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Neonatal Bladder Inflammation Alters Activity of Adult Rat Spinal Visceral Nociceptive Neurons

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Abstract

Purpose—This investigation examined the effect of inflammation produced by intravesical zymosan during the neonatal period on spinal dorsal horn neuronal responses to urinary bladder distension (UBD) as adults.

Methods—Female rat pups (P14–P16) were treated with intravesical zymosan or with anesthesia-only. These groups of rats were subdivided forming four groups: half received intravesical zymosan as adults and half received anesthesia-only. One day later, rats were anesthetized, the spinal cord transected at a cervical level and extracellular single-unit recordings of L6–S1 dorsal horn neurons obtained. Neurons were classified as Type I - inhibited by heterotopic noxious conditioning stimuli (HNCS) or as Type II - not inhibited by HNCS - and were characterized for spontaneous activity and responses to graded UBD (20–60 mm Hg).

Results—227 spinal dorsal horn neurons excited by UBD were characterized. In rats treated as neonates with anesthesia-only, Type II neurons demonstrated increased spontaneous and UBD-evoked activity following adult intravesical zymosan treatment whereas Type I neurons demonstrated decreased spontaneous and UBD-evoked activity relative to controls. In rats treated as neonates with intravesical zymosan, the spontaneous and UBD-evoked activity of both Type I and Type II neurons increased following adult intravesical zymosan treatment relative to controls.

Conclusions—Neonatal bladder inflammation alters subsequent effects of acute bladder inflammation on spinal dorsal horn neurons excited by UBD such that overall there is greater sensory neuron activation. This may explain the visceral hypersensitivity noted in this model system and suggest that impaired inhibitory systems may be responsible.

Keywords

visceral; urinary bladder; cystitis; zymosan; spinal

INTRODUCTION

Inflammation can result in short- and long-term alterations in an organism's responses to other noxious stimuli. When inflammation occurs early in life, particularly during the development of nociceptive and antinociceptive systems, these alterations may become permanent.

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Hospitalizations or medical visits, particularly during infancy and when coupled with painful events, are associated with chronic pain in adulthood [3,7,13,27]. Preclinical studies of cutaneous and gastrointestinal nociception in rodents have demonstrated long-lasting behavioral, physiological, gene-expression and neuroanatomical changes following exposure to inflammation during the neonatal period [1,6]. Recently, our laboratories have identified similar findings in association with inflammation associated with the urinary bladder. We [19,21] and others [2] have utilized the stimulus of urinary bladder distension (UBD) to evoke reliable and reproducible reflex and neuronal responses that are inhibited by analgesics. Zymosan, a mycotic inflammogen that produces a robust cystitis when administered intravesically, also mildly augments reflex responses to UBD [23] and produces complex excitatory/inhibitory effects on spinal dorsal horn neuronal responses to UBD [20]. When adult rats have experienced zymosan-induced bladder inflammation during the neonatal period they demonstrate magnified reflex responses to UBD [24] after re-inflammation. It is not known how neonatal bladder inflammation affects spinal neuronal responses to UBD.

In the naïve state (no neonatal treatments), the neurophysiological consequences of acute bladder inflammation can be predicted by responses of spinal neurons to heterotopic noxious conditioning stimuli (HNCS). We recently reported [20] that in adult rats spinal neurons inhibited by HNCS (Type I neurons) demonstrated reduced responses to UBD following acute inflammation of the bladder whereas neurons that were not inhibited by HNCS (Type II neurons) demonstrated highly augmented responses to UBD. This stratification of dorsal horn neurons according to their response to HNCS also has proven to be predictive of multiple neuronal characteristics [12] in addition to their response to pharmacological treatments [19] suggesting that classifying neurons in this fashion has great physiological relevance. The present study sought to determine whether neonatal inflammation altered the spinal neurophysiological mechanisms of urinary bladder sensory systems. This was done by determining the effect of acute bladder inflammation on spinal neurons in rats which had experienced bladder inflammation as neonates. These studies were approved by our local institutional animal use committee and adhered to the ethical guidelines established by the U.S. National Institutes of Health.

MATERIALS AND METHODS

Thirty-two newborn, female Sprague-Dawley rat pups (mothers from Harlan; Prattville, AL) were randomly assigned to Neonatal Zymosan or Neonatal Anesthesia groups and subsequently were halothane-anesthetized (5% induction, 1–2% maintenance) via mask on days P14–P16. On each day of treatment the Neonatal Zymosan groups had a 24-gauge angiocatheter placed transurethrally into the urinary bladder. Intravesical zymosan (0.1 ml; 1% solution in saline; Sigma-Aldrich, St. Louis) was administered, allowed to dwell for 30 min then drained prior to catheter removal. The Neonatal Anesthesia groups remained anesthetized for 30 min but had no intravesical catheter placement. All rats were given ampicillin (50 mg/kg, s.c.) on each treatment day to prevent secondary infection. As adults (12–15 weeks of age), these two groups were subdivided further into Adult Zymosan and Adult Anesthesia subgroups. The Adult Zymosan subgroups (designated AnZy & ZyZy with the first set of letters indicating the neonatal treatment and the second set of letters indicating the adult treatment; An=Anesthesia-only, Zy=Zymosan intravesically) were anesthetized with isoflurane and had 0.5 ml of a 1% zymosan solution in normal saline instilled into their bladders via a transurethral catheter for 30 min prior to awakening from anesthesia. Adult Anesthesia subgroups (designated AnAn & ZyAn) were anesthetized for 30 minutes. All adult rats received ampicillin (50 mg/kg s.c.) to prevent secondary infection. One day after their pretreatment, rats were again anesthetized with isoflurane. Jugular venous, arterial carotid, and tracheal cannulae were placed and the rats artificially ventilated. The upper cervical spinal cord was exposed at the level of the atlanto-occipital joint, infiltrated with 50 µl of 1% lidocaine and subsequently transected. The brain

was mechanically pithed and the anesthesia discontinued. Arterial blood pressure was continuously monitored and normal saline was administered as needed to prevent hypovolemia. The spinalized animals were kept warm with heating pads and overhead radiant heating and allowed to recover until they demonstrated brisk flexion reflex responses to tail and hindlimb pinch. Paralysis was then established with pancuronium bromide (0.2 mg/h i.v.) and a lumbar laminectomy was performed exposing the L6-S2 spinal cord segments. The rats were suspended by vertebral clamps and the dura mater was cut and removed and the exposed spinal cord covered with a protective layer of mineral oil.

Constant-pressure, graded UBD (20–60 mm Hg, 20 s) was the noxious visceral test stimulus employed and was produced by inflating the urinary bladder with air via a 22 gauge angiocatheter placed via the urethra and held in place by a tight suture around the distal urethral orifice. UBD was used due to its long history of use in electrophysiological studies [14–17, 22] and its utility in studies of bladder-related reflexes [2,21]. UBD was administered as a phasic stimulus (rapid onset, rapid offset) as previously described [19]. Although phasic distension does not mimic the slow filling that occurs during normal bladder function, the rapid onset and offset makes this a highly reproducible stimulus and allows for the observation and quantification of time-linked phenomena. Intravesical distending pressure was monitored via an in-line, low-volume pressure transducer. Multiple neuronal responses to a 60 mm Hg, 20 s UBD with intertrial interval of 4 minutes were determined and averaged as a “standard” response to UBD.

Tungsten microelectrodes (Micro Probe Inc., Gaithersburg, MD; 1.2–1.5 M Ω) were used for single-unit extracellular recordings 0–1.0 mm lateral to midline, 0.1–1.0 mm ventral to the spinal cord dorsum. To quantify neuronal responses, units were displayed on an oscilloscope for continuous monitoring, discriminated conventionally from background, converted into uniform pulses and saved by computer as peristimulus-time histograms. Spontaneous Activity was determined as the average rate of action potentials per second in the 10 second period prior to the onset of UBD. Total Activity was determined as the rate of action potentials in the 20 second period during the UBD stimulus. Evoked Responses were calculated as the difference between the Total Activity measure and the Spontaneous Activity. Responses (excitatory/inhibitory) to cutaneous inputs were determined following the presentation of multiple UBD trials using the following stimuli: brush with a cotton-tipped applicator (non-noxious mechanical), pinch with a rat-tooth forceps at sufficient intensity to produce pain in the investigator (noxious mechanical.) Cutaneous receptive fields were classified as Class 1,2 or 3 as described in Table 1. The effect on Spontaneous Activity of a 5 second duration application of noxious mechanical stimulus to cervical or upper thoracic dermatomes (HNCS) was determined in all units. If inhibited >20%, then the neuron was defined as a Type I neuron. If excited, unaffected or inhibited < 20%, then the neuron was defined as a Type II neuron. This 20% criterion was based on previous quantitative studies which have observed random changes in spontaneous activity up to 20% [19].

Female rats were employed in these studies for both technical and interpretative reasons: bladders are more easily manipulated in females and bladder pain is more prevalent in females. We have previously demonstrated that estrous cycle effects contribute to the variability of responses to urinary bladder distension [21] but chose not to formally control for these effects since the changes that occurred secondary to bladder inflammation were of sufficient magnitude to observe statistically significant effects despite the added variability. Descriptive statistics are reported as means $\pm$ SEM where appropriate. Statistical comparisons were made using paired t-tests and/or repeated measures analysis of variance (ANOVA) with post hoc analysis performed using Tukeys HSD. Incidence data were analyzed using a χ^2 analysis. $p \leq 0.05$ was considered significant.

RESULTS

Neuronal Characteristics

A total of 227 dorsal horn neurons responsive to UBD were characterized: 117 in the Neonatal Anesthesia Group and 110 in the Neonatal Zymosan Group. They were further subdivided into adult treatment subgroups and according to their neuronal classification. Characteristics of these neurons are described in Table 1. Approximately half of the neurons studied were inhibited by HCNS and so were classified as Type I neurons; the other half were classified as Type II neurons. All neurons characterized had a convergent cutaneous receptive field: Type I neurons were more likely to be of the Class 2 type (excited by noxious and non-noxious cutaneous stimuli) than Type II neurons which were more likely to be of the Class 3 type (excited only by noxious cutaneous stimuli). There were no significant differences in cutaneous receptive field characteristics between the Neonatal Anesthesia or Neonatal Zymosan groups. There were also no significant differences in the depth below cord dorsum of electrode penetration between any groups. All-in-all the neurons characterized in this study are similar to those of previous studies [19].

Effects of Adult Acute Bladder Inflammation in the Neonatal Anesthesia Group

There was a differential response of acute bladder inflammation on the Type I versus Type II neurons. The AnZy group had a significantly higher rate of Spontaneous Activity in Type II neurons (15.5 ± 2.1 Hz) when compared with the Spontaneous Activity of Type II neurons in AnAn rats (7.3 ± 1.3 Hz; difference $p < 0.05$). This is in contrast to the lower rate of Spontaneous Activity of Type I neurons in AnZy rats (2.9 ± 0.6 Hz) when compared with Type I neurons in AnAn rats (9.6 ± 1.5 Hz; difference $p < 0.01$). Evoked Responses to a 60 mm Hg, 20s UBD stimulus in this same sample of neurons followed the same pattern with greater activity in Type II neurons in AnZy rats (15.5 ± 1.5 Hz) than in AnAn rats (10.7 ± 2.2 Hz; difference $p < 0.05$) and lesser activity in Type I neurons in AnZy rats (8.5 ± 1.4 Hz) than in AnAn rats (18.0 ± 2.2 Hz; difference $p < 0.05$). Complete stimulus-response functions in a smaller sample of Type I and Type II neurons (Figure 1A&B; statistics in legend) suggest that the effects of acute bladder inflammation on neuronal responsiveness were present at a wide range of stimulus intensities.

Effects of Adult Acute Bladder Inflammation in the Neonatal Zymosan Group

In contrast to the neurons studied in the Neonatal Anesthesia group, there was no differential effect of acute bladder inflammation on the Type I versus Type II neurons. Namely, ZyZy rats had significantly higher rates of Spontaneous Activity in both Type I and Type II neurons (6.2 ± 1.0 Hz & 21.1 ± 2.5 Hz respectively) when compared with the Spontaneous Activity of Type I and Type II neurons in ZyAn rats (3.6 ± 0.7 & 8.3 ± 1.1 respectively; differences $p < 0.01$ for both comparisons). Notably, the Spontaneous Activities of the Type I neurons in both subgroups of the Neonatal Zymosan group rats differed significantly from the corresponding adult treatment subgroups in the Neonatal Anesthesia group (ZyZy>AnZy & ZyAn<AnAn; $p < 0.01$ for both comparisons) indicating the presence of baseline differences produced by the neonatal treatments in addition to altered effects of inflammation noted as adults. Similar baseline differences in neuronal activity produced by neonatal treatments has been noted in gastrointestinal pain model systems [1].

Evoked Responses to a 60 mm Hg, 20s UBD stimulus in this same sample of neurons followed the same pattern with greater responses in both the Type I and Type II neurons in ZyZy rats (14.3 ± 3.3 Hz and 18.7 ± 2.6 Hz respectively) than in ZyAn rats (5.8 ± 0.9 Hz and 10.0 ± 1.7 Hz respectively; difference $p < 0.05$ for both Type I and Type II neurons). Complete stimulus-response functions in a smaller sample of Type I and Type II neurons suggest that the effects of bladder inflammation on neuronal responsiveness were present at a wide range of stimulus intensities (Figure 1C&D; statistics in legend). Similar to the Spontaneous Activities, there

were significant differences in the Evoked Responses of Type I neurons in the Adult Anesthesia subgroups in the two different neonatal treatment groups (AnAn>ZyAn; $p < 0.01$) again giving evidence for baseline effects produced by early-in-life treatments.

DISCUSSION

The most important finding of the present study is the demonstration of a change in the phenotypic response of Type I neurons to acute bladder inflammation in adult rats which had experienced neonatal bladder inflammation. This finding gives a neurophysiological basis to observations of hypersensitivity in adult rats which experienced neonatal treatments. In the case of neonatal bladder inflammation, it is a correlate to the highly augmented visceromotor and cardiovascular reflex responses to UBD in adults when a second inflammatory event occurs as an adult [24]. Namely, the augmentation of reflex responses is likely produced by the overall increase in spinal neuronal activity that occurs due to the alteration in the effect of acute adult inflammation on Type I neuronal responses. In control rats, adult bladder inflammation leads to reduced Type I neuronal responses, whereas in neonatally bladder-inflamed rats, adult bladder inflammation leads to increased responses. Part of this augmentation is likely due to the inability of neonatally bladder-inflamed rats to engage a reactive opioidergic inhibitory system affecting spinal neurons that is normally activated by acute bladder inflammation. Such an inhibitory system (and its associated deficiency in neonatally bladder-inflamed rats) has been demonstrated using reflex responses to UBD [4]. Although effects of acute inflammation have been examined in other bladder-related neuronal systems [14,20], the present study is the first report of the neurophysiological effects of neonatal bladder inflammation and the effects that such inflammation may have on an organism's future ability to compensate for second exposure to inflammation later in life. It is unknown whether this lack of engagement of an inhibitory system is focal to the sensory structures which were affected by the neonatal inflammation or whether it is a more global effect that might alter the activity of all neurons that are normally inhibited by HNCS. The concept of a localized effect rather than a global one is supported to some extent because the inhibitory effect of a HNCS on Type I neurons was not wholly abolished as these neurons could be still classified according to the inhibitory effect of a distant noxious pinch. To determine this definitively would require extensive experiments that are outside the scope of the present investigation. The result of an impaired inhibitory system in the present urological model is an overall increase in spinal neuronal activation by primary afferent input from the bladder. It seems logical that this would culminate in magnified reflex responses to UBD that have been noted in previous studies [24]. Comparison of baseline Spontaneous Activity and Evoked Responses of Type I neurons in the ZyAn and AnAn groups suggests that adult acute inflammation may not appear to engage an inhibitory system because that system may already be partially engaged. As a consequence, that system is functionally impaired because of an inability to increase further. Ruda et al [25] has demonstrated reduced reflex responses in rats at baseline in adults treated as neonates with paw inflammation. Notably, their observed baseline hypoalgesic state became a hyperalgesic state following re-inflammation of the paw. A similar phenomenon could be occurring in this present visceral model system.

Impaired inhibitory antinociceptive systems have been proposed as one of the mechanisms of chronic pain [5] with demonstrated alterations in the pain-inhibiting effects of HNCS in irritable bowel syndrome, fibromyalgia, temporomandibular disorder, chronic fatigue syndrome and headache [8,11,18,26]. The results of the present study would suggest that alterations in inhibitory systems association with sensory processing might also be present in painful bladder disorders such as interstitial cystitis (IC).

The present study also gives further evidence that at least two populations of spinal dorsal horn neurons exist which encode for visceral stimuli and which likely serve differing sensory

functions including the perception of pain (nociception). It strongly suggests that an effect of neonatal inflammatory events is to alter the balance of activity within these neuronal populations thereby altering the flow of information into higher centers of processing within the central nervous system. IC has been highly associated with childhood urological “problems” such as infection [9] and so the neurophysiological changes noted in the present study may have a correlate in human disease where an early-in-life inflammatory event has been associated with adult hypersensitivity.

The present findings set the stage for future neurophysiological studies investigating the interaction of early-in-life visceral inflammatory events and the inhibitory antinociceptive systems that modify visceral sensations. Future investigations will be able to probe the effects early-in-life events on other endogenous pain control systems, such as those activated by stress or on central facilitatory mechanisms. These model systems may prove useful for the investigation of novel pharmacotherapy that would restore impaired antinociceptive systems or to suppress chronic pronociceptive mechanisms and so may prove useful in the treatment of human disease.

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Figure 1.

Stimulus-response functions relating group mean activity ($\pm$ SEM) of L6-S1 dorsal horn neurons excited by UBD in Neonatal Anesthesia group rats (A,B; circular symbols) and Neonatal Zymosan group rats (C,D; experienced neonatal bladder inflammation; square symbols) which were subsequently treated as adults one day prior to testing with either intravesical zymosan (filled symbols – adult inflamed bladders) or with anesthesia alone (open symbols – adult non-inflamed bladders). Arrows in each figure indicate effect of the adult bladder inflammation treatment. Neurons were stratified according to the effect of heterosegmental noxious conditioning stimuli (HNCS): Type I neurons (A,C) were inhibited by HNCS and Type II neurons (B,D) were not inhibited by HNCS. In the Neonatal Anesthesia group rats, there was a differential effect of adult zymosan treatment on Type I versus Type II neurons with a robust increase in Evoked Responses of Type II neurons and a statistically significant decrease in Evoked Responses of Type I neurons. An ANOVA revealed significant overall treatment effects [$F(1,46) = 8.859$; $p=0.005$ for Type I neurons; $F(1,45) = 4.454$, $p=0.04$ for Type II neurons] and no significant treatment $\times$ UBD pressure interactions. $N=23-25$ /group. In contrast, in the Neonatal Zymosan group rats, there was no differential effect of adult zymosan treatment on neuronal subgroups with robust increases in Evoked Responses of both

Type I and Type II neurons. An ANOVA revealed a significant overall treatment effect [$F(1,39) = 8.810$, $p=0.005$ for Type I neurons; $F(1,36) = 8.339$, $p=0.007$ for Type II neurons] and no significant treatment x UBD pressure interactions. $N=15-21/\text{group}$. * ($p<0.05$) and ** ($p<0.01$) indicate significant post-hoc comparisons of mean Evoked Responses.

Table 1

L6-S1 Spinal Dorsal Horn Neuronal Characteristics

GROUP ¹	NEONATAL TREATMENT	ADULT TREATMENT	NEURONAL CLASS ²	N	MEAN DEPTH ³	CUTANEOUS FIELD (1:2:3) ⁴
Zy/Zy	Zymosan	Zymosan	Type I	27	0.47 ± 0.06	0:22:5
Zy/Zy	Zymosan	Zymosan	Type II	30	0.35 ± 0.05	0:11:19
Zy/An	Zymosan	Anesthesia	Type I	30	0.52 ± 0.06	0:25:5
Zy/An	Zymosan	Anesthesia	Type II	23	0.52 ± 0.06	0:10:13
An/Zy	Anesthesia	Zymosan	Type I	28	0.55 ± 0.06	0:25:3
An/Zy	Anesthesia	Zymosan	Type II	30	0.52 ± 0.06	0:16:14
An/An	Anesthesia	Anesthesia	Type I	29	0.37 ± 0.05	0:29:0
An/An	Anesthesia	Anesthesia	Type II	30	0.46 ± 0.05	0:15:15

¹ Treatment Group designation indicates Neonatal Treatment as first letter and Adult Treatment as second letter

² Type I neurons are inhibited by heterotopic noxious conditioning stimuli (HNCS); Type II neurons are not inhibited by HNCS

³ Mean depth of electrode penetration (mm) below cord dorsum at site of neuron recording

⁴ Number of neurons in the following 3 Classes (1:2:3); Class 1 neurons are excited by non-noxious stimuli only; Class 2 neurons are excited by noxious and non-noxious stimuli; Class 3 neurons are excited only by noxious stimuli