Published in final edited form as:

Neuroscience. 2014 June 20; 271: 149–159. doi:10.1016/j.neuroscience.2014.04.035.

Neuropeptide Y in the rostral ventromedial medulla reverses inflammatory and nerve injury hyperalgesia in rats via non-selective excitation of local neurons

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Abstract

Chronic pain reflects not only sensitization of the ascending nociceptive pathways, but also changes in descending modulation. The rostral ventromedial medulla (RVM) is a key structure in a well-studied descending pathway, and contains two classes of modulatory neurons, the ON-cells and the OFF-cells. Disinhibition of OFF-cells depresses nociception; increased ON-cell activity facilitates nociception. Multiple lines of evidence show that sensitization of ON-cells contributes to chronic pain, and reversing or blocking this sensitization is of interest as a treatment of persistent pain. Neuropeptide Y (NPY) acting via the Y1 receptor has been shown to attenuate hypersensitivity in nerve-injured animals without affecting normal nociception when microinjected into the RVM, but the neural basis for this effect was unknown. We hypothesized that behavioral anti-hyperalgesia was due to selective inhibition of ON-cells by NPY at the Y1 receptor. To explore the possibility of Y1 selectivity on ON-cells, we stained for the NPY-Y1 receptor in the RVM, and found it broadly expressed on both serotonergic and non-serotonergic neurons. In subsequent behavioral experiments, NPY microinjected into the RVM in lightly anesthetized animals reversed signs of mechanical hyperalgesia following either nerve injury or chronic hindpaw inflammation. Unexpectedly, rather than decreasing ON-cell activity, NPY increased spontaneous activity of both ON- and OFF-cells without altering noxious-evoked changes in firing. Based on these results, we conclude that the anti-hyperalgesic effects of NPY in the RVM are not explained by selective inhibition of ON-cells, but rather by increased spontaneous activity of OFF-cells. Although ON-cells undoubtedly facilitate nociception and

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Competing Financial Interests: none

Disclosures

Supported by grants from NINDS: NS070374 (DRC), NS066159 (MMH), and NS065406 (MMH). DRC was supported by the Neurobiology of Disease Fellowship from the OHSU Brain Institute. Confocal microscopy was carried out at the Advanced Light Microscopy Core at the Jungers Center for Neuroscience Research, and was supported by NINDS (P30 NS061800).

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contribute to hypersensitivity, the present results highlight the importance of parallel OFF-cell mediated descending inhibition in limiting the expression of chronic pain.

Keywords

descending pain modulation; nociception; ON-cells; OFF-cells; NPY; raphe magnus

1.0 Introduction

Treatments effective against acute pain are often less useful in the management of persistent pain, an observation that has driven investigations into the mechanisms separating acute from chronic pain. Alterations in descending nociceptive modulation are now recognized to have a significant role in chronic pain (Heinricher et al., 2009, Heinricher and Fields, 2013), but defining the specific function of these systems is made difficult by the fact that the descending pathways can inhibit as well as enhance nociceptive processing.

The best studied pain-modulating system has its primary efferent node in the rostral ventromedial medulla (RVM, Heinricher and Ingram, 2008, Heinricher et al., 2009). This region exerts bidirectional control of nociception through two physiologically defined populations of neurons, the “ON-cells” and “OFF-cells.” ON-cells are activated by stimuli that evoke nocifensive withdrawal responses, and direct pharmacological activation of the ON-cell population produces behavioral hyperalgesia (Fields and Heinricher, 1985, Heinricher and Neubert, 2004, Neubert et al., 2004, Kincaid et al., 2006). By contrast, OFF-cells are inhibited by stimuli that evoke nocifensive withdrawal responses, and pharmacological activation of these neurons produces hypoalgesia or analgesia (Heinricher et al., 1994, Heinricher et al., 2010). Considerations of the role of the RVM in persistent pain have therefore focused on the ON-cells. Lesions, inactivation of the RVM, and other manipulations assumed to block the ON-cell output can attenuate allodynia in animals subjected to nerve injury or persistent inflammation of visceral and deep tissues (Pertovaara et al., 1996, Porreca et al., 2001, Burgess et al., 2002, Chen et al., 2004, Sugiyo et al., 2005, Vera-Portocarrero et al., 2006, Taylor et al., 2007, Da Silva et al., 2010a, Da Silva et al., 2010b). However, the response properties of OFF-cells as well as ON-cells are altered in chronic inflammation or following nerve injury (Carlson et al., 2007, Gonçalves et al., 2007, Cleary and Heinricher, 2013), and a significant body of evidence indicates that descending inhibition, presumably mediated by OFF-cells, is enhanced during chronic inflammation (Guan et al., 2002, Miki et al., 2002, Terayama et al., 2002, Vanegas and Schaible, 2004). Consistent with this latter idea, we recently showed that mechanical allodynia in lightly anesthetized rats with chronic localized inflammation of the hindpaw is worsened rather than relieved by non-selective inhibition of both ON- and OFF-cells, indicating that a net descending inhibitory influence limits hypersensitivity in these animals (Cleary and Heinricher, 2013).

To dissect out the RVM contributions of descending inhibition versus facilitation, in the present experiments we used neuropeptide Y (NPY) as a pharmacological tool. When microinjected into the RVM, this peptide reverses persistent hyperalgesia following nerve injury without producing measurable hypoalgesia in normal animals (Taylor et al., 2007).

Since the direct post-synaptic effects of NPY have been previously reported as inhibitory (Michel, 1991), we hypothesized that NPY reverses hyperalgesia by selectively inhibiting RVM ON-cells. We found instead that, despite the concurrent activation of ON-cells, the anti-hyperalgesic action of NPY in the RVM is best explained by the increased activity of OFF-cells.

2.0 Experimental Procedures

2.1 Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Procedures 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. All experiments were performed on male Sprague-Dawley rats (Charles River, Hollister, CA) weighing between 250 and 350 g.

2.2 Spinal nerve ligation procedure

The spinal nerve ligation (SNL) surgery is a well-established model for nerve injury hyperalgesia, and was performed using sterile technique, as previously described (Kim and Chung, 1992, Carlson et al., 2007). Animals were deeply anesthetized (3–4% isoflurane in oxygen). The back was shaved and swabbed with iodine, and a longitudinal incision made just off midline near the pelvic rim. The paraspinal muscles were separated to reveal the L5 and L6 nerve roots. These nerve roots were individually ligated with 4.0 silk suture, the muscle and skin layers closed separately with dissolvable suture, and an antibiotic ointment applied over the incision. Animals were allowed to recover for 2–3 weeks after surgery, during which period they were closely monitored for signs of distress, infection, or motor deficit, any of which would have precluded the animal from use in the experiment.

2.2 Chronic inflammation

Chronic inflammation was induced with subcutaneous injection of Complete Freund's Adjuvant (CFA), which has been used extensively to study chronic inflammatory pain and descending pain modulation (Hurley and Hammond, 2000, Guan et al., 2002, Ren and Dubner, 2002, Sykes et al., 2007). As described previously (Cleary and Heinricher, 2013), animals were briefly anesthetized with 5% isoflurane in oxygen, and 0.1 ml of CFA (heat-killed *Mycobacterium tuberculosis* in mineral oil, 1 mg/ml, 0.1 ml, Sigma-Aldrich) was injected subcutaneously into the plantar surface of the left hindpaw using a 25-gauge needle. After injection, the hindpaw showed signs of inflammation, including redness, swelling, and heat. The inflammation was allowed to progress for 3–10 days before behavioral testing or electrophysiological recording. This time frame was chosen because of functional and neurophysiological evidence that RVM-mediated descending control is established and stable across this period (Ren, 1999, Ren and Dubner, 2002, Hamity et al., 2010, Cleary and Heinricher, 2013).

2.3 Lightly Anesthetized Animals

Studies of cell activity and nociceptive behavior were conducted under light anesthetic in a stereotaxic frame. Animals were first deeply anesthetized with either pentobarbital (60

mg/kg, i.p.) or inhaled isoflurane (5% in oxygen). An incision was made to expose the external jugular, and a catheter inserted for subsequent infusion of barbiturate anesthetic. The animal was transferred to a stereotaxic frame, a craniotomy drilled, and the dura removed. Upon removal to the stereotaxic frame, anesthetic was switched to intravenous methohexital, a short-acting barbiturate. The dose was gradually titrated over an hour until a brisk withdrawal could be consistently elicited from noxious thermal or mechanical stimuli without spontaneous movement or vocalization. Following pentobarbital induction, testing was not initiated for at least 2.5 h after the initial injection, due to lingering anti-hyperalgesic effects of that agent (Cleland et al., 1994, Carlson et al., 2007). With either method of induction, experiments were started after the methohexital dose was unchanged for at least 45 min and heart rate, respiratory rate, and core temperature were stable.

2.4 RVM drug administration

In experiments without cell recording, drugs were injected directly into the RVM using pulled glass capillary pipettes with 70–80 μm tips. NPY (Tocris Bioscience, Ellisville, MO) was dissolved in either artificial cerebrospinal fluid (aCSF) or saline, and 200 nl of solution injected over 4–6 min using a 1- μL Hamilton syringe. Injection sites were marked either with a low concentration of green fluorescent beads included in the solution (Fluospheres; Invitrogen, Eugene, OR) or marker dye injected at the end of the experiment. The presence or absence of fluorescent beads in the injectate did not affect behavioral or electrophysiological results, so results with the two conditions were combined and reported together.

2.5 Single-unit electrophysiology of RVM neurons

In experiments on the neurophysiological effects of NPY, simultaneous RVM microinjection and neuronal recordings were conducted. A gold- and platinum-plated microelectrode was glued parallel to a microinjection pipette, with a horizontal separation of 100–250 μm and a vertical separation of 50–100 μm (with the injector the more ventral). This configuration has been validated and extensively used for studying local neuronal effects of microinjecting small, but behaviorally relevant, drug volumes (Heinricher and Neubert, 2004, Neubert et al., 2004, Martenson et al., 2009). Signals from the microelectrode were amplified 10,000-fold, band-pass filtered (100 to 15,000 Hz), and then digitized (Spike2 Software; CED, Cambridge, UK). Waveforms were monitored continuously to ensure proper discrimination of a single, unambiguous waveform throughout the experiment.

RVM neurons were classified as ON-cells, OFF-cells, or NEUTRAL cells, based on cell responses during withdrawal from noxious stimuli (Fields et al., 1983). When an animal withdraws, ON-cells fire a volley of action potentials, starting in the hundreds of milliseconds before the actual withdrawal. OFF-cells slow or stop firing at the withdrawal. RVM neurons of a third class, the NEUTRAL cells, do not modulate their firing in response to acute noxious stimulation, and have an unclear role in pain modulation.

2.6 Nociceptive testing in anesthetized animals

Thermal and mechanical testing were used to examine changes in nociceptive threshold following RVM microinjection. To test mechanical sensitivity, ascending strengths of von Frey (vF) fibers (4, 8, 15, 26, 60, and 100 g) were applied to the interdigital area of the hindpaw. In animals with nerve injury or inflammation, testing started at 4 g, whereas in naive animals testing started at 15 g. A positive response was counted following a visible withdrawal or leg movement in at least two of three trials. A delay of at least 30 s was given between trials, and three positions on the foot were rotated so that the same site was never tested twice in a row.

Thermal nociception was assessed using contact heat. A Peltier plate (1×1 cm) at a holding temperature of 35 °C was applied to the plantar surface of the hindpaw with the ankle extended. The plate was heated from 35 °C to a maximum of 53 °C at a rate of 1.5 °C/s. The plate was removed at the first sign of withdrawal, which in lightly anesthetized animals is normally between 47 and 49 °C. Thermal thresholds were measured every 4–5 min. The precise temperature at the withdrawal was quantified using EMG electrodes placed 1 cm apart in the calf. The thermal withdrawal threshold was considered to be the temperature at the first upward deflection on EMG after the application of stimulus.

2.7 Experimental protocols

The first set of experiments focused on the effect of NPY in the RVM on *mechanical* hypersensitivity in animals that had undergone SNL or CFA injection. Following three baseline measurements of paw withdrawal threshold, NPY or aCSF was injected into the RVM. Post-treatment measurements of withdrawal threshold were started at 5–10 min after the RVM injection. In untreated controls, mechanical withdrawal threshold was examined on only one side; in all other animals, thresholds were measured on both sides.

For a second group of behavioral experiments, the effect of RVM microinjection of NPY on *thermal* withdrawal thresholds in normal animals was quantified. Baseline thermal withdrawal thresholds were measured over three trials, NPY or aCSF was injected into the RVM, and then thermal testing was repeated every 4–5 min for approximately 1 h.

In a third group of experiments, single-unit recording was conducted in conjunction with NPY microinjection in normal animals. The thermal withdrawal thresholds were used as the measure of nociceptive responsiveness. In these experiments, spontaneous activity of the neuron was recorded in the first 10 min of the experiment, during which time no overt stimulation was applied. After three baseline trials, drug or vehicle was injected into the RVM. Repeated thermal testing continued until at least 1 h after injection, and the recording site was then marked with an electrolytic lesion.

2.8 Histology

Experimental sites were marked with an electrolytic lesion, fluorescent microbeads, or dye. After all experiments, animals were euthanized with an anesthetic overdose, perfused transcardially with saline and 10% formalin. The brainstem was removed and allowed to post-fix for at least 48 h. The RVM was blocked and sectioned at 60 μ m. Sites were

identified using an Olympus BX51 fluorescence microscope. Cells or injections outside the RVM were not included in subsequent analyses (Figure 1).

2.9 Immunohistochemistry and cell counting

For immunohistochemical studies, animals were euthanized with an overdose of pentobarbital and perfused transcardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brain was extracted and allowed to postfix for 24 h. Coronal brain sections were cut on a cryostat at 40 μ m thicknesses.

Immunohistochemical processing was done on a shaker at room temperature. For antigen retrieval, sections were first incubated in sodium citrate buffer at pH 6.0 at 67 °C for 10 min, and then washed in 0.1 M Tris 5 min. Sections were then incubated in 1% sodium borohydride in 0.1 M Tris saline for 30 min, washed three times in 0.1 M Tris, and blocked with 10% normal donkey serum (NDS, Jackson ImmunoResearch) in Tris for 30 min. After blocking, the sections were again washed three times in 0.1 M Tris and then incubated in 5% NDS Tris with the first primary antibody (polyclonal goat NPY1-R IgG, 1:100, Santa Cruz SC-21990) on a shaker at 4 °C for 48 h.

After incubation, tissue was washed three times in 0.1 M Tris for 20 min then incubated for 1 h at room temperature in 5% NDS Tris with the secondary antibody (AlexaFluor 555 donkey anti-goat IgG, 1:700, Invitrogen). Tissue was washed three times in 0.1 M Tris, blocked with 10% NDS in 0.5% Triton-X in Tris for 30 min, and then washed again. To co-stain for tryptophan hydroxylase, the tissue was incubated with the second primary antibody (polyclonal rabbit anti-TPH₂ IgG, 1:200, Santa Cruz SC-134774), in 5% NDS and 0.5% Triton-X in Tris on a shaker at room temperature for 24 h. Sections were washed three times in 0.1 M Tris and then incubated with the secondary antibody (AlexaFluor 488 donkey anti-rabbit IgG, 1:500, Invitrogen), in 5% NDS and 0.5% Triton X in Tris for 1 h at room temperature. After incubation, sections were washed three times in 0.1 M Tris and coverslipped while wet using Prolong Gold (Invitrogen). Controls performed for the immunohistochemical staining included omission of the primary antiserum and addition of a blocking peptide against which the antibody was raised. For both these negative controls, staining was eliminated.

The RVM was imaged with a Zeiss LSM 710 confocal microscope. Tile images (2×1) of the RVM and adjacent pyramids and facial nucleus were taken with a 5x objective. Focus stacking (z-stacking) was used to produce 5 images per tile, with a pinhole size of 1 airy unit. To reduce potential cross talk, argon and DPSS lasers were activated serially, and laser intensity levels were limited to maximum values determined by background and cross-talk controls. Images were flattened, and neurons were counted with ImageJ (Schindelin et al., 2012).

For the purposes of cell counting and quantifying overlap between Y1-like immunoreactivity and TPH₂, the RVM was defined as the nucleus raphe magnus and the adjacent reticular formation at the level of the facial nucleus. The boundaries of the RVM were marked as a midline rectangular region, bounded on the side by the edges of the pyramidal tract and the top by the dorsal edge of the facial nucleus (Figure 2A). A

quantitative analysis of TPH₂/Y1-LI overlap was performed by counting the number of stained cells within the sample area of the RVM. Only clearly immunopositive neurons with a full cell body were included in the counts. To avoid bias in data gathering, counts were conducted first on the independent colors channel, then overlaid to assess for co-labeling. Immunopositive cells for TPH₂ and Y1 were counted in six brains. In each brain, sections for counting were selected from seven representative levels as demarcated by Paxinos and Watson within the range of –1.04 mm to –2.6 mm from the interaural line (Paxinos and Watson, 1997). For each level, the total numbers of single and double-labeled neurons were averaged across all brains. No statistical analyses were performed on the data from immunohistochemistry experiments.

2.10 Data analysis and statistics

All experimental data were digitized using Spike2 and stored off-line for later analysis. Cell responses associated with withdrawals were quantified as the absolute change in firing rate between the 10 s prior to the onset of stimulation and the 3 s around the withdrawal, starting 0.5 s prior to the initiation of the withdrawal. Spontaneous activity was measured as the mean firing rate in the 30 s prior to each stimulus trial.

Mechanical thresholds before and after RVM drug injections were compared using a Kruskal-Wallis test followed by Dunn's post-hoc test. Thermal withdrawal thresholds before and after RVM drug injections were compared using a paired *t*-test or repeated measures ANOVA with Dunnett's post-hoc test. The NPY dose-response curve was calculated using a non-linear curve-fitting algorithm with a Hill slope of 1.0 for log(dose) versus mean effect. Mechanical withdrawal thresholds, and evoked and spontaneous neuronal activity following RVM injections were compared to baseline using a Wilcoxon rank-sum test or Friedman's test followed by a Dunn's post-hoc test. All analyses were accomplished using GraphPad Prism. Data are presented as mean + SEM.

3.0 Results

3.1 NPY Y1-like immunoreactivity labels a subset of RVM neurons

Previous work shows anti-hyperalgesia produced by NPY acting at the Y1 receptor in the RVM (Taylor et al., 2007). We hypothesized that this receptor would be expressed by a subset of pro-nociceptive neurons in this region, and so we stained for the Y1 receptor. As RVM serotonin is linked to hyperalgesia and a subset of RVM serotonergic neurons respond like ON-cells, we also co-labeled for TPH₂, the primary enzyme controlling serotonin synthesis in the central nervous system (Suzuki et al., 2004, Gau et al., 2013).

Cells with NPY1-like immunoreactivity (Y1-LI+), TPH₂-like immunoreactivity (TPH-LI+), and double labeling were found along the entire rostral-caudal extent of the RVM. Y1-LI+ neurons were widely dispersed, with positive labeling throughout the medulla and a higher concentration of labeled neurons in the RVM (Figure 2B, E). As expected, neurons showing TPH-LI+ were concentrated in the raphe regions (magnus, pallidus) and, to a lesser extent, in nucleus reticularis gigantocellularis pars α (Figure 2C, F, Skagerberg and Björklund, 1985). Although these distributions were distinct, some overlap existed, with a subset of neurons showing both Y1-LI+ and TPH-LI+ (Figure 2D, G). Neurons showing Y1-LI+ were

more numerous than those positive for TPH-LI (Figure 2H). Averaged across all levels, nearly half of all TPH-LI+ cells also expressed Y1-LI ($47.7 \pm 1.3\%$). Conversely, a smaller proportion of the Y1-LI+ cells co-expressed TPH₂ ($16.6 \pm 0.6\%$).

3.2 NPY injected into the RVM reverses mechanical hyperalgesia in animals subjected to nerve injury or persistent inflammation

NPY microinjected did not significantly change mechanical thresholds in control animals or that of the contralateral, untreated paw in either treatment group (Figure 3). However, animals subjected to either SNL surgery or CFA injection displayed increased sensitivity to mechanical stimulation on the treated (ipsilateral) side, and microinjection of NPY into the RVM reversed this mechanical hypersensitivity in both models at doses comparable to those previously reported to be effective in awake behaving animals (Figure 3, Taylor et al., 2007). Here, doses ranging from 1 to 10 μg were found to have similar effect regardless of dose, so these results are presented together. Injection of aCSF into the RVM did not change hindpaw withdrawal thresholds in animals subjected to SNL surgery (ipsilateral: 9.3 g vs. 9.0 g, $p > 0.05$; contralateral: 76.7 g vs. 67.7 g, $p > 0.05$; $n = 4$). These results are consistent with previous findings showing no effect of aCSF microinjection on animals subjected to CFA injection (Cleary and Heinricher, 2013).

3.3 NPY injected into the RVM produces thermal hypoalgesia in naïve animals

The experiments described above showed that although NPY reversed hyperalgesia in models of inflammatory and neuropathic pain, the drug did not affect *mechanical* withdrawal threshold in naïve controls or for the unaffected limb. However, thermal threshold was slightly elevated following microinjection of a low dose of NPY (0.5 μg) in the RVM (Figure 4). At this dose, the average baseline thermal withdrawal threshold was 47.6 ± 0.2 °C, compared to 48.3 ± 0.2 °C, averaged over the hour after NPY injection ($n = 21$, $p < 0.001$, paired t -test). Expressed as percent of maximal percent effect (MPE), this elevation was modest ($11.3\% \pm 2.3\%$). For comparison, a low dose of DAMGO injected into the RVM produces greater than 60% MPE on hindpaw withdrawal under similar experimental conditions (Phillips et al., 2012). Artificial CSF injection did not significantly alter the withdrawal threshold (Figure 4, MPE: $1.9 \pm 1.9\%$).

Consideration of a wider range of doses (0.005 to 50 μg) did not reveal increased thermal antinociception at higher doses of NPY, as measured by the average withdrawal threshold in the 45 min following NPY injection (Figure 5). The lowest dose considered (0.005 μg) produced an effect comparable to that of aCSF, while the highest dose (50 μg) produced only modest hypoalgesia, comparable to the effect of a dose of 0.5 μg . An unconstrained, non-linear dose-response curve was fit to the NPY data, with an EC₅₀ of 0.14 μg (95% CI: 0.0058 μg to 33 μg , $R^2 = 0.12$), the bottom value of the curve at 0.01 ± 0.29 °C, and the plateau of the curve at 0.74 ± 0.15 °C. These results show an imprecise fit, but they indicate that the doses used for thermal hypoalgesia and reversal of mechanical hyperalgesia both produced near maximal effect in normal animals. Based on this analysis, we combined reporting of doses of NPY between 0.5 μg and 10 μg for subsequent electrophysiology experiments.

3.4 NPY non-selectively increases spontaneous activity without altering reflex-related changes in firing

To examine the neural basis for NPY-induced anti-hyperalgesia and hypoalgesia, NPY (0.5 to 5 μg) was microinjected into the RVM while simultaneously recording from physiologically identified RVM neurons in naïve animals. Examples of the effect of local NPY (1 μg in each example) on the activity of ON-, OFF-, and NEUTRAL-cells are shown in Figure 6A. The peptide produced a sustained increase in spontaneous firing in every neuron tested, with a consistent spontaneous firing rate increase of 2- to 3-fold for each class of RVM neuron (Figure 6B, left). Injection of vehicle (aCSF) had no significant effect on spontaneous firing rates for any cell class (Figure 6B, right).

Although the *spontaneous* firing rate was elevated, NPY did not alter the *withdrawal-related change* in ON- or OFF-cell firing. That is, the ON-cell firing rate at rest was significantly elevated after NPY injections, but at the withdrawal the ON-cells still increased their firing above the spontaneous levels by the same amount, regardless of treatment (Figure 6C, left). Similarly, reflex-related slowing in OFF-cell activity was unchanged by NPY. Despite OFF-cells' spontaneous activity being increased, cell firing still slowed by a comparable amount at the time of withdrawal. Microinjection of aCSF into the RVM did not alter withdrawal-related changes in firing of either ON- or OFF-cells (Figure 6C, right). NEUTRAL-cells displayed no changes in firing rate at the withdrawal either before or after microinjection (data not shown).

4.0 Discussion

Although plasticity in ascending nociceptive transmission pathways is widely accepted as a major factor in chronic pain, changes in descending modulatory pathways are increasingly recognized as playing a significant role. The RVM is necessary for the development and maintenance of persistent pain, but understanding its role has been complicated by the fact that it has both pro- and anti-nociceptive components. The present experiments showed that mechanical hyperalgesia induced by either nerve-injury or chronic inflammation can be reversed by increasing the excitability of all neurons within the RVM. While descending facilitation from the RVM can initiate and maintain hyperalgesia (Neubert et al., 2004, Vera-Portocarrero et al., 2006, Cleary and Heinricher, 2013), these findings support the idea that descending inhibition (or the lack thereof) also plays a significant role in the development and maintenance of hyperalgesia (De Felice et al., 2011, Leong et al., 2011).

4.1 Activation of OFF-cells explains the anti-hyperalgesic actions of NPY in the RVM

Understanding ways in which chronic or abnormal pain can be ameliorated may also shed light on the mechanisms contributing to pain. NPY has been reported to have a reliable anti-hyperalgesic effect in animal models of neuropathic pain when applied directly into the RVM, without altering normal responses to noxious stimulation (Taylor et al., 2007). This profile exemplifies the long sought-for treatment for pathological pain that preserves "normal" pain and its protective function. One goal of the present studies was to investigate the mechanism of the anti-hyperalgesic action of NPY at the circuit level by recording the

activity of physiologically identified RVM neurons during nociceptive behaviors, and determining how the neuronal activity is altered by local infusion of NPY in the RVM.

At the behavioral level, our results with NPY microinjections in lightly anesthetized animals were consistent with those of Taylor and colleagues (2007) with awake animals, showing a reversal of mechanical allodynia produced by nerve injury. We saw a similar anti-hyperalgesic effect of the peptide in animals subjected to chronic inflammation (CFA injected into the hindpaw), demonstrating that the anti-hyperalgesic effect of NPY is not restricted to neuropathic states. We also observed no change in basal nociceptive responding with discrete mechanical testing. However, thermal stimulation, which allows a continuous rather than discrete measure of nociceptive threshold, revealed a modest but statistically significant increase in withdrawal threshold, consistent with the known hypoalgesic influence of spontaneous OFF-cell firing (Heinricher et al., 1989).

We originally hypothesized that the anti-hyperalgesic effect of NPY in the RVM would be explained by a selective suppression of ON-cell firing. This idea was based on the demonstrated pro-nociceptive influence of these neurons (Heinricher and Neubert, 2004, Neubert et al., 2004), and on evidence that ON-cells contribute to neuropathic pain states as part of a positive feedback loop (Porreca et al., 2001, Heinricher et al., 2009). Further, in Taylor and colleagues' studies in nerve-injured animals (2007), block of the RVM with lidocaine produced an anti-hyperalgesic effect, leading to the hypothesis that NPY and lidocaine have a common mechanistic underpinning. However, in that work NPY had a greater anti-hyperalgesic effect than lidocaine, suggesting that NPY was not producing therapeutic benefit via the same mechanism as lidocaine. We found that NPY microinjected at behaviorally relevant doses did not selectively inhibit ON-cells, but instead resulted in a several-fold *increase* in the spontaneous firing of all three classes of RVM neurons, including ON-cells.

These unexpected physiological findings are paralleled by results from our immunohistochemical experiments. Although serotonin from the RVM has been tied to both pain facilitation and inhibition (LeBars, 1988, Suzuki et al., 2004), recent evidence showed that a significant subset of RVM serotonergic neurons display increased firing in response to noxious stimuli (Gau et al., 2013). We initially hypothesized that staining for the Y1 receptor would selectively label a subpopulation of RVM neurons with strong TPH₂ co-localization (presumably the ON-cells), but the Y1 receptor was broadly expressed and did not selectively co-localize with TPH₂.

Given the known influence of ON- and OFF-cells on nociception and the fact that antinociception dominates when ON- and OFF-cells are co-activated (Neubert et al., 2004, Phillips et al., 2012), the *anti-hyperalgesic* effect of NPY microinjection in RVM is best explained by the increase in the *ongoing* activity of OFF-cells. The lack of substantial *analgesia* is explained by the fact that the magnitudes of *reflex-related changes* in OFF-cell (and ON-cell) firing were not altered following NPY. Following NPY administration, OFF-cells continued to display the same relative decrease in firing at the withdrawal, suggesting that the NPY-mediated increase in spontaneous activity is primarily post-synaptic. This observation of increased spontaneous activity with a preserved reflex-related change in

firing lends strength to the idea that drugs must block the GABAergic input mediating the OFF-cell pause for RVM-mediated analgesia (Heinricher et al., 1991, Heinricher et al., 1994, Heinricher et al., 2010). At the same time, this finding also shows that an increase in the output of the OFF-cell population is sufficient to suppress hyperalgesia without producing analgesia. The distinction between anti-hyperalgesic and analgesic effects of OFF-cell output has important implications for drug development, as well as for our understanding of the physiology of pain-modulating neural circuits

4.2 Physiology and pharmacology of exogenous NPY in the RVM

The EC50 concentration and dose of peptide microinjected into the RVM used here were on par with previous microinjection studies, and within the physiological range for NPY in plasma in rat (Zukowska-Grojec and Wahlestedt, 1993). Significant interest exists in altered expression of NPY and its receptor in chronic pain models as potentially limiting abnormal pain (Smith et al., 2007, Solway et al., 2011) or contributing to abnormal activity in pathways transmitting tactile information (Ossipov et al., 2002). Previous work noted that direct injection of a Y1 receptor antagonist in the RVM by itself had no effect on mechanical allodynia or thermal hyperalgesia following nerve injury (Taylor et al., 2007). This finding implies that endogenous NPY neither limits nor promotes hypersensitivity through an action in the RVM. Regardless, here we focused not on understanding the physiology of endogenous NPY in the RVM, but on using the behavioral and physiological effects of NPY as a route to better understand chronic pain and RVM physiology.

Every cell tested, regardless of dose, showed increased spontaneous activity in response to microinjected NPY. This finding was surprising, as not all RVM neurons showed strong Y1-LI, and the direct post-synaptic actions of NPY in the central nervous system were previously reported to be inhibitory (Michel, 1991). The effects observed here then could reflect presynaptic actions, circuit-level effects, or even local vascular effects of the peptide. NPY receptors have been identified on cerebral microvessels (Cauli et al., 2004), and NPY-positive fibers are associated with intracerebral microvasculature in rat (Abounader and Hamel, 1997). The peptide constricts isolated microvessels (Dacey et al., 1988), and reduces local blood flow when applied in various brain regions (Suzuki et al., 1989, Tuor et al., 1990). Activation of RVM neurons secondary to hypoperfusion may be a physiological process, since there is evidence that this region plays a role in chemoreception (Nattie and Li, 2001, Dias et al., 2008). If vasoconstriction proves to be the underlying cause of the changes in neuronal activity, NPY would not present an ideal target for analgesic drug development.

4.3 Independent Contributions of ON- and OFF-cells to chronic pain states

Considerations of the role of the RVM in chronic pain states have largely focused on the ON-cells. Selective ablation of μ -opioid receptor-expressing neurons, generally thought to be ON-cells, interferes with ongoing hypersensitivity following nerve injury (Porreca et al., 2001), and non-selective block of all RVM neurons has been shown by several groups to attenuate mechanical allodynia following nerve injury (Pertovaara et al., 1996, Burgess et al., 2002, Chen et al., 2004, Ossipov et al., 2005, Bee and Dickenson, 2007, Taylor et al., 2007). However, the idea that decreased OFF-cell activity also contributes to the

development of hyperalgesia need not conflict with existing ideas on descending facilitation. In models of chronic inflammation and nerve injury, both ON- and OFF-cells have been shown to be sensitized to non-noxious stimulation (Carlson et al., 2007, Cleary and Heinricher, 2013). Moreover, descending inhibition can limit expression of hypersensitivity in nerve injury (De Felice et al., 2011). Further, SNL surgery has been shown to drive increased RVM cell death in concert with the development of hyperalgesia, and blocking this cell death attenuates increased nociceptive responsiveness in these animals (Leong et al., 2011). Finally, in chronic inflammation, inactivation of the RVM generally potentiates hypersensitivity, rather than reversing it (LeBars, 1988, Guan et al., 2002, De Felice et al., 2011, Cleary and Heinricher, 2013). Collectively, these findings, obtained in both awake and anesthetized animals, imply that the loss of the normally protective OFF-cell activity can play an important role in the development of both neuropathic and inflammatory pain, and suggest that loss of descending inhibition is at least as important as gain of facilitation.

4.4 Technical Limitations

In this study, behavioral testing was done in animal models of pathological pain, but the neuronal responses to NPY were determined in normal animals, which may limit the extrapolation of the results. However, the behavioral effects were consistent across multiple models, and NPY doses were within the same ranges in each group. Taken together with the consistent effects of NPY across all neurons tested for all three cell classes, drastic differences in the neuronal response to NPY across the different models seem unlikely.

Similarly, the consistency of the behavioral and neuronal responses to NPY also downplays variability within the data set. By combining data within the 1 μ g to 10 μ g dose range for electrophysiology, the possibility exists for missing a subtle dose-related effect. Likewise by combining data from animals between 3 and 10 days post-inflammation, a time-dependent anti-hyperalgesic effect may be missed. However, in both these cases, the effect of NPY was so reliably predictable (either strongly increased spontaneous activity or potent behavioral anti-hyperalgesia) that the combination of data is unlikely to have overlooked a physiologically relevant effect that would change the interpretation of the data.

4.5 Conclusion

The present study argues that hypersensitivity exhibited in chronic pain models is due to a failure of descending inhibition in addition to the previously described contributions of increased descending facilitation and enhanced sensory transmission. Understanding plasticity of both pro- and anti-nociceptive RVM outputs and how the influence of each can change with different pain states is critical for developing better treatments for chronic pain.

Abbreviations

aCSF	Artificial cerebrospinal fluid
CFA	Complete Freund's adjuvant
MPE	Percent of maximal effect

NPY	Neuropeptide Y
RVM	Rostral ventromedial medulla
SNL	Spinal nerve ligation

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Highlights

NPY in the RVM reverses mechanical hyperalgesia from nerve injury and inflammation.

In naïve animals, NPY in the RVM produces mild thermal hypoalgesia.

NPY increases firing of both pain-inhibiting and pain-facilitating RVM neurons.

Pain relief from NPY in the RVM is best explained by increased OFF-cell activity.

Figure 1.

Location of RVM injections and cell recordings. Left are the locations at which NPY was microinjected into the RVM in behavioral studies. Right are the locations for which cells were recorded during simultaneous NPY microinjection. Numbers on the left represent distances from the interaural line (mm).

Figure 2.

Identification of TPH+ and Y1-LI+ neurons in the RVM. A. Outline of area considered the RVM, and shown in B, C, and D, with higher magnification view shown in E, F, and G. Y1-LI: red, TPH-LI: green, overlay: yellow. H: number of RVM cells with Y1-LI, TPH-LI, or both at different distances from the interaural line.

Figure 3.

NPY microinjection (1 to 10 μ g) into the RVM reverses mechanical hyperalgesia in SNL and CFA models of chronic pain. In normal animals, microinjection of NPY did not significant change responses to mechanical stimuli ($p = 0.098$, $n = 7$, Wilcoxon test). NPY also failed to alter response thresholds for the uninjured paw in either model. However, hyperalgesia ipsilateral to nerve-injury and inflammation was reversed by RVM NPY (Compared to pre-injection baseline, SNL: $n = 6$, $p = 0.009$; CFA: $n = 9$, $p = 0.014$. Kruskal-Wallis analysis of variance by rank, followed by Dunn's post-hoc test.)

Figure 4.

NPY microinjection (0.5 μ g) in the RVM slightly increases the thermal withdrawal threshold compared to baseline, while aCSF had no effect. Repeated-measures ANOVA with Dunnett's post-hoc test for comparison to baseline. No significant changes occurred with aCSF microinjection. $**p < 0.01$ vs. middle baseline value. NPY: $F_{11, 220} = 5.50$, $n = 21$; aCSF: $F_{11, 209} = 1.77$, $n = 20$.

Figure 5.

Dose response curve for thermal antinociception for NPY. Circles show the average change in nociceptive threshold over the 45 minutes following NPY injection into the RVM, and the line shows a non-linear dose-response curve fit to these points. (From left to right, n's = 20,4,3,21,15, and 3 in the different dose groups).

Figure 6.

RVM microinjection of NPY increased spontaneous neuronal activity without compromising the pause or the burst. In representative examples, (A) RVM microinjection of 1 μ g NPY increased spontaneous activity of neurons from each cell class. (B) Across all cells tested, NPY significantly increased spontaneous activity (Wilcoxon pre- vs. post-drug; $n = 10$, $p = 0.006$ for NEUTRAL cells; $n = 11$, $p = 0.001$ for OFF-cells; $n = 7$, $p = 0.016$ for ON-cells), whereas aCSF did not change the activity of any cell class ($n = 7$, $p = 0.11$ for NEUTRAL cells; $n = 6$, $p = 0.18$ for OFF-cells; $n = 7$, $p = 0.68$ for ON-cells). (C) When

looking at the relative change in firing at the withdrawal, NPY did not significantly change the magnitude of the ON-cell increase ($p = 0.94$) or the OFF-cell decrease in activity ($p = 0.054$).