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Activation of Descending Pain Facilitatory Pathways from the Rostral Ventromedial Medulla by Cholecystokinin Elicits Release of PGE₂ in the Spinal Cord

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Abstract

Cholecystokinin (CCK) has been suggested to be both pro-nociceptive and anti-opioid by actions on pain modulatory cells within the RVM. One consequence of activation of RVM CCK₂ receptors may be enhanced spinal nociceptive transmission but how this might occur, especially in states of pathological pain is unknown. Here, *in vivo* microdialysis was used to demonstrate that levels of RVM CCK increased by approximately 2-fold following ligation of L₅/L₆ spinal nerves (SNL). Microinjection of CCK into the RVM of naïve rats elicited hypersensitivity to tactile stimulation of the hindpaw. Additionally, RVM CCK elicited a time-related increase in PGE₂ measured in cerebrospinal fluid from the lumbar spinal cord. The peak increase in spinal PGE₂ was approximately 5-fold and was observed at approximately 80-min post-RVM CCK, a time coincident with maximal RVM CCK-induced mechanical hypersensitivity. Spinal administration of naproxen, a non-selective COX-inhibitor, significantly attenuated RVM CCK-induced hindpaw tactile hypersensitivity. RVM-CCK also resulted in a 2-fold increase in spinal 5-HIAA, a 5-HT metabolite, as compared to controls, and mechanical hypersensitivity that was attenuated by spinal application of ondansetron, a 5-HT₃ antagonist. The present studies suggest that chronic nerve injury can result in activation of descending facilitatory mechanisms that may promote hyperalgesia via ultimate release of PGE₂ and 5-HT in the spinal cord.

Keywords

rostral ventromedial medulla; descending facilitation; cholecystokinin; serotonin; PGE₂; microdialysis; tactile hypersensitivity; nerve injury

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Introduction

Chronic pain is thought to be associated with engagement of descending pain facilitatory mechanisms from areas of the brainstem including the rostral ventromedial medulla (RVM) [4,17,67]. However, it is unclear how descending facilitation may alter the release of transmitters that may act to promote nociception at the level of the spinal cord under physiological or pathological pain states. A number of studies suggest that cholecystokinin (CCK) can activate the descending pain facilitatory system from the RVM [18,22,23,65]. CCK is co-localized in pain related regions of the CNS including the RVM [19,66] and produces its action at CCK₂, G_{as}-coupled receptors. Recently, we have demonstrated CCK₂ receptors are co-localized on putative RVM pain facilitatory neurons with mu opioid receptors [66]. Microinjection of CCK into the RVM produces hyperalgesia *via* activation of CCK₂ receptors [65].

RVM CCK antagonists can enhance the antinociceptive effects of acute opioid administration [10,16]. Hyperalgesia that is observed following sustained systemic administration of morphine [56,57] is reversed by blockade of RVM CCK₂-receptors [65]. Studies performing RVM microdialysis have demonstrated that CCK is increased 4 fold following sustained morphine administration [65] suggesting that endogenous CCK may drive descending pain facilitation. CCK₂ antagonists within the RVM are effective in reversing hypersensitivity resulting from nerve injury or visceral hyperalgesia suggesting endogenous CCK is up-regulated in the RVM in pathological pain states [18,30]. CCK has been demonstrated to be both pronociceptive by directly activating ON cells and “antiopioid” by inhibiting the activation of OFF cells [23,46]. While a role for endogenous RVM-CCK in states of pathological pain have been suggested through antagonist studies, it is not known whether increased expression of CCK occurs and the possible consequences at the level of the spinal cord.

Prostaglandin-E₂ (PGE₂) is known to either directly activate or sensitize nociceptors and/or spinal dorsal horn neurons via EP receptors [7,36]. PGE₂ and the associated EP₂ and EP₄ receptor levels are significantly elevated under conditions of inflammation and nerve damage [5,6,11,34,37,38,43,62]. Administration of PGE₂ antagonists have been shown to attenuate chronic pain in animals [25,29,32,45]. It remains unknown if CCK activation of descending facilitatory pathways alters spinal levels of PGE₂.

Chronic pain states are associated with an enhanced descending facilitatory control, mediated in part through activation of excitatory, spinal 5-HT₃-receptors [55]. Studies have suggested that 15–25% of RVM neurons are serotonergic [52], and significant portions of these serotonergic neurons are CCK-positive [35]. Small clinical studies have suggested that the 5-HT₃ antagonist ondansetron can attenuate both acute and chronic pain states [1, 41]. Multiple preclinical studies have suggested that 5HT₃ receptor blockade [55] can attenuate nerve injury induced pain yet some studies have demonstrated a lack of an effect [51]. The effects of activating the RVM pain facilitatory pathways on spinal 5-HT/5-HIAA levels are unclear.

Here, we used both *in vivo* microdialysis and behavioral studies to demonstrate for the first time that nerve injury significantly increases endogenous CCK in the RVM, and that CCK drives descending facilitatory pathways of the RVM in naïve animals to increase spinal PGE₂ and 5-HT/5-HIAA.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA), 250–350 g at the time of testing, were maintained in a climate-controlled room on a 12 hr light/dark cycle (lights on at 7:00 A.M.), with food and water available *ad libitum*. All of the testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals and received approval from the Institutional Animal Care and Use Committee of the University of Arizona.

RVM Cannulation

Rats were anesthetized with ketamine-xylazine (vol/vol: 80/20; 100 mg/kg, 1 ml/kg, i.p.) for stereotaxic placement of bilateral cannula in the RVM. The skull was exposed, and two 26-gauge guide cannula separated by 1.2 mm (Cat # C235G-1.2/SP, Plastics One, Roanoke, VA) were directed toward the lateral portions of the RVM using the atlas of Paxinos and Watson (anteroposterior: –11.0 mm from bregma; lateral: ± 0.6 mm; dorsoventral: –7.5 mm from the dura mater). The guide cannula were cemented in place and secured to the skull by small stainless steel machine screws. The animals were allowed to recover for 4–7 days after surgery before any pharmacological manipulations were made. Drug administration into the RVM was performed by slowly expelling 0.5 μ l of drug solution through a 33-gauge injection cannula (cat # C235I/SP, Plastics One, Roanoke, VA) inserted through a guide cannula and protruding an additional 1 mm into fresh brain tissue preventing backflow. Either a 2% Pontamine blue or 70% India ink (v/v) solution was injected into the RVM injection site at the termination of the experiments and cannula placement verified histologically. Data from animals with incorrectly placed cannula were not included within the experimental data analysis. Histological data from animals with misplaced cannula were utilized as off-site controls.

Spinal Nerve Ligation

Tight ligation of the L₅/L₆ spinal nerves was performed in accordance with the method described by Kim and Chung [28]. Anesthesia was induced with 5% isoflurane and maintained with 2.5% isoflurane in O₂ anesthesia delivered at 2 L/min. Following surgical preparation of the rats and exposure of the dorsal vertebral column from L₄ to S₂, the exposed L₅ and L₆ spinal nerves were tightly ligated with 4-0 silk suture. Sham control animals were subjected to the same operating procedure minus the actual nerve ligation. Rats that demonstrated motor difficulties or lacked behavioral hypersensitivity were excluded from further testing.

Intrathecal Cannulation

While under ketamine/xylazine (v/v: 10/1, 100 mg/kg, i.p) anesthesia/paresthesia, rats were implanted with either a chronic intrathecal catheter (PE-10, 8.0 cm) for drug administration or a double lumen, intrathecal cannula fitted with a looped microdialysis membrane (polycarbonate membrane with a 20kDa molecular cutoff) that terminated at the lumbar level of the spinal cord (7.8–8.0 cm length) for PGE₂ and 5-HT sampling at the level of the lumbar spinal cord, based on the previously described procedure by Yaksh and Rudy [64]. The loop catheter was inserted through an incision in the atlanto-occipital membrane and advanced caudally to the rostral edge of the lumbar enlargement. Animals were allowed 5–10 days of recovery before experiments were conducted and animals exhibiting motor impairment were excluded from experimentation. In some cases, placement of intrathecal

cannula occurred 4 days prior to RVM cannula implantation for assessing the effects of exogenously applied CCK.

In vivo Intrathecal Microdialysis

One hour before the experiment, rats were placed in a recording chamber with access to food and water *ad libitum* for habituation. After acclimation, the microdialysis system was connected to the animals with inflow and outflow tubing comprised of polyethylene 10 tubing connected to a polyethylene-50 joint connected to the exposed polyethylene-10 microdialysis catheters extending out from the base of the rat's neck. Microdialysis of the cerebrospinal fluid (CSF) in the lower lumbar spinal cord was performed in freely moving, non-anesthetized animals, as based on previous studies, which have utilized *in vivo* microdialysis to analyze endogenous levels of norepinephrine, 5-HT, 5-HIAA, and PGE₂ in the spinal cord at a flow rate of 10 µl/min, the microdialysis probe was continuously perfused with artificial CSF (aCSF; in mM: 138 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 11 NaHCO₃, and 1 NaH₂PO₄, pH = 7.4) containing 0.2% bovine serum albumin (BSA), 0.2% glucose, and 0.03% of the peptidase inhibitor Bacitracin; aCSF used in 5-HT/5-HIAA studies was prepared with an antioxidant cocktail to preserve the integrity of the samples over time. The 40x cocktail stock solution contained 6.0 mM L-cysteine, 2.0 mM oxalic acid (Sigma-Aldrich, St Louis, MO, USA) and 1.3% glacial acetic acid. Samples were prepared as described by Hubbard et al., 2010 and diluted to 1x at the time of high performance liquid chromatography (HPLC) analysis [24]. To allow the dialysis to reach a steady state after connection, the probe was perfused for a 30-minute washout period before collection of the first baseline sample. Forty-minute fraction collections began after the washout period to establish baseline levels of PGE₂ or 5-HT in the lumbar spinal cord. Microdialysate was then collected for 40-minute fractions over the entire 160-minute collection period. In all experiments, four dialysate fractions of 400 µl (40-min of perfusion each) were collected at 4°C and then immediately frozen at -80°C until their PGE₂ and 5-HT content could be measured via EIA or HPLC-electrochemical (EC) detection, respectively. At no point during the microdialysis did rats exhibit any behavioral signs of discomfort, abnormal behavior, or motor hyperactivity and they were able to move freely for the duration of the collection.

In vivo RVM Microdialysis

Three hours before the experiment, rats were placed in a recording chamber with access to food and water *ad libitum*. After acclimation, a microdialysis probe [CMA/12 (CMA Microdialysis, North Chelmsford, MA); polycarbonate membrane; outer diameter of 0.5 mm; length of 2 mm; molecular cutoff of 20 kDa] was inserted into the guide cannula such that the probe membrane extended 2 mm into the RVM. Microdialysis of the RVM was performed in freely moving, awake animals. At a flow rate of 3.5 L/min, the microdialysis probe was continuously perfused with aCSF (in mM: 138 NaCl, 5 KCl, µL MgCl₂, 1 CaCl₂, 11 NaHCO₃, and 1 NaH₂PO₄, pH 7.4) containing 0.2% BSA, 0.2% glucose, and 0.03% of the peptidase inhibitor Bacitracin. To allow the dialysis to reach a steady state after insertion, the probe was perfused for a 1-hr washout period before collection of the first sample. In all experiments, nine dialysate fractions of 100 µL (30-min of perfusion each) were collected at 8°C and then immediately frozen at -20°C until their CCK-LI content could be measured using the radioimmunoassay (RIA) procedure described below. Thirty-minute fraction collections began after the washout period to establish baseline levels of CCK-LI in the RVM.

CCK RIA

CCK-LI was determined using a modified RIA procedure with improved sensitivity developed especially for the determination of neuropeptide concentrations in microdialysates [61]. An increased sensitivity of the RIA was achieved by minimizing the

total incubation volume and limiting the amount of radioligand (≈ 1000 cpm/assay tube), as well as a sequential incubation procedure. This modified protocol yielded an eight-fold increase in the sensitivity of the assay, as indicated by a shift in the average IC_{50} value, which was reduced from 105.6 ± 10.1 ($n = 6$) to 12.9 ± 1.5 pM ($n = 5$; mean $\pm$ SEM). The microdialysates and standard samples (100 μ L) were preincubated for 24-hr at 4°C with the C-terminal-directed CCK/gastrin antiserum (2609/10) in 0.1 M barbital buffer containing 0.2% BSA [53]. After addition of the radioligand (≈ 1000 cpm/assay tube of 125 I-gastrin-I; specific activity, 70 MBq/nmol), all samples were incubated at 4°C for another 72-hr. Free radioligand was separated from antibody-bound radioligand in all RIA samples, except those used for determination of the total radioactivity, by the addition of 250 μ L sheep anti-rabbit antibody-coated Sepharose suspension. After 30-min of incubation and centrifugation at 2600 μ g for 10-min, the supernatant was discarded by aspiration, and the radioactivity in the pellets containing the bound fraction was measured in a gamma counter.

Tactile Thresholds

The withdrawal threshold of the hindpaw in response to probing with a series of eight calibrated von Frey filaments (Stoelting, Wood Dale, IL) in logarithmically spaced increments ranging from 0.41 to 15 g (4–150 N) was determined [56]. Each filament was applied perpendicularly to the plantar surface of the left hindpaw of rats kept in suspended wire-mesh bottom cages. Measurements were taken both before and after administration of drug or vehicle. Withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength (“up and down” method), analyzed using a Dixon nonparametric test, and expressed as the mean withdrawal threshold. Significant changes from baseline control values were detected by ANOVA, followed by Fisher’s least significant difference test. These evaluations were all performed with the aid of the statistics package FlashCalc (Dr. Michael H. Ossipov, University of Arizona). Statistical significance was set at $p < 0.05$.

PGE₂ Analysis

PGE₂ concentrations were assayed using a commercially available kit (Amersham Biosciences RPN222, Buckinghamshire, England). This kit uses a monoclonal antibody to PGE₂ (anchored to the plate via anti-IgG antibody) and PGE₂-peroxidase conjugate. The PGE₂ antibody has the following cross-reactivities: 7.0% with PGE₁, 4.3% with PGF_{2 α} and 5.4% with 6-Keto PGF_{1 α} , no other cross-reactivities exceeding 2% are known (manufacturer’s specifications). After incubation of the reagents and spinal CSF sample in the EIA plate, activity bound to the solid phase is measured as directly proportional to the HRP-TMB (3,3',5,5'-tetramethylbenzidine) substrate reaction as absorbance at 450 nm. Due to the competition between the PGE₂ in the sample and the HRP-conjugate reagent, the amount of PGE₂ can be calculated by an inversely proportional relationship to the absorbance measured.

5-HT Analysis

Initially, 5-HT concentrations were determined using a modified HPLC-*fluorometric detection* (Waters 2695 HPLC Separations Module coupled with 2475 Fluorescence Detector) method [24,26] developed to improve sensitivity for the indoleamines, 5-HT and its primary oxidative metabolite, 5-HIAA occurring at picomole levels in spinal CSF. A 3.0 mm internal diameter (i.d.) $\times$ 100 mm narrow-bore analytical column (Waters Atlantis dC18 3 μ m particle-size packing) was used to optimize sensitivity, with a flow rate of 0.6 mL/min. Waters Empower 2 Software was used for processing and analysis. Internal standards for both 5-HT and 5-HIAA were used to confirm the molecular identity of chromatographic peaks of interest in conjunction with electrospray-ionization mass spectrometry (ESI-MS analysis) to provide further molecular confirmation of the sample being measured.

5-HT/5-HIAA levels were also assessed using an HPLC-*electrochemical* (EC) system consisting of an Agilent 1100 quaternary pump, thermostat controlled column compartment, thermostat autosampler, and autoinjector (Agilent Technologies, Palo Alto, CA) coupled with an ESA Coulochem III Multi-Electrode Detector (model no. 5300; ESA Biosciences, Chelmsford, MA, USA) with an ESA Microdialysis Cell 5041(model 5014B; Chelmsford, MA, USA). Data were acquired and analyzed using HP ChemStation software for LC 3D, (Rev. B.04.01 (SP1), Agilent Technologies, Palo Alto, CA).

The initial conditions of the HPLC-EC method were chosen from a previously published method for determination of neurotransmitter metabolites 5-HIAA and HVA in human CSF [24,26]. The ESA guard cell (EG; model 5020), which was connected in line before the injector and used to remove oxidizable impurities in the mobile phase, was set at +250 mV. The first analytical cell (E1) was used as an oxidative screen to eliminate interfering compounds that oxidize at lower potentials than the analytes and set to -150 mV. The second analytical cell (E2) was set to +220 mV to be at the maximum oxidation potential of the analytes. Separation of the analytes (5-HIAA/5-HT) was obtained on an ESA MD-150, 3.2 mm column coupled with an ESA C18 guard cartridge (Chelmsford, MA, USA) kept at room temperature. Flow rate was 0.9 mL/min with a mobile phase consisting of acetonitrile/aqueous (10/90: v/v) containing 75 mM monobasic sodium phosphate buffer, 25 μ M EDTA, 1.7 mM sodium octylsulfonate (OSA; Sigma-Aldrich, St Louis, MO, USA) and 100 μ L/l triethylamine (TEA, Sigma-Aldrich, St Louis MO, USA). The mobile phase buffer was adjusted to pH 3.0 with 85% phosphoric acid (Fisher Scientific, Fairlawn, NJ, USA) and after addition of acetonitrile, was filtered with a 0.2 μ m PALL Membrane (Port Washington, NY, USA). The mobile phase was degassed for approximately 10 min prior to overnight equilibration on the HPLC-EC system. The temperature of the autoinjector was set at 8°C. Samples were injected at a volume of 20–40 μ L and each represented a single fraction collected from an individual animal. All fractions were analyzed by HPLC-EC in triplicate and compared to a standard curve.

Drugs

CCK-8(s) (American Peptide, cat # 46-1-32; Sunnyvale, CA, USA) was purchased and dissolved in MilliPore H₂O. The CCK₂ antagonist, YM022 (Tocris, cat # 1408; Ellisville, MO, USA), was dissolved in 1% DMSO in MilliPore H₂O was given 5 min prior to CCK-8(s). Naproxen (Sigma Aldrich cat # N5160; St. Louis, MO, USA) and ondansetron (Sigma Aldrich cat # O3639; St. Louis MO, USA) were dissolved in 0.9% physiologic saline.

Data Analysis

PGE₂ and 5-HT/5-HIAA baseline concentrations were measured and normalized to 100%, and used as a basis of comparison for all post-baseline measurements, which were calculated as percent change of baseline values. Significant changes from baseline control values were detected by ANOVA, followed by Fisher's least significant difference test. These evaluations were all performed with the aid of the statistics package FlashCalc; statistical significance was set at $p < 0.05$.

Results

Endogenous CCK levels are increased in the RVM in nerve-injured animals

Using continuous infusion of aCSF by *in vivo* microdialysis, endogenous CCK-Li levels in the RVM were measured in both sham and nerve injured rats. Microdialysate fraction collections were taken for one-hour intervals over three hours in either sham or nerve injured animals 7 days after surgery (Figure 1A). All animals that underwent nerve injury

demonstrated significant mechanical hypersensitivity prior to microdialysis studies. Individual one hr dialysate fractions were summed and the area under the curve (AUC) calculated and plotted (Figure 1B). Basal levels of CCK-Li in sham animals did not change significantly over a period of 3-hrs resulting in a total of 6.1 ± 0.8 pM CCK-Li (Figure 1A, B). However, the basal levels of CCK-Li in L₅/L₆ spinal nerve injured animals resulted in over a 2-fold significant increase ($p < 0.05$) when measured on day 7 after injury. Basal levels at hours 1 through 3 in L₅/L₆ SNL animals (day 7) were elevated with a total AUC over the entire 3-hr period of 12.6 ± 0.4 pM (Figure 1B).

The selective CCK₂ receptor antagonist YM022 in non-injured animals attenuates RVM CCK-induced mechanical hypersensitivity

CCK-8(s) (30 ng) was administered bilaterally into the RVM in conjunction with either vehicle or the selective antagonist YM022 in non-injured animals. CCK-8(s) administered into the RVM induced time related mechanical hypersensitivity with the time of peak effect occurring 45 min post- injection (5.9 ± 0.3 g; $p < 0.05$, $n=6$) as compared to controls (Veh/Veh) (15.0 ± 0.0 g; $n=6$) (Figure 2). YM022 (25 pg), given bilaterally into the RVM 5 min prior to CCK-8(s) administration into the RVM attenuated CCK-8(s)-induced mechanical hypersensitivity (14.8 ± 0.03 g; $p < 0.01$, $n=6$) at the same time point (Figure 2). RVM administered, CCK-8(s)-induced mechanical hypersensitivity returned to baseline levels within 120 min after induction. Pre-treatment with YM022 (25 pg), administered bilaterally into the RVM did not significantly alter mechanical paw withdrawal thresholds in animals receiving vehicle into the RVM bilaterally compared to baseline values ($n=6$) (Figure 2).

CCK in the RVM evokes the release of PGE₂ in the lumbar spinal cord

As spinal nerve injury may itself cause alteration of spinal neurotransmitters, studies to determine the contribution of the RVM descending system were performed in non-injured rats utilizing CCK-8(s) to activate the pain facilitatory system. Basal levels of PGE₂ in spinal CSF were first measured and normalized to 100%, and used as the basis for comparison for all measurements of PGE₂ following application of CCK-8 into the RVM (presented as percent change from the normalized baseline). Administration of CCK-8(s) via bilateral RVM microinjection resulted in a time-dependent increase in the release of PGE₂ in the lumbar spinal cord. The dose of CCK-8(s) (30 ng) was based on our previous studies [65]. CCK-8(s) microinjection into the RVM resulted in the following percent of baseline measurements of PGE₂ in the spinal CSF; 40 min: $198 \pm 20\%$, 80 min: $469 \pm 73\%$ and 120 min: $555 \pm 79\%$ (Figure 3A). Total PGE₂ spinal release due to RVM CCK administration in the presence of either vehicle or the CCK₂ antagonist YM022 was plotted over the entire 120 min as the area under the curve (AUC) (Figure 3B). PGE₂ release into the spinal CSF was significantly attenuated ($p < 0.05$) with RVM pre-administration of the CCK₂ receptor antagonist, YM022, given 10 min prior to RVM-CCK (Figure 3). No significant increase in PGE₂ release was seen with vehicle (diH₂O) injection into the RVM or in conjunction with the CCK₂ receptor antagonist, YM022 (25 pg, bilaterally), as compared to non-injection baseline controls levels.

Intra-RVM CCK-induced mechanical hypersensitivity is attenuated by the nonselective COX inhibitor, naproxen (i.th.)

In a separate set of animals, CCK-8(s) (30ng) was administered bilaterally into the RVM in conjunction with either intrathecal naproxen (100 or 200 μ g in 5 μ L) or saline vehicle (5 μ L). Animals were tested for behavioral signs of mechanical hypersensitivity using graded von Frey filaments before and after RVM or spinal manipulations. Intra-RVM CCK-8(s) resulted in a time related mechanical hypersensitivity that returned to baseline within 120 min as observed previously. RVM CCK-8(s) produced its maximum nociceptive effect ($p < 0.001$, $n=6$) at 45 min with an average paw withdrawal threshold of 3.9 ± 0.6 g (Figure

4A). The RVM CCK-8(s)-induced mechanical hypersensitivity was significantly attenuated by the pre-administration (i.th., -5 min) of the nonselective COX inhibitor, at both the 100 μ g and 200 μ g doses administered into the spinal cord ($p < 0.01$, $n = 5$) (Figure 4A). Paw withdrawal thresholds at the time of peak effect for spinal naproxen (30- and 45-min) were as follows: 12.7 ± 0.4 g and 13.9 ± 0.3 g at the 100 μ g and 14.4 ± 0.3 g and 14.1 ± 0.4 g at the 200 μ g doses, respectively. Neither vehicle nor naproxen alone altered paw withdrawal thresholds in naïve animals when compared to baseline mechanical thresholds. Spinally applied vehicle prior to RVM administered CCK-8(s) did not attenuate the resulting mechanical hypersensitivity. Calculating total area over the curve between spinal vehicle/RVM CCK-8(s) group and the spinal naproxen/RVM CCK-8(s) groups resulted in a significant attenuation of the RVM CCK-8(s) paw withdrawal thresholds ($p < 0.01$, $n = 5$) (Figure 4B).

CCK in the RVM increases the detectable release of serotonin into the lumbar spinal cord

Basal levels of the serotonin metabolite (5-HIAA) in the spinal CSF were first measured and normalized to 100%, and used as the basis for comparison for all post-injection measurements of 5-HIAA as a representation of spinal 5-HT release. All post-drug measurements of 5-HIAA in the spinal CSF are reported as percent change from a normalized baseline. Administration of CCK-8(s) bilaterally (30ng) into the RVM resulted in significant increases in the release of 5-HIAA in the lumbar spinal cord beginning 40 min after application ($n = 12$, $p < 0.05$) (Figure 5A). CCK-8(s) microinjection into the RVM resulted in the following percent changes from baseline measurements of 5-HIAA in the spinal CSF; 40 min: $123 \pm 7\%$, 80 min: $139 \pm 11\%$ and 120 min: $148 \pm 15\%$ (Figure 5A). Total 5-HIAA spinal release due to RVM CCK administration in the presence of either vehicle or the CCK₂ antagonist YM022 was plotted over the entire 120 min time-course as the area under the curve (AUC) (Figure 5B). 5-HIAA release into the spinal CSF was significantly attenuated ($p < 0.05$, $n = 5$) with RVM pre-treatment with the CCK₂ receptor antagonist, YM022, given 10-minutes prior to RVM-CCK (Figure 5). No change in 5-HIAA release was seen with vehicle (diH₂O) or YM022 (25 pg) administration into the RVM as compared to non-injection baseline controls levels (Figure 5).

RVM/CCK-induced mechanical hypersensitivity is attenuated by the 5-HT₃ antagonist, ondansetron (i.th.)

Animals were tested for baseline paw withdrawal thresholds using calibrated von Frey filaments (15.0 ± 0.0 g). CCK-8(s) (30ng) was administered into the RVM 15 minutes prior to either intrathecal ondansetron (5-HT₃ antagonist) or saline (vehicle). Animals were then evaluated for behavioral signs of mechanical hypersensitivity to probing with calibrated von Frey filaments. Administration of CCK into the RVM produced its maximum nociceptive effect at 45 min with a paw withdrawal threshold of 5.2 ± 0.7 g (Figure 6A). The RVM CCK-8(s)-induced mechanical hypersensitivity was significantly ($p < 0.05$, $n = 10$) attenuated by spinal administration of ondansetron at all three doses tested: 0.1, 1, and 10 μ g (Figure 6A). Paw withdrawal thresholds at the 45-min time point (ondansetron peak effect) were as follows for the three doses used: 0.1 μ g dose: 8.7 ± 0.8 g; 1 μ g dose: 10.1 ± 0.8 g; 10 μ g dose: 10.3 ± 0.7 g (Figure 6A). A dose-dependent attenuation of CCK-induced nociception was observed for ondansetron, with significance achieved ($p < 0.05$) for all doses of ondansetron over the entire time-course and with the greatest effect observed with the 10 μ g dose (Figure 6B). Neither vehicle alone nor, ondansetron had effects on baseline mechanical thresholds in naïve animals. Spinal vehicle administered prior to RVM CCK-8(s) did not attenuate the CCK8(s)-induced mechanical hypersensitivity.

Discussion

In vivo microdialysis and behavioral testing were utilized to further elucidate the role of CCK in the RVM in facilitating chronic pain states. We identified a significant increase in endogenous CCK in the RVM after ligation of the L₅/L₆ spinal nerves suggesting that CCK may act to promote descending pain facilitation. Injecting CCK-8(s) into the RVM of naïve rats produced robust mechanical hypersensitivity of the hindpaw that was blocked by the RVM administration of a selective CCK₂ receptor antagonist. We also demonstrated that a single injection of CCK-8(s) administered into the RVM resulted in a significant increase of PGE₂ and 5-HIAA in spinal CSF as measured by *in vivo* microdialysis. Further, the CCK-induced increases in spinal PGE₂ and 5-HIAA were blocked by RVM-pretreatment with a CCK₂ antagonist. Lastly, we report that CCK-8(s) induced hypersensitivity was attenuated by spinal application of either naproxen or ondansetron. These data support a role for RVM CCK to drive descending pain facilitation leading to increases in spinal PGE₂ and 5-HIAA and behaviors consistent with hypersensitivities observed in rodent models of chronic pain.

CCK is thought to act as a pronociceptive transmitter and to exert “anti-opioid” actions [16]. Recent studies have demonstrated that CCK receptor antagonists can enhance opioid antinociception in animals [18,65] and analgesia in humans [40,41]. Considerable overlap exists in the distribution of CCK, opioid peptides and their receptors within the pain modulating nuclei of the central nervous system [19,60] including the RVM [66]. Behavioral and electrophysiological studies have demonstrated that RVM-CCK may contribute to neuropathic pain, opioid antinociceptive tolerance, and opioid-induced tactile and thermal hypersensitivity [22,23,30,65]. Three types of cells have been characterized in the RVM: two of which, the ON- and OFF- cells, are believed to function directly in descending pain modulation and possess facilitatory and inhibitory activity, respectively [4,17,67]. The third type, NEUTRAL have no defined activity with respect to pain modulation to date. CCK-8(s) microinjected into the RVM is known to promote ON cell activation coinciding with behaviors including thermal hyperalgesia, suggesting that CCK activity in the RVM is pronociceptive and likely drives descending facilitation to contribute to pain hypersensitivity [23,30,65]. Conversely, it has been shown that opioid-induced disinhibition of OFF cells with ensuing antinociception can be blocked by microinjection of the CCK-8(s) into the RVM revealing an additional anti-opioid action [22,46].

Here we report that spinal nerve ligation results in a significant, two-fold increase of CCK within the RVM over control levels. This supports previous observations demonstrating a significant reversal of both mechanical and thermal hypersensitivity in nerve-injured rats by microinjection of a selective CCK₂ antagonist into the RVM [30]. We also show that exogenous administration of CCK into the RVM of non-injured rats mimics this nerve-injured state eliciting hypersensitivity of the hindpaws that is attenuated RVM application of a CCK₂ receptor antagonist as previously reported [18,65]. These data support the possibility that CCK at CCK₂ receptors drives pain facilitatory pathways in the RVM possibly promoting abnormal pain.

Descending pain modulatory regions of the brainstem have also been shown to be important in both the manifestation and maintenance of certain chronic pain states [42,56]. For example, experimentally produced neuropathic pain can be reversed in a time-dependent fashion by lidocaine or a CCK₂ antagonist microinjection into the RVM, supporting the critical role of descending facilitation in chronic pain [30,50]. However, knowledge of the neurotransmitter(s) altered in spinal CSF after activation of the descending pathway are largely unknown and complicated by nerve injury. Following the observation that endogenous CCK is upregulated in the RVM after spinal nerve ligation, we administered CCK-8(s) into the RVM to drive the descending pain facilitatory pathway in non-injured

animals and measured, by *in vivo* microdialysis, the levels of two proinflammatory mediators- PGE₂ and 5-HIAA- in the spinal cord.

Prostaglandins such as PGE₂ are known to function as important mediators in the development of central sensitization and when applied to the spinal cord, produce an allodynic and hyperalgesic state [3,21,34,46,60]. PGE₂-induced spinal inflammatory hyperalgesia is mediated by EP receptors residing in either the dorsal root ganglia (DRG) and/or the spinal cord [3,7] suggesting that prostaglandins influence the release of neurotransmitters from primary afferent terminals. Topical application of PGE₂ or activation of EP₂ receptors in spinal cord neurons have been shown to activate rat spinal dorsal horn neurons inducing a state of hyperexcitability in dorsal horn neurons analogous to peripheral inflammation [3]. Spinal application of a COX inhibitor, which limits the production of prostaglandins, attenuates the generation of inflammation-evoked central sensitization [58]. Specifically, the COX-1 and COX-2 enzymes, responsible for the synthesis of PGE₂, are present both in the spinal cord and DRG with extensive upregulation of COX-2 during peripheral inflammation [5,6,21] coinciding with enhanced spinal release of PGE₂ [11]. Furthermore, behavioral studies suggest that PGE₂ may modulate nociceptive transmission in the spinal cord and serve as a major contributing factor to central sensitization [43,61,63]. Here we demonstrated that CCK-8(s) administered bilaterally into the RVM of non-injured animals resulted in a significant, five-fold increase in spinal PGE₂ levels over basal levels prior to RVM CCK-8(s). Our findings suggest that the activation of descending pain pathways from the RVM can induce the upregulation of spinal PGE₂ resulting in enhanced pain sensitivity that can be attenuated by a COX inhibitor. Although descending facilitation is important in a number of pain states including inflammatory, visceral and dysfunctional pains [48], the clinical utility of NSAIDs, including COX inhibitors, for chronic pain is lacking.

In states of neuropathic pain, NSAIDs lack analgesic efficacy most likely due to reduced CNS access. For the majority of NSAIDs, the vascular corrected brain concentration at steady state is only 1 to 5% of that of the total plasma concentration [44]. NSAIDs circulate in plasma with high affinity to serum albumin reducing the free fraction [9]. The remaining fraction circulates as organic anions such that CNS uptake of NSAIDs is highly restricted by both the small, un-ionized fraction and the large number of organic anion transporters expressed by the blood brain barrier [31]. Epidural or intrathecal injections of NSAIDs in humans and animals have demonstrated attenuation of chronic pain [34,49]. Recently, studies with intrathecal ketorolac have demonstrated mixed results suggesting that spinal delivery is safe and lack side effects in human volunteers while attenuating hyperalgesia and allodynia whereas using a single dose of intrathecal ketorolac in patients undergoing surgery or suffering from chronic nonmalignant pain provided negative results on spontaneous pain [12–14]. Many factors may contribute to the lack of efficacy in this single dose trial including the pharmacokinetics of a particular NSAID [2]. Ultimately, new discoveries in the prostaglandin field have resulted in novel drugs that act directly at the prostaglandin receptors to avoid general COX inhibition-induced side effects to achieve efficacy throughout sustained dosing. Efforts to selectively target enzymes in the sole synthesis of PGE₂ such as mPGEs and cPGEs are currently underway to evaluate their role in pain inhibition [27] resulting in advanced NSAIDs and increased utility in treating conditions of chronic pain.

The monoamine serotonin can elicit pronociceptive effects through the excitatory 5-HT₃ ligand-gated receptor [12] and has been implicated in the descending facilitation. We investigated levels of the serotonin metabolite 5-HIAA after intra-RVM CCK-8(s) in non-injured animals and observed a significant, 2-fold increase in spinal levels. Multiple studies have demonstrated that ondansetron, a 5-HT₃ antagonist, can block the consequences of

descending facilitation when given spinally resulting in antinociception in rodents and possible pain-relief in humans [41,55]. Additionally, the pronociceptive contribution of serotonin has been demonstrated in many animal models including intraplantar formalin, spinal cord injury and opioid-induced hyperalgesia [8,20,47,51,55,59]. Here, CCK-induced allodynia was significantly attenuated by intrathecal ondansetron, preceding a dose of CCK into the RVM in non-injured rats. These data further support the idea that CCK in the RVM drives descending nociceptive facilitation leading to the activation of 5-HT₃ receptors in the spinal cord.

The microdialysis and behavioral data presented here suggest that chronic pain states may result in elevated endogenous CCK in the RVM consistent with our previous suggestions [32]. Peripheral nerve injury induced increases in RVM CCK levels in which may result in evoked release of PGE₂ and 5-HIAA in the spinal cord. This enhanced release of PGE₂ and 5-HT mediators may be one mechanism whereby the RVM, through CCK₂ receptors, exerts pronociceptive effects on afferent fibers in settings of inflammatory or neuropathic pain. Presently, increases in spinal CSF levels of PGE₂ (500%) and 5-HIAA (148%) were observed after CCK microinjection into the RVM in non-injured animals, supporting the idea that the increased levels of spinal pronociceptive mediators were driven from brainstem nuclei and not from the periphery. RVM CCK-8(s) administration in naïve animals resulted in mechanical hypersensitivity that was abolished by central administration of a CCK₂ selective antagonist, a non-selective COX inhibitor or a selective 5-HT₃ receptor antagonist. These data demonstrate the neurochemicals that mediate in part the pronociceptive pain facilitatory pathways of the RVM to the spinal cord and reinforce the possibility that new therapies may arise by targeting mechanisms associated with descending modulation.

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Figure 1.

Nerve injury increases endogenous CCK levels in the RVM. **A)** Spinal nerve ligated (L₅/L₆ SNL) and sham operated male Sprague-Dawley rats were subjected to a 3-hr time-course via *in vivo* microdialysis sampling of basal CCK-Li in the RVM (n=6/group). **B)** The three, one-hour microdialysis fractions were pooled for both the sham and the L₅/L₆ SNL animals, and the area under the curve was summed for the two groups (*p<0.05) with the L₅/L₆ SNL group demonstrating over a 2-fold increase in basal CCK-Li in the RVM.

Figure 2.

Effect of the CCK₂ receptor antagonist, YM022 (25 pg/0.5 μ L) bilaterally injected into the RVM 5 min pre-CCK administration into the RVM. CCK-8(s) (30 ng/0.5 μ L) bilaterally into the RVM induced significant mechanical hypersensitivity 45 min post injection (* p <0.05; n =6). RVM CCK-8(s)-induced mechanical hypersensitivity was attenuated by the pre-administration of YM022 (** p <0.01; n =6) into the RVM. RVM YM022 and vehicle (1% DMSO or diH₂O) alone had no significant effect when compared to baseline thresholds (n =6).

Figure 3.

RVM CCK promotes spinal PGE₂ release. **A)** Male SD rats received CCK-8(s) (30 ng/0.5 μ L) bilaterally into the RVM and PGE₂ levels were determined through intrathecal microdialysis collection of the spinal CSF in the lumbar spinal cord. A single injection of CCK-8(s) into the RVM resulted in a significant increase in spinal PGE₂ within 80 and 120 mins as compared to baseline levels (* $p < 0.05$, $n = 5$). **B)** Area under the curve over the entire 120 minutes of spinal microdialysate collection demonstrating CCK-8(s)-induced spinal PGE₂ release. The CCK₂ receptor antagonist, YM022 (25 pg/0.5 μ L) administered 5-min prior to CCK bilaterally into the RVM resulted in a statistically significant attenuation of CCK-8(s)-induced PGE₂ release (** $p < 0.05$, $n = 6$).

Figure 4.

RVM CCK-induced allodynia is blocked by pretreatment with spinal naproxen. **A)** Male SD rats received naproxen (100 or 200 µg in 5 µL) intrathecally followed by CCK-8(s) (30 ng/0.5 µL, 5min post naproxen) bilaterally administered into the RVM. RVM CCK-8(s) resulted in significant tactile hypersensitivity (* $p < 0.001$; $n = 6$), which was significantly attenuated by the pre-administration of the non-selective COX inhibitor, naproxen (** $p < 0.01$; $n = 5$). Naproxen alone in naïve animals had no effect on mechanical thresholds. **B)** Area under the curve comparing animals over the entire 90 min period demonstrates a dose related reversal of RVM CCK-induced tactile hypersensitivity by spinal naproxen versus vehicle (* $p < 0.001$; ** $p < 0.01$; $n = 6$).

Figure 5.

RVM CCK enhances spinal levels of 5-HT. Male SD rats received CCK-8(s) (30 ng/0.5 μ L) bilaterally into the RVM, and spinal CSF was collected by intrathecal microdialysis. Levels of the 5-HT metabolite, 5-HIAA, were determined by HPLC. CCK-8(s) administered into the RVM resulted in a significant increase of 5-HIAA levels in vehicle pre-treated rats compared to baseline controls (n=12, $p < 0.05$). Bilateral YM022 (25 pg/0.5 μ L) pretreatment (5 min) blocked RVM CCK-mediated release of 5-HIAA in the lumbar spinal cord compared to basal measurements or levels in vehicle pre-treated animals (* $p < 0.05$).

Figure 6.

CCK-8(s) induced mechanical hypersensitivity is blocked by spinal ondansetron (Ond). **A)** Male SD rats received Ond (0.1, 1.0, 10 µg in 5 µL, i.th.) followed by CCK-8(s) (30 ng/0.5 µL) administered bilaterally intra-RVM. CCK-8(s) administered into the RVM resulted in a significant increase in tactile hypersensitivity (* $p < 0.05$; $n = 6$), which was significantly attenuated by the pre-administration of the 5-HT₃ antagonist, Ond (** $p < 0.05$; $n = 10$). Ond alone had no effect on mechanical thresholds in naïve animals. **B)** RVM CCK-8(s)-induced tactile hypersensitivity over the entire 90 min period as shown by AUC. Spinal administration of Ond dose-dependently attenuates CCK-8(s)-induced mechanical hypersensitivity.