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Increased c-fos Expression in Spinal Neurons after Irritation of the Lower Urinary Tract in the Rat

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This study utilized neuronal c-fos expression to examine the spinal pathways involved in processing nociceptive and non-nociceptive afferent input from the lower urinary tract (LUT) of the urethane-anesthetized rat. C-fos protein was detected immunocytochemically in only a small number of cells (<2 cells/L_6 section) in control animals. However, chemical irritation with 1% acetic acid or mechanical stimulation of the LUT markedly increased the number of c-fos-positive neurons (56-180 cells/L_6 section) in four regions of the caudal lumbosacral (L_6-S_1) spinal cord: medial dorsal horn (MDH), lateral dorsal horn, dorsal commissure (DCM), and sacral parasympathetic nucleus (SPN). Only small numbers of c-fos-positive cells were detected in rostral lumbar segments, a region that is thought to receive nociceptive input from the LUT via afferent pathways in sympathetic nerves. The distribution of c-fos-positive cells in the L_6 spinal cord varied according to the stimulus (i.e., urethral catheter, bladder distension, or chemical irritation). Distension of the urinary bladder increased the number of c-fos-positive cells mainly in DCM and SPN regions of the cord. In contrast, irritation of the LUT increased c-fos expression largely in DCM and MDH areas. Spinal cord transection (T_8 level) did not alter the c-fos expression induced by a catheter or chemical irritation, indicating that gene expression was mediated by spinal pathways. Denervation experiments showed that c-fos expression was induced by activation of afferent pathways in the pelvic and pudendal nerves. These results suggest that neurons in several regions of the spinal cord are involved in processing afferent input from different parts of the LUT. Neurons in the DCM appear to have an important role since they respond to both nociceptive and non-nociceptive inputs and to visceral (pelvic nerve) and somatic (pudendal nerve) afferent pathways. Thus, these neurons may be involved in the mechanisms of visceral-somatic referred pain.

Electrophysiological studies have identified several types of afferent neurons innervating the urinary bladder (Janig and Morrison, 1986; Janig and Koltzenburg, 1990). One type is mechanosensitive and responds to bladder distension or bladder contractions. In the cat, these afferent neurons have myelinated axons and are activated by both low (non-nociceptive) and high (nociceptive) intravesical pressures. Other types of afferents do not respond to bladder distension but are activated by cold temperatures (Fall et al., 1990) or chemical irritation of the bladder mucosa (Janig and Koltzenburg, 1990). The latter types have unmyelinated axons and are believed to have primarily nociceptive functions (Habler et al., 1990).

The majority of bladder afferent neurons are located in the sacral or caudal lumbosacral dorsal root ganglia and send axons into the periphery through the pelvic nerves (Sharkey et al., 1983; de Groat, 1986; Steers et al., 1991; Keast and de Groat, 1992). A smaller group of bladder afferent neurons that are located in rostral lumbar dorsal root ganglia have axons that travel in sympathetic (hypogastric) nerves (Steers et al., 1991; Keast and de Groat, 1992).

WGA-HRP tracing studies in the rat revealed that in the L_6-S_1 spinal cord, bladder afferent neurons project to discrete regions of the dorsal horn and intermediate gray including Lissauer's tract, lateral lamina I, laminae V-VII, and the dorsal commissure (DCM) (Jansco and Maggi, 1987; Steers et al., 1991). Similar afferent projections have been noted in the cat (de Groat, 1986; de Groat et al., 1986). Neurons at these sites in the spinal cord respond to non-nociceptive mechanical stimulation of the urinary bladder (de Groat, 1971; de Groat et al., 1981; McMahon and Morrison, 1982a; Honda, 1985). Some of these spinal neurons send axons rostrally into the lateral funiculi and are presumably tract neurons that transmit information to supraspinal centers involved in visceral sensation or in the initiation of voiding reflexes (de Groat et al., 1979; McMahon and Morrison, 1982b). The spinal processing of nociceptive input from the urinary bladder has not been analyzed using electrophysiological techniques. However, WGA-HRP tracing experiments indicate that capsaicin-sensitive, small-diameter bladder afferents, presumably including the nociceptive population, exhibit central projections similar to those of non-nociceptive afferents (Steers et al., 1991).

The present study was undertaken to obtain more detailed information about the spinal pathways that process nociceptive and non-nociceptive afferent input from the lower urinary tract (LUT) of the rat. Spinal neurons activated by bladder distension or chemical irritation of the LUT were identified by increased expression of *c-fos*, an immediate-early gene (Curran, 1988). Previous studies have shown that an increase in c-fos protein in spinal neurons can be detected with immunocytochemical techniques within a few hours following chemical irritation of somatic structures, such as the paw (Hunt et al., 1987; Menetrey et al., 1989; Bullitt, 1990). The present experiments revealed that noxious or non-noxious stimulation of the LUT increased c-fos expression in L_6-S_1 spinal neurons in discrete regions of

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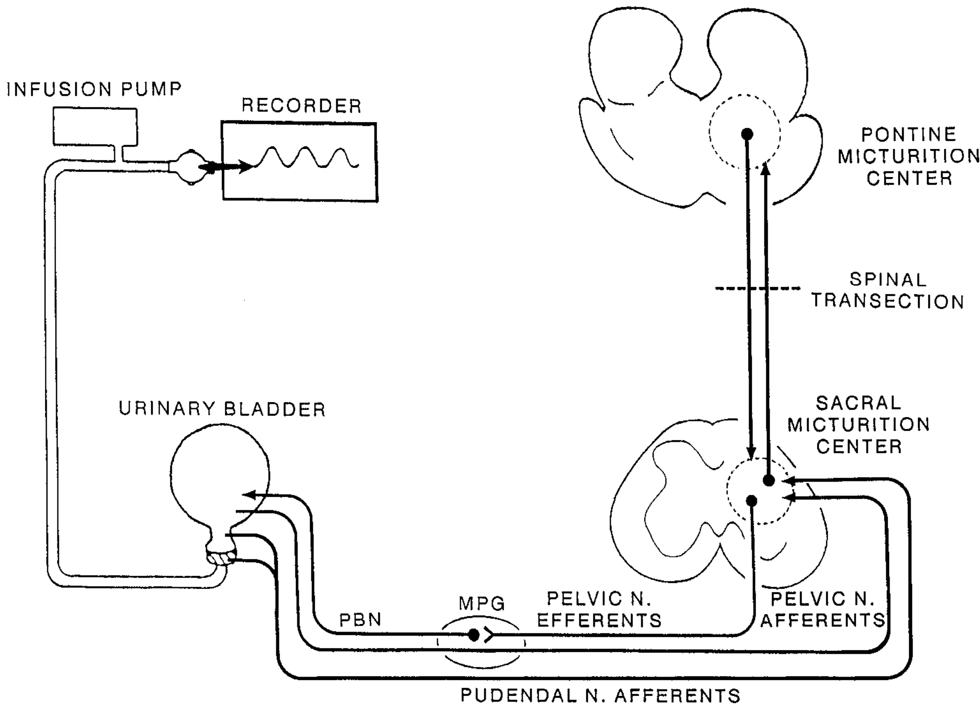

Figure 1. Diagram showing the organization of the spinobulbospinal micturition reflex pathway and the method for infusing fluids into the urinary bladder via a transurethral catheter. The urethral outlet was open during a continuous cystometrogram to allow fluid to drain out from the bladder or was closed by a tie at the urethral orifice to allow recordings of isovolumetric bladder contractions. In some preparations, the spinal cord was transected to block the micturition reflex pathways. *PBN*, postganglionic bladder nerves; *MPG*, major pelvic ganglion.

the cord that overlap with those areas receiving afferent input from the urinary bladder. It is noteworthy, however, that more rostral lumbar segments that also receive sympathetic afferent projections from the LUT did not exhibit c-fos–positive neurons.

Preliminary results of some of these observations have been reported previously (Birder et al., 1989, 1990).

Materials and Methods

Experiments were performed on 92 urethane-anesthetized (1.2 mg/kg, i.p.) adult female Wistar rats; 78 had intact spinal cords and 14 had spinal transections at the T_8 level 4–7 d prior to the experiment. Experiments were also conducted in rats ($N = 14$) with intact spinal cords in which either pelvic or pudendal nerves were bilaterally transected 1 week prior to the experiments.

The urinary bladder was catheterized through the urethra to infuse fluids and to measure bladder pressure. Intravesical pressure was measured using a strain gauge transducer and recorded on a polygraph (Fig. 1). Two different preparations were used for physiological studies: (1) animals in which the urethral outlet remained open (continuous infusion) or (2) animals in which the urethra was closed via a ligature placed around the urethral orifice (isovolumetric conditions). In rats with an intact spinal cord, physiological saline solution or a chemical irritant (1% acetic acid in physiological saline) was infused into the bladder (0.12 ml/min) for a 2 hr period. When the urethra was open, the infusion solution could leak out or be expelled during a bladder contraction, thereby preventing overdistension of the bladder. Mineral oil was applied to the area around the urethral orifice to eliminate the possibility of chemical irritation of the perineal skin and vaginal mucosa in this region. When the bladder outlet was closed, the chemical irritant was infused (0.12 ml/min) until the peak of the first bladder contraction, at which time the infusion was stopped, and the bladder was maintained under isovolumetric conditions. Rhythmic bladder contractions then continued for the remainder of the experiment. The volume necessary to induce the first bladder contraction will be termed the micturition volume threshold (Fig. 1). In spinal animals, the bladders did not exhibit reflex contractions and were unable to expel fluids; thus, to avoid overdistension, the bladders were partially filled with 1 ml of irritant solution and maintained at this volume. Two hours after exposure to the irritant, when the number of c-fos–positive cells in the spinal cord had reached a maximum (Birder et al., 1989), the animals were killed via intracardiac perfusion first with Krebs buffer followed by 8% paraformaldehyde fixative. Alternate sections (42 μm) of the spinal cord (L_1–S_2) were processed by an avidin-biotin method for the c-fos protein (Hsu et al., 1981) using antibodies either purchased from Cambridge, Inc. (catalog number OA-11-823), or provided by Dr. M. Iadarola (NIH). The two antibodies yielded similar results. Since the animals were killed 2 hr after the onset of urinary tract stimulation, it is likely that the majority of the immunoreactivity with the Iadarola antibody represents fos protein rather than fos-related proteins (Sonnenberg et al., 1989; Sharp et al., 1991; Young et al., 1991). Cells exhibiting c-fos immunoreactivity were counted in four spinal cord regions: medial dorsal horn (MDH), lateral dorsal horn (LDH), dorsal commissure (DCM), and lateral laminae V–VII including the sacral parasympathetic nucleus (SPN) (Fig. 2). The latter region contains preganglionic neurons as well as interneurons and spinal tract neurons.

Counts of c-fos–positive cells are presented as average numbers of cells per section or percentage change in numbers of cells per section. The changes in cell counts were evaluated statistically using analysis of variance (univariate and multivariate with repeated measures) to examine the interactions between preparations (spinal intact or spinal transected) and areas of cord (MDH, LDH, DCM, SPN). The Mann–Whitney *U* test was used to analyze differences in distribution of cell

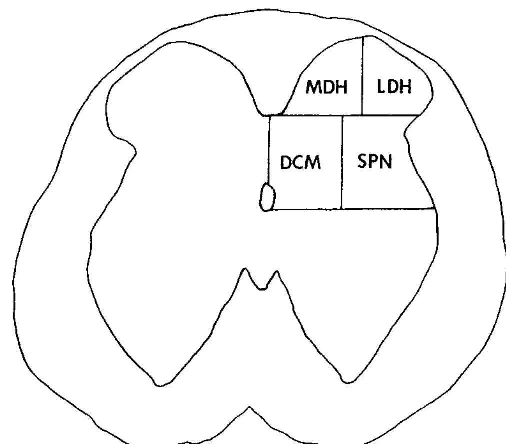

Figure 2. Drawing of a section from the L_6 spinal cord in the rat depicting four regions where c-fos–positive neurons were identified following stimulation of the LUT.

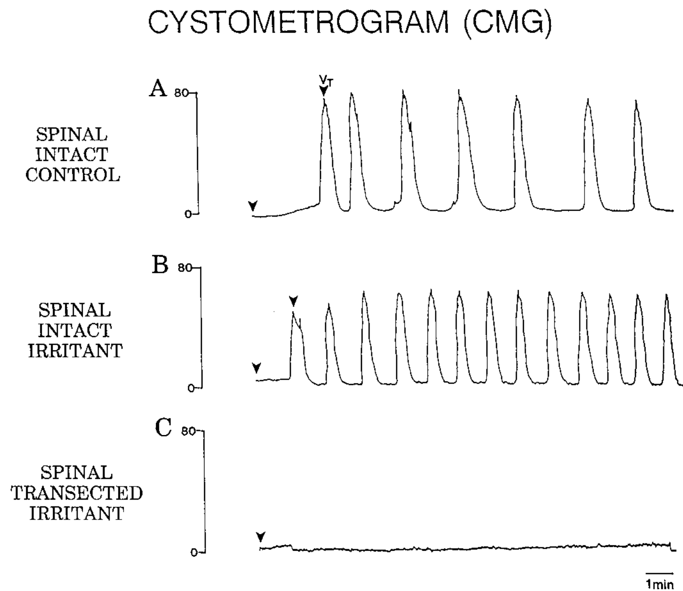

Figure 3. Effect of bladder irritation on the isovolumetric cystometrogram. *A*, Rhythmic bladder activity after physiological saline infusion at a rate of 0.12 ml/min. *B*, Rhythmic bladder activity after infusion of 1% acetic acid at a rate of 0.12 ml/min. *C*, Cystometrogram after infusion of 1% acetic acid at a rate of 0.12 ml/min in a spinal-transected rat. The *ordinate* indicates bladder pressure in cm H_2O; the *abscissa* indicates time of infusion in minutes (calibration, 1 min). *Arrowheads* indicate both start and stop of infusion; V_T, volume threshold.

counts in specific areas of the spinal cord. Control experiments were also conducted in animals (1) with or without a urethral catheter, (2) with the spinal cord intact or transected, and (3) with either the pelvic or pudendal nerves transected bilaterally prior to the experiment. In addition, a few animals were studied in which the urinary bladder was exposed through a midline abdominal incision, so that irritants could be applied directly to the serosal surface of the bladder.

In three urethane-anesthetized (1.2 mg/kg, i.p.) animals, c-fos expression was also evaluated following activation of somatic nociceptive afferents in the left rear hindpaw (plantar surface) by injection of 0.1 ml of a 10% buffered formalin solution (Hunt et al., 1987; Menetrey et al., 1989; Bullitt, 1990). The animals were perfused with fixative 2 hr after the injection of the irritant. These experiments provided control data that could be compared to observations made previously in other laboratories using unanesthetized animals and different c-fos antisera.

Sections of the bladder wall (28 μm) from saline control and chemically irritated animals were examined for inflammatory changes following hematoxylin/eosin (H/E) staining. The tissue was fixed in 8% paraformaldehyde and then sectioned and counterstained using the Meyer's technique for H/E (Luna, 1968).

In another series of experiments, the integrity of bladder urothelium was examined using the indigocarmine method (Perzin et al., 1991), following a 2 hr exposure to either saline or a chemical irritant. In these animals (N = 8: 3 normal controls, 5 chemically irritated), which were anesthetized with urethane, the urinary bladder was exposed to a 1% indigocarmine solution (0.3–0.5 ml); after 1 hr, the bladder was removed and homogenized. After precipitation of proteins with trichloroacetic acid (TCA), the absorbance of the supernatant was measured with a UV spectrophotometer at 610 nm wavelength to determine the amount of dye that passed through the urothelium into the bladder wall.

Results

Physiological changes induced by chemical irritation of the LUT

Spinal-intact animals. Since a variety of irritant chemicals can activate nociceptive afferents and induce an inflammatory reaction in the urinary bladder (Maggi et al., 1988; Habler et al., 1990; Morikawa et al., 1990), an initial series of physiological experiments was conducted to examine the effects on bladder activity of several chemical irritants (1% acetic acid, 25% turpentine, or 100% xylene) administered intravesically to determine which of these irritants would be most useful for the anatomical studies. Several parameters of bladder activity were evaluated, including (1) the volume threshold for inducing a micturition reflex during slow filling of the bladder, and (2) the frequency and (3) amplitude of rhythmic bladder contractions recorded in the distended bladder under isovolumetric conditions. Although all of the agents were found to enhance bladder activity, acetic acid was most useful since it is water soluble. Turpentine oil and xylene are not water soluble and therefore were difficult to remove from the urinary bladder.

In normal animals with an intact neuraxis, infusion of 1% acetic acid (0.12 ml/min) with the urethra either open or closed facilitated bladder reflexes (Fig. 3). In animals with the outlet closed by urethral ligation, chemical irritation decreased the volume threshold by 55% (range, 14–83%) and increased the frequency of isovolumetric contractions by 30% (range, 6–55%); however, baseline pressure during filling was unchanged (0–10 cm H_2O), as was the amplitude of bladder contractions. This effect occurred within a few minutes after infusion of the irritant. When examined with the urethral orifice ligated, the effect of irritation persisted for the length of the experiment (2 hr). However, ligating the urethral orifice activated somatic afferents from this region and increased c-fos expression in spinal cord (see below). Therefore, the majority of anatomical experiments were conducted using the continuous cystometrogram with the urethral outlet open. In this preparation, continuous infusion of a chemical irritant decreased by 30% (range, 19–46%) the micturition volume threshold and increased by 60% (range, 35–79%) the frequency of bladder contractions. In some animals, bladder contractions ceased approximately 20–30 min after the start of the irritant infusion. The reason for this is not known.

Spinal-transected animal. Transection of the spinal cord at the lower thoracic level in the rat produces an initial bladder areflexia lasting for 1–2 weeks (Mallory et al., 1989). Following this period, bladder reflexes recover. In the anatomical experiments, we wanted to examine c-fos expression in some animals in absence of bladder reflexes; thus, acute spinal animals (4–7 d, T_8 level) were used. In these animals, infusion of 1–2 ml of saline into the bladder did not evoke reflex bladder contractions or the release of urine around the catheter. Similarly, distension of the bladder with 1–2 ml of saline under isovolumetric conditions did not evoke reflex contractions. Injection of 1% acetic acid into the bladder in either experiment also did not increase baseline intravesical pressures, evoke rhythmic contractions, or induce voiding (Fig. 3).

Measure of the noxious action of chemical irritants on urothelial integrity

Exposure of the urothelium to noxious substances such as acetic acid or dimethyl sulfoxide (DMSO) can produce an acute irritation and break down the urothelial mucosal barrier (Horita and Weber, 1964; Hohlbrugger and Lentsch, 1985). The degree of damage to the mucosal barrier can be determined by measuring the penetration of a dye, indigocarmine, through the urothelium into the bladder wall (Perzin et al., 1991). In the present experiments, indigocarmine uptake was measured in animals whose urinary bladders were exposed to either saline, 1% acetic acid, or 70% DMSO for 2 hr. The quantity of indigocarmine in the bladder wall was determined by UV spectro-

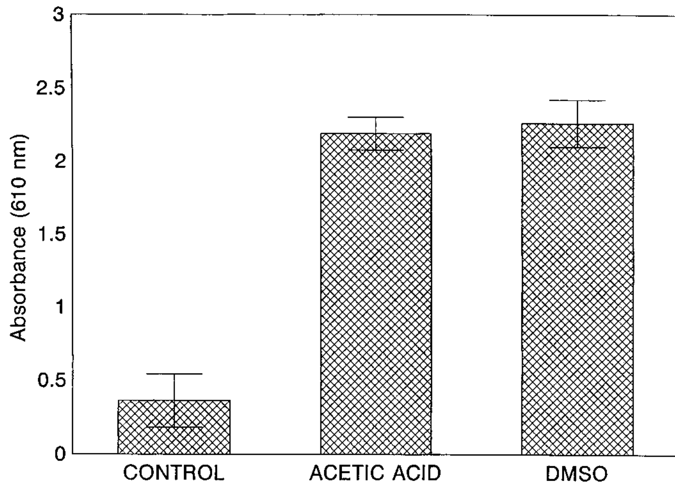

Figure 4. Histogram illustrates the degree of urothelial damage induced by chemical irritation as evidenced by uptake of indigocarmine dye into the bladder wall. The *ordinate* indicates absorbance of dye in control animals following saline distension ($N = 3$) or in animals in which the urinary bladder was chemically irritated with 1% acetic acid ($N = 3$) or with 70% DMSO ($N = 2$) for 2 hr. Error bars indicate SE.

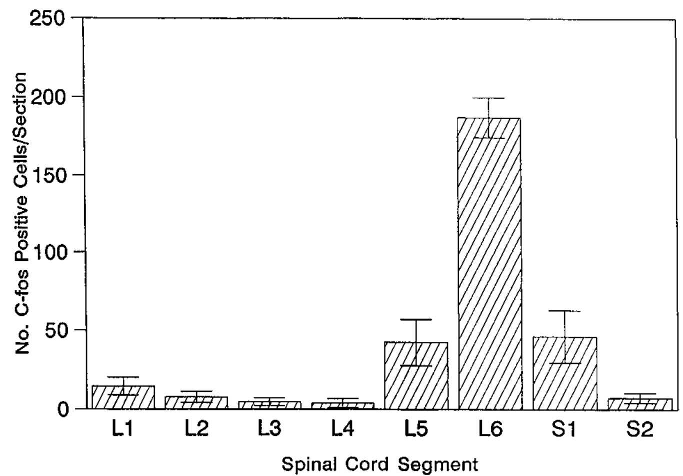

Figure 5. Histogram showing the segmental distribution of c-fos–positive cells (number/section) in the rat spinal cord (L_1–S_2) after chemical irritation of the LUT ($N = 7$). Error bars indicate SE.

photometric analyses of the supernatant fluid following homogenization of the bladder. To determine if UV absorbance was proportional to increasing concentrations of dye, measurements were performed on a range of concentrations of indigocarmine in a solution of TCA to establish linearity in the range of 0.001–1%. Control bladders ($N = 3$) exhibited small quantities of dye (Fig. 4), while exposure to acetic acid markedly increased dye concentration above saline controls ($N = 3$). DMSO (70% v/v solution in saline), which has been shown to enhance the permeability of the urothelium (Horita and Weber, 1964; Hohlbrugger and Lentsch, 1985), also increased the concentration of indigocarmine in the bladder ($N = 2$) (Fig. 4).

Bladders that were exposed to 1% acetic acid for 2 hr under isovolumetric conditions showed acute signs of inflammation as evidenced by regions of edema, infiltration of polymorphonuclear cells, plasma cells, and mast cells, and disruption of the epithelial layer.

C-fos immunocytochemistry: spinal-intact preparation

Preliminary experiments to establish optimal conditions. A number of preliminary studies were conducted to determine (1) the influence of prior surgical procedures on the baseline levels of c-fos, (2) the optimal interval following exposure to a chemical irritant for killing the animals, (3) the optimal duration of exposure to the irritant, (4) the type of anesthetic that would be most appropriate for studying c-fos expression, and (5) the segmental distribution of the c-fos–positive cells.

In control animals ($N = 5$) that were anesthetized with urethane but not subjected to any other treatment, the number of c-fos–positive cells in the lumbosacral spinal cord was low (2 cells/section). Animals in which either the pelvic or pudendal nerves were bilaterally transected exhibited small increases in the number of c-fos–positive cells in the L_5–S_1 spinal cord (mean, 2.5 and 10.5 cells/section, respectively) 1 week after nerve transections. Infusion of 1% acetic acid into the bladder via a urethral catheter markedly increased the number of c-fos–positive cells in the L_5–S_1 spinal cord (range, 42–187 cells/section) but produced relatively small increases (range, 4–15 cells/section) in adjacent segments (L_1–L_4, S_2) (Fig. 5). The c-fos immunoreactivity was detected in the nucleus of the cells as reported by other investigators (Fig. 6) (Weinberg, 1985; Curran, 1988; Sager et al., 1988; Sassone-Corsi et al., 1988).

Previous studies using noxious somatic stimuli in spinal-intact, unanesthetized rats have demonstrated that the increased expression of c-fos in spinal neurons was a transient phenomenon reaching a maximum in a few hours and decaying within 24 hr (Hunt et al., 1987). In the present experiments, a small set of animals was killed at various times (30 min to 24 hr) after bladder irritation (1% acetic acid) to determine the optimum time for detecting c-fos protein in urethane-anesthetized preparations. A large number of cells was detected after continuous exposure to the irritant for 1–2 hr, but the numbers declined at 6 hr and were almost back to control levels 24 hr after exposure (Fig. 7). Thus in the remainder of the experiments, animals were killed 2 hr after the initiation of noxious or non-noxious stimulation in the LUT.

The irritant was also applied for 30 min or 2 hr to determine whether the duration of the stimulus altered the number of c-fos–positive cells in the L_6 segment. In one group of animals, after a 30 min exposure the irritant was flushed out of the bladder by slowly infusing 2 ml of saline and the animals were maintained for an additional 1.5 hr before death. The number of c-fos–positive cells in these animals was 75% (144 cells/section) of the number in animals exposed to irritant for 2 hr (183 cells/section). It was concluded that duration of irritation would not be a critical factor at intervals greater than 30 min. A 2 hr duration was used in the remainder of the experiments.

Three anesthetics were studied in spinal-intact animals. No differences were noted in the number of c-fos–positive cells induced by chemical irritation of the urinary bladder in urethane- (1.2 gm/kg, i.p.) and halothane- (1%; $N = 2$) anesthetized animals. However, pentobarbital anesthesia (40 mg/kg, i.p.; $N = 2$) completely suppressed c-fos in all regions of the L_5–S_1 spinal cord. Urethane anesthesia was used in the remainder of the experiments.

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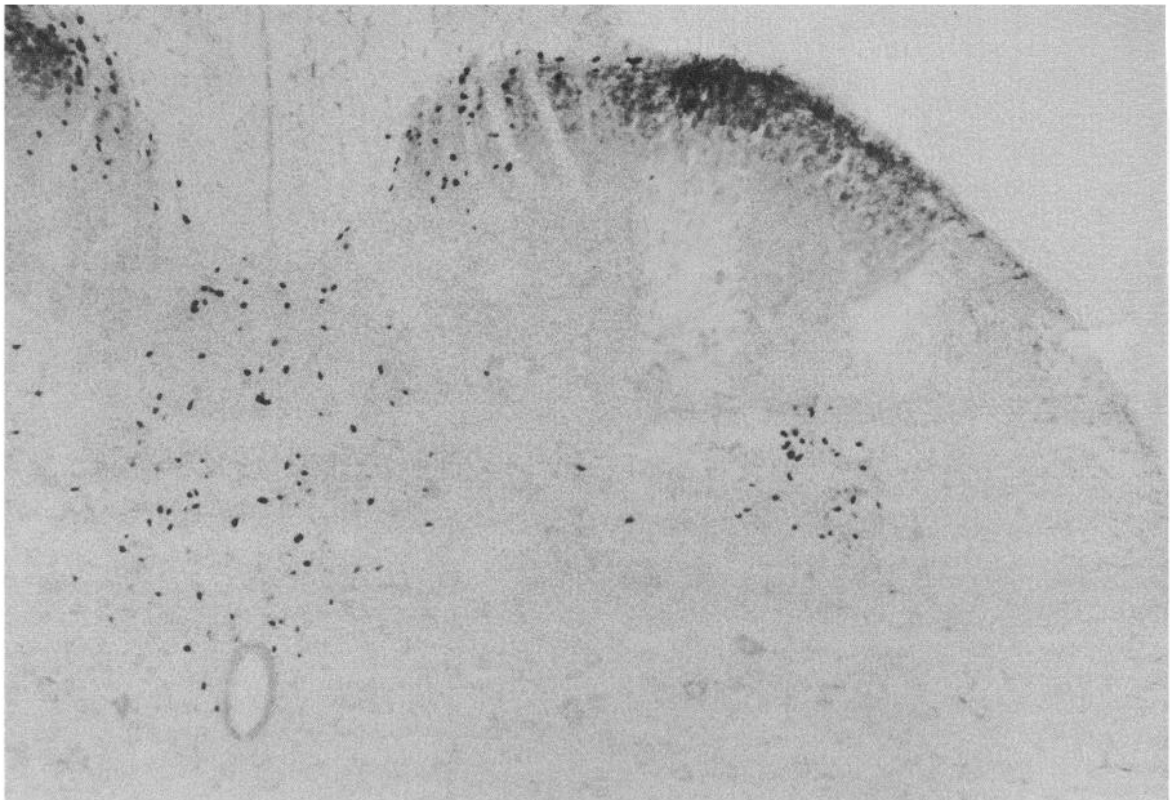

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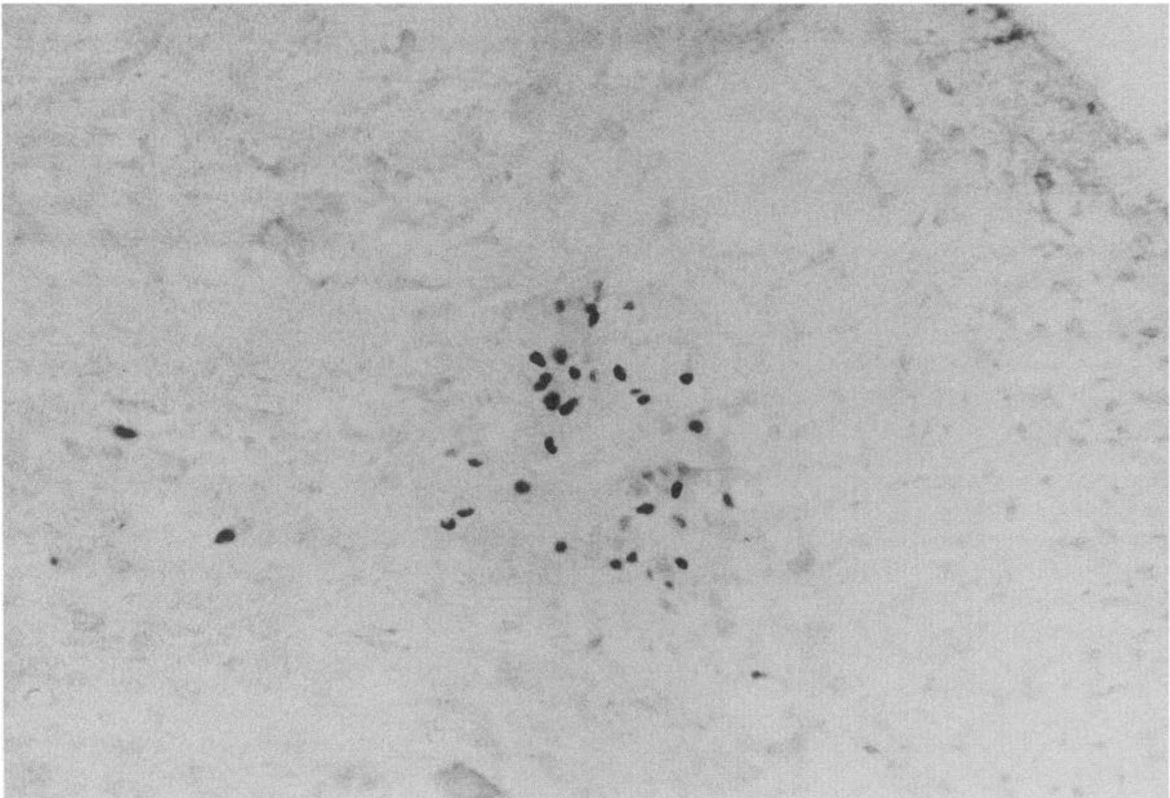

In contrast to the segmental distribution of c-fos in spinal cord neurons after LUT irritation, somatic irritation induced by injection of a 10% buffered formalin solution into the left rear footpad of the rat increased c-fos in cells mainly in the L_4–L_5 spinal cord (average, 40–60 cells/section) (Fig. 8). The cells were distributed throughout the MDH (laminae I–V) ipsilateral to the injection site, as noted previously by other investigators (Hunt et al., 1987; Menetrey et al., 1989; Bullitt, 1990).

Stimuli inducing c-fos expression. A series of experiments were conducted in which the urethral outlet was open and the bladder could empty. In these experiments, fluid was infused continuously (0.12 ml/min) into the bladder through a urethral catheter. Saline infusion induced voiding reflexes at 2–5 min intervals. Infusion of an irritant induced bladder hyperreflexia, increased the frequency of voiding, and typically produced a constant slow leak of fluid. This is most likely indicative of a tonically contracted bladder. At least three aspects of the experimental preparation could contribute to an increase in c-fos expression following manipulation of the LUT: (1) insertion of a catheter through the urethra into the bladder, (2) distension of the bladder by the infused solution, (3) chemical irritation of the bladder and urethral mucosa. The influence of each of these stimuli was examined by conducting control experiments in which (1) a catheter was inserted for 2 hr or (2) saline was slowly infused through a catheter for 2 hr.

The insertion of a catheter produced a considerable increase in the number of c-fos cells (56 ± 9.6 cells/section) in the L_6 segment, and slow infusion of saline through the catheter further increased the number (128 ± 4.1 cells/section). Infusion of acetic acid increased the number to an average total of 183 ± 26 cells/section (Table 1, Fig. 9). Thus, the response to chemical irritation must include a substantial component due to mechanical stimulation of bladder and urethral afferents; that is, 30% of the number of c-fos–positive cells is attributable to mechanical stimulation by the catheter, 33% to bladder distension and/or reflex contractions or voiding, and 37% to chemical irritation.

Topographical distribution of c-fos–positive cells. Following chemical irritation, c-fos–positive cells were located in four areas of the spinal cord: MDH, LDH, DCM, and SPN. No cells were present in the ventral horn. The distribution of c-fos–positive cells in four regions of the L_6 segment of the spinal cord was determined for each stimulus (i.e., catheter, catheter plus saline infusion, and catheter plus acetic acid infusion). The majority of cells activated by insertion of a catheter occurred in the MDH (42.9%) and DCM (40.9%) areas, whereas the cells activated by saline infusion through the catheter occurred in DCM (42.2%), SPN (29.5%), and MDH (25.6%). Infusion of acetic acid solution induced c-fos expression primarily in cells in the DCM (43.5%), SPN (21.9%), and MDH (27.4%).

To estimate the specific contribution of (1) tension receptor afferents activated by distension and reflex bladder contractions and (2) nociceptive afferents activated by acetic acid, a process of sequential subtraction was used. For example, the numbers of cells in each region induced by the catheter (N_c) were subtracted from the numbers induced by catheter plus saline infusion ($N_c + N_s$) to estimate the numbers attributable to saline

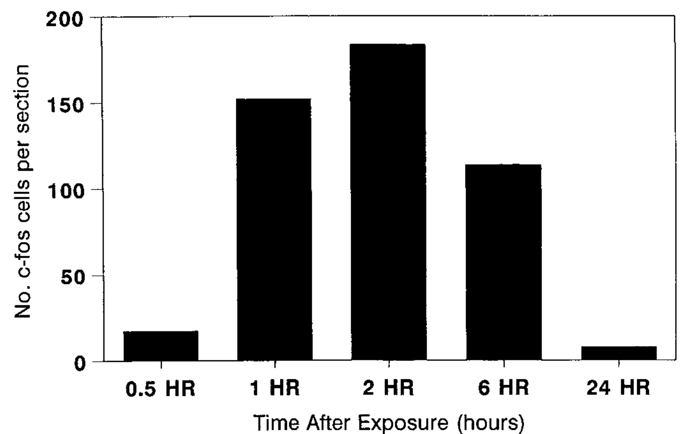

Figure 7. Histogram showing the number of c-fos–positive cells per section in the L_6 rat spinal cord, 30 min to 24 hr after LUT irritation.

infusion alone (N_s). Similarly, the numbers attributable to chemical irritation alone (N_i) were obtained by subtracting $N_c + N_s$ from the numbers produced by catheter plus infusion of irritation solution ($N_i + N_c + N_s$). These calculations revealed that saline infusion that activated repeated micturition reflexes produced a cell distribution that differed significantly from that produced by chemical irritation. While the DCM region contained a similar percentage of neurons following both stimuli (41% and 48%), the SPN area had a higher percentage of cells following saline distension (41% vs 9.5%; $p < 0.05$). On the other hand, the LDH and MDH regions had significantly ($p < 0.05$) higher percentages of neurons following chemical irritation (Fig. 9, Table 1).

Input from pelvic and pudendal afferents. Afferent pathways from the LUT to the L_6–S_1 spinal cord pass through the pelvic and pudendal nerves (de Groat and Steers, 1990). Nerve transection experiments were conducted to determine the relative contributions of pathways in each of these nerves to the chemical irritation–induced c-fos expression in the spinal cord.

Experiments were conducted in animals with either pelvic ($N = 5$) or pudendal ($N = 8$) nerves transected bilaterally 1 week prior to infusion of 1% acetic acid via a transurethral catheter. Nerve transections did not induce c-fos expression as noted following transections of the trigeminal (Sharp et al., 1989) and sciatic nerves (Chi et al., 1990). However, in pelvic nerve–transected animals, the number of c-fos–positive cells induced by urinary tract irritation was reduced to 39% of control (72 ± 20 cells/section) and the cells were located primarily in the DCM and MDH regions of the cord: DCM (46%), MDH (33.6%), and SPN (18.6%). In the pudendal nerve–transected animals, the number of cells was reduced to 47% of control (88 ± 16 cells/section) and the cells were located in DCM (48.4%), SPN (32.6%), and MDH (11%). The percentages of cells in the MDH, SPN,

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Figure 6. Bright-field photomicrographs of an L_6 spinal cord section from the rat showing the distribution of c-fos–positive neurons after LUT irritation. *A* depicts cells in dorsal horn, DCM region, and SPN area. *B* is a photomicrograph at a higher magnification depicting cells in the SPN region. Scale bars: *A*, 110 μm; *B*, 55 μm.

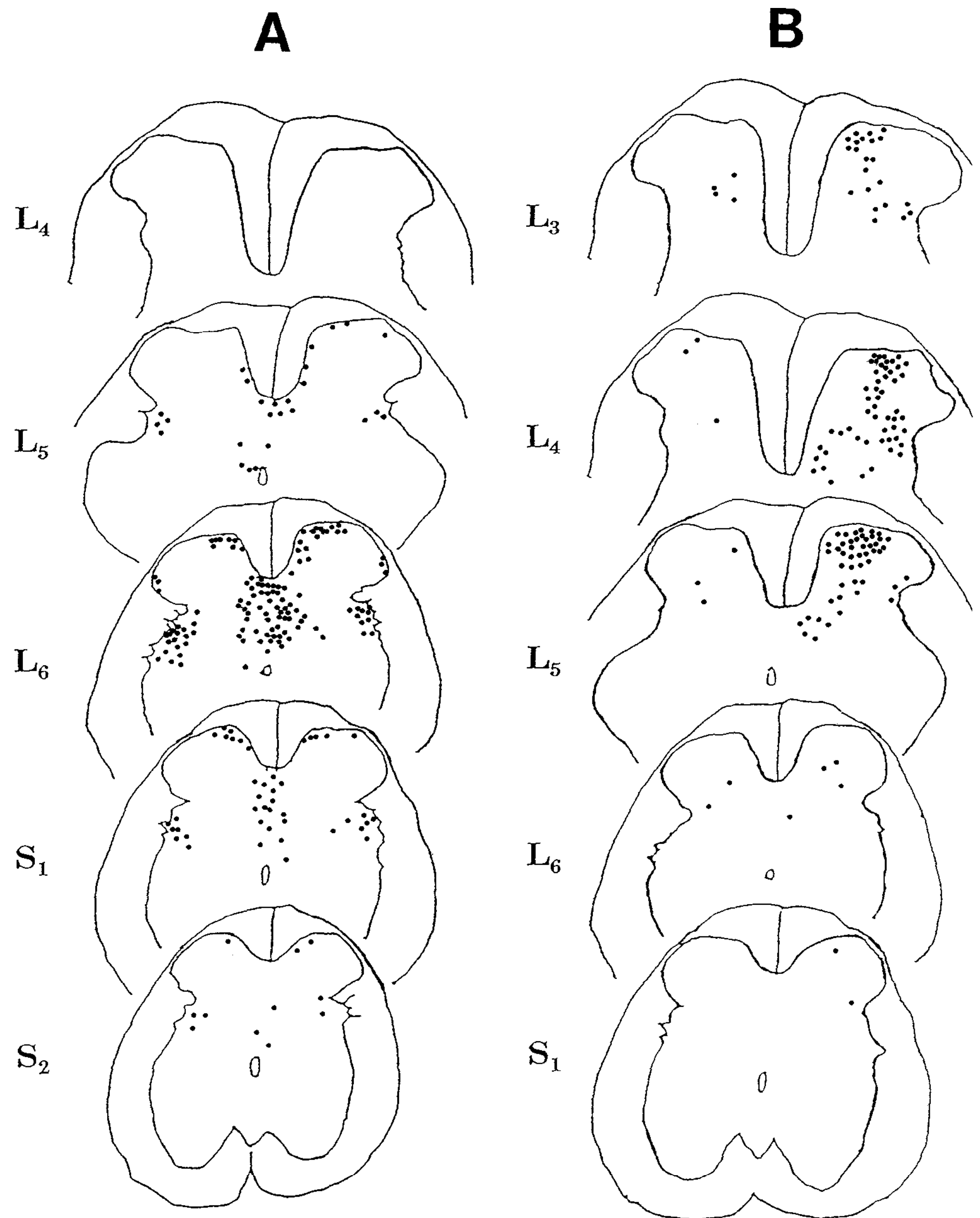

Figure 8. *A*, Camera lucida drawings from L_4–S_2 rat spinal cord showing c-fos immunoreactivity localized to four spinal cord regions after LUT irritation. *B*, Camera lucida drawings from L_3–S_1 rat spinal cord showing c-fos immunoreactivity localized to the dorsal horn after chemical irritation of the right hindpaw. Each drawing shows the distribution of cells in one representative section from each segment.

and LDH regions were significantly different in these two groups of animals ($p < 0.05$) (Fig. 10). Animals in which both pelvic and pudendal nerves were bilaterally transected prior to infusion of 1% acetic acid exhibited low levels of c-fos–positive neurons in the L_6 spinal cord (6 cells/section), indicating that these nerves contain the majority of the bladder–urethral afferents.

C-fos immunocytochemistry: spinal-transected preparation

Since approximately 30% of the c-fos–positive cells following LUT irritation appear to be induced by distension or distension-induced reflex contractions of the urinary bladder, several types of experiments were conducted to determine whether these cells were activated by reflexes. In order to determine the contribution of activity in the spinobulbospinal micturition reflex pathway (Kuru, 1965; de Groat, 1975; Mallory et al., 1989) to the induction of c-fos in spinal neurons after LUT irritation, an acute spinal-transected preparation was used in which the reflex pathway was interrupted. In spinal-transected animals that were anesthetized with urethane (1.2 mg/kg, i.p.) but not exposed to a noxious stimulus, c-fos protein was not detected in spinal neurons. Placement of a urethral catheter increased the number of cells (52 ± 16 cells/section), whereas instillation of 1 ml of a 1% acetic acid solution into the urinary bladder markedly increased c-fos expression in all four areas of the lumbosacral spinal cord (146 ± 7 cells/section; $N = 3$). As noted above, these animals did not exhibit large-amplitude reflex bladder contractions in response to the chemical irritant. The total number of c-fos–positive neurons occurring after irritation on the average was 17% less (range, 0–30%) than the number in intact animals; however, this change was not statistically significant ($p > 0.05$).

Serosal irritation

Previous studies by other investigators revealed that capsaicin applied to the serosal surface of the bladder enhanced reflex bladder contractions (Santicioli et al., 1986) and induced an inflammatory reaction in the bladder (Maggi et al., 1987), which

was particularly prominent in the region of the bladder neck and proximal urethra. To determine if chemical irritation of serosal afferents can increase c-fos immunoreactivity at the spinal cord level, two groups of animals were tested: (1) control animals in which the urinary bladder was exposed by an abdominal incision and (2) animals in which the bladder was exposed and a 1 cm cotton square soaked with an irritant (1% acetic acid or 2% capsaicin) was placed on the serosal surface for a period of 2 hr. In sham-operated animals, the number of c-fos cells was not significantly increased over that occurring in control animals with the abdomen closed (3 cells/section; $N = 2$). Serosal irritation of the urinary bladder either with acetic acid or with capsaicin did not increase the number of c-fos-positive cells over control.

Discussion

The present experiments revealed that non-nociceptive as well as nociceptive afferent input from the LUT increases c-fos expression in neurons in the lumbosacral spinal cord of the urethane anesthetized rat. The increased c-fos expression occurred in discrete regions of the spinal cord and was initiated via activation of both pelvic and pudendal nerve afferents innervating the urinary bladder and urethra.

The largest numbers of c-fos-positive neurons (183 ± 26 cells/section) were detected following chemical irritation of the bladder and urethral mucosa by injecting 1% acetic acid solution through a urethral catheter. This response reflected the activation of several types of afferent systems including (1) mechanoreceptor afferents responding to the transurethral catheter, (2) mechanoreceptor afferents responding to bladder distension and/or contractions, and (3) nociceptive afferents responding to chemical irritation of the vesicourethral mucosa. Based on control experiments in which the animals received only a urethral catheter or a catheter plus bladder distension, it is estimated that each of the first two stimuli accounts for approximately 30% of the total population of c-fos-positive cells in the irritated preparation, whereas chemical irritation activates the remaining 37% of the cells.

The segmental distribution of c-fos-positive spinal neurons induced by each stimulus (i.e., catheter, bladder distension, chemical irritation) was similar, indicating that nociceptive and non-nociceptive afferents project to the same level of the cord. In each instance, the majority of cells were located in the L_6–S_1 segments, which receive afferent input from the pelvic and pudendal nerves (Nadelhaft and Booth, 1984; McKenna and Nadelhaft, 1986). Very few cells were located in rostral lumbar segments (L_1–L_4). This was unexpected since it is generally believed that the rostral lumbar segments receive nociceptive input from the LUT via afferents traveling in sympathetic (hypogastric) nerves (Janig and Morrison, 1986). The localization of c-fos cells in L_6–S_1 may simply reflect the larger number of urinary tract afferents projecting to these segments or may indicate that sympathetic afferent pathways to L_1-L_2 are ineffective in inducing c-fos expression. This may be due to differences in synaptic transmission at the two levels of the spinal cord or possibly to the greater susceptibility of nociceptive transmission at L_1–L_2 to urethane anesthesia.

It should be noted, however, that the negative results with the c-fos technique are difficult to interpret. For example, although increased expression of c-fos implies that neurons have received synaptic input, the failure to detect c-fos expression does not necessarily mean that neurons have not been synaptically activated. Some neurons may not express c-fos under any condition, or may produce levels of c-fos protein below the threshold for detection with immunocytochemical techniques.

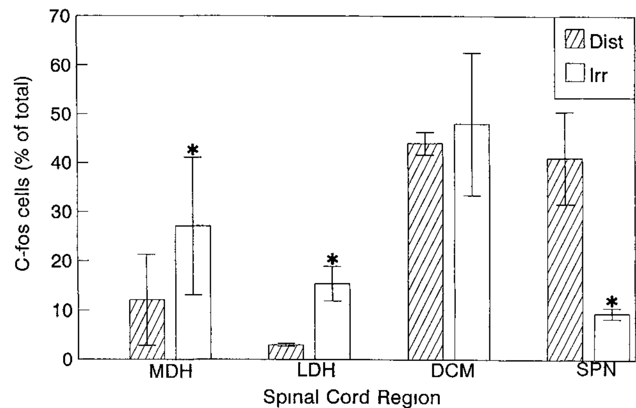

Figure 9. Histogram showing the distribution of c-fos-positive cells in four regions of the L_6 spinal cord following bladder distension (*Dist, hatched bars*; $N = 3$) or chemical irritation (*Irr, open bars*; $N = 7$). Values represent the percentage of the total population of c-fos cells induced by each stimulus. Cells activated by bladder distension were estimated by subtracting the number of cells induced by insertion of a urethral catheter. Cells activated by chemical irritation were estimated by subtracting the number of cells induced by catheter and bladder distension. Error bars indicate SE. *Asterisks* indicate statistically significant ($p < 0.05$) difference between percentages of cells induced in each area by distension or irritation.

Table 1. Percentage of c-fos neurons in regions of the L_6 spinal cord

Treatment	Total	MDH	LDH	DCM	SPN
CATH	56.4	42.9 ± 7.1	2.0 ± 1.3	40.9 ± 2.6	14.4 ± 7
DIST	127.9*	12.1 ± 9.2*	3.0 ± 0.3	44.0 ± 2.3	41.1 ± 9.4*
IRR	183.5**	27.1 ± 14**	15.4 ± 3.5**	48.0 ± 14.6	9.5 ± 1.1**

This table indicates the distribution expressed as percentages of c-fos-positive cells in four regions of the L_6 spinal cord (MDH, LDH, DCM, and SPN) in a spinal-intact rat following (1) urethral catheterization (CATH; $N = 3$), (2) bladder distension (DIST; $N = 3$), and (3) chemical irritation (IRR; $N = 7$). Cells activated by bladder distension were estimated by subtracting the cells induced by the catheter. Cells activated by chemical irritation were estimated by subtracting those induced by catheter and bladder distension. Total represents the total number of c-fos-positive cells per L_6 section. Asterisks indicate a statistically significant difference in each area ($p < 0.05$) between CATH and DIST (*) or between DIST and IRR (**).

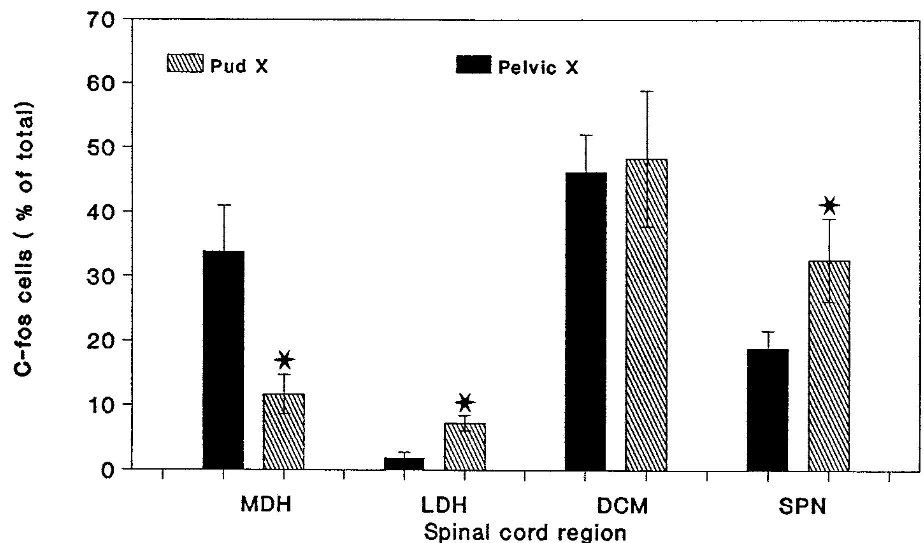

Figure 10. Histogram showing the distribution of c-fos–positive cells in four regions of the L_6 spinal cord after LUT irritation in animals in which pelvic nerves were bilaterally transected (*Pelvic X, solid bars*) 1 week prior to experimentation and in animals in which pudendal nerves were bilaterally transected (*Pud X, hatched bars*) 1 week prior to experimentation. Error bars indicate SE. *Asterisks* indicate statistically significant differences ($p < 0.05$) between the percentages of cells in each area in the two types of experiments.

In addition, certain types of synaptic activation may increase c-fos expression, whereas other types may not (e.g., descending activation of spinal inhibitory interneurons, Jones and Light, 1990; light activation of retinal neurons, Sagar and Sharp, 1990).

This problem is clearly illustrated by the failure of sphincter motoneurons in the present experiments to express c-fos in response to a constant infusion of saline into the bladder. Prominent external sphincter contractions occur during voiding in the rat (Maggi et al., 1986; Kruse et al., 1990), indicating that sphincter motoneurons are synaptically activated. However these cells did not exhibit detectable levels of c-fos protein. Other reports have also indicated that c-fos levels are low in motoneurons (Jenkins and Hunt, 1991). Thus, it seems reasonable to conclude that increased neuronal levels of c-fos protein do indicate synaptic activation; however, negative immunocytochemical findings do not always indicate an absence of synaptic activity.

Following activation of LUT afferents, c-fos–positive cells were identified in L_6–S_1 spinal segments primarily in four areas: MDH, LDH, DCM, and lateral laminae V–VIII in the region of the SPN. These areas also exhibit HRP- or WGA-HRP-labeled afferent projections either from the urinary bladder (Jansco and Maggi, 1987; Steers et al., 1991), or from afferent pathways in the pelvic or pudendal nerves, which contain, respectively, the innervation to the bladder and urethra (Morgan et al., 1981; Nadelhaft and Booth, 1984; McKenna and Nadelhaft, 1986; Thor et al., 1989). Bilateral transection of both pelvic and pudendal nerves almost completely abolished c-fos expression in response to LUT irritation, reducing the number of cells to 3% of control. Selectively blocking afferent inputs either from the pelvic or pudendal nerves also markedly reduced the number of c-fos–positive neurons to 39% or 47% of control, respectively, and altered the distribution of c-fos–positive cells in the four spinal cord regions. These selective denervation experiments indicated that pudendal nerve afferents activated cells primarily in DCM (46%) and MDH (34%) regions, whereas pelvic nerve afferents activated cells in DCM (48%) and SPN (33%) regions. The pelvic nerve (a visceral nerve) carries afferents primarily from the bladder and proximal urethra, whereas the pudendal nerve (a somatic nerve) carries afferents from the distal urethra, urethral sphincter, and perineum. Since the DCM region exhibits the highest percentage of c-fos–positive cells induced by either pelvic or pudendal afferents, it is likely that this area is important for viscerosomatic interactions and for processing of nociceptive information from different parts of the LUT. On the other hand, the SPN and MDH areas exhibit more specificity and seem to be important for processing visceral and somatic inputs, respectively.

This view is also supported by differences in the distribution of c-fos cells induced by other stimuli to the LUT. For example, introduction of a urethral catheter increased c-fos primarily in the MDH (42.9%) and DCM (40.9%) regions. In unpublished experiments, we noted that ligation of the urethral orifice without insertion of the catheter induced c-fos primarily in the MDH region (84% of the cells), indicating that this area receives a prominent input from the distal urethra presumably via pudendal nerve afferents. In the same experiments, insertion of a catheter followed by ligation of the urethral orifice, which should activate afferent input from bladder neck as well as distal and proximal urethra, activated similar percentages of cells in the MDH (43%) and DCM (45%) regions. These observations raise the possibility that distal urethral inputs project to MDH and proximal urethra inputs project primarily to DCM.

The number of c-fos–positive cells attributed to afferent input from bladder tension receptors was estimated by subtracting the number of cells produced by catheter alone. This method indicated that distension of the bladder produced a large percentage of cells in the SPN (41.1%) and DCM (44%) and a smaller percentage in the MDH (12.1%) region (Fig. 9, Table 1). The total number of c-fos–positive cells in MDH was similar to that induced by catheter alone. This suggests that tension receptor afferents activated by distension of the bladder or by reflex bladder contractions preferentially activates cells in the SPN and DCM. Using a similar approach, it was estimated that

chemical irritation of the LUT also activated cells in all four spinal regions, with the largest percentage in the DCM region (48%) followed in descending order by the MDH (27.1%), LDH (15.4%), and SPN (9.5%). These results suggest that the DCM area may be important in processing information concerning nociception as well as distension in the LUT.

The convergence of inputs from different types of afferents (nociceptive and mechanoreceptive) and from different areas of the LUT (bladder and urethra) to the same regions of the spinal cord raises a number of potential complications in interpreting the data particularly when estimating the size and distribution of certain populations of cells by subtracting the number of cells activated by one stimulus from that induced by a combination of stimuli. For example, this approach does not take into account convergence of two inputs onto the same population of cells, which could produce synergistic interactions (i.e., either input alone is ineffective in activating c-fos but two inputs acting synergistically induce gene expression) or occlusion where two inputs both maximally activate c-fos expression. Physiological experiments indicate that different stimuli do interact to influence micturition (Maggi et al., 1988; Morikawa et al., 1990). Since nociceptive stimuli facilitate bladder reflexes, nociceptive input from the urethra (via introduction of a catheter) (Abelli et al., 1991) could modulate the expression of c-fos induced by physiological distension of the bladder. In addition, different afferent inputs from the LUT elicited by distension or nociceptive stimuli can initiate reflex bladder contractions, which can in turn activate mechanoreceptor afferents. Thus, the presence of a reflex loop in animals with an intact nervous system could complicate the analysis of the effects of specific afferent pathways on c-fos expression. A more ideal experimental paradigm would allow the effect of each type of stimulus to be examined in isolation. However, this was not possible in the present experiments since catheterization and fluid distension of the bladder were necessary to induce chemical irritation.

Supraspinal micturition reflex pathways are essential for the control of bladder activity and the initiation of voiding (de Groat, 1975; de Groat and Steers, 1990). To determine if an intact supraspinal reflex has a role in the activation of c-fos in spinal cord neurons during LUT irritation, spinal-transected rats were studied. Following transection, the bladder was areflexic before and after a 1% acetic acid was introduced via a transurethral catheter. However, irritation (both catheterization and chemical irritation) induced c-fos in the same spinal cord areas as in the intact preparation (DCM, 47.4 $\pm$ 5.1%; MDH, 23.7 $\pm$ 4.5%; SPN, 22.8 $\pm$ 1.9%; LDH, 5.8 $\pm$ 1.7%). In these experiments, the number of c-fos–positive cells was less but the reduction was not statistically significant. The fact that the bladder was areflexic in the spinal-transected preparation indicates that the supraspinal reflex that is essential for voiding is not necessary for the increased expression of c-fos by chemical irritation. This suggests that c-fos is expressed in spinal neurons via an increase in primary afferent input and segmental synaptic mechanisms.

Experiments were conducted to determine if activation of serosal afferents via a chemical irritant could increase the expression of c-fos at the spinal level. It has been shown that capsaicin applied to the surface of the urinary bladder produces an increase in motility and an inflammatory reaction (increase in plasma extravasation) that is localized to the bladder neck and urethra (Maggi et al., 1987). Application of 1% acetic acid or capsaicin solution onto the serosal surface of the urinary bladder did not increase the expression of c-fos in spinal cord neurons, whereas irritation of the mucosal surface of the urinary bladder did increase c-fos. In studies in which 9% acetic acid was injected into the peritoneal cavity of the rat (Menetrey et al., 1989), a large number of nociceptive afferents were presumably activated, including serosal afferents from the urinary bladder. C-fos expression was slightly increased in the superficial dorsal horn at the L_6/S_1 level, but was markedly increased at the level of the caudal thoracic and rostral lumbar segments in the dorsal horn (superficial and deep areas) and in lamina X, regions that are implicated in nociceptive processing. These observations are consistent with the present negative results with bladder serosal irritation in the L_6–S_1 segments and suggest a difference in the spinal mechanisms for processing inputs from serosal and mucosal nociceptive afferents.

Noxious and non-noxious stimulation of the colon and rectum of the rat induced by distension with graded pressures (20–80 mm Hg) increases *c-fos* expression in the L_5–S_1 segments of the spinal cord (Traub and Gebhardt, 1990). Similar to the present results, more intense colonic stimuli produced greater numbers of c-fos–positive cells, and the cells were located bilaterally in laminae I, II, V, VI, and X and the region of the SPN. It is interesting, however, that in the superficial dorsal horn (laminae I and II), colonic distension induced the largest number of cells in the lateral region, whereas LUT irritation produced a larger number in the MDH. This difference might be explained in part by the effect of urinary tract irritation to activate pelvic and pudendal afferents. The latter are known to terminate in the MDH (Thor et al., 1989). On the other hand, colon–rectal distension primarily activates visceral afferents in the pelvic nerve, which have a prominent projection to the LDH (Nadelhaft and Booth, 1984; Steers et al., 1991).

Noxious stimulation of the LUT and colon induced c-fos expression in a small number of cells in lamina II. This is inconsistent with electrophysiological experiments that have indicated that cells in lamina II are unresponsive to visceral afferent input, but is consistent with anatomical tracing studies (Morgan et al., 1981; Sugiura et al., 1989) that have identified visceral afferent terminations in lamina II. These differences might be explained by an effect of weak synaptic inputs to increase gene expression but not increase cell firing. In addition, LUT irritation activates urethral afferents in the pudendal nerve. Afferents in this somatic nerve are known to project to deeper laminae of the dorsal horn (Thor et al., 1989).

The distribution of c-fos–positive neurons induced by LUT irritation differed from that induced by activation of somatic afferents (footpaw), in which c-fos–positive neurons were located in L_4–L_5 spinal segments ipsilateral to the site of stimulation throughout laminae I–V of the dorsal horn (Fig. 8). The latter distribution corresponds to that seen by other investigators (Menetrey et al., 1989; Bullitt, 1990) and indicates that urethane anesthesia does not produce a quantitative or qualitative difference in c-fos expression. These observations also indicate that neurons in laminae III and IV receive primarily inputs from somatic receptive fields whereas neurons in lamina I and V receive convergent inputs from visceral as well as somatic structures (Cervero and Tattersall, 1986).

In summary, the measurement of *c-fos* gene expression in spinal neurons has provided new insights into the central processing of afferent input from the LUT of the rat. The present results indicate that nociceptive and non-nociceptive afferent pathways from the urinary bladder and urethra activate neurons

in similar regions of the spinal cord. However, there were also differences in the relative distributions of cells according to the stimulus modality. The types of neurons that express c-fos in response to LUT irritation are unknown; however, the laminar distribution of these neurons suggests that they may participate in voiding reflexes and in nociceptive processing, and therefore may be part of ascending projection systems to the pontine micturition center and to the thalamus. This is supported by other studies (Birder et al., 1990) indicating that a percentage of the cells that are c-fos positive after bladder irritation are spinal tract neurons projecting to the brain.

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