells. **b**. OFF-cell pause duration: force ($F_{5,50} = 22.61$, $p < 0.0001$), paw ($F_{1,10} = 21.31$, $p = 0.0010$), force × paw ($F_{5,50} = 7.12$, $p < 0.0001$), n = 11. **c**. OFF-cell response threshold was significantly lower in the inflamed paw ($t_{12} = 7.88$, $p < 0.0001$). **d**. ON-cell burst: force ($F_{5,55} = 28.34$, $p < 0.0001$), paw ($F_{1,11} = 28.5$, $p = 0.0002$), force × paw ($F_{5,55} = 4.26$, $p = 0.0024$), n = 12. **e**. ON-cell peak firing rate: force ($F_{5,55} = 31$, $p < 0.0001$), paw ($F_{1,11} = 21.06$, $p = 0.0008$), force × paw ($F_{5,55} = 3.81$, $p = 0.0049$), n = 12. **f**. ON-cell response threshold was significantly lower in the inflamed paw ($t_{10} = 6.77$, $p < 0.0001$).

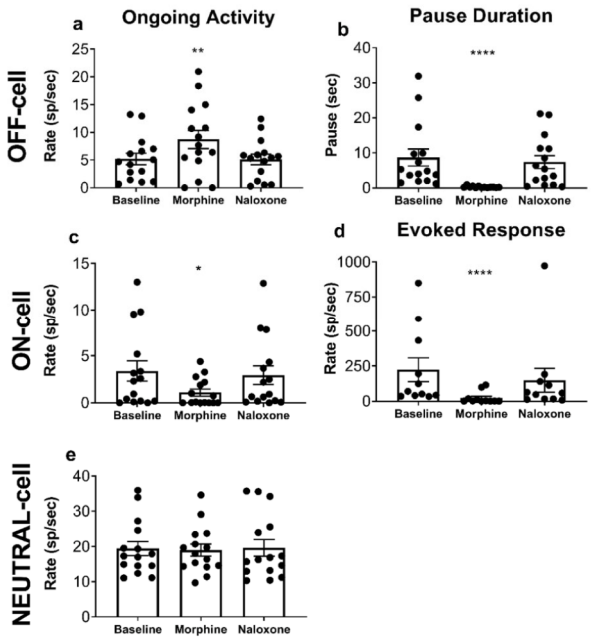

Fig. 9. Effects of systemic morphine administration on ongoing cell activity and withdrawal-evoked cell behaviors in naïve females. **a**. Systemic morphine administration significantly changed OFF-cell ongoing activity ($F_{2,28} = 7.76$, $p = 0.0021$), with post-morphine increased compared to baseline ($p = 0.0043$). **b**. Morphine significantly decreased the OFF-cell pause ($F_{2,26} = 25.43$, $p < 0.0001$) with post-morphine significantly different from baseline ($p < 0.0001$). **c**. There was a significant change in ON-cell ongoing activity ($F_{2,28} = 5.078$, $p = 0.013$), and morphine significantly decreased ongoing activity compared to baseline ($p = 0.016$). **d**. There was a significant difference in the total evoked spikes in the ON-cell burst ($F_{2,16} = 21.30$, $p < 0.0001$) with the post-morphine time point depressed compared to baseline ($p < 0.0001$). **e**. No significant change in NEUTRAL-cell ongoing activity ($F_{2,28} = 0.18$, $p = 0.84$). One-way ANOVA with repeated measures and *post-hoc* Dunn's multiple comparisons test, n = 15 OFF-cells, 15 ON-cells, 15 NEUTRAL cells. There was no significant difference between baseline and naloxone for any cell measure.

responding (Fillingim et al., 2009; Loyd et al., 2008; Mogil, 2012; Racine et al., 2012a; Wang et al., 2006).

We next looked at the effects of persistent inflammation on RVM output and behavioral sensitivity in female animals. When tested 3 to 6 days after localized injection of CFA in a single hindpaw, mechanical hyperalgesia was prominent in the CFA-treated paw, consistent with previous reports in lightly anesthetized males (Chen & Heinricher, 2019b; Cleary & Heinricher, 2013; Montagne-Clavel and Olivéras, 1994; Pinto-Ribeiro et al., 2008). We did not observe thermal hyperalgesia in female animals at the time points studied here, which again is in agreement with previous findings in male animals, that thermal hyperalgesia begins to resolve within the first 24 h after CFA injection (Almarestani et al., 2011; Cleary & Heinricher, 2013; Guan et al., 2003; Okun et al., 2011; Pinto-Ribeiro et al., 2008; Ren & Dubner, 1996; Wei et al., 1999). The observation that both ON- and OFF-cells are sensitized in females, as in males, is consistent with limited evidence for substantial sex differences in CFA-induced hyperalgesia (Armendariz & Nazarian, 2018; Bradshaw et al., 2000; Craft et al., 2013; Loyd et al., 2008; Wang et al., 2006).

Despite the similarity in behavioral endpoints and RVM sensitization in persistent inflammation in females and males, there are differences in pain-modulation circuitry between the sexes that could in principle underlie observed discrepancies in prevalence and presentation of chronic pain disorders in humans. For example, Loyd and colleagues (2008) reported increased activation of PAG-RVM output neurons in males compared to females during persistent inflammation. Nonetheless, these authors also saw no differences in inflammation-induced *hyperalgesia* between the two sexes. One possible explanation for this discrepancy is that the similar *behavioral* outcome in males and females ultimately reflects comparable recruitment of RVM ON- and OFF-cells by the PAG during inflammation. Our finding that ON- and OFF-cells are sensitized in females, as in males, is consistent with this idea. This argument would further imply that molecular and anatomical differences between males and females at the level of the PAG are compensated for at the level of the RVM, leading to similar output from the PAG-RVM system and explaining comparable behavior.

We also investigated the effects of systemic opioid administration on behavioral analgesia and RVM cell responses in female animals. Opioids are thought to produce analgesia in both sexes in part by engaging the PAG-RVM descending modulatory system. Thus, sex differences in the pharmacological properties of this circuit could contribute to differential opioid effects in men and women (Bernal et al., 2007; Bobeck et al., 2009; Loyd & Murphy, 2009). However, animal and human literature related to the impact of sex on opioid analgesia is not entirely consistent (Mogil, 2020; Nasser & Afify, 2019). Notably, μ-opioids are more potent in women (Niesters et al., 2010; Sarton et al., 2000), while male rats are generally found to be more sensitive to the antinociceptive properties of morphine (Baker & Ratka, 2002; Boyer et al., 1998; Cicero et al., 1996). The goal of the present experiments was to determine if RVM OFF- and ON-cells in female rats responded to acute systemic opioid administration as shown previously in males, where effectively every ON- and OFF-cell shifts to its own active (OFF-cell) or inactive (ON-cell) state *when morphine is given systemically at a dose that produces analgesia in that animal* (Barbaro et al., 1986; Heinricher et al., 2001a; Heinricher et al., 2001b). Here, we took the same approach in females and found that, with a few exceptions, ON-cells shifted to their inactive state and OFF-cells to their active state after morphine, producing a net increase in OFF-cell firing and net decrease in ON-cell firing. As in males, this was seen at a dose that produced analgesia. Although a small number of cells showed disparate results, this may be due to the cumulative dosing approach, or more likely, the greater sensitivity of females to the methohexital anesthetic than males, which could attenuate morphine effects. Nevertheless, our findings in females are generally consistent with previous reports in males (Barbaro et al., 1986; Fields et al., 1983b), and suggest that recruitment of OFF-cells and suppression of ON-cell firing contribute to analgesia following systemic morphine administration, as in males. This does not mean that differences in the sensitivity of ON- and OFF-cells to opioid administration might not explain differences in analgesic potency. However, an in-depth analysis of full neuronal and behavioral dose–response curves in both sexes would be required to determine this, and the present experiments provide a necessary foundation for such a study.

Finally, it should be noted that NEUTRAL-cells exhibited ongoing activity that was unchanged following morphine administration. It therefore seems unlikely that NEUTRAL-cells contribute to the antinociceptive actions of morphine, as already suggested for males (Barbaro et al., 1986).

It is worth considering that while RVM responses to morphine at an analgesic dose were consistent with what has been reported in males, and that the dose required to produce analgesia in females was within the range reported to produce analgesia in previous studies in male rats (Barbaro et al., 1986; Heinricher et al., 2001a; Heinricher et al., 2001b), it is possible that sex differences would be revealed with chronic administration. Although behavioral tolerance is not seen with focal application of morphine in the RVM itself (Boyer et al., 1998), exploration of RVM cell responses to chronic morphine administration, which would require recording from an identified brainstem neuron over a period of days, would certainly be of interest (Loyd et al., 2008).

In sum, the present results are consistent with limited evidence for sex differences in acute experimental pain in humans and rodents. Effects of sex in humans are small, with a host of confounding factors

(Fillingim et al., 2009; Hashmi & Davis, 2014; Kennedy et al., 2014; Racine et al., 2012b). Studies of sex differences in basal nociceptive sensitivity in rodents have also reported conflicting results. Sex differences are found in either direction, or not at all, and results are highly variable, both between different nociceptive assays, and within a given nociceptive assay (Mogil, 2020). Results also appear to depend on methodological details such as animal strain, laboratory environment, and experimenter. Moreover, the field is likely highly biased by the difficulty of publishing results that show no difference. On the whole, it appears unlikely that there are fundamental sex differences in basal nociceptive sensitivity. The present physiological evidence that the same neural "machinery" for descending control of pain exists at the level of the RVM in females, as in males, is consistent with this.

Despite the lack of strong sex differences in acute nociception in humans or rodent, there is no question that women are more greatly impacted by chronic pain than men (Fillingim et al., 2009; Mogil, 2012; Racine et al., 2012a). Although ON- and OFF-cells were sensitized in a persistent inflammatory state, as previously seen in males, it is possible that chronic pain impact in women is related to population differences, e.g., that females have a greater number of ON-cells or fewer OFF-cells than males. Determining this would require a unique genetic marker for each RVM cell class, since recording techniques cannot be used to quantify the number of cells of a specific physiological class without an unbiased search stimulus that would be able to recruit every neuron in the region during the recording session.

Overall, our findings provide a foundation for the use of female animals in understanding the RVM and pain-modulation more generally. Given the strong evidence for altered descending control in individuals with chronic pain, and the greater prevalence of chronic pain in women, an important task of future studies will be to determine whether and how this system is differentially recruited in the two sexes in relevant models of chronic pain.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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