Convergence of sensory pathways in the development of somatic and visceral hypersensitivity

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VISCERAL PAIN has several unique characteristics, including referral to distant somatic sites, which is due to viscerosomatic convergence of afferent pathways at the level of the spinal cord and higher centers within the central nervous system. Interestingly, visceral pain syndromes affecting different organs often coexist. Patients with functional bowel disorders often also complain about pelvic pain or symptoms consistent with interstitial cystitis (61). Conversely, many patients with interstitial cystitis also suffer from functional bowel disorders (2, 14). Patients with visceral pain syndromes can thus have a coexistence of pain syndromes in patients with functional diseases.

Visceral sensation; nerve growth factor; viscerosomatic convergence

METHODS

Animals. All experiments were performed using adult male B6129 mice (Taconic, Germantown, NY) weighing 20–30 g. Animals were housed singly on wood shavings with free access to water and food. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Iowa and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Surgical preparations. Mice were pretreated with 40 μg/kg ip atropine (Fujiwasa, Deerfield, IL) 10–60 min before being anesthetized with ketamine (87.5–175 mg/kg) and xylazine (12.5–25 mg/kg ip) (Phoenix Pharmaceutical, St. Joseph, MO). To obtain electromyographic (EMG) recordings from the abdominal musculature, Teflon-coated stainless steel wires (Cooner Wire Sales, Chatsworth, CA) were sewn into the external oblique abdominal musculature proximal to the inguinal ligament. The wires were tunneled subcutaneously to exit at the back of the neck as described previously (19). For some gene transfer experiments, the bladder was exposed using a midline incision. After viral vectors were injected into the bladder wall, the abdomen was closed in layers, and animals received 0.3 ml of 5% dextrose to replace fluid loss. All animals were monitored for distress and allowed to recover for 3 days.

Viral gene transfer. Replication-deficient adenoviral vectors (10⁹ plaque-forming units/ml) with the Rous sarcoma virus promoter were used for gene transfer. The NGF-encoding vector (Ad5RSVngf) was a generous gift of Dr. G. M. Smith (University of Kentucky) and Regeneron Pharmaceuticals (Tarrytown, NY). An adenoviral vector carrying the gene for green fluorescent protein (GFP; Ad5RSVgfp, University of Iowa Gene Transfer Vector Core Laboratory) served as the control. Viral vectors were suspended in 5% sucrose solution and diluted 2:1 in PBS containing 1% Evans blue to allow monitoring of the injection for leakage. Mice were anesthetized as described above, and resuspended virus was injected into four separate sites (2 μl/site) within the bladder wall or the glabrous skin of the right hindpaw or front paw using a 30-gauge needle as described previously (23).
Physiological testing and tissue harvesting were performed on day 5 after gene transfer.

**Quantitative determination of NGF.** Animals were euthanized with a pentobarbital sodium overdose, and the urinary bladder or glabrous skin of the right hindpaw was quickly removed, rinsed in ice-cold normal saline, frozen in liquid nitrogen, and stored at −80°C for further analysis. Tissue samples were homogenized in ice-cold lysis buffer containing 137 mM NaCl, 20 mM Tris·HCl (buffered to pH 8), 1% Nonidet P-40, 10% glycerol, 1 mM phenothemansulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate. The tissue protein concentration was determined with the Biuret method. The NGF concentration was measured using a commercially available assay following the manufacturer’s instructions (NGF E\text{max} Immunoassay, Promega, Madison, WI).

**Induction of localized inflammation.** To induce cystitis, we injected cyclophosphamide (CYP; 50 mg/kg ip every 3 days for a total of 2 doses) (8). Inflammation of the right hindpaw or front paw was caused by injecting 25 μl of complete Freund’s adjuvant (CFA) underneath the plantar skin of the paw (3). Physiological testing and tissue harvesting were performed on day 5 after the induction of inflammation or appropriate control treatment.

**Mechanical sensory threshold.** Mice were placed individually on an elevated plastic mesh floor covered with a clear plastic cage top (21 × 27 × 15 cm) and allowed to acclimate for 1 h. Baseline withdrawal thresholds to punctate mechanical stimuli were determined by applying calibrated von Frey filaments from underneath the mesh (openings 12 × 12 mm²) to the glabrous skin of the paw until a withdrawal response was obtained (64). Both hindpaws and front paws were examined in triplicate separated by 5 min per test to obtain the averaged withdrawal threshold.

**Thermal sensory threshold.** Mice were placed individually on the elevated mesh as described above. The heat stimulus was generated by a 50-W projector lamp, with an aperture diameter of 6 mm, and applied to the glabrous skin of the paw. Paw withdrawal latencies were measured in triplicate to the nearest 0.1 s separated by 5 min. To prevent thermal injury, the stimulus was automatically turned off at 30 s.

**Visceromotor response to colorectal distension.** To determine changes in colonic sensation, we measured the visceromotor response to distension (19). Mice were briefly sedated with halothane to introduce a small balloon catheter (3-cm polyethylene plastic balloon attached to PTFE-24 tubing, Cole-Parmer Instruments, Vernon Hills, IL) into the rectum. The distal tubing was taped to the tail to secure proper placement. Mice were then placed into small restraining devices and allowed 30 min for recovery and acclimation before being tested. With the use of a custom-made pressure distension device, the colorectal balloon was distended for 20 s to 15, 30, 45, or 60 mmHg separated by 4-min intervals. To determine the visceromotor response to colorectal distension, EMG activity was amplified, rectified, and recorded 10 s before and during the stimulus. The EMG signal was integrated and normalized as the change over baseline activity (Spike2 software, Cambridge Electronic Design, Cambridge, UK).

**Cystometrogram.** Mice were anesthetized with halothane (1–1.5% in 100% O2 at 2 l/min, Halocarbon Laboratories, River Edge, NJ), and the urinary bladder was exposed through a suprapubic incision. A 30-gauge needle was inserted into the dome of the bladder and affixed with cyanoacrylate. After 15 min, the bladder was drained. Two minutes later, prewarmed (37°C) saline was infused into the bladder at a rate of 20 μl/min for 20 min. Micturitions were observed, and the bladder pressure was continuously monitored using a pressure transducer. The time interval between the onset of bladder infusion and the first micturition (micturition onset) and the number and peak amplitude of micturition contractions were analyzed by a person blind to the treatment.

**Data analysis.** All data are given as means ± SE. Results were analyzed using ANOVA or two-way ANOVA for repeated measures where appropriate, followed by Tukey’s, Holm-Sidak’s, or Dunn’s test for multiple comparisons if warranted. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

A series of experiments was conducted to successively examine the effects of bladder inflammation on colon and somatic sensitivity [or the effects of somatic (hindpaw) inflammation on visceral sensitivity] to determine the extent of viscerovisceral, viscerosomatic, and somatovisceral convergence, after which NGF was evaluated as a potential mediator contributing to the development of cross-sensitization produced by tissue inflammation.

**Effects of cystitis on micturition, somatic sensory thresholds, and colorectal sensation.** We first established that the CYP treatment protocol employed produced bladder inflammation and altered bladder function. In control (saline treated) mice, a saline infusion into the bladder led to filling with a slow pressure increase, eventually triggering a bladder contraction followed by visible micturition. CYP treatment shortened the latency to the first micturition (Fig. 1A), lowered the peak micturition pressure (Fig. 1B), and increased the micturition frequency (Fig. 1C) compared with controls. We next examined the effects of CYP-produced cystitis on sensitivity in an adjacent viscus, the colon. Colon sensitivity was assessed quantitatively by measuring the visceromotor response to colorectal distension (15–60 mmHg), which in CYP-treated mice was significantly increased relative to saline-treated controls (Fig. 2). That is, bladder inflammation produced significant colon hypersensitivity of a magnitude not dissimilar to that produced by intracolic treatments that produce colon hyper-sensitivity (19). Bladder inflammation also affected somatic sensitivity at nearby (hindpaw) but not distant (forepaw) sites. CYP treatment significantly increased the sensitivity of responses (i.e., decreased response threshold and response latency) to mechanical and thermal stimulation of both hindpaws compared with controls (Figs. 3 and 4). The effects of CYP treatment did not extend to an anatomically distant site in the upper body. We examined the responses to mechanical and thermal stimulation of the right front paw after the induction of cystitis. Neither the withdrawal threshold to mechanical stimulation [control: 6.24 ± 0.09 mN and cystitis: 6.32 ± 0.07 mN, not significant (NS)] nor the withdrawal latency to thermal stimulation differed from controls (control: 9.8 ± 0.7 s and cystitis: 9.7 ± 0.8 s, NS), suggesting that anatomic proximity in the lumboasacral spinal cord of terminations of afferent innervations of the tissues studied is important in the development of cross-sensitization.

**Effects of somatic inflammation on micturition and colorectal sensation.** To address this further, we examined the effects of a well-established model of hindpaw inflammation and hyperalgesia, intraplantar injection of CFA, on colon and bladder sensitivity. An intraplantar injection of CFA, but not saline, produced robust hyperalgesia to both mechanical (Fig. 3) and thermal (Fig. 4) stimulation of both the ipsilateral (injected) hindpaw as well as the contralateral (uninjected) hindpaw. The magnitude of hyperalgesia in the CFA-injected right hindpaw, however, was significantly greater compared with hyperalgesia in the contralateral (left) hindpaw [mechanical stimulation: t-test ($t$) = 11.9, $P < 0.01$; thermal stimulation: $t = 10.1, P < 0.01$]. We also examined both mechanical and thermal sensitivity of the right front paw in CFA hindpaw-
treated mice. CFA-stimulated inflammation of the right hindpaw significantly reduced the mechanical withdrawal threshold of the front paw (6.4 ± 0.05 mN in control vs. 5.7 ± 0.14 mN with CFA, \( t = 4.46, P < 0.01 \)) without affecting the withdrawal latency of the right front paw to thermal stimulation (9.5 ± 0.09 s in control vs. 9.3 ± 0.23 s with CFA, NS).

With respect to the visceral sensitivity after an intraplantar injection of CFA and development of hindpaw inflammation, the visceromotor response to colon distension was significantly enhanced (Fig. 2), whereas reflex micturition was unaffected (Fig. 1, A–C), by hindpaw hyperalgesia. These experiments reveal robust viscerovisceral, viscerosomatic, and somatovisceral cross-sensitization. To examine these issues further, we then studied the contribution of NGF, a potential mediator of the effects of inflammation.

**Effects of inflammation and gene transfer on NGF tissue concentration.** We first determined whether the CYP and CFA treatment protocols employed affected NGF content in tissue. CYP-induced cystitis caused a significant increase in NGF content within the bladder wall (control: 14.4 ± 1.3 pg/mg protein and cystitis: 82.0 ± 5.7 pg/mg protein, \( n = 4 \) mice/group, \( t = 18.96, P < 0.01 \)). Similarly, NGF content increased in the right hindpaw after the CFA injection (control: 10.8 ± 2.0 pg/mg protein and CFA: 83.6 ± 2.6 pg/mg protein, \( n = 4 \) mice/group, \( t = 16.3, P < 0.01 \)).

To provide a basis for assessing the relationship between tissue inflammation, increased NGF content, and hypersensitivity (see below), we used replication-deficient adenoviral vectors to overexpress NGF (or GFP as a control) within the bladder or hindpaw. An injection of GFP-expressing virus did not alter NGF content in the bladder or hindpaw (bladder: 19.6 ± 1.3 pg/mg protein and paw: 11.8 ± 2.0 pg/mg protein, NS compared with saline controls). In contrast, the injection of the NGF-expressing virus significantly increased the NGF content (bladder wall: 95.2 ± 3.1 pg/mg protein, \( t = 17.0 \); hindpaw: 81.7 ± 4.3 pg/mg protein, \( t = 15.7; P < 0.01 \).
compared with GFP controls). The increase in tissue content produced by the viral vector matched the increases in tissue content produced by tissue inflammation.

Effects of NGF overexpression on micturition, colorectal sensation, and somatic sensory thresholds. Transient NGF overexpression within the bladder wall, like CYP-produced cystitis, led to earlier onset of the first micturition (Fig. 1D), lower peak micturition pressure (Fig. 1E), and produced more frequent micturitions (Fig. 1 F). Correspondingly, the injection of the Ad5RSVngf vector into the bladder wall also significantly enhanced visceromotor responses to colorectal distension (Fig. 5), replicating the viscerovisceral cross-sensitization evident after CYP-produced bladder inflammation. There was no difference in responses to colorectal distension between saline-treated mice and mice transected with the GFP-encoding adenoviral vector.

To examine viscerosomatic cross-sensitization, we overexpressed NGF in the bladder wall and documented significantly enhanced sensitivity (reduced thresholds for withdrawal) to mechanical and thermal stimulation of both hindpaws (Figs. 3 and 4). The sensitivity of the right front paw to mechanical (6.54 ± 0.05 mN with Ad5RSVgfp vs. 6.43 ± 0.06 mN with Ad5RSVngf, NS) or thermal (9.3 ± 1.9 s with Ad5RSVgfp vs. 9.7 ± 0.13 s with Ad5RSVngf, NS) stimulation was unaffected, however. As a control for the injection of the viral vector into the bladder wall, the injection of Ad5RSVgfp into the bladder wall did not significantly alter responses to mechanical or thermal stimulation of the hindpaws (Figs. 3 and 4).

Effects of somatic NGF gene transfer on micturition and colorectal sensation. To establish the validity of the model, we overexpressed NGF in the hindpaw. Compared with the control vector (GFP), NGF gene transfer into the right hindpaw led to the development of thermal hyperalgesia (Fig. 4) in both hindlimbs (as produced by intraplantar CFA), with a significantly longer latency in the right (injected) hindpaw compared with the untreated left hindpaw (t = 8.47, P < 0.01). Mechanical hyperalgesia, however, only developed in the NGF-injected hindpaw (Fig. 3). We also examined the responses to stimulation of the right front paw after a hindpaw injection of viral vectors. Compared with the control virus injection, the injection of Ad5RSVngf into the right hindpaw increased the sensitivity (lowered withdrawal threshold and latency) of the front paw to mechanical (6.3 ± 0.08 mN with Ad5RSVgfp vs. 5.8 ± 0.25 mN with Ad5RSVngf, t = 3.21, P < 0.01) and thermal (10.3 ± 0.3 s with Ad5RSVgfp vs. 9.5 ± 0.14 s with Ad5RSVngf, t = 3.45, P < 0.01) stimulation.

An injection of the NGF-encoding virus in the right hindpaw also decreased peak micturition pressures (P < 0.01; Fig. 1E), but neither the latency to the first micturition nor the frequency of micturition changed significantly compared with treatment with control virus Ad5RSVgfp (Fig. 1, D and F), which did not affect reflex micturition (Fig. 1, D–F).

To further examine somatovisceral sensitization, we examined the effects of NGF gene transfer into the right hindpaw on responses to colon distension. A hindpaw injection of the control virus did not alter visceromotor responses to colon distension, but NGF gene transfer into the right hindpaw significantly increased visceromotor responses to colorectal distension (F = 9.8, P < 0.01; Fig. 5). Given the effects of hindpaw inflammation (CFA) and NGF gene transfer into the hindpaw on responses to colorectal distension, we treated a distant site not sharing spinal convergence of afferent input
with pelvic organs (right front paw). Neither saline, CFA, nor Ad5RSVngf injection into the right front paw affected the responses to colon distension ($F < 1.5$ and $P > 0.05$ in all cases; Fig. 6).

**DISCUSSION**

Viscerosomatic and viscerovisceral convergence of sensory pathways play an important role in the manifestation of acute and chronic visceral pain syndromes, contributing to referral of pain to somatic sites and to the coexistence of pain states affecting more than one organ (15, 44). The present results confirm previously published findings showing that cystitis sensitizes responses to mechanical and thermal stimulation of hindpaws (17, 18). We now show that CYP-induced cystitis also shifts the stimulus-response function to colorectal distension, demonstrating the importance of viscerovisceral convergence of afferent pathways. Inflammation within the area of referred somatic hyperalgesia during cystitis similarly enhanced responses to colorectal distension. Finally, transient overexpression of NGF mimicked the effects of visceral or...
somatic inflammation on sensory function, further supporting a role of this growth factor in the pathogenesis of hyperalgesia.

Viscerosomatic convergence and hypersensitivity. Noxious stimulation of viscera triggers pain referred to somatic sites, which is generally attributed to viscerosomatic convergence at the level of the spinal cord (9, 13, 31, 50). The present results are consistent with prior studies demonstrating enhanced responses to stimulation of abdominal wall or hindpaw after acute irritation or inflammation of the colon, urinary bladder, ureter, or uterus (8, 15, 17, 18, 21, 60). Plasticity of viscerosomatic pain referral has also been observed in humans. For example, colon distension in healthy volunteers produced significant increases in both reported discomfort and in the size of the area of referred sensation over the course of 10 consecutive distensions (40). In another study (50), chemical stimulation of the esophagus with hydrochloric acid sensitized both the esophagus and chest wall to electrical stimulation without affecting thresholds at a site distant from the referral area. Similarly, acute appendicitis increased cutaneous sensitivity only in the referral area (52).

While supported by reports from other groups, the pattern of referred pain is surprising, as it affects areas outside the dermatomes that most clearly overlap with visceral afferents supplying pelvic organs. Afferents innervating the bladder or colon project to the thoracolumbar (T13–L2) and lumbosacral (L6–S1) segments of the rodent spinal cord (10, 26, 38, 59). Direct recordings from spinal neurons responsive to the bladder or colorectal distension revealed convergent cutaneous receptive fields in the area of the medial thigh, perineum, or genitalia with only a minority being excited by stimulation of the hindpaw (35, 37, 39). This is consistent with descriptions of dermatomes in rodents, showing perineal labeling or activation of lumbosacral neurons, while nerves innervating plantar skin of the hindpaw projected primarily to L4 and L5 spinal segments (48, 53). However, colonic application of irritants, such as zymosan, leads to a significant activation of spinal neurons (48, 53). However, colonic irritation of the colon or colorectal distension revealed convergent cutaneous receptive fields in the area of the medial thigh, perineum, or genitalia with only a minority being excited by stimulation of the hindpaw (35, 37, 39). This is consistent with descriptions of dermatomes in rodents, showing perineal labeling or activation of lumbosacral neurons, while nerves innervating plantar skin of the hindpaw projected primarily to L4 and L5 spinal segments (48, 53). However, colonic irritation of the colon or colorectal distension revealed convergent cutaneous receptive fields in the area of the medial thigh, perineum, or genitalia with only a minority being excited by stimulation of the hindpaw (35, 37, 39).

Viscerovisceral convergence and hypersensitivity. Cystitis and transient overexpression of NGF within the bladder wall altered the micturition reflex, which confirms prior results (8, 23, 33). We now show that bladder inflammation also sensitizes mice to colorectal distension. We relied on a commonly used animal model of cystitis, induced by the toxic cyclophosphamide metabolite acrolein, which is eliminated through the kidney (11, 20, 22, 58, 63). While it is possible that CYP directly affected the colon or that extension of the inflammatory process onto the neighboring organ contributed to this finding, the essentially identical results obtained with NGF gene transfer argue against this as the primary mechanism. Moreover, our results are consistent with a recent study (44) showing that acute bladder irritation sensitizes responses to colorectal distension in rats. Our experiments are the first to provide evidence that this sensitization is not simply an acute response to the local irritant. The afferent innervation of the bladder and colon overlap onto the same segments, if not also the same second-order neurons, within the spinal cord (4, 38, 45, 59). Recent reports have suggested that dichotomizing axons may innervate both visera in mice (10, 25), although direct physiological evidence in support of dichotomizing afferents is still missing (51). Taken together, the anatomic and present behavioral data provide a potential explanation for the clinical observation that patients with functional disease of the bladder or colon often complain about symptoms affecting more than one organ system (2, 27, 42, 61).

Somatovisceral convergence and hypersensitivity. In view of the hindpaw hypersensitivity after the induction of cystitis, we tested whether inflammation within this somatic referral site also triggered visceral hypersensitivity. While the micturition reflex, which is regulated in Barrington’s nucleus located in the pons, remained unchanged, we saw a significant change in the visceromotor response to colorectal distension, demonstrating visceral hypersensitivity. This apparent discrepancy may in part be due to differences in technique and end points. We monitored micturitions during constant infusions into the bladder, noting that peak pressures did not exceed 20 mmHg. However, pressures in the noxious range (>20 mmHg) are required to trigger clear visceromotor responses in response to colorectal distension (32, 36). An alternative approach with urethral catheterization and occlusion of the urinary outflow tract cannot be performed in male mice. Considering sex-related differences in nociceptive responses to visceral stimulation (19), we decided against obtaining cystometrograms in female mice after a CFA injection into the hindpaw.

The present results are consistent with recent reports (32, 43) showing visceral hypersensitivity after repeated injections of acid into the gastrocnemius muscle in rats, associated with a sensitization of spinal neurons that receive convergent input from the colon and affected muscle. Blockade of spinal ionotropic glutamate receptors abolished this cross-sensitization in a model of acute, noninflammatory muscle, suggesting that spinal mechanisms may contribute to the coexistence of different pain disorders in patients with fibromyalgia, interstitial cystitis, or functional gastrointestinal disorders (32). While sensitization of peripheral afferents underlies primary hyperalgesia, i.e., an increased excitability within the receptive field, central mechanisms with activation of descending facilitatory pathways and/or a decrease in descending inhibition likely contribute to enhanced sensitivity in adjacent regions (secondary hyperalgesia) or areas of pain referral (34, 50, 55–57). Changes in these central modulatory influences clearly play an important role in the coexistence of different chronic pain disorders (1, 2, 5). However, such mechanisms cannot explain our findings, because neither inflammation nor NGF overexpression at a distant site (front paw) triggered visceral hyper-sensitivity within the colon, arguing against a generalized effect on nociceptive processing as the main mechanism for the present results.

Role of NGF. Several studies (6, 24, 30, 54, 62) have demonstrated that NGF tissue content increases during inflammation and modulates the function of primary sensory neurons, contributing to the development of hyperalgesia. Consistent
with results obtained in rats, gene transfer of NGF into the bladder wall caused bladder hyperactivity in mice (23). Similarly, we noted hypersensitivity to thermal and mechanical stimulation of the affected extremity after an injection of the NGF-encoding virus into the hindpaw, further supporting the role of this growth factor in peripheral sensitization. The effects of NGF on visceral and somatic sensory function mimicked the results seen after cystitis or paw inflammation, providing evidence that it also contributes to the sensitization of convergent viscero-somatic and viscerovisceral afferent pathways. NGF alters the excitability and expression of transmitters/modulators in primary neurons (28, 46). In addition, longer lasting increases in NGF expression may lead to sprouting of nerve terminals within the spinal cord, thereby potentially contributing to the changes in receptive field size, a mechanism unlikely to play a significant role in our findings considering the relatively short time course (12, 47, 49).

In conclusion, the present results provide novel insights into peripheral mechanisms leading to the development of hypersensitivity affecting neighboring organs or referral sites. The plasticity of these convergent sensory pathways may contribute to the coexistence of pain syndromes. Conversely, it is conceivable that interventions affecting such converging pathways could be employed therapeutically to modulate sensation in less accessible areas, such as the viscera.

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