Application of Nucleus Pulposus to L₅ Dorsal Root Ganglion in Rats Enhances Nocticeptive Dorsal Horn Neuronal Windup

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Cuellar, J. M., P. X. Montesano, J. F. Antognini, and E. Carstens. Application of nucleus pulposus to L₅ dorsal root ganglion in rats enhances nociceptive dorsal horn neuronal windup. J Neurophysiol 94: 35–48, 2005; doi:10.1152/jn.00762.2004. Herniation of the nucleus pulposus (NP) from lumbar intervertebral discs commonly results in radiculopathic pain possibly through a neuroinflammatory response. NP sensitizes dorsal horn neuronal responses, but it is unknown whether this reflects a central or peripheral sensitization. To study central sensitization, we tested if NP enhances windup—the progressive increase in the response of a nociceptive spinal neuron to repeated electrical C-fiber stimulation—a phenomenon that may partly account for temporal summation of pain. Single-unit recordings were made from wide dynamic range (WDR; n = 36) or nociceptive-specific (NS; n = 8) L₅ dorsal horn neurons in 44 isoflurane-anesthetized rats. Subcutaneous electrodes delivered electrical stimuli (20 pulses, 3 times the C-fiber threshold, 0.5 ms) to the receptive field on the hindpaw. Autologous NP was harvested from a tail disc and placed onto the L₅ dorsal root ganglion after recording of baseline responses (n = 22). Controls had saline applied similarly (n = 22). Electrical stimulus trains (0.1, 0.3, and 1 Hz; 5-min interstimulus interval) were repeated every 30 min for 3–6 h after each treatment. The total number of evoked spikes (summed across all 20 stimuli) to 0.1 Hz was enhanced 3 h after NP, mainly in the after-discharge (AD) period (latency >400 ms). Total responses to 0.3 and 1.0 Hz were also enhanced at ≥60 min after NP in both the C-fiber (100- to 400-ms latency) and AD periods, whereas the absolute windup (C-fiber + AD – 20 times the initial response) increased at ≥90 min after treatment. In saline controls, windup was not enhanced at any time after treatment for any stimulus frequency, although there was a trend toward enhancement at 0.3 Hz. These results are consistent with NP-induced central sensitization. Mechanical responses were not significantly enhanced after saline or NP treatment. We speculate that inflammatory agents released from (or recruited by) NP affect the dorsal root ganglion (and/or are transported to cord) to enhance primary afferent excitation of nociceptive dorsal horn neurons.

INTRODUCTION

Intervertebral disc disease is a common cause of radiculopathy, which can result in severe pain and suffering. In addition, disc pathologies may be an important generator of low back pain (Toyone et al. 2004), a major source of disability that requires considerable health care resources. Although mechanical nerve root compression by a herniated disc may be an important factor in the generation of inflammation (Garfin et al. 1991; Kobayashi et al. 2004a,b; Olmarker et al. 1989, 1995) and radiculopathy (Hu 1998; Kawakami 1994a,b; Song et al. 1999; Tabo 1999; Winkelstein et al. 2002), many recent studies have shifted the focus toward possible involvement of inflammatory (Igarashi et al. 2000; Kang et al. 1996; Kawakami et al. 2003; Kayama et al. 1998; Marshall et al. 1977; McCarron et al. 1987; Olmarker et al. 1993, 1998, 2001, 2003; Takada et al. 2004; Takahashi et al. 1996; Yabuki et al. 2001) or autoimmune (Bobeckho et al. 1965; Gertzbein et al. 1975; Habtemariam et al. 1996; Marshall et al. 1977; Naylor et al. 1975; Pennington et al. 1988; Takenaka et al. 1986) processes initiated by contact of nucleus pulposus (NP) with the nerve root and surrounding tissues. NP sensitizes primary afferent neurons when applied to the dorsal root ganglion (DRG) (Takebayashi et al. 2001), and NP applied to the nerve root or DRG sensitizes spinal dorsal horn neuronal responses (Anzai et al. 2002; Cuellar et al. 2004; Onda et al. 2003). It is not known, however, whether this sensitizing effect of NP on responses of dorsal horn neurons reflects a central sensitization, is secondary to a peripheral sensitization, or a combination of both.

Windup is the progressive increase in nociceptive neuronal responses to repeated C-fiber stimulation of constant intensity and is thought to result from the temporal summation of N-methyl-D-aspartate (NMDA)- and neurokinin-1 (NK-1)-receptor-mediated depolarizations of spinal neurons evoked by C-fiber input (Baranauskas et al. 1998; Davies et al. 1987; Dickenson et al. 1987; Herrero et al. 2000; Ji et al. 2003; Mendell et al. 1965; Suzuki et al. 2003). Windup might be the source of temporal summation of pain sensation that has been observed experimentally in humans (Price 1972; Staud et al. 2003), and its enhancement is thought to reflect central sensitization. For example, the dose of vincristine which causes pain and hyperalgesia in rats also enhances windup of spinal wide-dynamic range (WDR) neurons (Weng et al. 2003). In addition, carrageenan-induced inflammation enhances windup of C-fiber-evoked flexion reflexes (Herrero et al. 1996), which has been used extensively for assessment of spinal neuronal excitability (Herrero et al. 1991, 1993; Laird et al. 1995; Woolf et al. 1991). We therefore utilized the phenomenon of windup to study the possibility that NP applied to the L₅ DRG in rats induces central sensitization of L₅ spinal dorsal horn neuronal responses, which we hypothesize would be reflected by enhanced windup.

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METHODS

Animals

Forty-four adult male Sprague-Dawley rats weighing 458 ± 41 (SD) g (Harlan, San Diego, CA) were used. The experimental procedures were approved by the UC Davis Animal Use and Care Advisory Committee. Rats were housed in a room with controlled temperature (22 ± 1°C) and lighting (lights on from 0800 to 2000 h), with food and water ad libitum.

Surgery

Rats were anesthetized with isoflurane 3–4% delivered in a balance of oxygen at 1 l/min in a chamber and then moved to mask anesthesia (isoflurane 2–2.5%) during surgery. Isoflurane concentration was adjusted as needed so that a strong tail or paw pinch failed to evoke a withdrawal response. A tracheostomy tube was implanted, the jugular vein was cannulated with PE-50 tubing for fluid delivery, and wound clips were used to close the incision. The carotid or femoral artery was cannulated in some animals for monitoring of arterial blood pressure. The L₅-S₁ intervertebral space was identified by palpation of the spinous processes and the posterior superior iliac spines, and a midline skin incision was made from S₁ to ∼L₅ spinous processes.

L₅ dorsal root ganglion exposure

The paraspinal muscles were dissected free from the L₅-L₆ spinous processes on both sides, and the transverse processes were exposed by scraping off attached connective tissue. The L₅ spinous process was cut and removed, and a partial (caudal) left side L₅ laminectomy was performed under a dissection microscope with microrongeurs. The left L₆ inferior articular facet was removed, but the superior articular process of L₆ was left intact to minimize DRG trauma and bleeding. This procedure allowed visualization of most of the dorsal side of the left L₆ DRG. The ligamentum flavum was removed between L₅ and L₆ on the left side, but the dura was left intact. Any bleeding was swabbed with clean cotton, and the site was filled with sterile saline to prevent desiccation.

L₅ spinal cord exposure

The midline incision was extended to the T₁₁ spinous process. The T₁₂-L₁ spinous processes were exposed as described above. L₁ and T₁₃ spinous processes were cut and removed, and a bilaminatec- tomy was performed at both levels under a dissection microscope with microrongeurs.

Coccygeal disc exposure

A coccygeal disc at the base of the tail was exposed by removing a square section of skin ∼10 mm in length and 7 mm in width on the dorsal surface of the tail (Cuellar et al. 2004). Connective tissue overlaying the disc was removed until the outermost surface of the annulus fibrosus was visible. Cotton was placed on the isolation site until incision and nucleus pulposus harvest at a later time.

Recording and unit characterization

The animal was placed in a stereotaxic frame with vertebral clamps on T₁₂ and L₁ vertebral bodies and S₁ spinous process. The dura overlaying the exposed spinal cord was opened with fine forceps and microscissors, and warm agar was placed over the site to a depth of 2–3 mm to prevent cord movement on respiration and desiccation of the tissue. Core body temperature was monitored rectally using a thermometer (BAT-12, Physitemp, Clifton, NJ) and maintained at 37 ± 0.2°C with a lamp and heating pad. Anesthesia was maintained by delivery of 1.1–1.2% isoflurane (∼0.9 MAC; minimum alveolar concentration required to prevent movement in response to a noxious stimulus) in a balance of oxygen, and was always kept constant for the duration of the experiment. The animal was ventilated using a positive-pressure pump (Harvard Apparatus, Holliston, MA) and end expired pCO₂ was monitored by an Ohmeda Rascal II gas analyzer (Helsinki, Finland) and maintained between 30 and 40 mmHg by adjustment of respiratory rate and/or tidal volume. The animal was often paralyzed (Pancuronium bromide, 0.2–0.3 ml of 1 mg/ml iv; Baxter, Deerfield, IL) to reduce respiration against the ventilator if present. In no case was the end tidal isoflurane <1%, a concentration well above that which suppresses consciousness (Dutton et al. 2001).

An 8- to 11-MΩ teflon-coated tungsten microelectrode (FHC, Bowdoinham, ME) was advanced into the dorsal horn of the spinal cord using a hydraulic microdrive (Kopf Instruments, Tujunga, CA) to record single-unit activity of dorsal horn neurons. Action potentials were amplified and displayed by conventional means and recorded (along with ECG and blood pressure when monitored) using a Pwerlab interface and Chart software (AD Instruments, Grand Junction, CO). Spike software (Forster et al. 1990) was used on a second computer for action potential display and data back-up recording. Data were analyzed off-line using Chart 5, and a Microsoft Excel macro was used to count spikes and subdivide windup responses into the various time windows.

Single units were searched for and isolated using innocuous mechanical stimulation of the plantar surface of the ipsilateral hind paw. Units isolated for study were always at depths <1 mm. Only units with receptive field areas corresponding to the L₅ spinal cord were chosen, based on prior dermatomal mapping studies (Takahashi et al. 1994, 1995, 1996). Of these, only units that responded to graded nonnoxious (brushing, 4–12 g von Frey) and noxious (76 g von Frey, pinch; WDR neurons) or to only noxious (nociceptive-specific; NS neurons) mechanical stimuli were considered for further study. Cells were further tested with constant-current electrical stimulation using an S48 stimulator (Grass, West Warwick, RI) and stimulus isolation unit with constant current output (Grass model PSIU6), administered by subcutaneous needle electrodes inserted within the receptive field area. Only units exhibiting a reproducible discharge occurring 100–400 ms (“C-fiber latency”) (Li et al. 1999) after the stimulus were studied further. The C-fiber threshold was determined by delivering an ascending series of paired electrical pulses (∼1-s interval between pulses, ∼3 s between pairs) at 1- to 2-V intervals. The intensity that evoked at least one spike within the C-fiber latency range in one of the two trials was considered the C-fiber threshold (Suzuki et al. 2003). A 0.3-Hz stimulus train (15–20 pulses; 0.5-ms pulse duration) was delivered at three times the C-fiber threshold (Dickenson et al. 1987; Flatters et al. 2003; Li et al. 1999) to assess windup. The cutaneous mechanical receptive field area was mapped using von Frey filaments with bending forces of 4, 12, and 76g in ascending order (Tabo 1999) and recorded onto a standardized paper template of the rat hind paw. Pretreatment responses of the unit to a von Frey filament stimulation series (4, 12, and 76g; 10-s duration, 1.5-min interstimulus interval) and pinch with blunt forceps (10 s) were recorded. This was followed by an electrical stimulation series consisting of either two or three stimulus trains (20 pulses; 5-min interval between each train): 0.1, 0.3, and 1.0 Hz (n = 22), 0.1, and 0.5 Hz only (n = 22). The order of stimulus frequencies was held constant as stated, whereas the stimulus protocol administered was randomly selected. The von Frey stimuli were delivered to the center of the receptive field area, whereas the electrical stimulation needles were placed on either side of the center.

NP harvest and application

While holding the single unit, the L₅ DRG was rinsed with sterile saline and gently swabbed dry so that accurate placement of the treatment substance could be performed. The annulus fibrosus of the coccygeal disc exposed earlier was incised, and the NP was removed. The treatment was randomly assigned at this time so as to avoid cell
selection bias. The animal received either of the following treatments: 1) NP group (n = 22), -2 mg autologous NP freshly harvested from the animal was carefully placed onto the L5 DRG; 2) control group (n = 22), sterile saline-soaked gel-foam that was approximately equivalent in mass and volume to NP was placed onto the L5 DRG. The stimulus series described above was performed, and this procedure was repeated at 30-min intervals for ≥3 h. On completion of the experiment, the animal was killed by an overdose of intravenous pentobarbital sodium.

Data analysis

ELECTRICAL STIMULATION. We considered action potentials arriving at a latency of 0–100, 100–400, and 400–1,000 ms to be in the A-fiber, C-fiber, and AD range, respectively, consistent with previous studies of dorsal horn neuronal windup in the rat (Dickenson et al. 1987; Flatters et al. 2003; Li et al. 1999; Svendsen et al. 1999b). Absolute windup was calculated as the total train response minus 20 times input, where input equals the number of action potentials evoked by the first stimulus (Chapman et al. 1994; Svendsen et al. 1999a). The evoked action potentials occurring during the C-fiber and AD range (100–1,000 ms) were combined for this calculation (Flatters et al. 2003; Suzuki et al. 2003; You et al. 2003). For analysis of the responses to 0.1- and 0.3-Hz stimulation, additional AD periods were also quantified: 1–3.33 s for both frequencies and 3.33–10 s for 0.1-Hz stimulus trains. However, these additional periods were not included in the absolute windup calculations so that an equivalent comparison could be made across all three frequencies.

MECHANICAL (VON FREY) STIMULATION. Responses to von Frey stimuli were quantified by summing the total number of action potentials recorded during the 10-s stimulation period (termed “response”) and the 30 s after the offset of the stimulus (termed AD). Responses to electrical and mechanical stimulation were plotted versus stimulus number and force, respectively, and compared over time for each treatment group. Responses from WDR neurons only were used for analysis of 4 and 12 s from the stimulus trains, whereas the remaining 13 and 11 animals in the NP- and saline-treated groups, respectively, were tested with 0.1 and 0.3 Hz only. The effect of time on windup responses to 0.1 and 0.3 Hz was not significantly different between the groups stimulated with all three versus only two frequencies. Therefore the two groups of NP-treated animals were pooled, as were the two saline-treated groups.

Electrical stimulation

Figure 1A provides an example of raw data recorded during 0.1-Hz electrical stimulation pre-NP to the 1st, 4th, 8th, 12th, and 16th electrical stimulus. The annotated scale above the time scale, at the bottom, delineates the latency windows used for quantification of A- and C-fiber–evoked responses as well as AD (AD1, AD2).

Saline group

Absolute windup values did not increase significantly over time during 0.3-Hz stimulation (Fig. 2, middle). There was no change in absolute windup pre versus postsaline during 1- (Fig. 2, right) or 0.1-Hz stimulation (Fig. 2, left). Figure 3 provides mean responses to 0.1-Hz electrical stimulation before and after saline treatment. There were no changes at any time-point postsaline. Figure 4 shows mean responses to 0.3-Hz stimulation. Although mean responses to 0.3-Hz stimulation increased during all latency windows (except A-fiber), these changes did not significantly differ from presaline baseline. Mean responses during 1-Hz electrical stimulation did not change significantly over time (Fig. 5).

NP group

Before NP treatment, there was no absolute windup during 0.1-Hz stimulation (Figs. 1 and 2, left). Absolute windup increased from pre- to 180-min post-NP, but the increase was not significant. There was a significant increase in the total number of spikes [area under the curve (AUC)] during the 1–3.33 and 3.33–10 s AD latency windows 180 min after NP versus pretreatment (P < 0.005 for both latency windows; Fig. 6D). Mean AUC for the total response (0–10 s) during 0.1-Hz stimulation was enhanced only at 180 min after NP (P < 0.005; Fig. 6E).

Figure 7 provides mean responses during 0.3-Hz electrical stimulation before and after NP treatment. There was a significant increase in the mean AUC 90 (P < 0.05), 120 (P < 0.05), and 180 (P < 0.001) min after NP for the C-fiber (100–400 ms) latency window (Fig. 7B). The mean AUC was significantly increased during the 400–1,000 ms AD period 60 (P < 0.05), 90 (P < 0.005), 120 (P < 0.005), and 180 (P < 0.001) min after NP (Fig. 7C). Mean AUC was also significantly elevated 180 min after NP (P < 0.005) during the 1–3.3 s AD period (Fig. 6D). Mean AUC for the total response to 0.3-Hz (0–3.3 s) stimulation was significantly enhanced 60 (P < 0.05), 90 (P < 0.05), 120 (P < 0.05), 150 (P < 0.05), and 180 (P < 0.001) min after NP (Fig. 7E). There was a significant increase in absolute windup 120 (281 ± 54; P < 0.05), 150 (299 ± 52; P < 0.005), and 180 min (391 ± 65; P < 0.001) after NP compared with pre-NP (144 ± 20; Fig. 2, center). One unit in this group was followed >6 h after NP treatment. The responses continued to rise by small increments throughout the
entire recording period for the 0.1- and 0.3-Hz windup (unit not tested to 1 Hz; data not shown).

Figure 8 provides mean responses during 1-Hz electrical stimulation before and after NP. Mean AUC was significantly enhanced during the C-fiber latency window 60 (P < 0.05), 90 (P < 0.05), 120 (P < 0.005), 150 (P < 0.001), and 180 (P < 0.005) min after NP (Fig. 8D). There was a significant enhancement of absolute windup 90 (P < 0.005), 150 (P < 0.01), and 180 (P < 0.01) min after NP treatment (Fig. 2, right). The C-fiber latency response to the first stimulus in each electrical stimulation train was enhanced only during 1-Hz stimulation 60 (P < 0.05), 120 (P < 0.005), and 150 (P < 0.01) min after NP (Fig. 8).

**Mechanical (von Frey) stimulation**

There were no changes in the spontaneous activity or responses to graded von Frey stimuli after saline treatment. In the NP group, there was a numeric increase in mean response to the 12 g von Frey stimulus [288.3 ± 67.3 (SE) spikes/10 s

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**FIG. 1.** Representative example of response to 0.1-Hz electrical C-fiber strength stimulation in a wide dynamic range (WDR) neuron and enhancement after nucleus pulposus (NP) application. A: vertically aligned raw tracings of responses (over 5 s) of a WDR unit’s responses to the 1st, 4th, 8th, 12th, and 16th (of 20) C-fiber strength electrical stimuli, applied to the ipsilateral hindpaw before application of NP. The annotated scale above the time scale, at the bottom, details the latency windows used for quantification of A-fiber, C-fiber, and afterdischarge responses [after-discharge (AD1): 400–1,000 ms; AD2: 1–3.3 s], but excludes the 3.33- to 10-s latency window. B: latency histograms (bin width: 10 ms) of each response shown in A. Note that there was little if any windup. C: responses of the same unit to the corresponding electrical stimuli shown in A, 2 h after application of NP to the L5 dorsal root ganglion (DRG). D: latency histograms as in B for the responses shown in C. Note the prominent windup post-NP predominantly within the AD1 and AD2 periods.
or responses to the graded von Frey stimuli after NP. There was no significant change in the mean spontaneous firing rate post-NP for C-fiber response only. Overall, however, there was no significant change in the mean spontaneous firing rate or responses to the graded von Frey stimuli after NP.

**DISCUSSION**

The following main observations were made in this study: 1) enhanced windup during 0.1-Hz electrical stimulation 180 min after NP treatment, but only of the late AD periods; 2) enhanced windup during 0.3-Hz electrical stimulation of the C-fiber and AD periods as soon as 60 min after NP treatment; 3) enhanced windup during 1-Hz electrical stimulation of the C-fiber and AD periods as soon as 60 min after NP treatment; 4) during only 1-Hz electrical stimulation, there was an upward shift of the C-fiber latency response curve and a significant enhancement of the C-fiber initial response; both occurring as soon as 60 min after NP treatment; 5) saline treatment induced a similar (although not statistically significant) pattern of enhancement over time only during 0.3-Hz electrical stimulation; 6) no change in responses to mechanical (von Frey) stimulation after either NP or saline treatment; and 7) no change in spontaneous firing.

Windup as a model of central sensitization

Electrical stimulation was performed at three times the threshold necessary to evoke dorsal horn neuronal action potentials at latencies consistent with those evoked by C-fibers. In using this method to study central sensitization, it must be assumed that the sensitivity of the primary afferent fibers being stimulated is irrelevant. This is a reasonable assumption because, at this intensity, all nerve fibers in proximity to the stimulation electrodes should be recruited, and this spatial recruitment should remain unchanged over time with constant current stimulation. However, this assumption might be violated if a developing peripheral sensitization increased the excitability of nociceptor fibers such that electrical stimulation recruited more fibers over time. If this were to occur, the increase in dorsal horn neuronal firing over time might reflect an increase or prolongation of firing of sensitized primary afferent neurons to successive stimuli. However, because windup of DRG neurons has not been shown to occur, sensitization of nociceptors in this scenario would be expected to cause an upward shift in the windup curve of dorsal horn neurons without a change in the slope, contrary to our observation of increased windup slope after NP in most cases (Figs. 6–8).

That no significant enhancements of windup of dorsal horn neurons were observed after application of saline to the DRG suggests windup is a valid model for testing the effects of sensitizing agents and that saline is an appropriate control. However, the observation that windup was modestly enhanced during 0.3-Hz stimulation after the first hour suggests that the initial windup stimulus may have induced a small amount of sensitization. This is consistent with previous studies suggesting windup may lead to signs of central sensitization (Cook et al. 1987; Li et al. 1999; Vatine et al. 1998; Woolf 1996; but see Svendsen et al. 1999c).

Central versus peripheral sensitization by NP

It was apparent that the AD period was the most significant contributor to windup, because very little windup of the C-fiber response was observed. Although some authors use only the C-fiber latency response to calculate windup (Li et al. 1999), whether explicitly stated or not, it is apparent that most calculate windup by combining the C-fiber and AD periods (Davies et al. 1987; Dickenson et al. 1987, 1990; Flatters et al. 2003; Passmore et al. 2003; Suzuki et al. 2001, 2003; Svendsen et al. 1999a; Urch et al. 2001; You et al. 2003). Furthermore, the AD was the most influenced time component during NP-induced sensitization. This was most pronounced at the lowest frequency of stimulation, where an enhancement was only observed during the late AD period (Fig. 6). This raises into question the significance of the AD period; is it a function of the slow removal of peptide neurotransmitters (e.g., substance P) from the synaptic cleft (De Koninck et al. 1991; Gerber et al. 1989, 1991; Radhakrishnan et al. 1991; Urban et al. 1984)? It is inter-
FIG. 3. Mean responses to 0.1-Hz electrical stimulation (3 times the C-fiber threshold; 0.5-ms pulses) of the receptive field area on the hindpaw before (open symbols) vs. 60 (left), 120 (middle), and 180 min (right) after (filled symbols) saline ($n = 22$). Each row contains data from a different latency window as indicated within the legend. A: A-fiber response (0–100 ms); B: C-fiber response (100–400 ms); C: after-discharge (AD; 400–1000 ms); D: after-discharge (AD; 1–3.3 s); E: total response (0–3.3 s). Error bars are SE. *$P < 0.05$; total number of spikes is significantly different from pretreatment (posthoc LSD test).
**FIG. 4.** Mean responses to 0.3-Hz electrical stimulation (3 times the C-fiber threshold; 0.5-ms pulses) of the receptive field area on the hindpaw before (open symbols) vs. 60 (left), 120 (middle), and 180 min (right) after (filled symbols) saline ($n = 22$). Each row contains data from a different latency window as indicated within the legend (format as in Fig. 3). Error bars are SE.
interesting to note that the cumulative depolarization thought to underlie windup does not occur at stimulation frequencies <0.2 Hz in vitro (Sivilotti et al. 1993), which is consistent with the lack of windup presently observed during 0.1-Hz stimulation in control animals. However, the observation that there was windup of the AD period during 0.1-Hz stimulation only after NP, with no increase in the response to the first stimulus, suggests that perhaps the NP-induced
sensitization involves or is caused by an alteration in the ability of the cell to repolarize within a normal time-course. This possibility would be consistent with central sensitization.

We also observed an upward shift in the stimulus C-fiber latency response curve during 1-Hz stimulation, which is consistent with an increase in general excitability of the dorsal horn neuron and/or with nociceptor sensitization. It may be that...
an enhancement of windup during dorsal horn neuronal sensitization is a result of increased release of peptides from sensitized primary afferent neurons (Baranauskas et al. 1998; Gardell et al. 2003; Urban et al. 1984) or interneurons synapsing with WDR and NS neurons.

It is also of interest that there was an increased initial response and an upward shift of the C-fiber latency response curve during only 1-Hz electrical stimulation. This might be partially explained by the experimental protocol—1 Hz was always tested last. Therefore the previous two stimulus trains...
FIG. 8. Mean responses to 1-Hz electrical stimulation (3 times the C-fiber threshold; 0.5-ms pulses) of the receptive field area on the hindpaw before (open symbols) vs. 60 (left), 120 (middle), and 180 min (right) after (filled symbols) saline ($n = 9$). Each row contains data from a different latency window as indicated within the legend (format as in Fig. 3 excluding AD 1–3.3 s). Error bars are SE. *$P < 0.05$, **$P < 0.005$; total number of spikes is significantly different from pretreatment (posthoc LSD test). #* $P < 0.05$, ###$P < 0.005$; initial C-fiber response is significantly different from pretreatment (posthoc LSD test).
could have augmented the sensitization of the cell caused by NP, but were inadequate to sensitize the responses of the saline-treated preparation.

**Mechanisms of action of NP**

Although the mechanism by which NP induces sensitization of spinal dorsal horn and primary afferent neurons is currently unknown, recent studies have provided evidence for cytokine involvement. Of the several cytokines and inflammatory mediators recently studied [i.e., tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1α, IL-1β, IL-8, transforming growth factor-β, etc.], TNF-α has been the most studied. TNF-α has been localized to NP cells (Olmarker et al. 1998), and selective blockers of TNF-α prevent NP-induced functional and structural (Olmarker et al. 1993) or behavioral changes (Olmarker et al. 2002, 2003). However, it is unclear whether the source of TNF-α or other cytokines is activated inflammatory cells, such as activated resident glial cells (Watkins et al. 2001), macrophages recruited to the injury site (Gronblad et al. 1994; Haro et al. 1996; Ito et al. 1996), or from the NP itself (Olmarker et al. 1998). Furthermore, the observation that doxycycline, which blocks the action of several cytokines, is more effective at preventing NP-induced neuropathy than specific TNF-α blockers of TNF-α suggests that other cytokines are likely to be involved (Olmarker et al. 1998).

That epidural application of NP to the DRG or nerve root can enhance dorsal horn neuronal responses on such a rapid time course is somewhat surprising but has been observed in other recent studies (Anzai et al. 2002; Cuellar et al. 2004; Onda et al. 2003). Although there is microangiographical evidence that large biomolecules may gain rapid access to the intraneuronal capillaries via small venules connecting to the epidural venous plexus (Byréd et al. 1995), we can only speculate the mechanisms by which responses of spinal dorsal horn neurons are enhanced within 1–2 h after application of NP to the L5 DRG some distance away. One possibility is that one or more cytokines in the NP gain rapid access to the spinal cord where it might act to sensitize dorsal horn neurons directly (Watkins et al. 2001). For example, TNF-α is rapidly (≤3 h) transported anterogradely from rat DRG to the spinal cord dorsal horn (Shubayev et al. 2002), where it may act at its receptor to affect signal transduction in neurons and glia (Vitkovic et al. 2000). TNF-α is also transported retrogradely (Shubayev et al. 2002), which could possibly reach and sensitize peripheral nociceptors. However, the relatively long transport distance to the periphery likely precludes this as a possible explanation for the present observations. If present in NP, the TNF-α protein may be able to insert itself into DRG cell membranes, forming a pH- and voltage-dependent cation (Na⁺) channel (Baldwin et al. 1996; Kagan et al. 1992), increasing nociceptor excitability. Another possibility is that cytokines act on DRG neurons via receptor-mediated mechanisms, increasing the excitability of primary afferent fibers, resulting in central sensitization due to increased peripheral input. For example, TNF-α rapidly (5–30 min) evokes transient Ca²⁺-dependent currents in cultured neonatal rat DRG neurons by a TNF-receptor-I and -2–p38 mitogen-activated protein kinase and c-Jun N-terminal kinase pathway (Pollock et al. 2002). Although these possibilities cannot be excluded in this study, we did not observe an increased spontaneous firing rate, arguing against the ectopic firing of primary afferent neurons (thought to be a source of neuropathic pain) as a possible explanation.

While responses to the 12 g von Frey stimulus increased numerically after NP, overall there was no significant change in graded mechanically evoked responses after NP. This result is largely consistent with our previous study (Cuellar et al. 2004) in which we showed that WDR neuronal responses to 12 g, but not 76 g, of von Frey stimuli were significantly greater at 2 h after NP. Responses to the 76 g stimulus were significantly enhanced at 3 h after NP in our prior study (Cuellar et al. 2004), a time-point that was not tested in this study.

The current finding of enhanced dorsal horn neuronal windup after exposure of the DRG to NP may partially explain the clinical manifestations of lumbar disc herniation-induced radiculopathy. For example, sufferers of radiculopathy often experience burning pain on sitting, which may continually escalate in its intensity until becoming intolerable, despite no change in the sensory stimulus over time (i.e., the pressure of sitting) and negligible change in intradiscal pressure on sitting from standing (Wilke et al. 1999). It is possible that, due to an enhanced dorsal horn neuronal excitability and windup response at low frequencies that do not normally elicit windup, the normally nonnoxious stimulus is inducing dorsal horn neuronal windup that is interpreted as pain and further enhances the dorsal spinal neuronal excitability to subsequent sensory input.

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