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# The double insult of neonatal cystitis plus adult somatic inflammation results in corticotropin releasing factor type 2 receptor-dependent bladder hypersensitivity in female rats

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## Abstract

The spinal mechanisms of visceral hypersensitivity are poorly understood, particularly when there is an interaction with somatic systems. Recently we demonstrated that rats which were pretreated with neonatal bladder inflammation (NBI) and subsequently pretreated as adults with bladder re-inflammation had augmented reflex and neuronal responses to urinary bladder distension via a corticotropin-releasing factor receptor type 2 (CRFR2) mechanism. Another insult producing similar augmented responses is somatic inflammation induced by Complete Freund's Adjuvant (CFA) in the hindlimb. Using neurochemical measures and both reflex and neuronal responses to urinary bladder distension as endpoints, the present study probed the role of CRFR2-related mechanisms in bladder hyperalgesia secondary to NBI and CFA-induced hindlimb inflammation. ELISA measures of the lumbosacral spinal cord demonstrated increased CRFR2 protein following pretreatment with NBI+CFA. Intrathecal CRFR2 antagonists blocked the augmentation of visceromotor responses to distension following pretreatment with both NBI+CFA. Lumbosacral dorsal horn neuronal responses to bladder distension in rats pretreated with NBI+CFA were attenuated by the spinal topical administration of a CRFR2 antagonist. These findings are the first demonstration of a somatovisceral interaction working via CRFR2 receptors and support the therapeutic value of these agents in the treatment of painful bladder disorders, particularly when triggered by somatic events.

### Keywords

visceral pain; hypersensitivity; interstitial cystitis/bladder pain syndrome; somatic inflammation

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AUTHOR CONTRIBUTIONS

All authors have contributed to the intellectual content of this manuscript. TJN and AR were responsible for study design, data collection, data analysis, manuscript generation/revision. CW was involved in data collection and manuscript generation/revision.



Declaration of Competing Interest

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## INTRODUCTION

Despite extensive research efforts, disorders associated with visceral hypersensitivity such as interstitial cystitis/bladder pain syndrome (IC/BPS) have limited effective treatment options. The neurochemical basis of these disorders is poorly understood and as a consequence, their treatment often lacks a mechanisms-based strategy. Preclinical study of visceral hyperalgesia has demonstrated a role for corticotropin releasing factor (CRF) -related mechanisms in the gastrointestinal tract[18,22–24,34,35] and musculoskeletal structures[1,21] but studies related to bladder sensation/function are few[e.g. 17]. Recently, we reported that CRF-related systems were altered in a model of bladder pain[29].

The bladder pain model which we used to test CRF-related mechanisms used a protocol developed by ourselves and others[e.g.,16,41,44] that results in adult female rats that have phenotypic features similar to those which have been noted for IC/BPS[36]. The primary insult in this model is neonatal bladder inflammation (NBI) which results in magnified nociceptive responses to urinary bladder distension (UBD) following a secondary insult, such as somatic inflammation, as an adult[28]. Using this protocol, we also demonstrated that NBI coupled with bladder re-inflammation as an adult resulted in augmented reflex and neuronal responses to UBD that were attenuated by the spinal administration of the CRFR2 antagonist aSVG30. It also resulted in elevated CRFR2 and urocortin 2 content in the lumbosacral spinal cord[29]. As the spinal mechanisms whereby bladder re-inflammation leads to visceral hyperalgesia in NBI-pretreated rats could easily differ from those associated with other secondary insults, we felt it was appropriate to probe in a focused fashion the role of CRFR2-related mechanisms of bladder hyperalgesia when Complete Freund's Adjuvant-induced inflammation of the hind limb (which will be abbreviated as CFA) was used as an alternative secondary insult in NBI-pretreated rats. To do this, lumbosacral spinal cord tissue measures of receptors, visceromotor reflex responses (VMRs) to UBD and lumbosacral spinal dorsal horn neuronal responses to UBD following NBI with and without CFA pretreatments were performed in the presence and absence of CRFR2 antagonists.

## METHODS

### General.

In order to allow a direct comparison, much of the methodology of these studies is identical to our previous report which used bladder re-inflammation as an adult insult[29] rather than hind limb inflammation. Anesthetics, drug doses, timing and other variables were therefore chosen to be consistent with that other report. Studies were performed in 171 female Sprague-Dawley rats and were approved by the University of Alabama at Birmingham's Institutional Animal Care and Use Committee and followed the ethical guidelines of the International Association for the Study of Pain[53]. To obtain female rat pups, timed pregnant females were obtained from Envigo Laboratories (Formerly Harland, Indianapolis, IN) and date of birth verified by daily observation of cages. As pups, separate groups of the female pups underwent treatments for three consecutive days on P14–16. Following each treatment, pups were returned to home cages. Subsequently, rats were raised using standard husbandry methods with weaning from the dams at three to four weeks of age. All rats were raised

to 12–15 weeks of age and then underwent additional testing. For statistical analyses, the SYSTAT 12 (SPSS, Inc. San Jose, CA, United States) software package was utilized.

### Neonatal Bladder Inflammation (NBI).

All rats underwent one of two pretreatments on days P14,15,16. For NBI pretreatment, rat pups were anesthetized with 2–5% isoflurane in oxygen, injected with ampicillin (50–100 mg/kg IP), their urethral orifice swabbed with an iodine-povidone solution and a 24 gauge angiocatheter passed transurethrally into their bladder. A solution of zymosan A (1% in normal saline; 0.1 ml; Sigma Aldrich, St. Louis, MO) was injected into the bladder and allowed to dwell for 30 minutes with the catheter left in place and the rat anesthetized. After 30 minutes the catheter was removed and the zymosan solution allowed to drain spontaneously upon emergence from anesthesia. Pups were kept warm on a heating blanket, allowed to recover and returned to their mothers. Control pretreatments for NBI consisted of a similar anesthetic for 30 minutes, iodine-povidone swabbing, ampicillin treatments and identical recovery protocols. Zymosan was used as an intravesical inflammatory agent due to our extensive characterization of responses to this substance and our previous demonstration[28] that NBI induced by other substances such as bacterial lipopolysaccharide do not produce the same long-lasting effects on reflex responses to UBD.

### Hind Limb Inflammation Using Complete Freund's Adjuvant (CFA).

Adult (12–15 weeks of age) female rats were anesthetized with 2–5% isoflurane in oxygen, injected with ampicillin (50–100 mg/kg IP) and the skin of the left lateral foot was treated with an iodine-povidone solution. Following published protocols[10] CFA pretreatment consisted of 100 microliters of CFA (Sigma Aldrich, St. Louis, MO) injected into the lateral foot's dorsal surface using a 30 gauge needle. Control treatments for CFA consisted of similar anesthetic, iodine swabbing and antibiotic treatment and in most cases 100 microliters of normal saline was injected. After pretreatment, rats were kept warm on a heating blanket, allowed to recover and returned to their home cages. CFA/Control treatments occurred only once and were performed approximately 72 hours prior to additional testing described below. Gross examination of the CFA-treated hind limbs demonstrated them all to be swollen and red and sensitive to light palpation, but no quantitative testing of somatic sensation was performed. To improve efficiency of animal utilization, data from some adult pretreatment control rats which were examined as part of other published studies[29,33] was combined with the present *de novo* data after statistical testing assessed no differences in responsiveness. In these rats, control pretreatments were performed 24 hours (rather than 72 hours) prior to study but also consisted of anesthesia, handling, antibiotic treatment and iodine/povidone swabbing.

### Enzyme-Linked ImmunoSorbent Assay (ELISA).

20 rats (n=5/group) were treated with NBI/Control pretreatment and CFA/Control pretreatment according to the protocols described above. As adults, 72 hours after their CFA/Control pretreatments, they were deeply anesthetized with 5% isoflurane and then euthanized via decapitation. Spinal cords were hydraulically extracted and lumbosacral (L6-S2) segments were isolated and processed according to ELISA kit instructions. Protein concentrations were determined using the Pierce BCA Assay Reagent Kit (Thermo Fisher

Scientific, Rockford, IL). CRFR2 content was quantified using an LSBio kit [Lifespan Biosciences, Seattle, WA]. Samples and serial dilutions of standards were processed according to kit protocols. A two-way ANOVA was used to identify treatment effects with Tukey's HSD for post hoc pairwise comparisons.

### Visceromotor Response (VMR) Measures.

Adult rats were initially anesthetized with 2–5% isoflurane and IP urethane (1.25 gm/kg), and a 22 gauge angiocatheter was placed transurethrally into the bladder and held in place by a tight suture around the distal urethral orifice. A 7.8 cm catheter made of PE10 tubing was inserted via an incision in the atlanto-occipital membrane following surgical exposure and threaded down through the subarachnoid space to the lumbosacral region. Silver wire electrodes were placed in the external oblique musculature immediately superior to the inguinal ligament. Isoflurane was then lowered until flexion reflexes were present (<1%). UBDs (20 s, 10–60 mm Hg) were produced using compressed air, and intravesical pressure was monitored using an in-line pressure transducer. Contraction of the abdominal musculature in response to UBD has been well characterized[8,32] and was quantified as electromyographic (EMG) activity measured via the external oblique electrodes using standard differential amplification (Grass, Inc. P511 AC amplifiers; 50 x amplification, 60 Hz clipping, low filter setting 10 Hz – high filter setting 3 KHz). The analog EMG signal was digitized with a sampling rate of 10KHz and saved on computer with digital rectification via software (Spike 4 software; Cambridge Electronic Design Limited, Cambridge, UK) allowing for calculation of mean EMG activity (in mV) during any defined time period. Notably, for each data set the same amplifier and filter settings were used for all rats. Prior to the presentation of any UBDs, the intrathecal catheter was dosed with one of the following in a 10 μl volume followed by a 10 μl normal saline flush: [1] normal saline; or [2] aSVG30 (12 μg, a selective CRFR2 antagonist; Phoenix Pharmaceuticals, Inc., Burlingame CA; dissolved in normal saline). After a 15 minute waiting period, three repeated 20 second duration UBDs at 60 mm Hg intensity were presented at 3 minute intervals and then followed by the measurement of responses to graded constant pressure stimuli (10–60 mmHg, 20 seconds, 1-minute intertrial intervals).

### Quantitative Analysis of VMRs.

Data are reported as means ± SEM unless otherwise stated. The vigor of VMRs are expressed as "Visceromotor Response" defined as increases in mean EMG activity during the 20 s period of UBD over the immediate prestimulus level of activity. Logistically, during each experimental run, the differential amplifier output is digitized and rectified by computer software (Spike 2, Cambridge Electronic Design, Inc., Cambridge UK) to positive values, which are then averaged over a period of time to give a mean voltage measure of amplified EMG activity. Measures of the Visceromotor Response were then treated as discrete data points and analyzed using a repeated measures ANOVA for each pretreatment group. We have previously demonstrated that discrete Visceromotor Response measures are normally distributed[28].

### Dorsal Horn Spinal Neuronal Responses to UBD.

These studies were performed using the same methods as we have previously reported[27,33]. Briefly, animals were anesthetized with isoflurane (5%) and a tracheal cannula placed allowing for mechanical ventilation. The cervical spinal cord was exposed surgically, injected with 50 μl of 1% lidocaine solution and subsequently transected using a sharp scalpel. The brain was then pithed mechanically, anesthesia discontinued and the rats allowed to recover until demonstrating robust hind limb flexion reflexes in response to paw pinching (typically 1–2 hours). Normal saline boluses were administered IP as needed. Paralysis was then initiated with rocuronium (10 mg boluses IP as needed). A laminectomy was performed and the dura incised exposing the L6-S2 spinal segments. The vertebral column was clamped both rostrally and caudally to the laminectomy for stabilization. Skin flaps were arranged to form a protective coating for exposed tissue except for the site of recording which was covered with warmed mineral oil. Tungsten microelectrodes (MicroProbe, Clarksburg, MD; 1.2–1.8 MOhm) were used for conventional extracellular single-unit recording. Signals were amplified using a preamplifier/amplifier system coupled with four factor discrimination of waveform shape (trigger voltage, time delay, maximal/minimal voltage [Bak Electronics, Inc., Umatilla, FL: Model A-1 preamplifier, Model MDA-2 differential amplifier, Model DDIS-1 dual waveform discriminator] from background noise while maintaining continuous oscillographic monitoring. Discrimination generated uniform digital pulses saved using Spike2 software [Cambridge Experimental Design, Cambridge UK]. The dorsal horn 0–0.5 mm from midline and 0–1.0 mm below cord dorsum was searched using microelectrodes positioned using a stereotaxic apparatus. Repeated UBD was used as the search stimulus and all units which responded, in a consistent excitatory fashion, to UBD were characterized. The UBD itself was delivered in a fashion identical to that used in the VMR experiments. The total number of unit action potentials (discriminated pulses) were counted in 10 s epochs before, during and after the UBD stimulus. Evoked Activity of the dorsal horn neurons was defined as the number of unit discharges during UBD minus the level of activity immediately preceding the onset of UBD. Measures of Evoked Activity were then treated as discrete data points and comparisons of groups performed using a repeated measures ANOVA. Noxious (cutaneous pinch) and non-noxious (cutaneous brush) somatic stimuli were also presented to each neuron and excitatory/inhibitory responses determined in a fashion similar to that which we have previously published[27] with stratification of neurons according to their response to a heterosegmental noxious stimulus: if inhibited by cutaneous pinch outside of their excitatory receptive field (upper body) they were defined as Type I; otherwise they were defined as Type II. Definitions of cutaneous receptive field sizes were adapted from a previous study[30]. Sizes were defined as small (long axis <2 cm), medium (long axis > 2 cm, including part of unilateral hind limb and/or tail) or large (involving > 10 dermatomes including contralateral input – coccygeal inputs treated as single dermatome). Prior to the presentation of any UBDs, 20 μg of the CRFR2 antagonist aSVG30, dissolved in 20 μl of normal saline, or an equal volume of normal saline (20 μl) was applied to the dorsal surface of the spinal cord, then dorsal horn neuronal responses to repeated and graded UBD (20, 40 or 60 mm Hg, 20 s; 32min intervals) were determined for two hours. Longer sampling periods were not employed since topically applied drug effects would be expected to fade with time.

A repeated measures ANOVA was employed comparing responses in each pretreatment subgroup.

## RESULTS

### General.

All rats received both neonatal and adult pretreatments. The labelling convention for pretreatment groups which will be followed will be to list according to all experimental pretreatments given. NBI indicates rats received Neonatal Bladder Inflammation as described above. CFA indicates adult pretreatment with Complete Freund's Adjuvant as an adult as described above. Control treatments were also given at neonatal and adult timepoints respectively. Data is presented as mean±SEM unless otherwise stated.

### Enzyme linked ImmunoSorbant Assays (ELISAs).

Spinal cord content of CRFR2 was assayed in the L6-S2 spinal cords of rats which had experienced NBI and/or CFA and their controls. This data is displayed graphically in Figure 1. The protein content of CRFR2 was increased in rats which experienced NBI plus CFA pretreatments when compared to the groups not treated with NBI but not with the NBI Only group. A 2 way ANOVA analysis demonstrated a statistically significant effect due to NBI and due to CFA pretreatments (for NBI effect $F_{1,1,17} = 7.571$, p=0.014; for CFA effect $F_{1,1,17} = 6.341$, p=0.022; individual paired comparisons stated in Figure 1).

### Interaction of NBI and CFA on the vigor of VMRs to UBD.

The interaction of NBI and CFA pretreatments on VMRs to UBD was assessed using data from the IT saline-treated rats which are represented by the filled circles in Figure 2A–D and consolidated in Figure 2E. In rats which experienced NBI, there was a robust, statistically significant increase in VMRs when also pretreated with CFA (repeated measures ANOVA comparison of NBI+CFA with NBI Only rats: $F_{1,20}$=8.852, p=0.008). In contrast, in rats which did not experience NBI, there was a small, statistically insignificant decrease in the vigor of the VMRs associated with CFA pretreatment (repeated measures ANOVA comparison of CFA only with Control Only rats: $F_{1,13}$=1.395, p=0.259).

### Effect of intrathecal CRFR2 antagonist on the vigor of VMRs to UBD.

To further examine the role of CRFR2s in the increased vigor of rats which experienced NBI plus CFA (the NBI+CFA group), the CRFR2 antagonist aSVG30 was administered intrathecally prior to the measure of VMRs to UBD. As apparent in Figure 2A, the CRFR2 antagonist was effective at reducing the vigor of the VMRs to UBD when compared with VMRs in rats administered intrathecal normal saline (repeated measures ANOVA for aSVG30 treatment: overall $F_{1,14}$=17.711, p=0.001, interaction $F_{5,70}$=15.935, p<0.001; pairwise comparisons are given in Figure 2A). To test whether this CRFR2 antagonist effect was specific to the NBI+CFA pretreatment group, studies in the other pretreatment groups using intrathecal aSVG30 were performed. Similar to what has been previously observed in NBI-pretreated rats[29], a statistically significant *augmentation* (as opposed to the inhibition noted above) of VMRs to UBD occurred in rats which had experienced NBI but which did not experience CFA (the "NBI Only" group; Figure 2B). A repeated measures

ANOVA indicated a statistically significant difference between the aSVG30-treated rats and the normal saline-treated rats in this pretreatment group (overall $F_{1,19}$=7.896, p= 0.01, interaction $F_{5,95}$=5.907, p=0.001; pairwise comparisons are given in Figure 2B). Such an augmentation suggests a tonic analgesic effect of the CRFR2 activation was present in the NBI Only group. No significant effects were observed in rats which had not experienced NBI but did experience CFA (the "CFA Only" group, Figure 2C) or in the rats which experienced neither NBI nor CFA (the "Control Only" group; Figures 2D). Direct evidence of the spinal site of action of CRFR2 mechanisms was given by the following studies of spinal dorsal horn neurons.

### Interaction of NBI and CFA on the vigor of dorsal horn neuronal responses to UBD.

The interaction of NBI and CFA pretreatments on dorsal horn neuronal responses to UBD was assessed using data from the IT saline-treated rats which are represented by the filled circles in Figure 3 panels. Following previous convention[27] a distinction was made in neuronal samples based on individual neurons' responses to a heterotopic noxious stimulus. Type I neurons are those which are inhibited when distant noxious stimuli (a.k.a. forelimb pinch) are presented. Type II neurons are not similarly inhibited by distant pinch stimuli. In rats which experienced NBI, there was a robust, statistically significant increase in Type I neuronal responses when also pretreated with CFA (repeated measures ANOVA comparison of NBI+CFA with NBI Only rats: $F_{1,60}$=14.691, p<0.001), but not in rats which did not experience NBI in which there was a small, but statistically insignificant decrease in the vigor of the neuronal responses (repeated measures ANOVA comparison of CFA only with Control Only rats: $F_{1,52}$=1.673, p=0.202). Type II neurons had similar responses. In rats which experienced NBI, there was a robust, statistically significant increase in Type II neuronal responses when also pretreated with CFA (repeated measures ANOVA comparison of NBI+CFA with NBI Only rats: $F_{1,62}$=15.349, p<0.001), but not in rats which did not experience NBI in which there was no change in the vigor of the neuronal responses (repeated measures ANOVA comparison of CFA only with Control Only rats: $F_{1,52}$=0.142, p=0.930).

### Effect of CRFR2 antagonist on excitation of spinal dorsal horn neurons by UBD.

Characteristics of the neurons studied are summarized in Tables 1 and 2, the former stratifying neurons using the Type I/Type II nomenclature and the latter using the Class 2/Class 3 nomenclature. Evoked neuronal responses are displayed in Figure 3 along with statistical comparisons. Notably, the only difference in baseline activity measures that was apparent between neuronal subgroups when comparing saline-treated and aSVG30-treated rats was in the NBI Only group where spontaneous activity was significantly greater in the aSVG30-treated rats (far right columns of the tables in Type II and Class 2 neurons). Convergent cutaneous receptive field sizes were larger in the aSVG30-treated rats in the CFA Only group (Type I and Class 2 neurons) but otherwise drug treatment had little effect on receptive field sizes. Likewise, Class 3 neurons were more common in aSVG30-treated rats in the Type I neurons of the NBI+CFA and Control Only groups. As the focus of the present studies was bladder hypersensitivity, limited conclusions can be drawn related to convergent cutaneous receptive field changes since these changes were not studied as rigorously. In regards to UBD-Evoked Activity, aSVG30 applied topically to the dorsal

surface of the exposed spinal cord resulted in a statistically significant reduction of neuronal responses evoked by UBD in rats which had experienced NBI plus CFA (the NBI+CFA group) when compared with responses in similarly pretreated rats following topical normal saline administration. This effect was present in both Type I (neurons inhibited by heterosegmental noxious stimuli; Figure 3A) and Type II neurons (neurons not inhibited by heterosegmental noxious stimuli; Figure 3B). The opposite proved true in rats which had experienced NBI but did not experience CFA (the "NBI Only" group): both Type I and Type II neuronal subgroups (Figures 3C&3D) had greater (rather than lesser) evoked neuronal responses in the aSVG30-treated rats. In rat pretreatment groups which had not experienced NBI (the CFA Only and Control Only groups; Figures 3E–H), there were no statistical differences in vigor between both Type I and Type II neuronal responses from aSVG30-treated rats and from those which were normal saline-treated indicating that perturbations of the spinal CRFR2 systems appeared uniquely related to the experience of NBI.

## DISCUSSION

The most important finding of the present study was that CRFR2-related mechanisms were associated with bladder hypersensitivity in rats following somatic inflammation if those rats had experienced NBI. This observation in rats has clinical relevance, because the disease which is being modelled, IC/BPS, often has its onset as well as "flares" in bladder sensitivity which appear to be triggered by nearby somatic inflammatory events[6]. As a disease entity, IC/BPS is still a clinical problem with a prevalence as high as 2–6% of the female population in the United States[5] so any improvement in preventative or reactive therapies would find clinical use. To the best of our knowledge, this is the first report of CRFR2-related mechanisms associated with a somatic trigger of visceral hypersensitivity and so these findings suggest a completely new avenue for therapeutics. Evidence for CRFR2 involvement included increases in spinal receptor protein content which were associated with both NBI and CFA treatments and the demonstration that a pharmacological antagonist of CRFR2 blocked augmentation of both visceromotor reflex responses to urinary bladder distension as well as bladder-associated spinal dorsal horn neuronal responses in rats which were pretreated with NBI and CFA.

Like the rat model used in these studies, IC/BPS has a clear relation to developmental insults as human epidemiological data suggest childhood bladder infections are significantly increased in incidence in individuals who subsequently develop IC/BPS[38]. We have hypothesized that a developmental event (such as NBI) is a necessary but not sufficient event for expression of IC/BPS since the adult onset of the disorder is typically prefaced by a separate antecedent event[3,50] such as bladder re-infection or nearby surgery/injury producing inflammation. By design, the current experiments are examining such a somato-visceral interaction. Most reports of cross-sensitization of differing sensory structures describe inflammation of visceral structures leading to sensitization of somatic structures[e.g., 13,20] or other visceral structures[7]. In the absence of other pathology, somatic inflammation/nociception appears to have limited effects on visceral sensations or is robustly inhibitory[e.g.,31]. The present study observed minimal effects of the CFA pretreatment in rats which had not experienced NBI (the CFA Only versus Control Only

groups), but the rats which had experienced NBI demonstrated a susceptibility to pro-nociceptive effects of the second pretreatment/insult. This suggests that the experience of NBI "primes" the animal for experiencing bladder nociception and the pharmacology of the present study suggests that this priming mechanism involves CRFR2s. Potential mechanistic interactions of somatic inflammation with urinary bladder sensory systems are numerous. CFA injected into the hind limb has many known physiological and behavioral effects associated with an extensive body of scientific literature since it represents one of the "standard" animal models of inflammatory pain[e.g.,9,26]. Biochemical effects that occur at a spinal level following CFA treatment include, but are not limited to, glutamatergic[12,39,47–49], purinergic[52], opioidergic[14], nicotinic[4] and TRP channel-related[2,12,47,48,51].

There is clearly a convergence of somatic and visceral sensory input at the level of the spinal dorsal horn neurons that occurs in the present study since all neurons characterized here which were excited by UBD were also excited by somatic stimulation. This suggests that spinal dorsal horn neurons were receiving neuroactive inputs, both as neurotransmitters and as other cytokine signals from the inflamed somatic primary afferents. Apparently, the signals from these somatic afferents produce secondary effects that are similar to the signals received from inflamed bladder primary afferent neurons which then leads to an alteration in CRFR2-related protein content and functional changes. One would expect that an interruption of any important component of the mechanisms of hypersensitivity would have clinical relevance.

As noted above, a comparison of the present findings with our recently published study in which rats experienced NBI and then had a secondary insult as an adult consisting of bladder re-inflammation, shows a remarkable similarity in neurophysiological responses and CRFR2 measures[29]. In both sets of studies, NBI followed by a secondary inflammatory insult resulted in increases in CRFR2 content and augmented VMRs and spinal dorsal horn neuronal responses that were reduced by the spinal application of a CRFR2 antagonist. Interestingly, in both sets of studies, when rats experienced NBI but were not exposed to an adult inflammatory insult (the NBI Only groups) the application of a CRFR2 antagonist resulted in *enhanced* VMRs and neuronal responses suggesting that there could be a baseline analgesic effect of CRFR2 activation which then switches to a pro-nociceptive effect following an inflammatory insult. These results, in turn, suggest that NBI produces a change in CRFR2-related spinal modulatory mechanisms that primes the system for further change by CFA. The precise nature of this change is yet to be delineated, but it is notable that both the VMR and dorsal horn neuronal data in the present study suggest that the first change (which is produced by NBI) is associated with a tonic analgesic effect of CRFR2 activation in rats that is the reversed to a pro-nociceptive effect following CFA. This manipulation produces an apparent "switch" in responsiveness to spinal CRFR2 antagonism such that aSVG30 results in *decreased* VMRs and dorsal horn neuronal responses to UBD. These changes are in contrast to CRFR2-related measures in rats which had not experienced NBI (the CFA Only and Control Only groups). These groups did not appear to have any changes to their CRFR2 systems as reflex and neuronal responses were not significantly affected by the administration of the CRFR2 antagonist and there was only a small change in CRFR2 content due to CFA pretreatment. We have previously reported, in bladder-related studies not involving NBI, that a similar "switch" from anti-nociception to pro-nociceptive

mechanisms occurs in spinal serotonergic and noradrenergic mechanisms following bladder inflammation[40].

Future studies may be able to explain how the observed *switch* in CRFR2 actions occurs following NBI plus CFA or bladder re-inflammation. Fitzgerald and colleagues[37,43] have reported that nociceptive systems go through a developmental switch in the inhibitory versus excitatory actions of various neurotransmitters (e.g., GABA) during the same neonatal time period in which the rats of the present study were subjected to bladder inflammation. It would seem reasonable that a disruption of normal developmental processes by an insult such as bladder inflammation might also result in alterations in neurotransmitter actions during adulthood. We speculate that this switch in receptor function occurs at the level of the primary afferent and have ongoing studies using calcium influx in isolated dorsal root ganglion neurons which are examining this possibility. However, it is just as plausible that CRFR2-related mechanisms are working at the level of second neurons or through interneurons, although direct testing of mechanisms at those sites is experimentally more complex due to the difficulty of differentiating post-synaptic mechanisms from those in primary afferents. Mousa and colleagues[25] specifically identified increases in CRFR2 expression/content following CFA-induced inflammation of the hind limb (a finding consistent with the present study) and demonstrated co-localization/analgesic interactions of CRFR2s with enkephalinergic spinal interneurons. Hence, the observation that alterations in opioid agonist content and pharmacological action are present in NBI-treated rats[45,46] increases even more, the potential for the involvement of interneurons.

Limitations of the present study include the exclusive use of female rats. Although done for practical reasons (females are more easily cannulated both as neonates and adults), the study of this group is justifiable as 60–90% of patients with interstitial cystitis/bladder pain syndrome are females[6,11,15,19]. Results must also be limited in interpretation due to the focus on CRFR2s. Currently available agents for use as CRFR2 antagonists are peptides[42] with all of the limitations that come with peptides (lack of oral bioavailability, limited CNS penetration) necessitating administration of study agents parenterally or via implanted intrathecal catheters if CNS effects are being studied. Development of compounds for clinical use with increased delivery options and better CNS penetration would facilitate the testing of the present observations in humans. There is clearly a need for CRFR2-related research in relation to pain therapy.

In conclusion, the augmented VMRs and neuronal responses that occur in rats which have experienced NBI plus CFA require activation of a spinal CRFR2-related mechanism. This is a novel finding related to somatovisceral interaction and suggests the potential for use of CRFR2 antagonists as therapies for visceral hypersensitivity disorders such as IC/BPS, particularly when triggered by antecedent events associated with somatic structures.

## DATA AVAILABILITY

The data related to these studies is available on request from the Corresponding Author.

## ABBREVIATIONS:

| | |
|---|---|
| **CFA** | Complete Freund's Adjuvant induced hind limb inflammation |
| **CRF** | corticotropin releasing factor |
| **CRFR2** | CRF receptor Type 2 |
| **ELISA** | enzyme linked immunosorbent assay |
| **EMG** | electromyogram |
| **IC/BPS** | interstitial cystitis/bladder pain syndrome |
| **NBI** | neonatal bladder inflammation |
| **UBD** | urinary bladder distension |
| **VMR** | visceromotor response |

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## HIGHLIGHTS

- Neonatal bladder inflammation (NBI) & adult Complete Freund's Adjuvant (CFA in hindlimb) interact
- NBI+CFA increases reflex and dorsal horn neuronal responses to urinary bladder distension
- The combination of NBI+CFA pretreatments is associated with increased spinal content of CRFR2
- Responses augmented by NBI+CFA are attenuated by the spinal application of a CRFR2 antagonist
- Following NBI, hind limb inflammation produced effects similar to re-inflammation of the bladder

**PERSPECTIVE:**

Bladder hypersensitivity occurs following neonatal cystitis and an adult insult such as somatic inflammation. This paper demonstrates that CRFR2-related mechanisms are associated with this hypersensitivity. This supports the therapeutic value of these agents in the treatment of painful bladder disorders, particularly when triggered by somatic events.

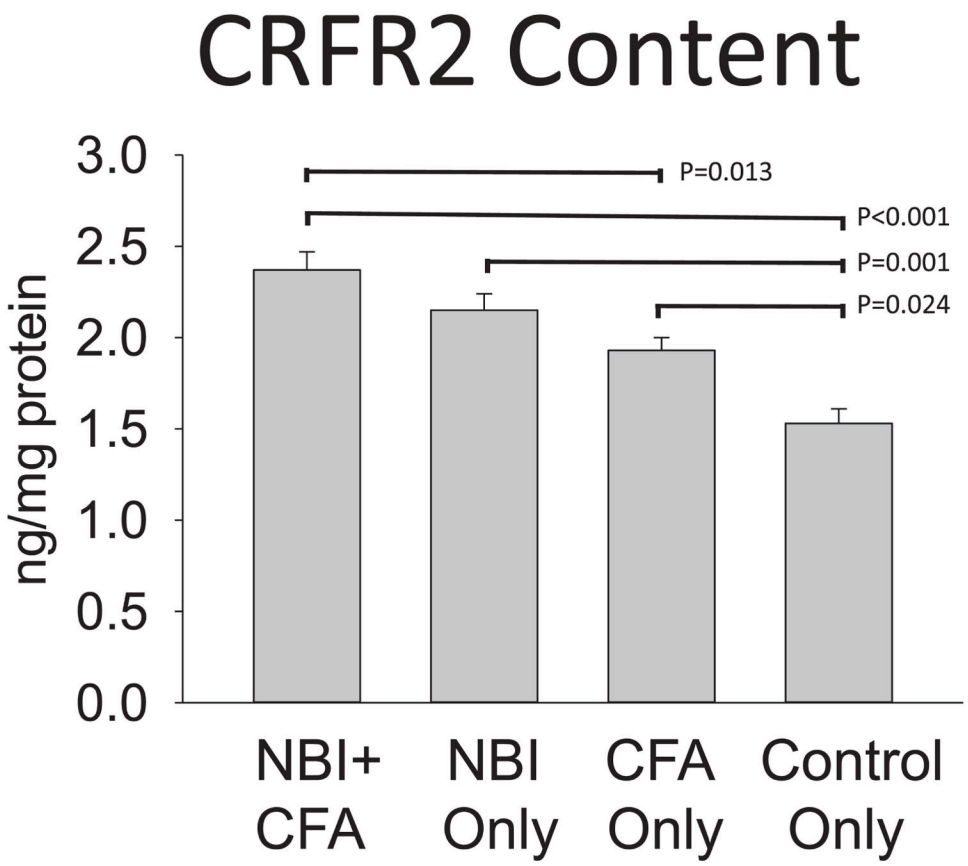


**Figure 1.**

Effect of NBI and/or CFA on CRFR2 content in lumbosacral spinal cord. CRFR2 indicates corticotropin-releasing factor receptors type 2. Data represent mean ± SEM with n = 5 rats per group. NBI indicates rats experienced Neonatal Bladder Inflammation. CFA indicates rats experienced hind limb inflammation induced by Complete Freund's Adjuvant as an adult. See text for more description. A 2 way ANOVA analysis demonstrated a statistically significant effect due to NBI and due to CFA pretreatments (for NBI effect $F_{1, 1, 17} = 7.571$, $P = .014$; for CFA effect $F_{1, 1, 17} = 6.341$, $P = .022$; post hoc comparisons (Turkey's HSD) are indicated by bars with associated statistics.

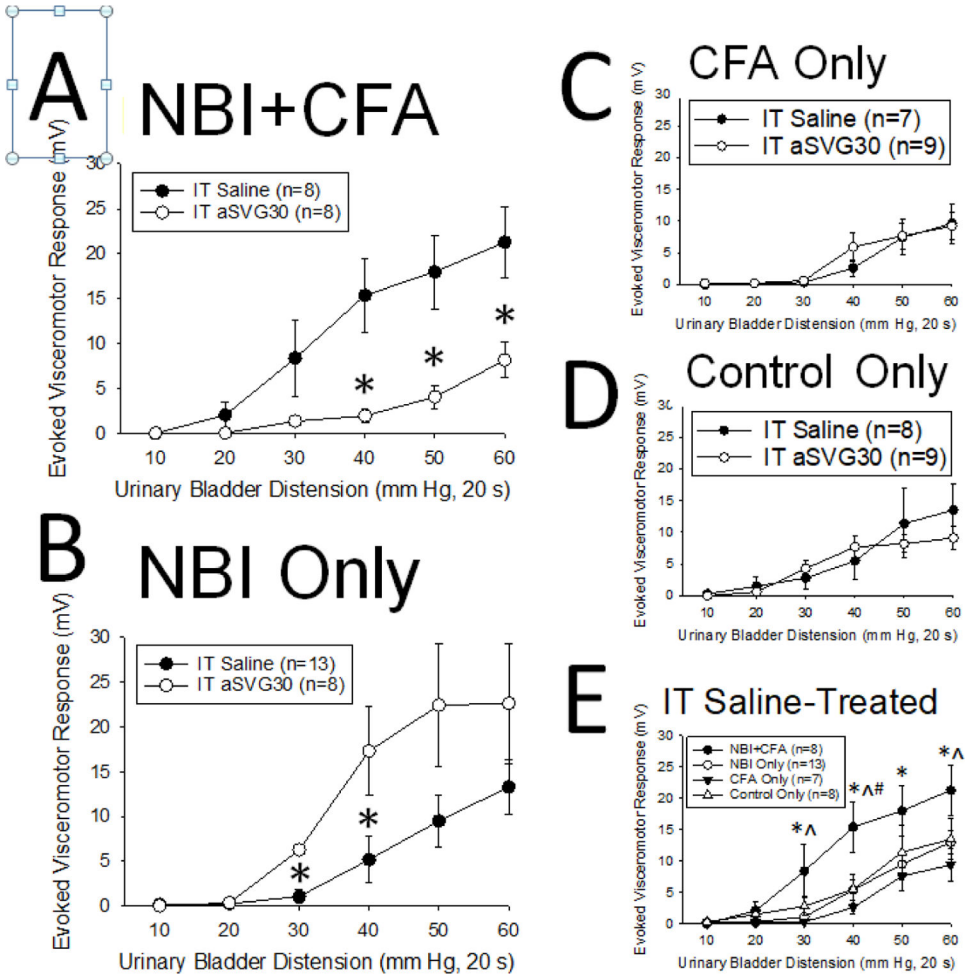


**Figure 2. Effect of intrathecal CRFR2 antagonist on visceromotor responses to urinary bladder distension (UBD).**

Data represent mean ± SEM. NBI indicates rats experienced Neonatal Bladder Inflammation. CFA indicates rats experienced CFA-induced hind limb Inflammation. See text for more description and ANOVA statistics. In Panel A, the CRFR2 antagonist aSVG30 inhibited VMRs to UBD in rats which had experienced NBI plus CFA. In Panel B, similar data presentation as Panel A in rats which experienced NBI, but not CFA. These rats had a statistically significant augmentation of VMRs to UBD with aSVG30 treatment. In Panels A&B post hoc pairwise comparisons (Tukey's HSD) for differences from IT saline group are indicated by asterisks with $p<0.05$. In Panels C&D, similar data presentation as in Panel A for rats which did not experience NBI. No statistically significant effects of aSVG30 treatment were noted in either of these two pretreatment groups. Panel E consolidates data related to IT saline-treated rats in Panels A-D in order to allow for a comparison of the effect of groups on the vigor of visceromotor responses. The "NBI+CFA" group had the most robust responses with post hoc pairwise comparisons (Tukey's HSD) for differences ($p<0.05$) of this group from the other groups indicated by * for comparison with the "NBI Only" group, by ^ for comparison with the "CFA Only" group and by # for the "Control Only" group..

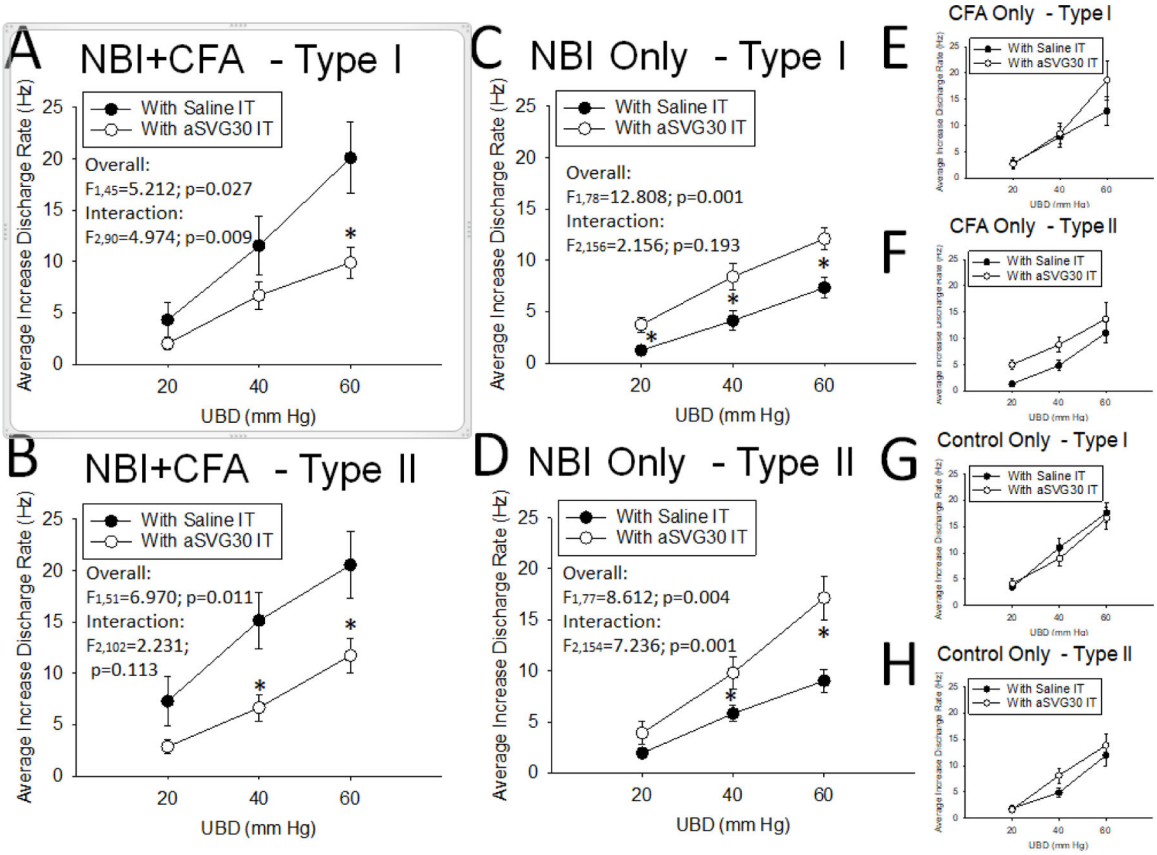


**Figure 3. Effect of aSVG30 on lumbosacral dorsal horn neuronal responses to UBD.**

Neurons excited by UBD were separated into two groups based on responses to heterosegmental noxious stimuli: Type I neurons were inhibited by noxious cutaneous pinch of the upper body; Type II neurons were not inhibited by noxious cutaneous pinch of the upper body. In data with open symbols, the CRFR2 antagonist aSVG30 was topically applied to the cord dorsum prior to initiation of the experiment; closed symbols are from rats that had normal saline similarly applied. Both Type I (panel A) and Type II neurons (panel B) in rats which experienced NBI+CFA were statistically reduced in rats which received aSVG30. Repeated measures ANOVA statistics are given as inset. Post hoc pairwise comparisons for differences from IT saline group are indicated by asterisks with $p<0.05$. In Panels C&D, similar data presentation as Panels A&B in rats which experienced NBI, but not CFA. Both Type I and Type II neurons of these rats had an augmentation of neuronal responses to UBD with aSVG30 treatment. In Panels E-H, similar data presentation as in Panels A&B for rats which did not experience NBI: secondary pretreatment with or without CFA made no difference as no statistically significant effects of aSVG30 treatment were noted in these four groups.

**Table 1.**

Characteristics of L6-S2 Dorsal Horn Neurons Responsive to UBD Stratified According to Type I/Type II Classification System

| PRETREATMENT | DRUG TREATMENT (# RATS) | TYPE | NEURONAL N | DEPTH (MM) | CLASS 2:3 | RF SIZE S:M:L | SPONTACT(HZ) |
|---|---|---|---|---|---|---|---|
| NBI+CFA | Normal Saline (8) | Type I | 22 | 0.49±0.06 | 20:2 | 7:12:3 | 14.1±2.3 |
| | | Type II | 26 | 0.54±0.07 | 17:9 | 4:4:18 | 15.6±3.6 |
| ASVG30 (8) | | Type I | 25 | 0.58±0.05 | 13:12^ | 9:14:2 | 14.7±2.5 |
| | | Type II | 27 | 0.55±0.06 | 14:13 | 3:3:21 | 11.5±2.0 |
| NBI Only | Normal Saline (12) | Type I | 40 | 0.51±0.05 | 29:11 | 12:18:10 | 7.1±1.3 |
| | | Type II | 38 | 0.51±0.05 | 16:22 | 2:7:31 | 8.0±1.5 |
| ASVG30 (11) | | Type I | 40 | 0.48±0.05 | 33:7 | 15:17:8 | 8.8±1.2 |
| | | Type II | 41 | 0.50±0.04 | 21:20 | 2:4:35 | 17.4±2.1* |
| CFA Only | Normal Saline (8) | Type I | 18 | 0.54±0.08 | 11:7 | 13:5:0 | 7.2±2.0 |
| | | Type II | 22 | 0.67±0.06 | 8:14 | 4:3:15 | 12.1±2.1 |
| ASVG30 (8) | | Type I | 18 | 0.65±0.06 | 10:8 | 3:12:3+ | 13.5±2.4 |
| | | Type II | 34 | 0.56±0.05 | 21:13 | 6:8:20 | 13.9±2.1 |
| Control Only Normal Saline (14) | | | | | | | |
| | | Type I | 37 | 0.47±0.05 | 37:0 | 10:20:7 | 10.4±1.4 |
| | | Type II | 32 | 0.55±0.05 | 18:14 | 3:10:29 | 8.1±1.6 |
| ASVG30 (12) | | Type I | 30 | 0.67±0.05 | 21:9^ | 8:16:6 | 14.0±2.5 |
| | | Type II | 35 | 0.61±0.05 | 10:25 | 3:9:23 | 9.8±2.0 |

Data represents Mean±SEM. UBD indicates urinary bladder distension. NBI indicates pretreatment with Neonatal Bladder Inflammation. CFA indicates pretreatment with Complete Freund's Adjuvant injected into the hind limb. Drug treatments consisted of either Normal Saline or aSVG30 (20 $\mu$mg) applied topically to the cord dorsum. Type I neurons were inhibited by heterosegmental noxious cutaneous stimuli. Type II neurons were not inhibited by heterosegmental noxious cutaneous stimuli. Depth indicate distance below cord dorsum. Class 2 indicates the neuron is excited by both noxious and non-noxious segmental cutaneous stimuli. Class 3 indicates neuron is excited by noxious segmental cutaneous stimuli but not non-noxious stimuli. RF Size indicates excitatory receptive field size stratified according to small, medium or large (s;m;l); see text for definitions. SpontAct indicates spontaneous activity measured during the 10 s interval prior to UBD.

* indicates statistical difference ($P = .0006$ unpaired t-test) from normal saline treated Type II neurons of the NBI Only pretreatment group.

+ indicates statistical difference ($P = .006$; Chi Squared test) from normal saline treated Type I neurons of the CFA Only pretreatment group.

^ indicates statistical difference ($P < .004$; Chi Squared test) from normal saline treated rats in the same neuronal type/pretreatment groups in distribution of Class 2 versus Class 3 neurons. Only statistical differences which were present after correcting for 8 repeated measures are shown.

**Table 2.**

Characteristics of L6-S2 Dorsal Horn Neurons Responsive to UBD Stratified Using Receptive Field Class Nomenclature

| PRETREATMENT | DRUG TREATMENT (# RATS) | TYPE | NEURONAL N | DEPTH (MM) | TYPE I:II | RF SIZE S;M;L | SPONTACT(HZ) |
|---|---|---|---|---|---|---|---|
| NBI+CFA | Normal Saline (8) | Class 2 | 37 | 0.50+0.05 | 10:17 | 6;16;15 | 15.1+2.4 |
| | | Class 3 | 11 | 0.56+0.10 | 2:9 | 5;0;6 | 14.2+5.4 |
| | ASVG30 (8) | Class 2 | 27 | 0.56+0.05 | 13:14 | 6;10;11 | 15.8+2.4 |
| | | Class 3 | 25 | 0.57+0.6 | 12:13 | 6;7;12 | 10.0+2.0 |
| NBI Only | Normal Saline (12) | Class 2 | 45 | 0.49+0.05 | 19:16 | 7;17;21 | 5.6+1.0 |
| | | Class 3 | 33 | 0.53+0.05 | 6:27 | 7;8;18 | 10.3+1.9 |
| | ASVG30 (11) | Class 2 | 54 | 0.46+0.04 | 33:21 | 16;14;24 | 11.9+1.7 * |
| | | Class 3 | 27 | 0.54+0.04 | 7:20 | 1;7;19 | 15.5+1.9 |
| CFA Only | Normal Saline (8) | Class 2 | 19 | 0.54+0.08 | 11:8 | 9;4;6 | 8.9+2.0 |
| | | Class 3 | 21 | 0.68+0.06 | 7:14 | 8;4;9 | 10.8+2.2 |
| | ASVG30 (8) | Class 2 | 31 | 0.57+0.05 | 10:21 | 1;13;17 + | 17.1+2.2 |
| | | Class 3 | 21 | 0.61+0.06 | 8:13 | 8;7;6 | 8.8+1.9 |
| Control Only | Normal Saline (14) | Class 2 | 53 | 0.48+0.04 | 36:17 | 11;26;16 | 9.0+1.2 |
| | | Class 3 | 14 | 0.59+0.08 | 0:15 | 2;4;8 | 10.5+2.2 |
| | ASVG30 (12) | Class 2 | 32 | 0.66+0.05 | 22:10 | 6;15;11 | 12.6+2.4 |
| | | Class 3 | 34 | 0.62+0.05 | 9:25 | 6;10;18 | 10.6+2.2 |

Data represents Mean±SEM for the same neurons described in Table 1 but stratified according to their convergent cutaneous receptive field class. UBD indicates urinary bladder distension. NBI indicates pretreatment with Neonatal Bladder Inflammation. CFA indicates pretreatment with Complete Freund's Adjuvant injected into the hind limb. Drug treatments consisted of either Normal Saline or aSVG30 (20 $\mu$mg) applied topically to the cord dorsum. Class 2 indicates a neuron is excited by both noxious and non-noxious segmental cutaneous stimuli. Class 3 indicates a neuron is excited by noxious segmental cutaneous stimuli but not non-noxious stimuli. Type I neurons were inhibited by heterosegmental noxious cutaneous stimuli. Type II neurons were not inhibited by heterosegmental noxious cutaneous stimuli. Depth indicate distance below cord dorsum. RF Size indicates excitatory receptive field size stratified according to small, medium or large (s;m;l); see text for more complete description. SpontAct indicates spontaneous activity measured during the 10 s interval prior to UBD.

+ indicates statistical difference ($P = .0008$ Chi Squared test) from normal saline treated neurons of the same Class/pretreatment group;

* indicates statistical difference ($P = .0031$; unpaired t-test) from normal saline treated neurons of the same Type/pretreatment group. Only statistical differences which were present after correcting for 8 repeated measures are shown.