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Differential Activation of Colonic Afferents and Dorsal Horn Neurons Underlie Stress-Induced and Comorbid Visceral Hypersensitivity in Female Rats

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Abstract: **Chronic Overlapping Pain Conditions, including irritable bowel syndrome (IBS) and temporomandibular disorder (TMD), represent a group of idiopathic pain conditions that likely have peripheral and central mechanisms contributing to their pathology, but are poorly understood. These conditions are exacerbated by stress and have a female predominance. The presence of one condition predicts the presence or development of additional conditions, making this a significant pain management problem. The current study was designed to determine if the duration and magnitude of peripheral sensitization and spinal central sensitization differs between restraint stress-induced visceral hypersensitivity (SIH) and chronic comorbid pain hypersensitivity (CPH; stress during pre-existing orofacial pain). SIH in female rats, as determined by the visceromotor response, persisted at least four but resolved by seven weeks. In contrast, CPH persisted at least seven weeks. Surprisingly, colonic afferents in both SIH and CPH rats were sensitized at seven weeks. CPH rats also had referred pain through seven weeks, but locally anesthetizing the colon only attenuated the referred pain through four weeks, suggesting a transition to colonic afferent independent central sensitization. Different phenotypes of dorsal horn neurons were sensitized in the CPH rats seven weeks post stress compared to four weeks or SIH rats. The current study suggests differential processing of colonic afferent input to the lumbosacral spinal cord contributes to visceral hypersensitivity during comorbid chronic pain conditions.**

Perspective: ***Chronic Overlapping Pain Conditions represent a unique challenge in pain management. The diverse nature of peripheral organs hinders a clear understanding of underlying mechanisms accounting for the comorbidity. This study highlights a mismatch between the condition-dependent behavior and peripheral and spinal mechanisms that contribute to visceral pain hypersensitivity.***

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Key words: *Stress, visceral pain, comorbid hypersensitivity, referred pain, primary afferents, dorsal horn neurons, female rats.*

Chronic pain is a major health problem affecting 50-100 million people in the United States.[30] Ten chronic pain conditions including irritable bowel syndrome (IBS) and temporomandibular disorder (TMD) are collectively known as Chronic Overlapping Pain Conditions and have minimal basis in underlying injury or disease.[38,61] One characteristic that sets these conditions apart is the propensity to overlap; having one condition significantly increases the odds of having multiple conditions.[14.54,64,65] In addition, stress is a significant risk factor and prevalence is significantly greater in women.[12,21,41] Mechanisms underlying these conditions individually and the mechanism underlying the comorbidity are unclear. The high comorbidity could result from central sensitization at multiple levels of the nervous system that increase sensitivity to visceral stimuli. In addition, many patients have pain conditions comorbid with anxiety or depression.

Previous studies in animals have shown that stress increases visceral sensitivity.[7,16,22,31,32,46,57,70,71] The visceromotor response (VMR) is a contraction of core muscles in response to hollow organ distension recorded as a change in magnitude of the abdominal muscle EMG. It is generally accepted that colonic inflammation following intracolonic administration of exogenous substances (eg, TNBS, mustard oil, capsaicin) increases the VMR in rats and mice to colorectal distension by sensitizing colonic afferents and visceroceptive dorsal horn neurons.[29,33] Likewise, neonatal stress or chronic stress in adults increases the magnitude of the VMR to visceral stimulation.[15,16,31,32,47,57,60] Stress activates the HPA axis and sympathomedullary axis increasing release of inflammatory mediators in the colon and altering corticotrophin releasing factor (CRF)/CRF receptor signaling, degranulation of mast cells and release of serotonin suggesting stress sensitizes colonic afferents and dorsal horn neurons contributing to visceral hypersensitivity.[4,31,55,66] However, the duration of stress-induced visceral hypersensitivity (SIH) is limited, generally resolving within weeks to a month. In addition, using the forced swim stress paradigm there is a sex difference in the duration of SIH, persisting days in male rats and several weeks in females.[32]

In rats, stress in the presence of preexisting orofacial pain significantly prolongs visceral hypersensitivity compared to stress alone.[57] This comorbid visceral pain hypersensitivity (CPH) models the pain reported by patients with TMD who present with symptoms of IBS.[1,65] We previously reported that CRF signaling and mast cell activation during the first few weeks following stress in CPH rats is similar to that observed in SIH rats.[31] Since the magnitude of visceral hypersensitivity in CPH rats is similar to hypersensitivity in SIH rats we asked 2 questions in the current study: 1) is there a difference in the response of primary afferents or dorsal horn neurons during the timeframe when both models exhibit visceral hypersensitivity; and 2) during the period of chronic visceral hypersensitivity in CPH rats, does peripheral sensitization persist?

Methods

Experiments were performed on cycling adult female Sprague-Dawley rats (Envigo, Indianapolis, USA; 10 weeks old on arrival at the University of Maryland School of Dentistry animal facility). Rats were acclimated to the housing facility at least 7 days prior to entering the study. Rats were not tested for estrous cycle stage since the excessive stress could alter our results due to prolonged daily handling for 7 weeks. All protocols were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee and conform to the guide for use of laboratory animals by the International Association for the Study of Pain. This study focused on intact female rats as we have shown that the currently used stress paradigm resulted in significantly shorter duration visceral hypersensitivity in both the stress and comorbid pain models in male SD rats,[32] Da Silva and Traub, unpublished observations. Rats were randomly assigned to one of three experimental groups: naÿve, stress-induced visceral hypersensitivity (SIH; stress alone) or comorbid pain hypersensitivity (CPH; orofacial pain plus stress).

Surgery

Rats were anesthetized with isoflurane (5% for induction, 2% for maintenance) and electromyogram (EMG) electrodes made from 40 AWG 10/50 stranded stainless steel wire (AS631; Cooner Wire Co., Chatsworth, CA) were implanted in the abdominal muscle and exteriorized at the base of the neck 10 days prior to recording. Rats were subsequently single housed to avoid interfering with cagemate's electrodes.

Restraint as the stressor for SIH and CPH rats: Rats were restrained in Broome style rodent restrainers (4.8 cm diameter, 20 cm length) preventing movement for 2 hours per day for 4 consecutive days [31,37,51]. During the two hours, rats were tilted at a 45 degree angle head up or head down in 15 min blocks alternating with 15 min blocks in the horizontal position [31]. The day after the last restraint session was designated Day 1.

Complete Freund's Adjuvant (CFA) Injection in Masseter Muscle

One day prior to starting the stress protocol, CPH rats were briefly sedated with isoflurane, and CFA (Sigma-Aldrich, F5881; 150 μL per side, 1:1 in saline) was injected bilaterally into the masseter muscles. This protocol (CFA+stress) produces the comorbid pain condition. We previously reported that saline injection into the masseter muscle followed by stress was similar to stress alone and CFA injection without stress did not induce visceral hypersensitivity.[31 57,71]

Visceromotor Response

The visceromotor response (VMR) is manifest as changes in the magnitude of the electromyogram (EMG) recorded from the abdominal muscles in response to colorectal distention. The EMG signal was recorded with a CED 1401 and analyzed using Spike 2 for windows software (Cambridge Electronic Design, UK). Starting two days before the baseline recording, rats were acclimated to the rodent restrainers (6.2 cm diameter, 21.5 cm length; larger than the stress restrainers) for two hours each day. Rats were then fasted overnight (water ad libitum) to facilitate balloon placement. On the day of the experiment, rats were briefly sedated with isoflurane and a 5-6 cm balloon (made from the finger of a surgical glove attached to Tygon tubing) was inserted through the anus into the descending colon and rectum. The distal end of the balloon was maintained 1 cm proximal to the anus by taping the tubing to the tail. Rats were then put in the restrainers and allowed 30 min to recover from the isoflurane. Three colorectal distention trials were run. Each trial consisted of inflating the distention balloon to 20, 40, and 60 mmHg, two times each (20 sec duration, 3 min interstimulus interval). Colorectal distention was produced by inflating the distention balloon with air using a computer controlled valve controller (B482CM-1, University of Iowa, Iowa City, IA). The recorded EMG signal was rectified and averaged over the 20 seconds of distension using Spike 2. The response to each pressure was the response during distention minus the signal during the 20 sec prior to distension. The data are presented two ways: as the stimulus response function (SRF) generated from the mean response from the second and third trials on each day and as the sum of the SRF each day giving the area under the curve (AUC). The VMR to colorectal distention was recorded prior to stress (baseline) and 1, 4 and 7 weeks after the last stress session.

Electrophysiology

Rats were anesthetized with urethane (1500 mg/kg, i.p.) and prepared for lumbosacral (LS: L6-S2) dorsal root teased fiber recordings or single unit recordings from the LS or thoracolumbar (TL: T13-L2) spinal cord as previously reported.[33,59,62]

The left carotid artery was catheterized for continuous arterial blood pressure monitoring and bolus administration of pancuronium bromide (0.2 mg/kg/hr). A tracheal cannula was inserted for artificial ventilation. End-tidal CO2 was maintained at 3.5-4.5%. Body temperature was maintained with a water-jacket heating pad and overhead lamp. The rat was placed in a head holder and suspended with thoracic vertebral and ischial clamps. The LS or TL spinal cord segments were exposed by laminectomy. The dura matter was cut and the spinal cord was bathed in warm mineral oil. The distention balloon was placed into the colon and the rat left undisturbed for 1 hour before recording.

Primary Afferents

Afferents were recorded from naÿve rats and SIH and CPH rats 7 weeks post stress. A pair of silver electrodes was used for recording. The dorsal roots were cut close to the root entry zone and the end in continuity with the periphery was carefully split into fine filaments until a single colorectal distention responsive fiber could be isolated. Signals were amplified (model 1800 AC amplifier; A-M systems, Carlsborg, WA) and passed through a dual time and voltage window discriminator (DDIS-1; BAK Electronics, Umatilla, FL) to isolate a single unit. Data were collected with a CED micro 1401 and Spike 2 for Windows software for online and offline analysis. The response to graded intensities of colorectal distention (20, 40, 60 mmHg) was recorded. The response to distention was quantified as the mean discharge frequency during the 20 sec distention minus the mean spontaneous activity in the preceding 20 sec.

Dorsal Horn Neurons

Neurons were recorded from naÿve rats and SIH or CPH rats 4 or 7 weeks post stress. The surgery was done as described above. Tungsten microelectrodes (1-2 MΩ; Micro probe, Potomac, MD) were used for extracellular single-unit recording in the TL or LS spinal segments (0-1.5 mm lateral to midline, 500-1500 m ventral to spinal cord dorsum). Signals were amplified, isolated and recorded as above. After identifying a neuron, two graded intensity distention trials to 20, 40, and 60 mmHg, two times each (20 sec duration, 3 min interstimulus interval) were averaged for each neuron.

Two phenotypes of dorsal horn neurons with excitatory responses to colorectal distension were identified on the basis of the response to 60 mmHg colorectal distention. Abrupt neurons increased firing with stimulus onset and ceased responding within 4 sec of terminating the stimulus; activity dropping below the mean plus 2 standard deviations of the spontaneous activity (mean of 20 sec prior to distention) for 2 sec. Abrupt unit activity was quantified as the mean discharge frequency during the 20 sec of the colorectal distention stimulus minus the mean spontaneous activity determined in the preceding 20 sec.

Sustained neurons increased firing with stimulus onset and had an after discharge that persisted longer than 4 sec after the stimulus ceased. Sustained unit activity was measured as the mean discharge frequency from the start of distention through the end of the after discharge (activity dropped below the spontaneous activity plus 2 standard deviations for 2 sec) minus the mean spontaneous activity.

A third phenotype, Inhibited neurons, had spontaneous activity that was inhibited by distension. The percent inhibition was calculated as the ((response minus background) divided by background) x100.

Referred Pain

Comorbid rats were tested for referred mechanosensitivity at baseline and pre- and post-intracolonic

lidocaine (1 mL, 2% jelly) 1 and 4 weeks post stress or benzocaine (1 mL, 18% jelly) 7 weeks post stress. The potency of these local anesthetics (LA) is comparable.[24,42] Rats were placed on an elevated tray (46 × 36 cm) with 1-cm sides and left for 30 minutes to acclimate. von Frey filaments were applied to the back at the level of the base of the tail. Each filament was tested 5 times at 10-second intervals. Filaments were tested with increasing force ranging from 4 to 100 g. Nonlinear regression was used to calculate the EF50.

Statistics

Data were analyzed in Prism 9. The data are expressed as mean ± SEM for each group. Data were analyzed by two-way ANOVA. Post-hoc comparisons following a significant ANOVA were conducted with a Dunnett correction for multiple tests. An adjusted P value < .05 was considered significant.

Results

Visceromotor Response (VMR)

The visceromotor response (VMR) was recorded from naÿve, SIH and CPH rats. We previously reported that not all rats responded to the restraint stress paradigm, some were resilient and failed to show visceral hypersensitivity over the subsequent weeks.[31] In order to compare the maximum effects of stress or comorbid conditions, rats that had a decreased VMR at 1 week post stress compared to baseline were excluded (ie, were resilient to stress, 3 SIH rats).

The VMR to graded intensities of colorectal distention was recorded up to 7 weeks post stress. In naÿve rats (n = 13), there was no significant change in the stimulus response curve over time (2 way ANOVA, time: $F_{(3,132)}$ = 2.088, P = .1048; pressure: $F_{(2,132)}$ = 41.69, P < .0001; Fig 1A). In SIH rats (n = 8), there was a significant increase in the magnitude of the VMR at 1 and 4 weeks, but it was no longer different from baseline at 7 weeks

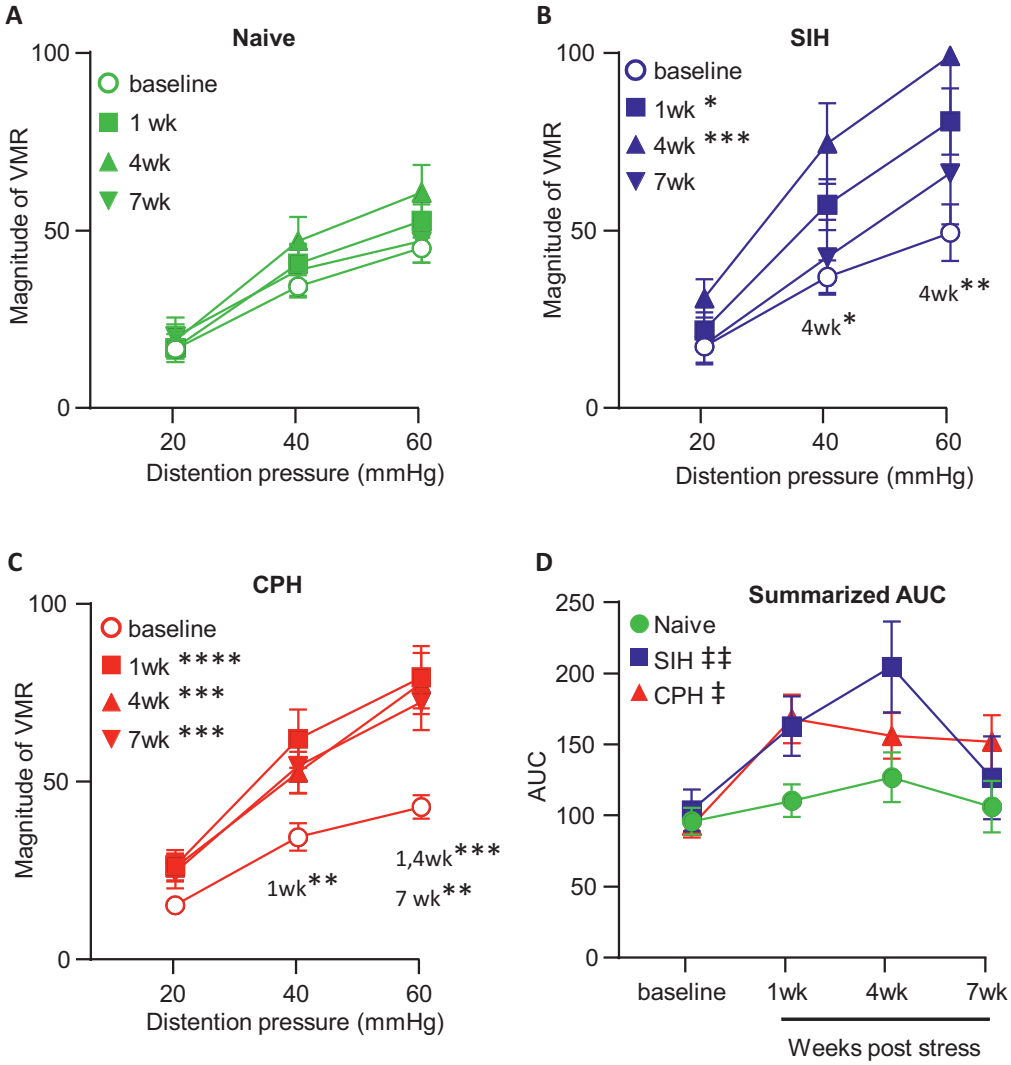

Figure 1. The magnitude of the VMR to graded intensities of colorectal distension. Stimulus response curves to graded intensities of colorectal distension at baseline (pre-treatment) and 1, 4 and 7 weeks post stress in Naÿve (A), SIH (B) and CPH (C) treated rats. Some error bars are shown in one direction to maintain the same Y axis scale. *,**,***,**** P < .05, .01, .001, .0001 vs. baseline. D: the summarized area under the curve at each timepoint for each treatment group. ‡, ‡‡ P < .05, .01 vs. naÿve. n = 8-14 rats/group.

(2 way ANOVA, time: $F_{(3,84)}$ = 6.976, P = .0003; pressure: $F_{(2,84)}$ = 29.60, P < .0001; Fig 1B). In contrast, stress in the presence of a preexisting orofacial pain condition (CPH, n = 12) induced visceral hypersensitivity that persisted at least 7 weeks (2 way ANOVA, time: $F_{(3,132)}$ = 9.933, P < .0001; pressure: $F_{(2,132)}$ = 54.18, P < .0001; Fig 1C). As previously reported, visceral hypersensitivity occurred only at higher distention pressures. When the summed AUC of the stimulus response curves was calculated, both SIH and CPH conditions were greater than naÿve (2 way ANOVA, treatment: $F_{(2,115)}$ = 5.856, P = .0038; Fig 1D).

Primary Afferents

The magnitude of the VMR to colorectal distention in SIH and CPH rats was similar through 4 weeks. Since stress increases CRF signaling in the colon evoking degranulation of mast cells and release of inflammatory mediators sensitizing colonic afferents,[11,52] the question was if colonic afferents were similarly sensitized in the SIH and CPH models. This was addressed by recording the response of colonic afferents in the L6 and S1 dorsal roots to graded intensities of colorectal distention. Since the VMR differed between SIH and CPH at 7 weeks, this was the first group tested. Recordings were made from fibers in naÿve rats (n = 13 fibers, 3 rats), and 7 weeks following the end of the stressor in SIH (n = 18 fibers, 3 rats) and CPH (n = 16 fibers, 5 rats) rats. Compared to naÿve, there was a significant increase in the response to distention in both the SIH and CPH rats (2 way ANOVA, pressure: $F_{(2,132)}$ = 22.57, P < .0001; treatment: $F_{(2,132)}$ = 10.31, P < .0001; interaction: $F_{(4,132)}$ = 2.736, P = .0315; Fig 2). In the SIH rats this was unexpected since the VMR to colorectal distention abated to the pre-stress level by 7 weeks. Since colonic afferents were sensitized in the SIH rats at 7 weeks post stress, it was deemed unnecessary to record at the 4 week timepoint.

Referred Pain

The presence of sensitized colonic afferents seven weeks post stress in the SIH rats when visceral hypersensitivity had resolved suggested the effect of the sensitized colonic afferent input was mitigated. In contrast, colonic afferent sensitization in the CPH rats during chronic visceral hypersensitivity suggests the colonic afferents facilitated, or at least helped maintain the hypersensitivity. This was tested by examining the effects of intracolonic injection of LA 1, 4 and 7 weeks post stress on referred pain in the lower back. In rats, hypersensitivity to mechanical stimulation of the lower back at the base of the tail is indicative of referred pain from a hypersensitive colon.[5,57,59]. Abatement of mechanohypersensitivity following injection of LA suggests the referred pain is dependent on persistent colonic afferent input. Referred pain was measured before and 30 min post intracolonic injection of LA 1, 4 and 7 weeks following CFA+stress in CPH rats. Compared to naÿve rats, the EF50 was significantly lower at 1, 4 and 7 weeks prior to LA injection indicating referred pain (1 way ANOVA, $F_{(3,21)}$ = 8.630, P = .0006; Fig 3A). At

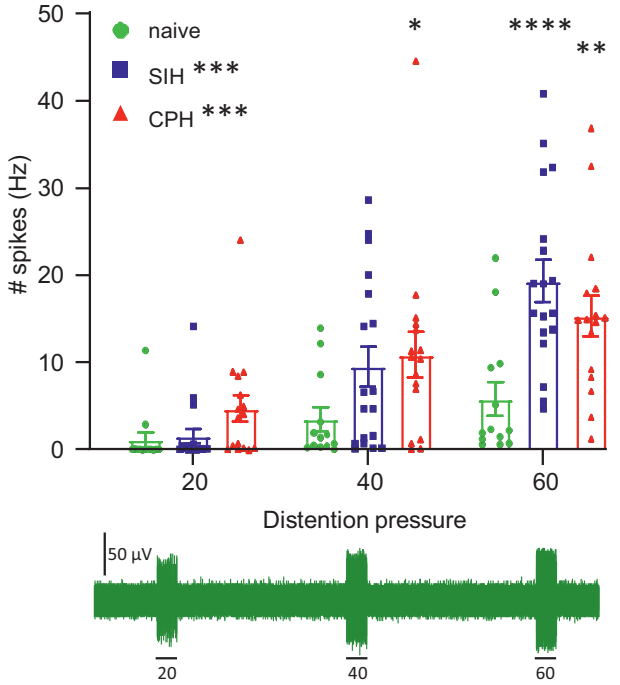

Figure 2. The response of colonic afferent fibers in the L6-S1 dorsal roots to graded intensities of colorectal distension from naÿve rats and 7 week SIH and CPH rats. Bars are mean ± SEM. An example of an afferent from a SIH rat responding to different distension pressures is shown below. *,**,***,**** P < .05, .01, .001, .001 vs. naÿve. n = 13-18 fibers/group.

both 1 and 4 weeks, LA normalized the mechanosensitivity (paired t-test: 1 week, P = .0256; 4 weeks, P = .0318). By 7 weeks, the effect of intracolonic LA was much more variable suggesting referred pain was present in some rats and absent in others. Overall however, there was no difference pre-LA compared to post-LA at 7 weeks (Wilcoxin matched pairs signed rank test, P = .4375).

In a separate group of rats, intracolonic injection of LA attenuated the VMR (RM ANOVA, $F_{(1.881, 7.523)}$ = 5.321, P = .0376, Fig 3B,C), confirming that intracolonic LA locally anesthetizes colonic afferents.

Dorsal Horn Neurons

The presence of lower back mechanohypersensitivity (referred pain) at 7 weeks in some CPH rats that is not alleviated by anesthetizing colonic afferents suggests the presence of central sensitization of dorsal horn neurons independent of colonic afferent activity. This was examined by recording the response of 149 LS and 67 TL dorsal horn neurons from 125 rats divided between naÿve rats, and 4 and 7 weeks post stress in rats with SIH or CPH. Neurons were classified as Abrupt (on/off with the stimulus), Sustained (prolonged afterdischarge) or Inhibited (inhibited by distention).[33,62] The magnitude of response significantly increased with pressure for all treatment groups for each phenotype.

Table 1 shows the number of neurons recorded in each treatment group for each phenotype. Recording from the LS spinal cord, comparing the response of Abrupt neurons between treatment groups and across

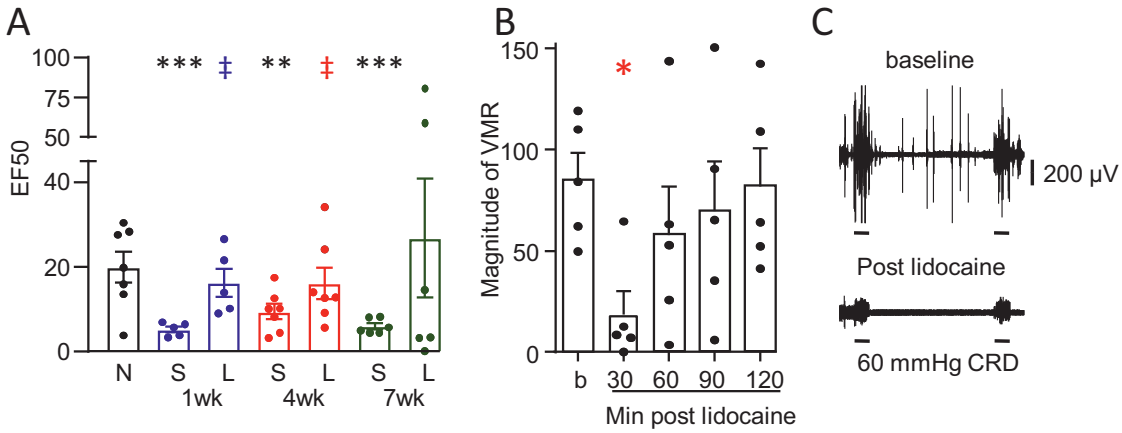

Figure 3. The effect of intracolonic local anesthetic on referred pain and the VMR in CPH rats. A: The EF50 to von Frey stimulation of the lower back in naÿve rats (N) and 1, 4 or 7 weeks following CFA + stress. S is the response at 1, 4, or 7 weeks following stress before intracolonic LA, L is the response 30 min following intracolonic injection of local anesthetic. **,*** $P < .01$, .001 vs. naive. ‡ $P < .05$ vs. weekly post stress (S) response. n = 5-7 rats/group. B: The magnitude of the VMR 30-120 min post intracolonic lidocaine. * $P < .05$ vs. baseline. n = 5. C: An example of the EMG recordings at baseline and 30 min post lidocaine injection.

Table 1. The Number of Cells (Rats) Recorded from Lumbosacral (L6-S2) Dorsal Horn in Different Experimental Groups at Different Times

	Abrupt	*Sustained*	*Inhibited*
Naÿve	19 (9)	12 (6)	6 (4)
SIH 4 wk	11 (7)	7 (6)	10 (5)
SIH 7 wk	7 (5)	8 (5)	13 (8)
CPH 4 wk	11 (5)	7 (6)	13 (5)
CPH 7 wk	11 (6)	8 (4)	6 (3)

time showed an overall significant effect of treatment and pressure, but not interaction (2 way ANOVA, treatment: $F_{(4,168)} = 6.588$, $P < .0001$; pressure: $F_{(2,168)} = 36.45$, $P < .0001$; Fig 4A). Multiple comparison within treatment showed a significant increase in response of neurons from SIH treated rats at 4 weeks and 7 weeks and CPH treated rats at 4 weeks compared to naÿve. The response of neurons from 7 week CPH rats was not different from naÿve.

In contrast, while there was an overall significant effect of treatment and pressure for Sustained neurons (2 way ANOVA, treatment: $F_{(4,102)} = 3.318$, $P = .0134$; pressure: $F_{(2,102)} = 17.62$, $P < .0001$; Fig 4B), only the 7 week CPH group was significantly different from naÿve.

Inhibited neurons have a steady rate of spontaneous activity that is inhibited by colorectal distention. Overall, there was a small, but significant change in the % inhibition between treatment groups, but no individual differences (2 way ANOVA, treatment: $F_{(4,129)} = 2.444$, $P = .0498$; pressure: $F_{(2,129)} = 12.00$, $P < .0001$; Fig 4C).

These results suggest that Abrupt neuron hyperexcitability is driven by colonic primary afferent input and increases transient visceral hypersensitivity. In contrast, sensitization of Sustained neurons appears associated with chronic visceral hypersensitivity.

Previous studies have shown that the increase in the VMR following acute colonic inflammation is partially due to an increase in activity in the TL spinal cord receiving colonic afferent input via the lumbar splanchnic nerve [27,56,62,63]. Since stress increases visceral sensitivity, the response of TL dorsal horn neurons to colorectal distension was examined. Surprisingly, there was no clear change in activity in TL neurons. Most neurons (43) had Abrupt responses. There was no change in response magnitude of TL neurons in SIH or CPH rats at any timepoint compared to naÿve (Fig 4D), suggesting TL Abrupt neurons do not contribute to the increase in visceral sensitivity in SIH and CPH rats. Ten Sustained neurons were recorded, but none from naÿve rats. This was consistent with previous findings where few Sustained neurons were recorded in the TL spinal cord of male rats in the absence of colonic inflammation.[62] Fourteen Inhibited neurons were recorded from naÿve, 4 week CPH and 7 week SIH rats, but due to the unbalanced distribution, no conclusions could be drawn.

Discussion

In the present study, we tested the hypothesis that peripheral sensitization would be a significant factor in developing visceral hypersensitivity following stress but would resolve as the hypersensitivity transitioned to a chronic state in rats with comorbid pain conditions. The hypersensitivity would then be maintained by central sensitization in the spinal cord and at higher levels of the neuraxis. In addition, central sensitization would persist in CPH rats mirroring the response to noxious visceral stimuli in awake animals. This was based in part on the results from previous studies reporting that peripheral CRF signaling partially mediated visceral hypersensitivity in both the SIH and CPH models.[9,31,34,55] This report focuses on the spinal cord and our results indicate that CPH and SIH are due to differential activation of colonic afferents and dorsal horn neurons, strongly suggesting differential modulation by descending activity.

Visceral hypersensitivity is a defining characteristic in the pathophysiology of IBS. Visceral hypersensitivity is reflected in increased perception of gut physiological signals and/or increased perception of experimental

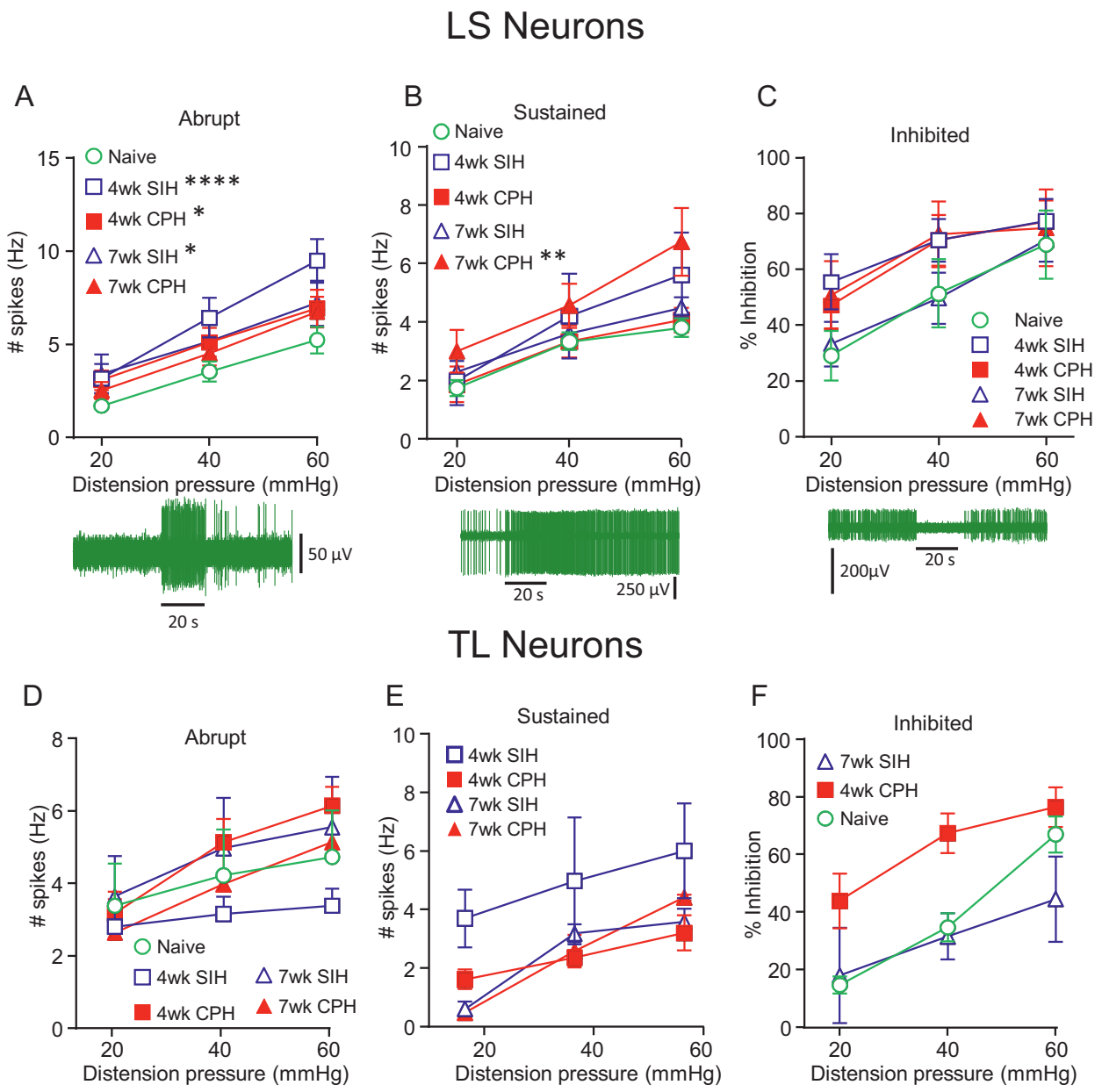

Figure 4. The response of Abrupt, Sustained and Inhibited dorsal horn neurons in the LS spinal cord (top row) and TL spinal cord (bottom row) to graded intensities of colorectal distension. Tracings are examples of neuronal responses. *,**,**** $P < .05$, .01, .0001 vs. naÿve.

visceral stimuli and a high degree of vigilance to these stimuli.[17,55] Epidemiologic studies have shown that stress (psychosocial and physiological) is a trigger for the first onset or exacerbation of IBS symptoms and that presentation of IBS is more prevalent in women.[10,18,25,39,41] Furthermore, women with high levels of anxiety or stress are more prone to idiopathic pain conditions including TMD and IBS that are collectively known as Chronic Overlapping Pain Conditions [3, 21, 40, 54]. As such, various animal models of stress including, but not limited to forced swim, restraint, water avoidance, neonatal maternal separation and prenatal (pregnant dam) plus adult stress have been developed to understand the mechanisms underlying visceral hypersensitivity and the effects of stress on visceral pain. For example, visceral hypersensitivity induced by stress is of greater duration or more robust in females and subject to hormonal modulation; it is facilitated by estrogen and attenuated by testosterone.[6,28,32,36,48,67] Stress in the presence of preexisting orofacial pain further increased the duration and visceral hypersensitivity, especially in females.[31,57] Therefore, stress is an exacerbating factor in visceral hypersensitivity that has more robust effects in females.

Peripheral Sensitization

It is well recognized that visceral hypersensitivity can occur due to sensitization of primary afferents innervating the viscera. The gastrointestinal (GI) tract from the esophagus to the transverse colon is innervated by vagal afferent fibers. The rest of the large bowel is innervated by pelvic nerve afferent fibers, originating from the LS

dorsal root ganglia, and projecting centrally to the sacral spinal cord. The entire GI tract is also innervated by afferent fibers in the splanchnic nerves projecting to the T5-L2 segments of the spinal cord. Both pelvic nerve and splanchnic nerve afferents are sensitized following release of inflammatory mediators in the viscera[8,19,53] and this activity converges on spinal neurons in the LS and TL segments evoking central sensitization of dorsal horn neurons.[27,33,35,49,50] Supraspinal projections including the postsynaptic dorsal column pathway, spinothalamic tract, and spinoparabrachial pathway convey visceral information to the brain.[2,43,58] Ascending fibers feedback to modulate descending control of nociceptive transmission.[20,35,50] The present study mainly examined how colonic afferent nerves and dorsal horn neurons contributed to SIH and CPH.

In the present study, the magnitude of the VMR to colorectal distention was similar in the CPH and SIH rats in the first 4 weeks, but the hypersensitivity resolved in the SIH rats and was still present in the CPH group 7 weeks following the last stressor. This is consistent with a previous report where CPH persisted at least 3 months in female rats.[31] One possible explanation was differential activation of colonic afferents. Seven weeks following restraint stress there was a significant increase in the response of colonic afferents to distention in both the SIH and CPH rats. This result was unexpected since the visceral hypersensitivity abated to the baseline by 7 weeks in the SIH rats. One possible explanation is that in SIH rats stress-induced dysregulation of descending inhibition recovered, inhibiting synaptic input from sensitized primary afferents to dorsal horn neurons normalizing visceral sensitivity. Indeed, 5-HT_{2C} receptor expression in the dorsal horn of the spinal cord was significantly down-regulated 2 days post stress in SIH and CPH rats,[68,69] suggesting the impairment of 5-HT mediated descending pain inhibition. It remains to be determined if 5-HT_{2C} receptor levels remain reduced 7 weeks post stress in CPH rats although RNAseq data indicate no difference in expression levels of 5-HT_{2C} vs. naÿve in 7 week CPH rats (Dorsey and Traub, unpublished observations). These results suggest that sensitization of colonic afferents and impairment of descending pain inhibition together lead to the occurrence of visceral hypersensitivity. Recovery of descending inhibition in the SIH rats could nullify the effects of persistent peripheral sensitization of colonic afferents. Since visceral hypersensitivity persists at least 7 weeks in the CPH rats and peripheral sensitization similarly persists, it suggests descending modulation might still be dysfunctional. Future studies will examine this possibility. Alternatively, dorsal horn neuron activity might differ between SIH and CPH rats at different time points.

Referred Pain

The role of colonic afferents in visceral hypersensitivity could be indirectly measured by examining referred pain.[5,59,72] Local anesthetic in the colon attenuates the VMR. In CPH rats LA reduced mechanosensitivity of the lower back at 1 and 4 weeks. This suggests that referred pain is dependent on sensitized colonic afferent input maintaining hyperexcitability of spinal neurons with convergent somatic input. At 7 weeks the effects of LA were much more variable suggesting some rats had central sensitization and referred pain independent of primary afferent input. It is possible that this would occur in all rats with more time.

Central Sensitization

The distal colon is innervated by the least splanchnic and pelvic nerves. Sensory signaling from the colon is relayed by primary afferent neurons within these nerves into the TL and LS spinal cord, respectively, activating viscerceptive dorsal horn neurons.[13,26,27,44,58,62] A sexually dimorphic phenotypic difference in the response to acute colonic inflammation of TL and LS dorsal horn neurons has been reported.[33,45,62,63] In females, acute colonic inflammation (hours after injury) increased the response of Abrupt neurons in the LS and TL spinal cord and decreased the response of Sustained neurons in the TL spinal cord.[33] Based on the response of dorsal horn neurons to colonic inflammation, and the fact that stress causes an immune response in the colon,[23,26] it was hypothesized that TL and LS visceroceptive dorsal horn neurons would be sensitized in SIH and CPH rats.

However, there was a differential effect of exclusively stress-induced verses comorbid conditions on different phenotypes of dorsal horn neurons in the LS spinal cord. In SIH rats at both 4 and 7 weeks and CPH rats at 4 weeks, there was an increase in the response of Abrupt neurons while in CPH rats 7 weeks following stress there was an increase in the response of Sustained neurons. The increased response of Sustained neurons parallels the VMR in the 7 week CPH rats, suggesting sensitization of Sustained neurons contributes to visceral sensitivity. Surprisingly, there was no obvious change in the response of TL neurons under any condition. Since TL processing of colorectal input is a major contributor to visceral hypersensitivity following acute colonic inflammation,[56] these data suggest that the TL spinal cord does not mediate visceral hypersensitivity several weeks following stress. However, in ovariectomized rats with estrogen replacement, CPH conditions increased Fos expression to colorectal distension in both the TL and LS spinal cord.[57] Alternatively, colonic afferent input over the pelvic nerve inhibits the response of TL dorsal horn neurons in male rats.[63] It is possible that the sensitized colonic afferents in the pelvic nerve inhibited the response of TL neurons following stress. These contradictory data indicate the role of the TL spinal cord in SIH and CPH needs further examination.

In conclusion, the present study demonstrated that colonic afferents and dorsal horn neurons had different effects in visceral nociceptive processing in SIH and CPH rats. We found that the visceral hypersensitivity existed at 1 and 4 weeks and recovered at 7 weeks following stress in the SIH group, while visceral hypersensitivity persisted at least 7 weeks in the CPH group. In SIH and CPH rats, the response to colorectal distention in LS colonic afferents significantly increased 7 weeks

following the end of the stressor. There was a significant increase in the response of Abrupt neurons in the LS dorsal horn to colorectal distention in SIH rats at 4 and 7 weeks, and CPH rats at 4 weeks. However, the response of Sustained neurons increased in CPH rats at 7 weeks. In combination with previous reports, these findings demonstrate that chronic visceral hypersensitivity is partly due to differential activation of colonic afferents and dorsal horn neurons, suggesting that similar mechanisms contribute to visceral hypersensitivity during the time when the effects of stress are prevalent, but central sensitization, independent of afferent input, contributes to chronic comorbid pain hypersensitivity.

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