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fMRI OF SUPRASPINAL AREAS AFTER MORPHINE AND ONE WEEK PANCREATIC INFLAMMATION IN RATS

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Abstract

Abdominal pain is a major reason patients seek medical attention yet relatively little is known about neuronal pathways relaying visceral pain. We have previously characterized pathways transmitting information to the brain about visceral pain. Visceral pain arises from second order neurons in lamina X surrounding the spinal cord central canal. Some of the brain regions of interest receiving axonal terminations directly from lamina X were examined in the present study using enhanced functional magnetic resonance imaging (fMRI) before and one week after induction of a rat pancreatitis model with persistent inflammation and behavioral signs of increased nociception. Analysis of imaging data demonstrates an increase in MRI signal for all the regions of interest selected including the rostral ventromedial medulla, dorsal raphe, periaqueductal grey, medial thalamus, and central amygdala as predicted by the anatomical data, as well as increases in the lateral thalamus, cingulate/retrosplenial and parietal cortex. Occipital cortex was not activated above threshold in any condition and served as a negative control. Morphine attenuated the MRI signal, and the morphine effect was antagonized by naloxone in lower brainstem sites. These data confirm activation of these specific regions of interest known as integration sites for nociceptive information important in behavioral, affective, emotional and autonomic responses to ongoing noxious visceral activation.

Keywords

morphine; visceral pain; nociception; central pain; pancreatitis

Introduction

Relatively little information has been available concerning how the brain processes visceral pain information [1]. Brain imaging techniques have become invaluable tools to assess activation of supraspinal areas. Using functional Magnetic Resonance Imaging (fMRI), for example, brain areas activated in patients with gastrointestinal disorders have been mapped

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[2–5], cortical representation of visceral and somatic sensations compared [6,7], and modulation of visceral sensations by emotions examined [8]. In general, data available reveal specific brain sites activated regardless of the source of pain, but visceral pain evokes greater fMRI signal intensity.

In the past few years, our group has characterized pathways relaying information to supraspinal areas about noxious stimulation of visceral structures including colon [9–14], duodenum [15] and pancreas [16–18]. The experimental evidence supports clinical studies in which dorsal myelotomies diminish or abolish pain in patients with pelvic and abdominal cancer [19–24]. In addition to the dorsal column midline ascending pathway, our anatomical studies have traced axons ascending in ventral white matter from visceral processing centers located around the spinal cord central canal directly to other supraspinal sites [25]. In addition to hypothalamic sites important in autonomic control, the axonal pathways terminate in the rostral ventromedial medulla (RVM), dorsal raphe (DR), periaqueductal gray (PAG), medial and lateral thalamus, and amygdala, known sites of nociceptive integration. The present study uses MRI signal to compare these brainstem sites known to receive input from these visceral pain pathways and selected cerebral regions of interest at two time points separated by one week, before and after development of a persisting visceral inflammatory pain state. An animal model of pancreatitis mimicking the clinical condition has been developed and the accompanying persistent pain state characterized [26]. The time course of the model (two weeks) is particularly useful for fMRI study and assumes a constant barrage of nociceptive information of visceral origin. The study reported here compares MRI signal before and after noxious pancreatic inflammation of one week duration. Morphine attenuation of the effect and naloxone reversal of the morphine reduction during the second session demonstrates opiate receptor involvement.

In initial animal studies, fMRI was used to assess brain regions after noxious events such as sciatic nerve stimulation [27], median nerve stimulation [28], and forepaw stimulation [29]. Fewer studies examined brainstem regions in animals after noxious visceral stimulation [13, 30]. Other more recent studies will be discussed. To our knowledge there are no animal MRI studies imaging persistent visceral pain responses of one week duration in specific brainstem and cortical regions.

Materials and Methods

Experimental Animals

All procedures were approved by the University of Texas Medical Branch Animal Care and Use Committee and adhere to the guidelines for the ethical treatment of experimental animals published by the International Association for the Study of Pain. The experimental subjects were male Lewis rats weighing 150–175 grams. Animals were kept two per cage until the day of the experiment when they were kept one per cage thereafter. Low soy content diet (Harlan Teklab 8626, Madison, WI) was provided, and the animals were kept under a standard 12:12 light: dark cycle (lights on at 7 am, lights off at 7 pm).

General Experimental Design

Male inbred Lewis rats (n=18; Harlan Sprague Dawley, Houston, TX), anesthetized with 1.2–1.5% isoflurane in a mixture of oxygen and nitrous oxide (30:70), were prepared for acquisition of baseline imaging data by inserting a catheter into the tail vein for contrast tracer injection. After the set of baseline images were collected and before the animals recovered from the anesthetic state, dibutyltin dichloride (DBTC, Aldrich, Milwaukee, WI) was injected to induce pancreatitis or vehicle was injected through the tail vein catheter already in place. After the injection, animals were allowed to recover for a week in their homecage with close monitoring and special care for those with developing pancreatitis. The DBTC induced

pancreatitis was maintained with Teklab diet 8626 and 10% alcohol in the drinking water as described previously [26]. On day 7 after injection of DBTC (n=12) or vehicle (n=6), animals underwent a second imaging acquisition series. After the acquisition of images, the rats received either morphine (n=3; 5 mg/kg, Paddock Laboratories, Minneapolis, MN) or saline (n=3) injected intravenously through the tail vein catheter. Another set of images was acquired 30 minutes after the morphine or saline injection. In a separate group of animals, naloxone (1 mg/kg, Endo Pharmaceuticals Inc. PA) was injected 15 minutes before morphine (n=5) or saline (n=3). Naloxone is a non-specific opioid receptor antagonist widely used to confirm that effects are opioid receptor-mediated [31].

Acquisition and Analysis of Imaging Data

Rats were anesthetized with 1.2–1.5% isoflurane in a mixture of oxygen and nitrous oxide (30:70). During the imaging procedures, each animal was artificially ventilated using a veterinary anesthesia ventilator (Hallowell EMC, Pittsfield, MA). A cannula was placed in the tail vein for the delivery of 2 mg Fe/kg of a superparamagnetic iron oxide tracer (SPIO; Combidex Advanced Magnetics, Inc). Combidex has a long vascular residence time ($t_{1/2} = 5$ hours), thereby enhancing the signal intensity decrease in proportion to increased cerebral blood volume [32]. To obtain coronal multislice spin echo images (TR = 3S, TE = 65ms, FOV = 6×6 cm, slice thickness = 1.6 mm) before and after SPIO injection, a 4.7 T magnet (Varian, INOVA) was used with a 5-cm diameter surface coil tuned to 200 MHz. The rats were placed in a supine position on a Plexiglas cradle with their heads positioned in the center of the surface coil. After the cradle was locked in place inside the magnet, a sagittal image of the rat brain using a fast low-angle shot gradient-echo imaging sequence, or FLASH [33] was acquired to define slice positions in the hemodynamic scans. The enhanced fMRI protocol consisted of two imaging regimens: 1) multislice T₂-weighted spin echo imaging (T₂WI); and 2) single slice haemodynamic bolus tracking gradient echo imaging in which animals are injected with superparamagnetic iron oxide. Steady state plasma volume images supplemented bolus transit experiments. Zero filling was used to increase image resolution, particularly when isolating signal intensity profiles from arteries and veins. Each fMRI protocol took about 50 minutes per animal. Statistical analysis was carried out using Sigma Stat 3.0 (Jandel, San Rafael, CA). For comparison purposes, repeated-measures ANOVA was used to identify areas where signal intensity changed significantly from baseline ($p \geq 0.05$), using Newman-Keul's post-hoc comparisons.

MRI Signal Intensity Measurements

MRI images were analyzed for areas of localized increases in signal intensity above threshold in the regions of interest. The T₂-weighted spin echo images were compared to the Paxinos rat brain atlas [34]. The use of anatomical landmarks insured that the animal was placed in the correct position from acquisition to acquisition and any variability produced by removing the animals from the magnet is reduced. Registration of the coronal brain MRI slices with the brain atlas was achieved by careful comparisons with known brain neuroanatomical landmarks that are easily visible in the MRI image, including ventricles, cerebral aqueduct, and hippocampus etc. Overlays of brain regions corresponding to various regions of interest were outlined by hand for each animal on the T₂-weighted baseline images using image processing software developed in-house (Figure 1A) and the overlay used at the subsequent one week time point. This method insured that the same regions of interests were compared between scans. The region of interest overlays were then analyzed by an observer blinded to the treatment groups. The digital image signal intensity in the MRI images was measured, where intensity was proportional to the amount of tracer in the region of interest taken as a relative circulating blood volume. In order to control for possible variations in plasma SPIO concentrations between MRI sessions, parenchymal signal intensity measurements were normalized by dividing

parenchymal signal intensity by that measured in large blood vessels within the slice of analysis. The large blood vessels consisted of either the sagittal sinus or the transverse sinus.

Regions of Interest

In this study, brain areas with increased signal intensity compared to baseline were overlaid on anatomical MRI setting a blood volume threshold equal to the highest parenchymal blood volume in baseline images. Those regions that rose above that threshold were mapped. Then, using the same threshold in the “activated” image, those regions of high signal intensity were overlaid on the anatomical images in order to confirm their location in the brain. These regions coincided with areas receiving axonal input from visceral processing areas in the spinal cord in our tract tracing studies [25] and higher brain regions known to process nociceptive information. The major neural structures analyzed in this study included the rostral ventromedial medulla, the dorsal raphe, periaqueductal gray, medial thalamus, lateral thalamus, central amygdala, midcingulate/retrosplenial cortex, and the parietal cortex (Figure 1). Signal in other sites known to process visceral nociceptive information and to receive lamina X projections, i.e. dorsal column nuclei, parabrachial nuclei, dorsal medullary reticular formation (SRD), and insular cortex, did not rise above the threshold set for this study most likely due to their small size and thus were not analyzed further. Other cortical activation sites not known to us when this study was done have also not been included in this study (e.g. anterior and subgenual cingulate cortex). Data are expressed as region/sinus ratio and percent of baseline.

In accordance with stereotaxic coordinates from the Paxinos rat brain atlas [34], four planes allowed analysis of signal intensity in the regions of interest (Figure 1A). The stereotaxic coordinates for each of the regions of interest are as follows: rostral ventromedial medulla (RVM, 9.6–9.8mm posterior to the bregma, LR 0.6mm, Depth 9.8–10.8mm), dorsal raphe (DR, 7.3–8mm posterior to the bregma, LR 0.5mm, Depth 5.5–6.5mm), periaqueductal gray (PAG, 7.3–8mm posterior to the bregma, LR 0.4–1mm, Depth 4.4–6.0mm), medial thalamus (M thal; 2.3–3.6mm posterior to the bregma, LR 0–1.5mm, Depth 5–6.7mm), lateral thalamus (L thal; 2.3–3.6mm posterior to the bregma, LR 2–3.6mm, Depth 5.4–7.5mm), central amygdala (AMG, 2.8mm posterior to bregma, Depth 7.9 mm, LR 4.6 mm), midcingulate/retrosplenial cortex (Cg, 1.3mm posterior to the bregma, LR 0–1mm, Depth 0.2–3mm) and parietal cortex, areas 1 and 2 (Par 1; 1–3.3mm posterior to bregma, , LR5.2 mm, Depth 2.7 mm and Par 2, - 3.3mm posterior to bregma, Depth 5.2 mm, LR 6.2 mm). The PAG, lateral thalamus, amygdala, and all cortical regions were analyzed bilaterally. The signal intensity of occipital cortex (OC, 7.3–7.8mm posterior to the bregma, LR 1–6.2 mm, Depth from 0.6–4mm) was measured as a negative control region (Figure 1B).

Statistical Analysis

The relative region/sinus ratios at baseline in the regions of interest were normalized to 100%. The experimental group data are expressed as a percent of baseline. An analysis of variance was conducted for each region of interest to compare the profiles of the groups followed by Newman Keuls multiple comparison tests. Statistical analysis was done using Prism software (San Diego, CA).

Results

In a previous study we showed that rats injected with the chemical irritant, DBTC, develop inflammation of the pancreas and sensitivity on the skin of the abdominal area [26]. The increase in sensitivity, referred to as secondary cutaneous mechanical and thermal hypersensitivity, is greatest at one week. Thus, in this study animals with pancreatitis were examined with MRI at this time point. There were no changes in signal above the set threshold

in the left or right occipital cortex of the anesthetized rats in any of the conditions, and thus this region served as a negative method control (Figure 1B). Images shown in Fig. 2–4 illustrate two representative animals at baseline, after one week with pancreatitis and then after morphine treatment (Rat 15) or after the combined naloxone and morphine treatment (Rat 17). Specific results for each region are provided in the text below and shown quantitatively in Figure 5. In the MRI images (Figures 2–4), color represents increase in signal within the region of interest that was above the threshold level. The yellow color is indicative of the greatest MRI signal intensity (blood volume), orange is intermediate and the red color indicates less signal.

Subcortical areas of activation after pancreatic inflammation

In rats injected with DBTC to induce pancreatitis, significant increases in MRI signal over baseline were evident in the specific brainstem regions of interest after one week. For example, the MRI signal greatly increased in RVM of rats with pancreatic inflammation compared to baseline (DBTC, 132.00 ± 5.1 % increase over baseline set at 100%; Figure 2, 5). There was no increase over baseline MRI signal in the RVM in animals given vehicle rather than DBTC (87.76 ± 8.82 %).

One week after induction of pancreatitis, MRI signal was also significantly increased in the DR (DBTC, 147.73 ± 14.40 ; Figure 2, 5). Results were similar with significant increases in signal in both the left and right sides of the PAG in animals with pancreatitis (L and R; 135.06 ± 6.87 and 129.34 ± 6.46 %, respectively). The medial and lateral divisions of the thalamus were also examined (Figures 3, 5). Analysis of the imaging data demonstrated a significant increase in MRI signal compared to baseline for both the medial thalamus (142.19 ± 9.52 %), and the left and right lateral thalamus (145.47 ± 4.14 and 135.06 ± 6.87 %, L and R respectively) in animals with DBTC-induced pancreatitis.

Amygdalar and cortical areas of activation after pancreatic inflammation

The central amygdala was examined in this study since it is known to be involved in nociceptive processing (Figure 3). The increases in MRI signal in the amygdala after one week of pancreatitis (L and R; 129.18 ± 12.51 and 125.62 ± 10.36 % of baseline) were increased but not significantly over MRI signal at baseline or in control animals in this small region with this method.

The cortical areas examined in the present study were the midcingulate/retrosplenial, parietal (area 1 and 2) and occipital cortices. Of these areas only the signal in the midcingulate/retrosplenial cortices (Figure 4, 5; L and R, 139.86 ± 7.27 and 142.64 ± 8.71 %) was significantly increased over the baseline and the controls after one week of pancreatitis.

MRI signal in the occipital cortex remained at baseline in animals with pancreatitis (Figure 1B; L and R, 99.83 ± 4.26 and 98.35 ± 6.68 % of baseline, respectively). Thus, the occipital cortex areas served as negative controls for these studies.

Morphine attenuation of activation due to pancreatic inflammation

Morphine (DM; 5 mg/kg) was able to significantly attenuate increase in MRI signal in the RVM compared to animals with pancreatitis (DBTC; 78.41 ± 7.78 %) (Figure 2, 5). When naloxone was injected 15 minutes before the morphine administration, the effect of morphine was antagonized, and MRI signal was not significantly different from animals with DBTC induced pancreatitis (DNM; 120.00 ± 9.14 %). The MRI signal was not increased in animals given vehicle followed one week later by naloxone and morphine (VMN; 86.54 ± 9.54 %). Morphine also significantly attenuated the MRI signal increase for the DR (DM; 74.34 ± 6.55 %). Pre-treatment with naloxone negated the morphine effect, and MRI signal in these animals was not significantly different from that in animals with DBTC induced pancreatitis.

(122.83±8.44%). For PAG, morphine significantly reversed the increase below baseline (76.52±5.08% and 70.27±8.85%). When naloxone preceded morphine, MRI signal in PAG was at baseline levels (106.96±3.87% and 99.89±3.76%) and was not significantly different from any of the controls. Morphine significantly attenuated the increase in MRI signal in all regions of the thalamus (56.54±1.79 medial; 63.33±3.99% and 69.02±0.78%, left and right lateral thalamus). With naloxone the MRI signal remained at baseline but the effect was not significantly increased over the morphine treatment (88.46±6.86, 93.81±6.58% and 83.95%±4.72, respectively).

The increase in MRI signal in the amygdala in animals with pancreatitis was reduced by morphine (76.91±2.75 and 70.18±1.92%), and this reduction was significant in the right amygdala. The morphine mediated reduction in MRI signal was returned to baseline in the presence of naloxone (102.11±4.23 and 97.35±6.20%). The decreases in MRI signal after morphine were also significant in the midcingulate/retrosplenial in animals with pancreatitis (L and R, 66.96±4.28 and 60.21±4.24%) and in parietal cortex regions (Area1: L and R, 62.38±6.35% and 69.3±2.60%; Area 2: L and R, 70.59±4.11% and 74.93±6.82%), with the exception of the left parietal Area 2. Unlike any other region studied, MRI signal in the cingulate/retrosplenial cortex was further reduced bilaterally when naloxone preceded morphine (50.62±8.46 and 47.1925±9.70%).

Summary of visceral activation sites

In summary, in animals with pancreatitis, MRI signal was increased in the RVM, dorsal raphe, PAG bilaterally, medial thalamus and lateral thalamus bilaterally, and the midcingulate/retrosplenial cortex in comparisons to signal in these regions in all the control groups (Figures 2–4). The greatest increases were noted in the dorsal raphe and the medial and lateral thalamus bilaterally (42–51% increases over baseline one week prior). The increases in signal in the sensory cortex of rats (parietal cortex I and II) did not reach significance. Some change was also noted in the right amygdala.

Morphine significantly abrogated the signal in all of the activated subcortical regions examined, and the morphine effect was limited in many regions in animals pre-treated with naloxone. Morphine and naloxone given in the same doses to vehicle treated animals did not significantly increase signal in any of the regions of interest compared to baseline or to animals with vehicle injections alone.

A summary diagram depicting the areas involved in the transmission and processing of visceral nociceptive information is shown in Figure 6. The pathways transmitting information about visceral pain from spinal cord to higher brain centers are known from previous electrophysiological, lesion and anatomical studies in animals and humans. Visceral nociceptive information is transmitted to the brainstem from lamina X via the dorsal columns, the spinoreticular and the spinothalamic tracts [19,25]. Cortical activation arises from the medial and lateral thalamus. In our previous anatomical study with small injections of an anterograde tracer (Phaseolus leucoagglutinin) made into lamina X, we demonstrated termination sites in the brainstem indicated in Figure 6 [25]. In the present study, we find that MRI signal is increased in these same brainstem sites in animals with pancreatitis of one week's duration confirming these regions receiving projections from lamina X are activated in this persistent visceral pain state. Many of the sites and the cortical regions responsible for integration and processing of visceral pain, including the thalamus, amygdala and limbic and sensory cortex, have also been described by others previously and will be discussed below.

Discussion

The data illustrate increased MRI signal in selected supraspinal and higher centers in rats with persistent noxious pancreatic inflammation of one week duration. The regions include the rostral ventrolateral medulla, dorsal raphe, periaqueductal gray, amygdala, thalamus, cingulate/retrosplenial cortex and parietal cortex. These regions were selected because they are sites known for integration of visceral nociceptive information. This includes brainstem sites and amygdala shown to be directly innervated by neuronal projections arising from lamina X neurons [25], where neurons are activated by visceral inflammation in our previous studies [9,19,36]. Studies have shown that amygdala, retrosplenial and parietal cortex have increased fMRI signal in patients with ongoing visceral pain [7,37,38,39]. In one recent fMRI study, signal intensity increase was found in the same brainstem nuclei in humans after electrical stimulation at either somatic or visceral sites [7]. Other highly relevant animal studies are discussed below.

In comparisons made to baseline and to control animals with vehicle injections, increases in MRI signal were observed in all of the selected visceral pain processing regions in animals with pancreatitis of one week duration. The increases reached significance in all regions except amygdala and parietal cortex. The present study demonstrates morphine attenuation of contrast enhanced fMRI signal increases in the selected regions after persisting pancreatic inflammation. Reductions are significant in all regions except the left parietal cortex, area 2 and left central amygdala. The effect of systemic morphine is naloxone-reversible in dorsal raphe and the rostral ventrolateral medulla, regions known to contain large numbers of opiate receptors, indicating specific involvement of opioid receptors affecting input to these sites. The lack of naloxone reversal at other sites could indicate that the reduction of the signal by morphine in those areas may be through other non-specific mechanisms which reduce the blood volume such as autonomic changes and/or the stringency of the statistical testing.

The increased fMRI signal in regions receiving axonal terminations from lamina X [25] establishes this pathway as an important link for transmission of visceral nociceptive input. The present findings indicate that the RVM, DR, PAG, thalamus (medial and lateral) and cingulate/retrosplenial cortex remain in a persistently sensitized state with pancreatic inflammation of 7 days duration. Few reports thus far have shown activation of small brainstem regions such as RVM and DR in a visceral pain paradigm in rat made possible with use of the 4.7 Tesla MRI. Electrophysiological studies have shown that the RVM, DR, PAG, thalamus and amygdala are involved in processing and descending modulation of somatic nociceptive information, and responses of cells in these regions are reduced by morphine [40]. Evidence of ongoing activation provides further relevance of these regions in integration of noxious visceral input important for attentional, autonomic, motor and emotive responses.

Rostral Ventrolateral Medulla

While all of the regions of interest are known to be important in pain processing, anatomical evidence for direct spinal input to the RVM was lacking prior to the spinal cord lamina X anatomical tract tracing study [25], despite a multitude of data demonstrating that this region is a primary integrative site for descending modulation of painful input. The RVM has also been shown to play a role in central sensitization after visceral stimulation [41], as well as in descending modulation of acute visceral pain [42–47]. Descending modulation from the RVM can either inhibit or facilitate visceral nociception.

Periaqueductal Gray

The PAG has been studied extensively in regard to its antinociceptive role in somatic pain and its central role in behavioral responses to stress. For example, stimulation of the lateral PAG

can produce avoidance and escape behaviors [48,49]. Stimulation of the ventrolateral PAG elicits a cessation of ongoing spontaneous activity (quiescence) and a profound hyporeactivity [50]. Therefore, it is no surprise to find PAG activated by the persisting pancreatic inflammation in this model in which animals have significantly reduced spontaneous behavioral activity. The PAG integrates information from different sensory modalities and triggers different appropriate behavioral responses to stressors. The PAG is activated in many visceral pain paradigms [50–52]. In imaging studies in humans, the PAG is activated by application of a heat thermode to the skin [53–55] in patients with chronic pain. The PAG also has been involved in functional imaging of micturition in humans as reviewed by Kavia and colleagues [37].

Amygdala

Studies correlating regional blood flow with other pain-related measures such as c-fos or electromyographic recordings indicate the amygdala as a site of activation in animal models of acute visceral pain [56,57]. Suppression of activation with morphine in the present study implies a role in nociceptive perception in addition to generalized anxiety/emotional processes. It is interesting to note the morphine reversal in the present study is only significant in the right amygdala since asymmetries in amygdalar activation are reported, for example with formalin-induced inflammatory and colorectal distension pain responses [56–59]. Thus, our findings support the assertion made in previous studies that the right amygdala plays a dominant role in pain processing.

Medial and Lateral Thalamus

The involvement of thalamic nuclei in visceral nociceptive information processing is well documented. Neuronal activity has been recorded in the lateral thalamus in response to nociceptive input from inflamed colon [10,11,12] and pancreas [16], and was greatly reduced by spinal morphine. The medial thalamic nuclei have also been shown to be activated after noxious visceral stimulation in previous studies [51,60–62]. Activation of these thalamic nuclei has been demonstrated by fMRI in humans [7,63–66] and monkeys [27]. The thalamus fulfills a central role in nociceptive information processing, receiving input from both somatic and visceral tissues. The spinothalamic system terminating ventral posterolaterally in the thalamus plays a role in processing discriminative aspects of pain, while the medial thalamus assigns affective characteristics of the pain. It was expected therefore that thalamic nuclei would remain activated with seven days of pancreatic nociceptive input.

Cingulate/Retrosplenial Cortex

The present study demonstrates increased MRI signal in the cingulate/retrosplenial cortex transition zone of rats with pancreatic inflammation. Functional significance of anterior cingulate cortex (ACC) activation is a matter of great debate. The region is responsive to many types of sensory activation related to affective, emotional and autonomic responses. The cingulate cortex is consistently activated in somatic pain paradigms [67], and in humans, the ACC is also activated in visceral pain paradigms [6,37]. Davis et al. [68] showed surgical lesion of the ACC in a patient removed the associated emotional responses and suffering but not the sensation of chronic pain. Units in the ACC in animals have response characteristics similar to units from the medial and intralaminar thalamic nuclei from which the ACC receives projections [69,70]. Enhanced responses of anterior cingulate cortex neurons, particularly in the anterior portion, have been recorded in response to colonic distension in rats pre-sensitized with albumin [71]. Unfortunately, the subgenual and rostral ACC were not analyzed in this study. However, the midcingulum and retrosplenium analyzed in this study have also been implicated in subjective pain responses. A study using repeated colorectal distensions found significant activation primarily in retrosplenial cortex with fMRI and c-fos localization [56]. In rats with conditioned aversion responses to formalin injections, Fos localization specific to

the condition was evident in cingulate, retrosplenial and parietal cortex, in addition to other cortical and hypothalamic sites [72]. In studies of long-term changes in regional cerebral blood flow in rats with chronic painful nerve constriction injury, the retrosplenial cortex was consistently activated at 2, 8, and 12 weeks, implicating this region is important in long-term adaptations of physiological responses to chronic pain [73].

In addition to classification as limbic structures, the anterior cingulate and retrosplenial cortices, as well as the medial thalamus, have been included as part of the medial pain system involved in cortical planning of responses to pain [74]. The visceral-related spinal ascending pathways shown in Figure 6 bring this information to the higher order systems through the dorsal column and ventral white matter [25,75]. The present study has shown increased MRI signal in many medial brain structures (RVM, DR, PAG, medial thalamus, cingulate/retrosplenial cortex) supporting consideration of these structures as the functional and anatomical sites in a medial pain system responsible for relaying information about visceral pain, as well as integrating and executive planning of responses to persistent noxious visceral input. In the context of visceral pain, as opposed to somatic pain, visceral pain is poorly localized but provides input evoking autonomic, motor, emotive, affective and attentional responses, as well as affecting working memory [74,76].

Nociceptive Specificity Through Morphine Attenuation

In the present study, morphine attenuated the MRI signal increase seen after seven days of pancreatic inflammation. The doses used have been reported to be effective in similar studies in rats [27,35]. The effect of morphine was significantly reversed by naloxone in dorsal raphe and rostral ventromedial medulla. These results provide confirmation that actions of morphine are specifically mediated by opioid receptors at these sites. Morphine is used extensively for its potent analgesic properties, but the dose of morphine used here did not affect MRI signal in the same sites in the vehicle control group. In this pancreatitis model, morphine attenuates nociceptive behaviors when given systemically in the same dose [26]. Opioid receptors have not only been shown to be abundantly present in the RVM and DR [25,77,78], but also in the PAG [79,80]; the thalamus [81] and the cingulate cortex [82–84]. It has been proposed that morphine treatment has a greater effect on affective-cognitive rather than sensory discriminative components of pain based on its effects in specific pain relay sites [85]. In previous behavioral and fMRI studies in rats and primates, similar doses of morphine reduced activation in selected regions involved in nociception including the anterior cingulate cortex and amygdala while activation continued in other regions [35,85].

Methodological Considerations

A study in normal humans indicates that gabapentin will reduce pain-induced signal at specific brain sites only after stimulation of capsaicin sensitized skin while otherwise having no effect on MRI signal [37,38]. Thus, fMRI is being used as a tool to determine specific brain sites affected by physiological stimuli and pharmacological treatments for pain in humans as it has been used in animal models [86]. The correlation of fMRI signals and neuronal activity is a matter of great discussion. The most commonly used method of imaging is BOLD (blood oxygenation level-dependent). This method measures neuronal activity indirectly via its assumed haemodynamic blood volume correlate to signal intensity. It has been demonstrated previously that blood pressure increases (30–80%), however, do not affect globally increase fMRI signal [87], which may be relevant to the morphine treatment used here. Nonetheless the fMRI signal correlates well with local field potential levels [87], and there is a tightly coupled correlation of neuronal synaptic activity and regional cerebral blood volume [88–94]. Recent studies have shown good correlation of fMRI signal with neuronal activation induced fos expression in nociceptive models with immunohistochemical methods [95,96]. The use of intravascular superparamagnetic iron oxide blood volume tracer has previously been employed

to map brain function by us [13] and others [97–99], and it also correlates well with function demonstrated physiologically and behaviorally.

A typical fMRI paradigm involves acquiring sequential hemodynamic-weighted scans during resting periods and during activated states. Most often, the whole fMRI protocol is carried out in a single scanning session, comparing relative hemodynamic changes during resting and activation. We employed an MRI technique that compares “resting” and “activated” states separated by one week, as well as during the same session before and after morphine. Thus, these studies rely on high reproducibility of the blood volume imaging technique and careful alignment of anatomical landmarks that we [13] and others [100] have previously demonstrated. This enhanced fMRI method allows us to assess persistent changes in nociceptive activation in response to visceral pain in our rat model of pancreatitis, as well as evaluate the effects of morphine analgesia in the anesthetized animal. The results of this study attest to the validity of the fMRI method in that no significant signal changes were observed above threshold in the control animals after morphine, whereas significant regionally specific CBV increases were observed in the rats with experimental pancreatitis. As a point of interest, the fMRI signal from the dorsal column nuclei and parabrachial nuclei appeared to be increased at one week when examined visually. The signal averages for this very small nucleus and this diffuse region, respectively, were quantitatively unchanged at the set threshold, and thus just beyond the level of resolution of this method.

Conclusion

In conclusion the present study provides evidence of increased MRI signal after one week of persisting visceral inflammation in specific brain areas directly innervated by the pathway originating in the spinal cord visceral processing region. The MRI signal increases were abrogated by morphine administration with a dose known to reduce nociceptive behavioral responses in the rat pancreatitis model. The morphine effect was directly attributable to opioid receptors in the dorsal raphe and rostral ventrolateral medulla where it was significantly attenuated by naloxone. These data confirm the functional relevance of the selected sites in the medial brainstem, medial and lateral thalamus, and cingulate/retrosplenial cortex, selected as pertinent to the physiological responses to ongoing pancreatic inflammation of one week duration because they are innervated by pathways bringing information about the visceral pain from spinal cord visceral pain processing regions. The data imply that the ascending input to the regions of interest examined in the present study are relevant to visceral nociceptive processes and likely the emotive, autonomic, motor, attentional and/or executive responses to visceral insult.

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Abbreviations

DBTC	dibutyltin dichloride
fMRI	functional magnetic resonance imaging

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A Regions of Study **B** Occipital Cortex

Baseline

DBTC

Figure 1.

A. MRI images of the four brain levels under study indicating the regions of interest where relative region/sinus ratios were determined. (RVM, rostral ventrolateral medulla; DR, dorsal raphe; PAG, periaqueductal gray; OC, occipital cortex; M thal, medial thalamus; L thal, lateral thalamus (left and right); Par1, Par2, parietal cortex areas 1 and 2; Cg, cingulate/retrosplenial cortex)

B. While subsequent Figures 2–4 will illustrate increases in MRI signal in most of the selected regions, signal was below threshold in the occipital cortex. Likewise, there were no increases in MRI signal in this region one week later in anesthetized rats with DBTC-induced pancreatitis. Thus, the occipital cortex served as a method control.

Rat 15 **Rat 17** **Figure 2.**

Graphical color representations of the relative region/sinus MRI signal ratios in regions of interest are shown as an overlay on the images. Yellow represents the areas of highest intensity. Baseline MRI signal in the rostral ventrolateral medulla (RVM), dorsal raphe and the periaqueductal gray (PAG) is shown in the top rows from two anesthetized animals. Using anatomical landmarks, these regions were re-imaged twice one week later in animals with DBTC-induced pancreatitis both before (middle row) and after morphine (bottom row). Rat 15 received i.v. morphine only, and Rat 17 received naloxone fifteen minutes prior to morphine.

Rat 15 **Rat 17** **Figure 3.**

Graphical color representations of the relative region/sinus MRI signal ratios in regions of interest are shown as an overlay on the images. Yellow represents the areas of highest intensity. Baseline MRI signal in the central amygdala (CeL), lateral and medial thalamus (left and right) is shown in the top rows from two anesthetized animals. Using anatomical landmarks, these regions were re-imaged twice one week later in animals with DBTC-induced pancreatitis both before (middle row) and after morphine (bottom row). Rat 15 received i.v. morphine only, and Rat 17 received naloxone fifteen minutes prior to morphine.

Rat 15 **Rat 17** **Figure 4.**

Graphical color representations of the relative region/sinus MRI signal ratios in regions of interest are shown as an overlay on the images. Yellow represents the area of highest intensity. Baseline MRI signal in the cingulate/retrosplenial and parietal cortex (Par1 and Par2) is shown in the top rows from two anesthetized animals. Using anatomical landmarks, these regions were re-imaged twice one week later in animals with DBTC-induced pancreatitis both before (middle row) and after morphine (bottom row). Rat 15 received i.v. morphine only, and Rat 17 received naloxone fifteen minutes prior to morphine.

Figure 5.

Bar graphs representing the relative region/sinus MRI signal ratios where baseline measurements are normalized to 100%. The experimental group data are expressed as a percent of baseline. The baseline (base), vehicle, and drug control (vehicle+morphine+naloxone, VMN) groups are shown to the left. The data for animals with DBTC-induced pancreatitis are shown in the three right columns, including animals with pancreatitis only (DBTC), animals with pancreatitis given morphine (DM), and animals with pancreatitis given both naloxone and morphine (DNM). The MRI signal was significantly increased in many of the visceral pain processing regions examined in animals with pancreatitis persisting for one week. Regions with increased signal after one week of pancreatitis included the rostral ventrolateral medulla (RVM), dorsal raphe (DR), periaqueductal gray (PAG-L (left) and PAG-R (right)), medial thalamus (Thalamus-M), lateral thalamus (Thalamus-L (left) and Thalamus-R (right)), and the cingulate/retrosplenial cortex (Cingulate Cortex-L and Cingulate Cortex-R).

Visceral Nociceptive Activation

Figure 6.

Schematic diagram illustrating regions with increased MRI signal in the present study after one week of pancreatitis and/or in previous studies by others after acute visceral stimulation indicating that these regions are activated by noxious visceral nociceptive input. The pancreatitis model produces hyperalgesic behaviors persisting for at least one week as shown in our previous studies. These data suggest that central sensitization producing hyperalgesic responses and increased MRI signals can persist in brainstem sites through at least one week with this persisting pancreatic insult.