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The dorsomedial hypothalamus mediates stress-induced hyperalgesia and is the source of the pronociceptive peptide cholecystokinin in the rostral ventromedial medulla

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Abstract

While intense or highly arousing stressors have long been known to suppress pain, relatively mild or chronic stress can enhance pain. The mechanisms underlying stress-induced hyperalgesia are only now being defined. The physiological and neuroendocrine effects of mild stress are mediated by the dorsomedial hypothalamus (DMH), which has documented connections with the rostral ventromedial medulla (RVM), a brainstem region capable of facilitating nociception. We hypothesized that stress engages both the DMH and the RVM to produce hyperalgesia. Direct pharmacological activation of the DMH increased sensitivity to mechanical stimulation in awake animals, confirming that the DMH can mediate behavioral hyperalgesia. A behavioral model of mild stress also produced mechanical hyperalgesia, which was blocked by inactivation of either the DMH or the RVM. The neuropeptide cholecystokinin (CCK) acts in the RVM to enhance nociception and is abundant in the DMH. Using a retrograde tracer and immunohistochemical labeling, we determined that cholecystokinin-expressing neurons in the DMH are the only significant supraspinal source of cholecystokinin in the RVM. However, not all neurons projecting from the DMH to the RVM contained cholecystokinin, and microinjection of the CCK₂ receptor antagonist YM022 in the RVM did not interfere with stress-induced hyperalgesia, suggesting that transmitters in addition to cholecystokinin play a significant role in this connection during acute stress. While the RVM has a well-established role in facilitation of nociception, the DMH, with its well-documented role in stress, may also be engaged in a number of chronic or abnormal pain states. Taken as a whole, these findings establish an anatomical and functional connection between the DMH and RVM by which stress can facilitate pain.

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Author Contributions

KW performed behavioral experiments, analyzed data, and contributed to writing of the manuscript. ZR and KD performed immunohistochemical staining and contributed to writing of the manuscript. AVB advised on immunohistochemical methods and contributed to writing of the manuscript. MMH and DRC designed experiments, analyzed data, and contributed to writing of the manuscript.

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Keywords

pain modulation; raphe; dorsomedial hypothalamus; stress; hyperalgesia; cholecystokinin

1.0 Introduction

Pain is a normal indication of acute tissue injury, but the intensity of pain experienced often fails to reflect the actual extent of damage (Beecher, 1959). Perception of an injury can be altered by cognitive and emotional factors, including anxiety and stress. Stress can either suppress or enhance pain, depending on the intensity of the stressor and level of associated arousal (Rhudy and Meagher, 2000, Meagher et al., 2001). Extreme stress can inhibit pain, producing “stress-induced analgesia,” whereas mild or prolonged stress often heightens pain, giving rise to “stress-induced hyperalgesia” (SIH).

The rostral ventromedial medulla (RVM) is a brainstem region shown to be critical for some forms of stress-induced *analgesia* (Watkins et al., 1983, Butler and Finn, 2009), but has also been implicated in stress-induced *hyperalgesia* (Imbe et al., 2006, Reynolds et al., 2011). The RVM integrates somatic information with influences from higher structures, and can facilitate nociception via activation of the pronociceptive “ON-cells” (Porreca et al., 2002, Heinricher and Ingram, 2008). Blocking ON-cell activity prevents behavioral hyperalgesia in acute injury, and also reverses hyperalgesia following inflammation and nerve injury (Heinricher and Ingram, 2008, Heinricher et al., 2009).

RVM-mediated hyperalgesia can occur through selective activation of ON-cells by the pronociceptive neuropeptide cholecystokinin (CCK; Kovelowski et al., 2000, Heinricher and Neubert, 2004). Conversely, blocking CCK receptors in the RVM reverses behavioral hypersensitivity in these and other abnormal pain states (Kovelowski et al., 2000, Xie et al., 2005, Edelmayer et al., 2009, Marshall et al., 2012). Levels of endogenous CCK are increased in the RVM following nerve injury and in opioid-induced hyperalgesia. Although the physiology of CCK in the RVM is well described, the origin of CCK-containing projections to the RVM has not been determined. Here, we used retrograde tract tracing combined with CCK immunohistochemistry to identify the dorsomedial hypothalamus (DMH), a site with abundant endogenous CCK (Innis et al., 1979), as the most prominent source of this pronociceptive neuropeptide in the RVM.

Functional and anatomical connections tie the DMH to the RVM, and link both regions to physiological responses to stress. Stress-activated neurons in the DMH project to the RVM, and stimulating the DMH mimics the physiological effects of external stressors, evoking tachycardia, tachypnea, and hyperthermia (DiMicco et al., 2002, Samuels et al., 2004, Sarkar et al., 2007, Martenson et al., 2009). Conversely, blocking the DMH interferes with these responses (DiMicco et al., 2006, Dampney et al., 2008, Fontes et al., 2011). Given the contribution of the DMH to stress-induced physiological responses, as well as the anatomical link between the DMH and RVM, the present study explored how a functional connection between these regions could contribute to stress-induced hyperalgesia. In our experiments, we used an air-puff stress protocol that has been previously employed in rodents as a paradigm of mild stress (DiMicco et al., 2002). We found mechanical hypersensitivity in awake, behaving animals that was dependent on both the DMH and the RVM. These results demonstrate a top-down pathway through which stress can facilitate the perception of pain.

2.0 Experimental Procedures

All surgeries and animal procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals 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 Oregon Health & Science University.

Adult male, Sprague-Dawley rats (250–350 g, Charles River Laboratories) were maintained in a 12-hour light/dark cycle with ad libitum access to food and water, and were monitored after surgeries and throughout the experiments.

2.1 CCK immunohistochemistry with retrograde tracers in the RVM

2.1.1 Tracer and Colchicine injections—To identify CCK-containing neurons projecting to the RVM, we used the retrograde tracer cholera toxin subunit B (CTB) combined with CCK immunohistochemistry. CTB was microinjected into the RVM, and CCK peptide concentration was enhanced in cell bodies using the microtubule inhibitor colchicine. For RVM tracer injections, rats were initially anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) and then placed in a stereotaxic apparatus. A glass injector with a 40–50 μ m tip diameter was used to inject 200 nl of CTB conjugated to AlexaFluor 488 (1 mg/ml) at a depth of 9.5 to 10 mm below the brain surface. The microinjector was left in place for 15 min to minimize tracking. Animals were subsequently housed individually.

Animals were re-anesthetized 6–8 days after the CTB injection, and colchicine (80 μ g/8 μ L in artificial cerebrospinal fluid, aCSF) was injected into the lateral ventricle. Twenty-four to 48 h after the colchicine injection, the rats were deeply anesthetized (sodium pentobarbital, 60–70 mg/kg, i.p.) and perfused transcardially with 100 ml of 0.1 M ice-cold phosphate-buffered saline (PBS) followed by 500 ml of ice-cold 4% paraformaldehyde, and then 100 ml of 30% sucrose in PBS. The brains were removed and post-fixed for 30 minutes in 4% paraformaldehyde, then equilibrated for 24 hours in 30% sucrose in PBS at 4 °C. Serial coronal sections (40 μ m) were made with a cryostat, and sections were stored in 30% sucrose or cryoprotectant prior to immunohistochemical processing.

2.1.2 Immunohistochemistry—Injection sites in the RVM, defined as the nucleus raphe magnus and surrounding reticular formation at the level of the facial nucleus, were confirmed using fluorescence microscopy prior to immunohistochemical processing. A dual fluorescence labeling procedure was performed on a representative selection of sections from –1 to –15 mm relative to bregma.

Unless noted otherwise, all steps were performed in a base solution of 0.3% Triton-X in Tris-buffered saline (TBS) and preceded by two to three washes in TBS. The sections were rinsed for 30 min in 1% sodium borohydride in TBS, and then gently shaken while in a blocking solution of 5% normal donkey serum (NDS) with 0.3% Triton-X in TBS for 45 min at room temperature. After blocking, the sections were incubated with two primary antibodies at 4 °C for 72 hours in 1% NDS. The two primary antibodies used were a mouse-derived monoclonal antibody to cholecystikinin-8, diluted 1:10,000 (Abcam 37274), and a rabbit-derived polyclonal antibody to CTB, diluted 1:2000 (Abcam 34992).

After incubating with the primary antibody, the sections were rinsed with 5% NDS blocking solution for 30 min at room temperature, then incubated for 60 min at room temperature in 5% NDS with the first secondary antibody, donkey anti-mouse AlexaFluor 555 diluted 1:400. The sections were rinsed in 5% NDS blocking solution for 30 min at room

temperature and then incubated in 10% NDS for 2 h at room temperature with donkey anti-rabbit Alexa Fluor 488, to aid visualization of the retrogradely transported CTB conjugate (dilution: 1:350). Slices were then mounted, air-dried, and cover-slipped with anti-fade mounting medium (ProLong Gold, Invitrogen).

Fluorescently labeled neurons were initially visualized with an Olympus BX51 fluorescence microscope, and neuroanatomical designations made using the Paxinos and Watson (2009) stereotaxic brain atlas. Relative numbers of single- and double-labeled neurons were counted in a single representative section from each brain. Optically sectioned images were taken with a Zeiss Apotome 2 microscope using a 10x objective, a z-stack of 5 images, and a 2×3 or 3×3 tile scan with proper settings for Alexa488 or 555 fluorophores. Stacks were combined with a maximum intensity z-projection. The resulting image was cropped around the region of interest but included all double-labeled neurons in the section. Labeled neurons with defined processes and a visible soma were counted with the ImageJ Cell Counter plugin (Preibisch et al., 2009). Neurons were counted independently in the CCK and CTB channels before counting double-labeled neurons.

2.2 Stress-induced hyperalgesia

2.2.1 Cannula Implantation—For implantation of cannulae into either RVM or DMH, adult male Sprague-Dawley rats weighing between 260 and 330 g were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) or ketamine/xylazine (60 and 10 mg/kg, respectively). A 26-gauge guide cannula was implanted stereotactically, 2 mm dorsal to either the DMH (3.2 mm caudal and 0.5 mm lateral to bregma, 7.0 mm ventral to the brain surface, *n* = 10) or RVM (10.8 mm caudal and 0 mm lateral from bregma, 7.3 mm ventral to the brain surface, *n* = 11). All DMH cannulae targeted the right DMH, based on previous reports of right-sided dominance for DMH-mediated autonomic effects (Xavier et al., 2009). The cannula was secured to the skull and two anchoring screws using orthodontic resin. A 30-gauge wire stylet was inserted into the guide cannula to prevent clogging. Animals were housed individually after awakening from anesthesia.

Animals were allowed seven days to recover before testing. The stylet was removed and replaced daily to ensure cannula patency. At least one day before experiments, a sham injector was inserted through the guide cannula into the area of interest.

2.2.2 von Frey Testing—Calibrated von Frey filaments were used to measure threshold for withdrawal from tactile stimulation using an up-down protocol (Chaplan et al., 1994). Rats were placed in a Plexiglas enclosure with a wire mesh bottom. Before each testing session, the animals were allowed to acclimate to the testing environment for at least 15 minutes, after which withdrawal thresholds were determined by applying the filaments to the webbing between the toes. Withdrawal thresholds were again measured at the end of the experiment. Pre- and post-treatment thresholds were measured three times on each paw, with 5-min intervals between measurements. Application sites were rotated to avoid sensitization. Reported measures are the averaged thresholds for both hindpaws.

2.2.3 DMH and RVM Microinjections—Intracerebral microinjections were made through the guide cannula using a 31-gauge injector attached to a 1- μ l Hamilton syringe. The injector extended 2 mm beyond the end of the cannula into the area of interest. Green fluorescent microbeads (0.1 μ m, Fluospheres, Invitrogen, Eugene, OR) were added to all solutions to allow injection localization on subsequent histology. The GABA_A receptor agonist muscimol (22 pmol/0.5 μ l, Tocris Bioscience, St. Louis) was injected into the DMH to inhibit constituent neurons. The GABA_A receptor antagonist bicuculline methiodide was injected to disinhibit DMH neurons (30 pmol in 0.3 μ l, Tocris Bioscience). Lidocaine (0.5

μl, 2%) was injected into the RVM to inhibit all neurons. The CCK₂ receptor antagonist YM022 was also injected into the RVM prior to the stress (100–300 pg in 0.2–0.6 μl DMSO, Tocris Bioscience). Muscimol, bicuculline, and lidocaine were dissolved in artificial cerebrospinal (aCSF). An equal volume of vehicle (aCSF) was injected as a control for all drug injections. Animals were loosely restrained during the injection. All injections were made at a rate of 200 nl/min, and the injector was left in place for a minimum of 20 s after the injection.

2.2.4 Experimental Protocol—To determine the effects of stress on nociceptive sensitivity in the absence of pharmacologic intervention, withdrawal thresholds were measured before and after a period of air stress. Briefly, the animal was placed in a loose plastic restrainer that limited movement, and a slow, continuous stream of air at room temperature was directed at the face for 30 min (Soltis and DiMicco, 1992, Stotz-Potter et al., 1996, Zaretsky et al., 2003). Control animals were returned to their home cages for 30 min in lieu of air stress. In a second set of experiments, baseline withdrawal thresholds were measured prior to inhibition of either the RVM or DMH using intracerebral injection; the injection was then followed by stress or control intervention. Thus, in experiments with injection into the RVM, either lidocaine or aCSF was injected after baseline, that is, immediately before the animal was subjected to the air-stress procedure or returned to the home cage (control). Following this intervention (stress or home cage), the animal was given 15 minutes to re-acclimate to the testing box, and the von Frey thresholds were re-determined. Effects of the intervention were thus compared to pre-drug baseline in animals treated with lidocaine or aCSF. In experiments with inhibition of the DMH, either muscimol or vehicle was injected after baseline, that is, prior to stress, and outcome measurements were taken as described above. Effects of the stress intervention were thus compared to pre-drug baseline in animals treated with muscimol or vehicle. In a third set of experiments, neurons in the DMH were disinhibited using bicuculline microinjection in the absence of stress or control intervention. Withdrawal thresholds were measured in baseline and following DMH injection.

Each animal participated in multiple experiments of stress or control in random order, and was given at least two days to recover between experiments. Autonomic responses to air stress are known to recover within an hour of the stress procedure (Zaretsky et al., 2003, de Menezes et al., 2008). Baseline withdrawal thresholds were not significantly different among experiments without cannulae, with cannulae in the DMH, and with cannulae in the RVM (Kruskal-Wallis ANOVA, $p = 0.47$).

2.2.5 Histology—After completion of the behavioral experiments, animals were overdosed with an intraperitoneal injection of pentobarbital (400 mg/kg) and perfused with 10% formalin. The brains were post-fixed for at least 3 days and then sectioned at 60 μm. Fluorescent microbeads at injection sites were identified using an Olympus BX51 microscope and known anatomy (Paxinos and Watson, 2009). Injection sites are shown in Figure 1.

2.2.6 Analysis—Statistical analyses were performed using Prism (GraphPad, La Jolla, CA). Pre- and post-treatment thresholds were compared using Wilcoxon signed-ranks test. Results are presented as mean ± SEM.

3.0 Results

3.1 Identification of CCK-containing projections to the RVM

Brains in which retrograde injections were limited to the RVM were used for a general survey to identify sites of double labeling. Three brains (CCK1, CCK2, and CCK3) had

retrograde tracer injections restricted to the RVM (including the nucleus raphe magnus and nucleus raphe pallidus at the level of the facial nucleus, Fig. 2B). The tissue was sliced at 40 μm thickness and surveyed for CTB-LI and CCK-LI neurons from -1 to -9 mm relative to bregma (CCK1) or from -1 to -15 mm relative to bregma (CCK2, CCK3). In our survey of possible CCK-containing inputs to the RVM, we gave particular attention to areas known broadly to project to RVM and to areas recognized to contain CCK (Innis et al., 1979, Beinfeld et al., 1981, Beinfeld and Palkovits, 1982, Schiffmann and Vanderhaeghen, 1991, Hermann et al., 1997).

CTB labeling closely resembled previous reports (Hermann et al., 1997). CTB-LI was widely distributed throughout the brain, with high concentrations in the medial preoptic nucleus, ventrolateral periaqueductal grey, the parabrachial complex, and the dorsomedial nucleus of the hypothalamus.

The locations of single-labeled CCK-positive neurons were consistent across all brains. As expected, high levels of CCK-LI were seen in the DMH, Edinger-Westphal nucleus, and solitary nucleus and tract (Innis et al., 1979, Beinfeld and Palkovits, 1982, Schiffmann and Vanderhaeghen, 1991). Bright labeling was notable in the compact dorsomedial hypothalamic nucleus. CCK-LI was seen in isolated fibers in raphe magnus and raphe pallidus, as shown previously (Skinner et al., 1997).

In these brains, consistently double-labeled neurons were found in only one location: the dorsal aspect of the DMH adjacent to the ventricle (Fig. 2C–H; this area is sometimes referred to as the “dorsal hypothalamic area”, Samuels et al., 2004). These neurons were in highest concentration at -3.12 mm relative to bregma and were distributed between -2.64 and -3.24 mm relative to bregma. Double-labeled neurons were typically clustered within a larger group of neurons showing CTB-LI labeling (Fig. 2). These neurons were brightly labeled and characterized by a well-defined soma and processes. Among the neurons with CTB-LI, only $5.6 \pm 1.2\%$ also showed CCK-LI, but among neurons with CCK-LI, $28.8 \pm 7.5\%$ were also positive for CTB-LI.

Occasionally, isolated double-labeled neurons were found outside the dorsal hypothalamic area of the DMH. In one brain (CCK1), in addition to the clustered neurons in the dorsal hypothalamic area, two double-labeled neurons were found in the more caudal DMH. In another brain (CCK2), an isolated double-labeled neuron was found more rostrally in the hypothalamic paraventricular nucleus, and another was found in the A7 region. These two neurons were both faintly labeled.

Some areas contained mixed single-labeled neurons, where neurons with CCK-LI were juxtaposed with neurons with CTB-LI, although no double labeling was found. Such areas included the parabrachial region, the medial preoptic area, and the Edinger-Westphal nucleus (Fig. 3), where the differentiation was particularly well defined.

In four additional brains in which the CTB injection extended beyond the borders of the RVM into nucleus reticularis gigantocellularis, bright double-labeled neurons were also observed in the DMH between -3.24 and -2.64 mm from bregma. Double-labeled neurons were not found in other regions, including A7 or the hypothalamic paraventricular nucleus.

3.2 Air stress induces hyperalgesia in awake animals

We first determined whether animals subjected to air stress exhibit hyperalgesia. Baseline withdrawal thresholds were established, after which animals were subjected to air stress or returned to the home cage for 30 min. Thresholds were then re-determined. Air stress

significantly decreased withdrawal threshold compared to baseline (Fig. 4A). Unstressed home-cage controls showed no change in threshold.

3.3 Role of the DMH in air stress-induced hyperalgesia

We next tested the hypothesis that pharmacological activation of DMH neurons produces hyperalgesia. DMH neurons were disinhibited using microinjection of the GABA_A receptor antagonist bicuculline. DMH injection sites ranged from -3.60 to -3.12 mm from bregma (Fig. 1). DMH activation resulted in decreased mechanical withdrawal thresholds (Fig. 4B). Control injections (aCSF) had no effect on withdrawal thresholds. This demonstrates that direct pharmacological activation of the DMH produces hyperalgesia in behaving animals, extending previous findings in a lightly anesthetized preparation (Martenson et al., 2009).

We then determined whether the DMH was required for hyperalgesia produced by the air-stress procedure. DMH block using the GABA_A agonist muscimol prevented stress-induced hyperalgesia (Fig. 5A). Control injections of aCSF prior to stress did not block the decrease in threshold. DMH block by itself had no effect in unstressed home-cage controls.

3.4 RVM modulates stress-induced hyperalgesia in awake animals

Autonomic responses to air stress are mediated by a connection from the DMH to the RVM (Zaretsky et al., 2003, Sarkar et al., 2007), raising the possibility that nociceptive behavior is modulated through this or a similar circuit. We therefore blocked activity in the RVM prior to the stress exposure to test whether this region, like the DMH, contributes to air stress-induced hyperalgesia. RVM microinjection sites ranged from -10.8 to -11.60 mm from bregma and were within 1 mm of midline (Fig. 1).

Microinjection of the local anesthetic lidocaine, but not aCSF, into the RVM blocked the subsequent decrease in withdrawal threshold produced by air stress (Fig. 5B). RVM block had no effect in unstressed home-cage controls. Finally, to determine whether the stress-induced hyperalgesia was mediated by CCK in the RVM, the CCK₂ receptor antagonist YM022 was microinjected into the RVM prior to stress. YM022 did not prevent the stress-induced decrease in withdrawal threshold (Fig. 5B).

4.0 Discussion

Exogenous application of the neuropeptide CCK in the RVM increases sensitivity to noxious stimuli, and endogenous CCK release in this region is implicated in hyperalgesia seen in some models of chronic pain (Kovelowski et al., 2000, Xie et al., 2005, Edelmayer et al., 2009, Ambriz-Tututi et al., 2011). However, the source of CCK-releasing inputs to the RVM had not previously been identified. We showed here that neurons in the dorsal aspect of the DMH are the only consistent supraspinal source of CCK-expressing cells that project to the RVM. We also demonstrated that pharmacological stimulation of the DMH increased sensitivity to mechanical stimulation in the awake animal, and conversely, that inhibiting either the DMH or the RVM blocked the mechanical hyperalgesia triggered by a mild stress. Previous work in the anesthetized animal found RVM function to be required for DMH-mediated hyperalgesic effects (Martenson et al., 2009). Collectively, these findings establish a physiological connection between the DMH and the RVM that mediates acute stress-induced hyperalgesia, and identify a distinct anatomical pathway that likely contributes to hyperalgesia in some chronic pain states.

4.1 A DMH-RVM connection in stress-induced hyperalgesia

Pharmacological activation of DMH neurons in awake, behaving animals lowers the threshold for response to von Frey stimulation. This observation reinforces previous work in

anesthetized animals, where thermal and mechanical hyperalgesia were evoked by disinhibition of DMH, and shown to be dependent on increased activity of the pronociceptive RVM ON-cells (Martenson et al., 2009). The conclusion that the DMH-mediated hyperalgesic effect requires a connection through the RVM is consistent with multiple other studies implicating activation of an RVM output, specifically the ON-cells, in thermal and mechanical hyperalgesia (Porreca et al., 2002, Heinricher et al., 2009). These pronociceptive neurons modulate withdrawal thresholds via descending projections to the dorsal horn of the spinal cord (Fields et al., 2006).

In addition to the hyperalgesic effect shown here, activation of the DMH has also been reported to modulate body temperature and cardiorespiratory function through sympathetic pathways. This sympathetic element of the connection between the DMH and the RVM has been investigated extensively (DiMicco et al., 2002, Samuels et al., 2002, Zaretskaia et al., 2003, Madden and Morrison, 2004, Samuels et al., 2004). The RVM, which includes portions of the nucleus raphe pallidus, mediates DMH-dependent increases in heart rate and core temperature (Morrison, 1993). Sites in the DMH where stimulation best elicits increases in heart rate coincide with a concentration of neurons that project to the raphe pallidus and surrounding RVM (Samuels et al., 2002, 2004). The CCK-positive, RVM-projecting neurons identified here were almost entirely located in this same subregion of the DMH. Our results thus tie RVM-mediated hyperalgesia to stress-induced tachycardia and hyperthermia through common sites and pathways, although the autonomic effects likely involve sympathetic premotor neurons and cannot be mediated solely by the RVM ON-cells responsible for hyperalgesia (Cao et al., 2004, Horiuchi et al., 2004, Martenson et al., 2009, Fontes et al., 2011, Reynolds et al., 2011, Tanaka et al., 2011, Phillips et al., in press).

The behavioral significance of the link between DMH and RVM was further supported by the experiments using air stress as a model of non-noxious anxiogenic stress. An air-puff stimulus elicits autonomic and neuroendocrine markers of stress in awake animals, and these increases can be reversed by block of either the RVM or the DMH (DiMicco et al., 2002, Zaretsky et al., 2003, Fontes et al., 2011). In the present studies, the same stress protocol resulted in mechanical hyperalgesia. Loosely restrained animals receiving 30 minutes of air stress exhibited significantly lower withdrawal thresholds, and this effect was prevented by inhibition of either the RVM or the DMH. Although many other models of stress-induced hyperalgesia have used protocols requiring repeated episodes of stress (e.g., Andre et al., 2005, Imbe et al., 2006, Rivat et al., 2010), the present experiments show that a single period of mild stress is sufficient to induce nociceptive hypersensitivity, and validate the use of DMH stimulation in the lightly anesthetized preparation as a model of “stress-induced” hyperalgesia (Martenson et al., 2009). These results are also consistent with the idea that mild stress leads to an enhanced experience of pain, and not simply altered reflex responses (King et al., 2007). Future studies of the DMN-RVM circuit should nevertheless include behavioral measures that tap into supraspinally organized aspects of pain.

DMH inactivation had no effect on withdrawal threshold in control animals not subjected to the air-stress procedure, which suggests that nociceptive testing *per se* was not a significant source of stress in these experiments. Also, while these data implicate the DMH in stress-induced hyperalgesia, contributions from surrounding regions should be considered. In the present experiments, DMH injections were made using a 31-gauge injector, which limits the precision with which specific brain regions can be targeted. However, our previous study in lightly anesthetized animals using a fine glass microinjection cannula found no effect of injections in areas surrounding the DMH (Martenson et al., 2009), and immediately adjacent areas such as the ventromedial hypothalamus and paraventricular nucleus have been linked to hypoalgesia rather than hyperalgesia (Oka et al., 1995, Hori et al., 2000, Condes-Lara et al., 2012), which argues for the DMH as the site of action of the injected drugs.

4.2 The DMH as the source of CCK in the RVM

Although CCK release in the RVM has been strongly implicated in behavioral hyperalgesia, the origin of the peptide was unknown. In the present experiments, we identified the DMH as the sole supraspinal source of CCK inputs to the RVM. This suggests that the DMH, with its well-documented role in stress (DiMicco et al., 2002), is engaged in a number of chronic or abnormal pain states.

Although the projection from the DMH to the RVM includes CCK, only a subset of projecting neurons were CCK-expressing, and microinjecting the CCK₂-receptor antagonist YM022 into the RVM did not block hyperalgesia after air stress. It is possible that the lack of effect of YM022 is because of an inadequate dose or an insufficient distribution of the antagonist throughout the RVM. However, the dose, volume, and timing used here were comparable with those employed in previous studies, so this explanation seems doubtful (Kovelowski et al., 2000, Xie et al., 2005, Edelmayer et al., 2009, Ambriz-Tututi et al., 2011).

More likely, stress-induced hyperalgesia is mediated by multiple neurotransmitters, possibly including CCK, thus limiting the behavioral effect of CCK receptor antagonism. Indeed, we previously showed that blocking excitatory amino acid receptors in the RVM prevented activation of RVM ON-cells and hyperalgesia produced by stimulation of the DMH (Martenson et al., 2009). DMH neurons that project to the RVM also show neuronal nitric oxide synthase immunoreactivity (Sarkar et al., 2007), and nitric oxide acting in the RVM has been implicated in hyperalgesia (Urban et al., 1999). In further support of this interpretation, the amount of CCK in the RVM is increased only modestly in other chronic pain models. High concentrations of CCK are required to activate RVM ON-cells and induce hyperalgesia (Kovelowski et al., 2000, Heinricher et al., 2001, Heinricher and Neubert, 2004, Marshall et al., 2012). Moreover, the majority of paradigms in which RVM CCK has been implicated were based on repeated or prolonged pain or stress, including nerve injury, opioid-induced hyperalgesia, social defeat, and post-formalin pain (Kovelowski et al., 2000, Andre et al., 2005, Xie et al., 2005, Rivat et al., 2010, Ambriz-Tututi et al., 2011). CCK may therefore have a prominent role only in chronic stress paradigms or in situations in which an acute stress (such as a single nociceptive testing session) is superimposed on a background of chronic stress (Bhatnagar et al., 2000). Future studies should focus on identifying the full scope of neurotransmitters released in the RVM during DMH-mediated hyperalgesia.

4.3 Pathways for stress-induced modulation of pain

Stress can induce analgesia or hyperalgesia. However, stress-induced analgesia occurs during an extreme acute stress, whereas stress-induced hyperalgesia typically arises during mild or persistent stress (Terman et al., 1984, Imbe et al., 2006, Rivat et al., 2007, Butler and Finn, 2009). Stress-induced hyperalgesia can be masked by stress-induced analgesia (Cornélio et al., 2012), and the two responses employ different neuronal mechanisms and pathways. However, in both these cases, the stress-induced changes in nociception require the RVM. Opioid-dependent stress-induced analgesia involves an amygdala-periaqueductal gray-RVM circuit, with activation of RVM antinociceptive neurons as the final output to the spinal cord (Helmstetter, 1992, Helmstetter and Terhner, 1994, McGaraughty and Heinricher, 2002, McGaraughty et al., 2004, Vianna et al., 2008). Similarly, electric shock can induce hyperalgesia dependent on the amygdala, periaqueductal grey, and bed nucleus of stria terminalis, although a role for the RVM has not been tested with this paradigm (McLemore et al., 1999, Crown et al., 2000). We have previously shown that stimulation of the DMH activates a distinct population of pain-facilitating RVM neurons, the “ON-cells”

(Martenson et al., 2009), and the present experiments establish the importance of a DMH to RVM connection in stress-induced hyperalgesia.

4.4 Conclusion

Given previous evidence that stimulation of the DMH results in thermal and mechanical hyperalgesia mediated by the RVM ON-cells (Martenson et al., 2009), the present results demonstrate that a defined anatomical and functional neural circuit between the DMH and the RVM is vital for stress-induced hyperalgesia. Thus, although the pathways of descending facilitation converge with those of descending inhibition in the RVM, they involve different RVM cell populations, and are separable at higher levels of the nervous system.

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Highlights

- Air-puff stress with restraint produces mechanical hyperalgesia in behaving animals.
- Stress-induced hyperalgesia is blocked by inactivation of the DMH or RVM.
- The DMH is the supraspinal source of the pro-nociceptive peptide cholecystinin in the RVM.
- A connection between the DMH and RVM allows stress to facilitate pain.

Fig. 1. Microinjection sites in the DMH and RVM

Markers indicate tip of injection cannula. Each labels the injection site in one animal, with each animal receiving multiple injections in a random order. DMH (left, $n=9$, one brain was lost to histology) and RVM (right, $n=11$). Distance from bregma noted on each section. DMH: dorsomedial hypothalamus, F: fornix, MT: mammillothalamic tract, VII: nucleus of the facial nerve.

Fig. 2. CCK-LI and CTB-LI in the dorsomedial hypothalamus

A) Schematic of the dorsomedial hypothalamus, section is 3.1 mm caudal to bregma.

B) Representative section showing CTB injection site in the RVM.

C,D): Retrograde labeling in the DMH is prominent in the dorsal hypothalamic area of the DMH. Higher magnification view in D.

E,F) DMH neurons showing CCK-LI. The area of strong CCK-LI in the lower right is the compact nucleus of the DMH. Higher magnification view in F.

G,H) Co-localization of CTB and CCK-like immunoreactivity in the dorsal aspect of the DMH. Higher magnification view in H. Arrowheads in D, F, and H mark two double-labeled neurons.

3V: third ventricle; DMH: dorsomedial hypothalamus; F: Fornix; MT: mammillothalamic tract, VMH: ventromedial hypothalamus.

Fig. 3. Edinger-Westphal nucleus shows both retrograde labeling (A) and CCK-LI (B). However, no double-labeled neurons were identified (C).

Fig. 4. Interventions producing hyperalgesia

A) Direct pharmacological activation of the DMH using the GABA antagonist bicuculline significantly lowered withdrawal thresholds compared to pre-stress baseline ($p = 0.016$). Microinjection of aCSF did not significantly change thresholds ($p = 0.94$ compared to baseline, number tested = 7 for both groups).

B) Air stress significantly lowered withdrawal thresholds compared to baseline ($p = 0.03$). Returning the animals to the familiar environment of their home cages for 30 min did not alter thresholds ($p = 0.63$, $n = 6$ for both groups). Baseline withdrawal thresholds were not different among groups in any of the experiments. * $p < 0.05$ compared to pre-treatment baseline.

Fig. 5. Inactivation of the DMH or RVM blocks hyperalgesia triggered by air stress

A) Injection of aCSF into the DMH did not block a stress-induced decrease in withdrawal thresholds compared to baseline ($p = 0.04$). However, stress failed to evoke a significant decrease in threshold following inactivation of DMH neurons by microinjection of muscimol prior to stress ($p = 0.25$ compared to baseline). DMH muscimol microinjection had no effect on withdrawal thresholds in animals returned to their home cages ($p = 0.28$). **B)** Injection of aCSF into the RVM did not block a stress-induced decrease in withdrawal thresholds compared to baseline ($p = 0.02$). Inactivating neurons in the RVM with a microinjection of lidocaine prior to stress blocked the decrease in threshold ($p = 0.83$). Lidocaine did not change thresholds in animals returned to their home cages ($p = 0.44$). Microinjecting the CCK₂ receptor antagonist YM022 in the RVM did not prevent lowered withdrawal thresholds following stress ($p = 0.02$). No difference among baseline thresholds, 5 – 8 animals tested/group. * $p < 0.05$ compared to pre-treatment baseline.