19. K. Scholich, A. J. Barbier, J. B. Mullenix, T. B. Patel, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2915 (1997).
20. S. Ohno, *J. Mol. Evol.* **44**, S23 (1997).
21. K. Ohmori, M. Hirose, M. Ohmori, *Plant Cell Physiol.* **33**, 21 (1992).
22. J. Felsenstein (Department of Genetics, University of Washington, Seattle, 1993).
23. M. Kasahara, K. Yashiro, T. Sakamoto, M. Ohmori, *Plant Cell Physiol.* **38**, 828 (1997).
24. M. Kasahara and M. Ohmori, *J. Biol. Chem.* **274**, 15167 (1999).
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Altered Nociceptive Neuronal Circuits After Neonatal Peripheral Inflammation

M. A. Ruda,* Qing-Dong Ling, Andrea G. Hohmann, Yuan Bo Peng, Toshiya Tachibana

Nociceptive neuronal circuits are formed during embryonic and postnatal times when painful stimuli are normally absent or limited. Today, medical procedures for neonates with health risks can involve tissue injury and pain for which the long-term effects are unknown. To investigate the impact of neonatal tissue injury and pain on development of nociceptive neuronal circuitry, we used an animal model of persistent hind paw peripheral inflammation. We found that, as adults, these animals exhibited spinal neuronal circuits with increased input and segmental changes in nociceptive primary afferent axons and altered responses to sensory stimulation.

Somatosensory development generally requires use-dependent activity during early postnatal times (*1*). However, noxious stimulation is normally absent or infrequent in the neonate. The early neonatal period is a time of great plasticity during which substantia gelatinosa neurons begin to develop their dendritic arbors, c-fibers are functionally immature, and there is as yet limited descending inhibitory modulation of spinal nociceptive neuronal circuits (*2*). The occurrence of persistent inflammation and pain during this period is likely to impact development across several critical time points. Medical procedures used in neonatal intensive care units often involve repeated and lengthy exposure to tissue injury where facial expressions, body movements, and physiological measures suggest a pain response (*3*, *4*) that may alter an individual's response to pain later in life (*5*, *6*).

To produce persistent inflammatory stimulation in newborn rat pups, we injected complete Freund's adjuvant (CFA) into the left hind paw (*7*). The pups exhibited distinct behaviors at the time of CFA injection that imply the presence of pain. These included immediate shaking and licking of the paw and occasional vocalization. The behavioral responses are identical to those seen in adult animal models of pain (*8*). Edema and erythema occurred shortly thereafter and persisted for 5 to 7 days. Injection of saline resulted in paw withdrawal but did not induce the exaggerated shaking or licking of the paw.

Spinal nociceptive neuronal circuits were examined by the selective uptake of wheat germ agglutinin–horseradish peroxidase (WGA-HRP) by a predominantly nociceptive population of unmyelinated and finely myelinated primary afferent axons in the sciatic nerve (*9*). Adult rats that experienced unilateral persistent hind paw inflammation starting on postnatal day one (P1) (n = 10) exhibited ipsilateral increases in the density of WGA-HRP–labeled dorsal horn primary afferents. The increase in laminae I and II labeling was strikingly apparent when afferents from the left and right sciatic nerves were viewed in the same tissue section (Fig. 1, A to D). This side-by-side comparison controlled for possible labeling differences related to animal variability in fixation and timing of the histochemical reaction. Motoneuron labeling, an indicator of comparable sciatic nerve uptake of the tracer, was similar on both sides (see Web fig. 1) (*10*).

Several spinal segments exhibited an increase in density of labeling on the treated as compared with the untreated side. The increase was greatest in caudal segments (Fig. 1, C and D). The most rostral lumbar segments to receive sciatic afferents exhibited the least change in terminal density (Fig. 1A). Quantification (*11*) of the density of WGA-HRP labeling identified a relative increase of 12, 23, and 20% in the L4-5, L5-6, and L6-S1 segments, respectively (Fig. 1E). In addition, a segmental difference in the location of labeled afferents was observed. WGA-HRP–labeled sciatic afferents on the neonatal treated side extended from spinal segments L2 through S1, whereas on the untreated side, these afferents extended from segment L2 only through the juncture of L5 and L6 (Fig. 1F). A similar increase in primary afferent labeling was found in adult rats that received a hind paw CFA injection on either P0 (n = 2) or P3 (n = 3). However, in rats that received a left hind paw CFA injection on postnatal day 14 (P14) (n = 3) (Fig. 2B), the adult distribution of WGA-HRP–labeled sciatic afferents was comparable on the left and right sides of the dorsal horn and did not differ from that of the untreated neonates (Fig. 2A).

To further characterize the population of dorsal root ganglia (DRG) neurons responding to neonatal inflammation (*12*), we used immunohistochemical labeling of the neuropeptide calcitonin gene–related peptide (CGRP) to identify the terminals of neurons that express the nerve growth factor (NGF) receptor, trkA. Isolectin B4 (IB4) binding was used to mark small-diameter DRG neurons that do not express trks (*13*). A distinct increase in staining for CGRP was observed in the dorsal horn on the neonatal treated side (Fig. 2C). No difference was seen in the IB4 labeling pattern on the treated and untreated sides of the spinal cord (Fig. 2D).

β-HRP was used to label nonnociceptive primary afferents that terminated in deeper spinal laminae. The central terminals of sciatic afferents showed comparable labeling of β-HRP on the neonatal treated and untreated side (Fig. 2, E and F) in adult rats that had received a hind paw CFA injection on P1 (n = 5).

The behavioral response to noxious thermal stimuli (*14*) was also studied. Comparable baseline withdrawal latencies were found with the left and right paws of neonatal treated or untreated rats (Fig. 3A). However, 24 hours after a unilateral injection of the inflammatory agent, CFA, into their left hind paws, there was a significant decrease in the paw withdrawal latency in the neonatal treated rats as compared with the untreated rats. Mean latencies were 2.63 ± 0.1 s and 3.13 ± 0.1 s for the treated and untreated groups, respectively (Fig. 3B).

Cellular Neuroscience Section, Pain and Neurosensory Mechanisms Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health (NIH), Bethesda, MD 20892, USA.

*To whom correspondence should be addressed. E-mail: maruda@dir.nidcr.nih.gov

We examined functional consequences of the altered spinal neuronal circuitry using a test of acute and tonic pain sensitivity (*15*). Adult behavioral responses to formalin were altered in the neonatal treated rats. The late phase of the formalin test occurred earlier in treated rats (average median ± SEM: 30.5 ± 1.7 and 37.1 ± 1.5 min for treated and untreated rats, respectively), as revealed by the leftward shift in the median of the late phase of pain behavior (Fig. 3C). This shift may be attributed to a reduction in active inhibition during the interphase and/or an earlier onset of neuronal activation. The area under the curve was similar for both groups (mean + SEM: 78.3 + 4.4 and 84.6 + 5.0 for adult neonatal treated and untreated rats, respectively).

The physiological responsiveness of single dorsal horn neurons was assessed (*16*). Adult neonatal treated rats exhibited increased evoked firing rates in response to brush and noxious pinch relative to the same intensities of stimulation applied to untreated rats (Fig. 3D). Spontaneous activity was also higher in adult neonatal treated rats. Neuronal responses to noxious pinch were maximally affected in the neonatal group ($P < 0.0002$). Also, there was a greater effect on the brush-evoked firing than spontaneous firing rates in the neonatal treated as compared with the untreated rats (*, $P < 0.003$).

These studies demonstrated that peripheral inflammation experienced during the neonatal period has long-standing consequences on nociceptive neuronal circuitry development. The activity evoked in an immature nervous system by peripheral inflammation may be uniquely interpreted, perhaps causing hyperexcitability and excitotoxicity. Neonatal rats that receive a subcutaneous injection of the acute excitotoxin capsaicin exhibit a loss of DRG neurons and a decrease in nociceptive primary afferents (*17*). This loss of afferents is in contrast to the increased primary afferent density found in our experiments and may be related to a difference in intensity and duration of nociceptor activity generated by the respective stimuli.

The changes in density and segmental distribution of sciatic nerve axons that innervate the neonatal inflamed hind paw are reminiscent of the axonal sprouting that occurs after peripheral nerve injury and

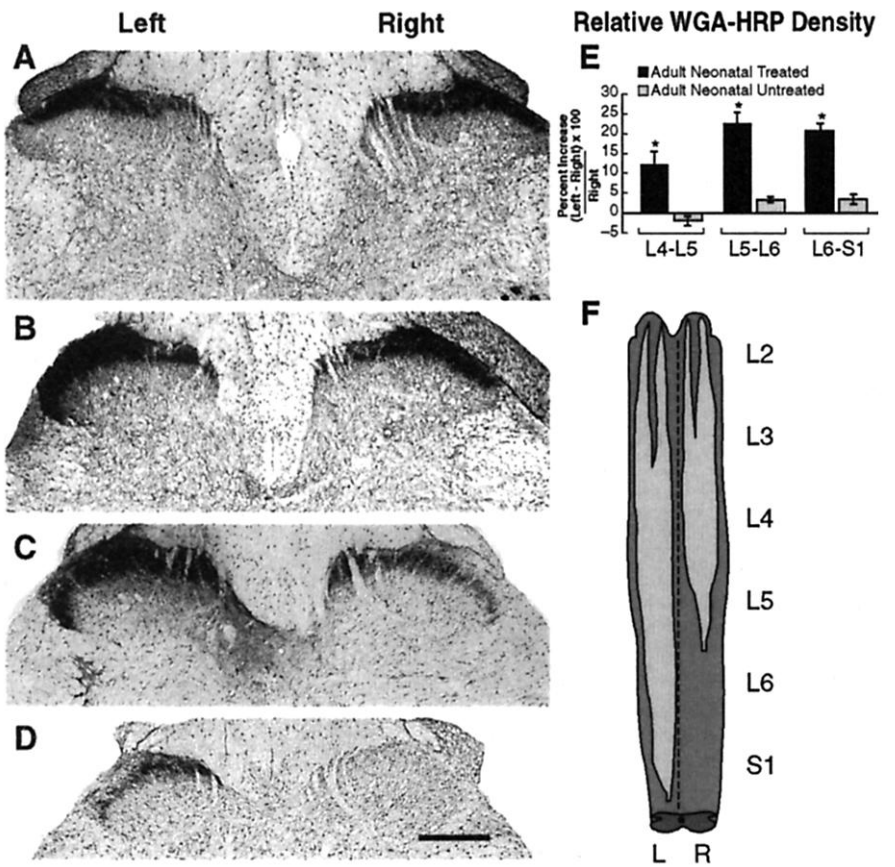

Fig. 1. Segmental comparison of WGA-HRP bilateral labeling of sciatic nerve primary afferents in adult rats exposed to peripheral inflammation of the left hind paw beginning on postnatal day 1 (P1). The greatest increase in density of terminal staining in the left dorsal horn was seen at L5 (**B**), L6 (**C**), and S1 (**D**) spinal segments, whereas the most rostral L4 segment (**A**) exhibited the least difference in labeling between the left and right sides. Also, on the left neonatal treated side, labeled sciatic afferents were found in more caudal sacral spinal segments than on the untreated right side (D). The relative density of adult neonatal treated WGA-HRP–labeled sciatic primary afferents (**E**) and untreated rats identified an increase in all segments of the treated as compared with untreated rats (*, $P < 0.0001$). The labeling on the left neonatal treated side extends further caudally than that seen on the right neonatal untreated side (**F**). Scale bar, 250 μm.

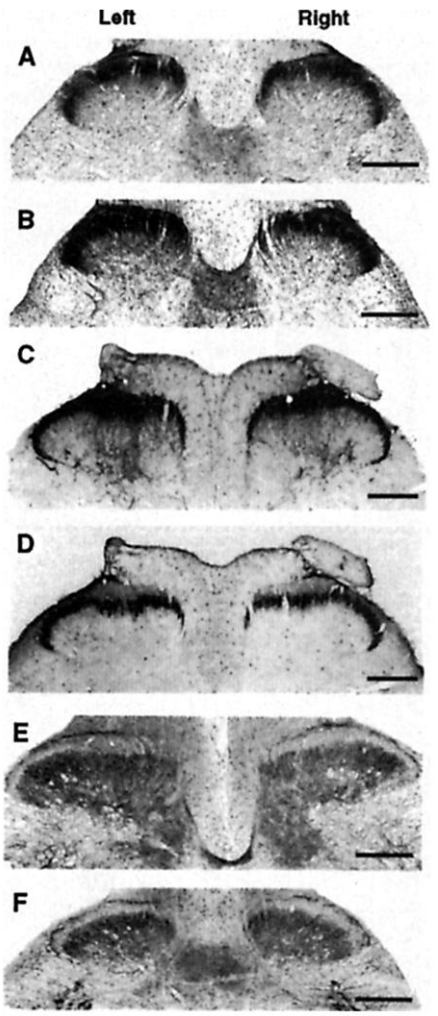

Fig. 2. (**A** and **B**) WGA-HRP bilateral labeling of sciatic nerve primary afferents in adult neonatal untreated rats (A) and those treated on P14 (B) exhibited a similar laminar distribution and density of labeled primary afferents on both sides of the L6 segment. CGRP immunoreactivity (**C**) and IB4 labeling (**D**) in the L5 spinal segment of adjacent sections of an adult rat that received an injection of CFA into the left hind paw on P1. CGRP immunoreactivity was increased in the middle of the dorsal horn on the left neonatal treated side, whereas IB4 labeling is comparable on the left and right sides. (**E** and **F**) β-HRP bilateral labeling of sciatic nerve primary afferents in adult rats exposed to peripheral inflammation of the left hind paw beginning at P1. Both sides of the spinal cord exhibited comparable β-HRP labeling in the L5 (E) and S1 (F) segments. Scale bars, 250 μm.

loss of afferent input (*18*, *19*). Because the normal sciatic termination area in the substantia gelatinosa of the neonate (*20*) is similar to that seen in the adult, the altered pattern observed in our studies suggests a sprouting of axons into new areas of the dorsal horn as opposed to a lack of "pruning" of axons related to novel activity during critical development times. This effect may result from the inflammation-induced release of peripheral growth factors such as NGF, which play a role in sensory neuron survival, growth of central terminals, and the terminal pattern in the periphery. In neonates, wounding of the skin increases NGF levels at the site (*21*). The dynamics of the peripheral inflammation in an immature immune system may alter the growth factor release pattern. The differential effect on the trk-expressing CGRP axons compared with the non–trk-expressing IB4 axons in this study suggests that trks may be related to the circuitry changes.

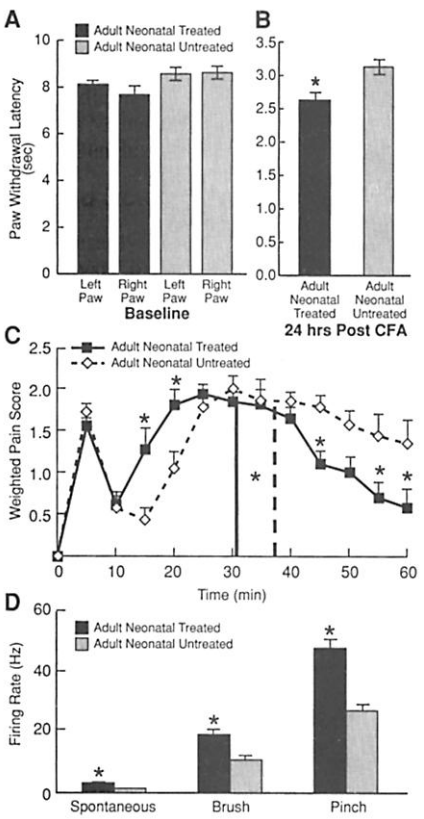

Fig. 3. Behavioral and physiological analysis. (**A**) Baseline paw withdrawal latency to a noxious thermal stimulus was comparable in the left and right hind paws of adult neonatal treated and untreated rats. (**B**) In the adult, 24 hours after CFA left hind paw injection, paw withdrawal latencies in the neonatal treated rats were decreased compared with the neonatal untreated rats (*, $P < 0.01$). (**C**) Adult neonatal treated rats showed an altered time course of formalin-induced pain relative to untreated rats (*, $P < 0.002$). Medians (vertical lines) revealed that the late phase of pain behavior occurred earlier in neonatal treated rats relative to untreated rats (*, $P < 0.02$). (**D**) Increased spontaneous activity and increased responsiveness to innocuous brushing and noxious pinch were observed in adult neonatal treated rats (*, $P < 0.05$).

Functional changes related to the altered neuronal circuits are an important consequence of neonatal inflammation. Enhanced responsiveness of dorsal horn neurons may translate into a permanent facilitated response to noxious stimulation. This response could be related to the increased density of nociceptive primary afferents that innervate the superficial dorsal horn laminae or to modified connections and altered cellular activity in neurons exposed to peripheral inflammation during the period of immature neuronal responses and reduced inhibitory control (*2*).

Advances in medical technology have substantially improved the chances for survival of medically compromised newborns through therapies that can cause tissue injury and pain. As shown in these experiments, peripheral inflammation in the neonate can result in lasting and potentially detrimental alterations in nociceptive pathways, which need further study.

References and Notes

1. J. R. Coleman, *Development of Sensory Systems in Mammals* (Wiley, New York, 1990).
2. M. Fitzgerald, *Br. J. Anaesth.* **75**, 177 (1995).
3. K. D. Craig, M. F. Whitfield, R. V. Grunau, J. Linton, H. D. Hadjistavropoulos, *Pain* **52**, 287 (1993).
4. K. J. S. Anand and P. J. McGrath, in *Pain in Neonates*, K. J. S. Anand and P. J. McGrath, Eds. (Elsevier, New York, 1993), pp. 1–18.
5. R. V. E. Grunau, M. F. Whitfield, J. H. Petrie, *Pain* **58**, 341 (1994).
6. A. Taddio, J. Katz, A. L. Ilersich, G. Koren, *Lancet* **349**, 599 (1997).
7. Sprague-Dawley male rat pups received a single injection of CFA (2:1, CFA:saline) or saline on either P0, P1, P3, or P14 (25 μl in P0, P1, and P3 rat pups and 50 μl in P14 pups) into the left hind paw, or they were untreated. The animals matured undisturbed. Procedures were approved by the National Institute of Dental and Craniofacial Research (NIDCR) Animal Care and Use Committee.
8. F. V. Abbott, K. B. J. Franklin, R. F. Westbrook, *Pain* **60**, 91 (1995).
9. Adult animals (8 to 12 weeks) were anesthetized with sodium pentobarbital, the sciatic nerves were exposed at midthigh, and WGA-HRP (250 μg) or β-HRP (30 μg) was microinjected bilaterally [C. C. LaMotte, S. E. Kapadia, C. M. Shapiro, *J. Comp. Neurol.* **311**, 546 (1991)]. Forty-eight hours later, they were anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The spinal cord was removed, postfixed, and transferred to 30% sucrose. Tissue was cut into 30-μm sections on a cryostat and processed with the tetramethylbenzidine method [M. M. Mesulam, *J. Histochem. Cytochem.* **26**, 106 (1978)]. Evaluation of labeling was performed blind. Sections were photographed onto Ektachrome 64T transparency film and scanned into Adobe Photoshop 5.0.
10. Web fig. 1 is available at www.sciencemag.org/feature/data/1051450.shl.
11. Tissue sections from the L4-5, L5-6, and L6-S1 spinal segments, six sections each, from neonatal treated ($n = 4$) and untreated rats ($n = 2$) were digitized (*9*) and analyzed with NIH Image 1.62. The relative density of labeling was measured in a 300 μm by 200 μm rectangle placed over the dorsal horn beginning at the dorsal root entry zone and continuing medially. The most caudal sections were not included in the analysis as the contralateral dorsal horn was devoid of labeling. To normalize the data, we measured background in lamina VII and subtracted it from the dorsal horn values. Relative density was graphed as the percentage of increase with the use of the formula left minus right side density divided by the right side density multiplied by 100. Data were analyzed with repeated measures analysis of variance (ANOVA).
12. At 8 weeks of age, neonatal CFA-treated rats ($n = 3$) were perfused and tissue sectioned (*9*). Adjacent sections were incubated in a rabbit polyclonal antibody to CGRP (1:200,000) or in biotinylated IB4 lectin (1 μg/ml). Labeling was visualized with standard avidin-biotin methodology with 0.05% 3,3′-diaminobenzidine tetrahydrochloride as the chromagen.
13. W. D. Snider and S. B. McMahon, *Neuron* **20**, 629 (1998).
14. At 8 to 10 weeks of age, baseline paw withdrawal latency of the left and right hind paw to a radiant heat source [K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, *Pain* **32**, 77 (1988)] was determined in neonatal treated ($n = 9$) and untreated ($n = 9$) rats. Withdrawal latency was tested again 24 hours after an injection of 200 μl of CFA (1:1) into the adult rat left hind paw for five times at intervals of 5 min. Latency was calculated as the mean excluding the first, familiarization trial and presented as means ± SEM. Significance was determined with ANOVA and a *t* test.
15. Pain behavior was assessed in adult neonatal treated ($n = 10$) and untreated ($n = 7$) rats after intraplantar injection of 1.5% formalin (50 μl) in the left hind paw. Weighted pain scores, representing the time spent licking, lifting, and favoring the injected paw, were quantified every 5 min for 60 min after injection (*8*). Data were analyzed by repeated measures ANOVA and Tukey post hoc tests. To quantify the shift in the time course of the response, we calculated the median of the late phase of pain behavior [the midpoint of the distribution from the local minimum (interphase) to the terminal observation interval] for each rat and subjected it to ANOVA.
16. Dorsal horn extracellular single-unit discharges were recorded in the lumbar enlargement of adult neonatal treated ($n = 5$; 120 neurons) and untreated ($n = 5$; 147 neurons) rats anesthetized with sodium pentobarbital, immobilized with pancuronium bromide, and mechanically ventilated. Spontaneous activity and activity evoked by nonnoxious brushing with a camel's hair brush and noxious pinch applied with an arterial clamp were assessed. Means of three stimulations (applied for 10 s at 10-s intervals) were calculated with Spike2 software. Data were analyzed by repeated measures ANOVA and Tukey post hoc tests.
17. S. H. Buck and T. F. Burks, *Pharmacol. Rev.* **38**, 179 (1986).
18. T. P. Doubell, R. J. Mannion, C. J. Woolf, *J. Comp. Neurol.* **380**, 95 (1997).
19. P. Shortland and M. Fitzgerald, *Eur. J. Neurosci.* **6**, 75 (1994).
20. M. Fitzgerald and J. Swett, *Neurosci. Lett.* **43**, 149 (1983).
21. J. Constantinou, M. L. Reynolds, C. J. Woolf, B. Safieh-Garabedian, M. Fitzgerald, *Neuroreport* **5**, 2281 (1994).

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