

# Age-dependent Responses to Nerve Injury—induced Mechanical Allodynia

Douglas G. Ririe, M.D.,\* James C. Eisenach, M.D.†

**Background:** Developmental differences in responses to acute and chronic nerve injury have received minimal attention. This study examines developmental differences in behavioral responses to a proximal (closer to the spinal cord) (L5 and L6 spinal nerve root ligation) or to a more distal (closer to peripheral innervation) (partial sciatic nerve ligation) nerve injury in rats paralleling the infant to young adult human.

**Methods:** Withdrawal thresholds to von Frey filament testing in the hind paw were determined before and various times after either spinal nerve root ligation or partial sciatic nerve ligation in rats aged 2, 4, and 16 weeks. Control rats of these ages were observed serially without surgery. Times for withdrawal thresholds to mechanical stimuli to return to 80% of that of the hind paw in the control animals were compared among the different ages in the two models.

**Results:** Baseline withdrawal thresholds in younger rats were lower ( $P < 0.05$ ). In the 2-week-old animals, distal injury partial sciatic nerve ligation did not cause a reduction in withdrawal threshold from baseline. This was different from the spinal nerve root ligation group and the older animals in the partial sciatic nerve ligation group. However, when compared with age-matched control animals, both nerve injuries resulted in reduced withdrawal thresholds ( $P < 0.05$ ). The resolution of hypersensitivity to mechanical stimulation, as measured by return of threshold to 80% of controls, occurred more quickly in 2-week-old than in 4- and 16-week-old animals in both injury models ( $P < 0.05$ ).

**Conclusion:** These data suggest that resolution of sensitization to A-fiber input occurs more rapidly in young animals. In addition, distal injury has less of a sensitizing effect on A-fiber input than proximal injury in the younger animals. The authors speculate that neuroimmune responses, especially at the site of injury, are developmentally regulated and less likely to produce chronic pain when injury occurs at a young age.

UNTIL only recently, there was debate over whether infants actually felt pain and whether there was any need to provide analgesia to them. It is now, however, generally accepted that infants and children do feel and respond to pain and benefit from alleviation of pain. Whereas interest in development of pain perception and modulation is growing, there is still limited knowledge on the physiology and pharmacology of pain and development. In particular, there is limited knowledge of the

role development plays in establishing and maintaining chronic neuropathic pain after injury to the peripheral nervous system.

Recently, using the paw incision model of acute pain, developmental differences in injury induced mechanical allodynia have been shown.<sup>1</sup> Allodynia in this model of postoperative pain resolved quicker in 2- and 4-week-old rats than in older ones. These results with a subacute model of pain and hypersensitivity suggest that responses to nerve injury leading to chronic pain may also vary with age.

Lumbar spinal nerve root ligation (SNL) as described by Chung is a well-established and characterized model of neuropathic pain,<sup>2</sup> and developmental aspects of this model have been probed.<sup>3</sup> In the 3-week-old rats, mechanical sensitivity developed to a similar extent as that seen in adult rats but dissipated more rapidly. To our knowledge, no other study has corroborated these results, nor has the role of location of nerve injury been investigated.

The pathophysiologic mechanism underlying neuropathic pain may differ depending on the site of injury. Previous studies have demonstrated differences in behavioral responses to the more peripheral injury of partial sciatic nerve ligation (PSL) compared with the more proximal SNL model.<sup>4-6</sup> Although both models involve direct injury and insertion of a foreign body (suture) around the nerve, the SNL has been shown to produce a more profound mechanical hypersensitivity.<sup>5</sup> There are differences in sympathetic fiber growth after the different injuries, which may play a role in the differential behavioral responses.<sup>6,7</sup> Furthermore, the dependence of the sympathetic growth is influenced by the growth factor production induced by the injury. The SNL model with a more proximal injury is different in that it is more uniform with all fibers in the L5-L6 nerve distribution being affected. This may deprive the cell body in dorsal root ganglia of a greater amount of the nerve axon, possibly reducing expression of mediators of pain distal to the injury, more akin to an avulsion-type nerve injury.<sup>8-10</sup> The PSL model, being a more peripheral injury and only part of the nerve, allows commingling of nerve fiber types of both injured and uninjured nerves, probably deprives the cell body of less traffic due to preservation of more axon, and may be more akin to peripheral nerve injury from trauma, surgery, or tumor. Because of the functional differences in these two models of neuropathic pain, there are differences in expression of both peripheral and central neurochemical mediators of pain making each model unique.<sup>8</sup> By studying the behav-

\* Associate Professor, † Professor, Department of Anesthesiology.

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Address correspondence to Dr. Ririe: Department of Anesthesiology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157-1009. dririe@wfbmc.edu. Individual article reprints may be purchased through the Journal Web site, [www.anesthesiology.org](http://www.anesthesiology.org).

ioral effects of age in both models, the features common to both, as well as differences between them, will allow better understanding of the role of age in response to these two types of nerve injuries.

The objectives of this study are to characterize the behavioral responses using mechanical thresholds in animals of different ages using two different models of chronic neuropathic pain and to evaluate developmental differences in responses to these injuries. We hypothesize that developmental differences in response to chronic pain beyond the neonatal period exist and result in altered behavioral responses with more rapid resolution of mechanical hypersensitivity in the younger animals.

## Materials and Methods

After review and approval from the Animal Care and Use Committee (Winston-Salem, North Carolina), male Sprague-Dawley rats were used to study the effects of lumbar SNL<sup>2</sup> or PSL on withdrawal thresholds.<sup>4</sup> Three groups of animals were studied using each model. Animals aged 2, 4, and 16 weeks representing preweanling, postweanling, and sexually mature animals were studied. Ten 2-week-old animals and eight 4- and 16-week-old animals were studied using each model of neuropathic pain.

### *Partial Sciatic Nerve Ligation*

After baseline testing, animals were anesthetized with 2% halothane and oxygen under spontaneous ventilation through a nose cone. As previously described,<sup>4</sup> the back was prepared in a sterile manner with a 10% povidone-iodine solution and draped. A No. 11 scalpel blade was used to make a linear incision over the left thigh from the sacrum perpendicular to the spine. Blunt dissection through the muscle planes was used to locate and reveal the sciatic nerve in its course to the lower extremity just proximal to the trifurcation. A 7.0 nylon suture on a BV-1 needle was used to tightly ligate the lateral third of the nerve. The fascia and skin were closed using 5.0 braided silk sutures with a running mattress stitch. The wound was covered with a mixture of polymyxin B, neomycin, and bacitracin ointment. After surgery, the animals were allowed to recover in their cages. The preweanling animals were allowed to recover in the cage with their mothers.

### *Lumbar Spinal Nerve Root Ligation Model*

After baseline testing, animals were anesthetized with 2% halothane and oxygen under spontaneous ventilation via a nose cone. As previously described,<sup>2</sup> the back was prepared in a sterile manner with a 10% povidone-iodine solution and draped. A No. 11 scalpel blade was used to

make a linear paravertebral incision. Blunt dissection through the muscle planes was used to locate the L5 and L6 nerve roots distal to their dorsal root ganglia. A small laminectomy was made in the 4- and 16-week-old animals to visualize these nerve roots. A laminectomy was not necessary to visualize these nerve roots in the 2-week-old animals. A 5.0, 6.0, or 7.0 braided silk suture (for the animals from oldest to youngest, respectively) was placed around the L5 and L6 nerve roots, and the nerve roots were ligated. After hemostasis with gentle pressure, the muscle layer was first apposed with a running mattress suture, and then the skin was apposed with a similar running mattress suture of 5.0 braided silk. The wound was covered with a mixture of polymyxin B, neomycin, and bacitracin ointment. After surgery, the animals were allowed to recover in their cages. For the preweanling animals, the animals were allowed to recover in the cage with their mother.

### *Withdrawal Threshold Testing*

Animals were placed in a plastic cage with a mesh floor. They were allowed to acclimate to the environment for 30 min before testing. Von Frey filaments were used to determine withdrawal threshold by placing the filament on the middle of the foot until the filament bent or the foot was withdrawn. The von Frey filaments used were 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, 5.18, 5.46, 5.88, 6.10, and 6.45, corresponding to 0.5, 0.9, 1.7, 3.7, 5.5, 8.0, 12.4, 21.5, 53.0, 72.0, and 129 g. This was done three times with a positive response determined by brisk withdrawal or lifting of the foot from the filament and a negative response being no movement. The threshold to withdrawal with a 50% probability was determined using the up-down method as previously described.<sup>11</sup> Mechanical withdrawal threshold was determined before surgery and at 24 h and every 7 days for a maximum of 14 weeks after surgery. This duration was determined by following up all animals until all animals returned to baseline thresholds for at least 1 week.

Withdrawal thresholds to mechanical stimulation are presented as mean  $\pm$  SE. The withdrawal thresholds ipsilateral to surgery were compared with the same hind paw for control animals that did not receive nerve injury. Time for withdrawal threshold to return to within 80% of that in age-matched controls was determined. These times to 80% recovery are presented as median with range and were compared across age groups using the Kruskal-Wallis test for overall differences followed by Wilcoxon rank sum test. Differences from baseline thresholds were compared using repeated-measures analysis of variance with the Fisher protected least significant difference. Multiple comparisons were accounted for using Bonferroni when necessary. Significance was considered at  $P < 0.05$ .

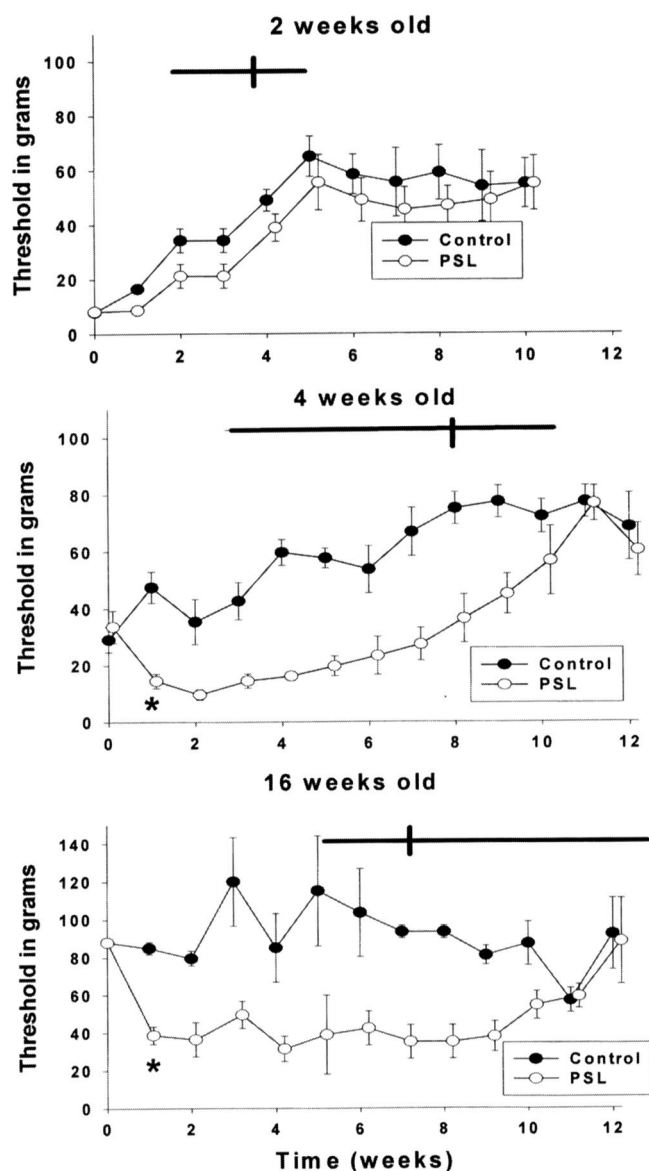


Fig. 1. Threshold change *versus* time in 2- ( $n = 10$ ), 4- ( $n = 8$ ), and 16- ( $n = 8$ ) week-old animals for partial sciatic nerve ligation (PSL). The left paw in the treated animals was compared to the left paw in the control animals with no surgery. The vertical solid line above the graph is the median time to return to 80% of control threshold, and the horizontal solid line is the range. The median time to return to 80% of control values was shorter in the 2-week-old animals than in either the 4- or 16-week-old animals ( $P < 0.05$ ). \* Significantly different from baseline threshold. The 2-week-old animals are the only group that did not have development of a threshold different from baseline.

## Results

### Partial Sciatic Nerve Ligation

In control animals without nerve injury, withdrawal threshold to mechanical stimulation was lowest in 2-week-old animals ( $8 \pm 0.4$  g,  $n = 12$ ), intermediate in 4-week-old animals ( $29 \pm 4$  g,  $n = 12$ ), and greatest in 16-week-old animals ( $88 \pm 9$  g,  $n = 10$ ;  $P < 0.05$ , all groups differ; fig. 1). The lower baseline thresholds observed in the younger animals increased rapidly in the control animals

to reach thresholds similar to those of the adults (16-week-old animals) by approximately 6 weeks of age. The thresholds in the contralateral paw in the partial nerve ligation animals were no different from that in the control animals for all ages.

After partial sciatic nerve ligation, the mechanical thresholds in the 2-week-old animals did not decrease from baseline threshold at any time after surgery (fig. 1). However, there was a significant decrease in thresholds in the 4- and 16-week-old animals after surgery when compared with baseline ( $P < 0.05$ ). The lowest mechanical threshold was 8.6 g seen at 1 week after injury in the 2-week-old animals, 9.7 g seen at 2 weeks in the 4-week-old animals, and 31.5 g seen at 4 weeks in the 16-week-old animals. This corresponds to no change from baseline in mechanical threshold for the 2-week-old animals (*vs.* 8.2 g baseline threshold) and a reduction of mechanical threshold of 72% in the 4-week-old animals and 65% in the 16-week-old animals when compared with the baseline threshold in the same foot. After partial sciatic nerve ligation, the mechanical thresholds decreased significantly in animals of all ages compared with age-matched controls ( $P < 0.05$ ). This is a reduction of 55%, 69%, and 65% in the 2-, 4-, and 16-week-old animals, respectively. The times of the maximum difference in thresholds between the control and the nerve-ligated animals were 3 weeks (13 g), 4 weeks (43 g), and 5 weeks (75 g) in the 2-, 4-, and 16-week-old animals.

The withdrawal thresholds are plotted over time in all ages of animals both treated and control (fig. 1). There was a significant difference as a function of age in the time for the mechanical allodynia response to return within 80% of that of the age matched controls, with the 2-week-old animals being 4 weeks (range, 2–5 weeks), the 4-week-old animals being 8 weeks (range, 3–11 weeks), and the 16-week-old animals being 7 weeks (range, 5–13 weeks) after the surgery. The differences between the 2- and 4-week and the 2- and 16-week-old animals were significant ( $P < 0.05$ ), whereas the difference between the 4- and 16-week-old animals was not.

### Lumbar Spinal Nerve Root Ligation

In control animals without surgery, withdrawal threshold to mechanical stimulation was lowest in 2-week-old animals ( $10 \pm 1$  g,  $n = 12$ ), intermediate in 4-week-old animals ( $33 \pm 5$  g,  $n = 12$ ), and greatest in 16-week-old animals ( $84 \pm 5$  g,  $n = 10$ ;  $P < 0.05$ , all groups differ; fig. 2). The lower baseline thresholds observed in the younger animals increased rapidly in the control animals to reach similar thresholds to the adult (16-week-old animals) by approximately 6 weeks of age. The thresholds in the contralateral paw in the lumbar spinal nerve ligation animals were no different from that in the control animals for all ages.

After L5–L6 nerve root ligation, in animals of all ages, the mechanical thresholds decreased significantly from



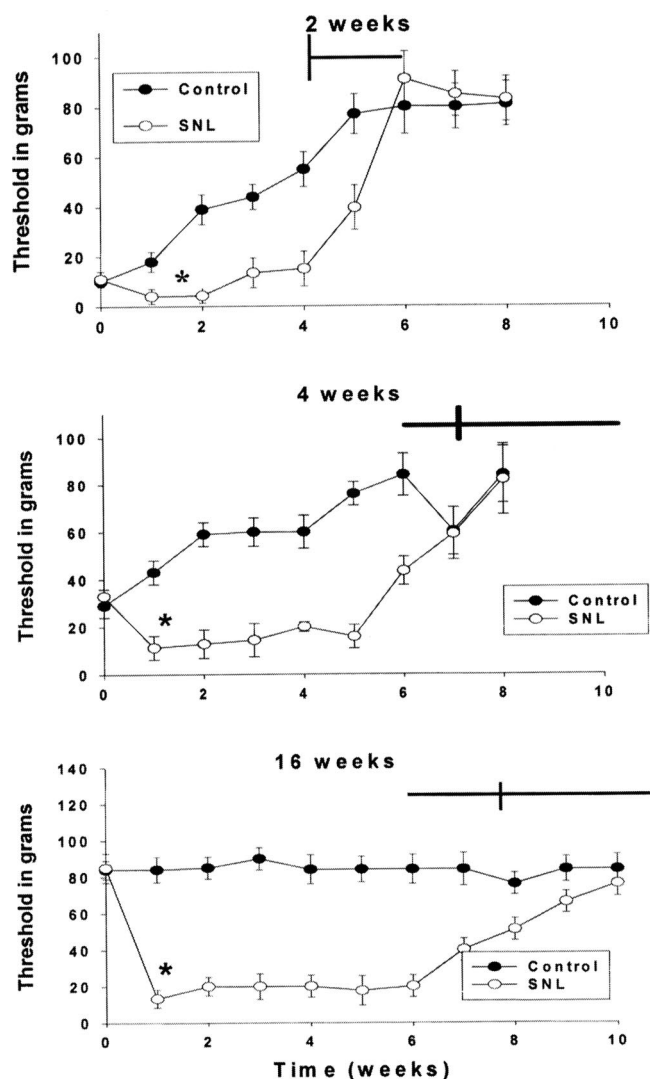


Fig. 2. Threshold change versus time in 2- ( $n = 10$ ), 4- ( $n = 8$ ), and 16- ( $n = 8$ ) week-old animals for L5–L6 lumbar spinal nerve root ligation (SNL). The left paw in the treated animals was compared with the left paw in the control animals with no surgery. The vertical solid line above the graph is the median time to return to 80% of control threshold, and the horizontal solid line is the range. The median time to return to 80% of control values was shorter in the 2-week-old animals than in either the 4- or 16-week-old animals ( $P < 0.05$ ). \* Significantly different from baseline threshold. All ages had development of a threshold different from baseline.

baseline ( $P < 0.05$ ; fig. 2). The lowest threshold was seen at 1 week in all three ages. This threshold was 4 g in the 2-week-old animals, 11.2 g in the 4-week-old animals, and 13.2 g in the 16-week-old animals. This corresponds to a reduction of mechanical threshold of 64% in the 2-week-old animals, 68% in the 4-week-old animals, and 84% in the 16-week-old animals when compared with the baseline threshold in the same foot. However, when compared with thresholds in age-matched control animals at the same time, this is a reduction of 78%, 73%, and 84% in the 2-, 4-, and 16-week-old animals, respectively. In all three ages, there was also a significant reduction in threshold after surgery when compared

with age-matched control animals from the age-matched control ( $P < 0.05$ ). The time of the maximum difference in thresholds between the control and the nerve-ligated animals was 4 weeks (40 g), 5 weeks (60 g), and 3 weeks (70 g) in the 2-, 4-, and 16-week-old animals.

The withdrawal thresholds are plotted over time in all ages of animals, both treated and control (fig. 2). There was a significant difference in the time for the mechanical allodynia response to return to within 80% of age-matched controls, with the 2-week-old animals being 4 weeks (range, 4–6 weeks), the 4-week-old animals being 7 weeks (range, 6–10 weeks), and the 16-week-old animals being 8 weeks (range, 6–11 weeks) after the surgery. The differences between the 2- and 4-week and the 2- and 16-week-old animals were significant ( $P < 0.05$ ), whereas the difference between the 4- and 16-week-old animals was not.

## Discussion

In this study, developmental differences in resolution of hypersensitivity to punctate mechanical stimuli were present in two models of neuropathic pain, the PSL and the SNL models.<sup>2,4</sup> The younger animals demonstrated earlier resolution of mechanical allodynia. This was similar for both models. However, the effect of the nerve injury on behavioral responses in the younger animals was more dramatic in the SNL.

Our results partially corroborate previous work on age of the chronic painful injury with the SNL model.<sup>3</sup> Our findings extend this by demonstrating that the PSL model also behaves similarly.<sup>4</sup> This suggests that between the second and third postnatal week, a mechanism extinguishes the response, or a maturational event occurs to permit the vulnerability to maintenance of pain. However, no studies have been performed using the SNL model to define the changes that occur between the second and third week postnatally responsible for these observations, and no study has reproduced these results in another nerve injury model.

Both PSL and SNL models have been extensively studied. SNL seems to provide a more consistent injury and more reliable responses with more allodynia.<sup>5,12</sup> This may be from greater uniformity of the injury.<sup>9,10</sup> Whether there is greater inflammation due to the uniformity of the injury, the greater number of sutures, or the more proximal injury is not clear. Inflammation might explain the differences in the degree of mechanical allodynia in the two models. This could be from peripheral differences in inflammation or centrally mediated differences in inflammation, possibly through activation of microglia.<sup>13</sup> The role of inflammation in initiation and maintenance of mechanical allodynia may be important in the differences seen during development. Differences in immune responses and inflammation occur as a func-

tion of age, and this may be responsible for age differences in models of neuropathic pain.<sup>14</sup> In using an inflammation model, nocifensive behavior seemed to be decreased between 15 and 20 days postnatally compared with older animals.<sup>15</sup> Differences could be related to differences in the inflammatory response. Further studies looking at the effect of aging suggest that the most vigorous response to neuropathic pain is seen around 20 weeks of age compared with older rats.<sup>16</sup> Similarly, the changes that occur with further aging which may be responsible for a reduction in the mechanical hyperalgesia have not been elucidated.

Our results demonstrate increasing withdrawal threshold as a function of age, which is consistent with previous studies.<sup>1,17,18</sup> This is also consistent with studies in humans examining developmental differences in withdrawal thresholds.<sup>18,19</sup> Although all age groups had development of mechanical allodynia after injury in our study, the mechanical allodynia in younger animals diminished much more rapidly. During the normal course of development, the mechanical threshold for withdrawal is increasing possibly related to A-fiber withdrawal from deeper lamina in the dorsal horn.<sup>20</sup> In the SNL model, the allodynia is thought to be from altered firing of A-fiber neurons in the absence of C-fiber activation.<sup>21</sup> After the nerve injury, the increase in threshold which normally occurs during development is stalled so that thresholds remain low. Then, after a period, the recovery begins, and the thresholds begin to increase until resolution of the allodynia occurs. Because the thresholds are changing over time in the younger animals, the more rapid resolution may be from A-fiber connections maturing in the spinal cord compensating for the altered input as permanent connections are established. This may reduce the impact of the ectopic discharge, allowing younger animals to more rapidly overcome the central stimulus, or the younger animals may have less ectopic discharge from the injury related to the anatomical differences.<sup>22</sup>

Nerve injury in the neonatal rat has been studied. Sprouting of sensory axons in the foot of the younger animal is greater, and this seems to occur more rapidly.<sup>23</sup> At the same time, nerve section results in increased receptive fields.<sup>24</sup> In the younger animals, cell loss is more rapid and more extensive in response to nerve section.<sup>25</sup> The apoptosis induced by the nerve injury is not confined to the sensory neurons, but also is induced in interneurons.<sup>26</sup> In conjunction with this, neurochemical function decreases more rapidly, but it also returns more rapidly in the young. Recovery of cellular function in the younger animals is of great interest because understanding this phenomenon may allow extension of it to older animals. Thus, there seems to be two separate processes: (1) initiation of injury behavior through cell death, change in neuroanatomical connections, and neurochemical changes, followed by (2) rees-

tablishment of cell functions and resolution of the pain behavior. Possibly they are related. For example, earlier cell death may allow more rapid turnover and compensatory mechanisms.

The reduced impact of the nerve injury in the younger animals is consistent with recently published data using two other models during development, the chronic constriction injury model and the spared nerve injury model.<sup>27</sup> However, neither of these models produced a significant nocifensive withdrawal response of any duration until after 33 days. This is quite different from our results in the 14-day-old pups whereby clear nocifensive withdrawal differences are seen but are of shorter duration than in the older animals. All models are consistent in that there is less nocifensive behavior (duration or presence) in the younger animals compared with the same injury in older animals. The variation in the amount of input arriving at the spinal cord with each of the models and their impact on subsequent withdrawal responses may explain overall differences. This will require further study to define the etiology of the differences in the four models of nociceptive pain and their relation to neuropathic pain in children.

Mechanisms for establishing pain after nerve injury as a function of age are still unclear. Even more unclear is the mechanism of extinguishing pain behavior in all ages. This would seem to be of great interest. It is possible that the increased number of cells with projections from the peripheral area arriving at the spinal cord and sending projections to multiple levels in the spinal cord may be responsible for the perceived effect of reduced pain.<sup>28</sup> Baseline behavioral responses were similar in the Ruda *et al.*<sup>28</sup> study in adulthood, but spontaneous and evoked firing of neurons were increased in animals injured in the neonatal period. Therefore, our results may still be consistent with others showing prolonged effects after neonatally experienced pain.<sup>29</sup> Our behavioral endpoints are gross measures of activity compared with electrophysiologic measures. However, it is not clear whether a prolonged response can be demonstrated with injury as far out as 2 weeks after birth or only after neonatal injury. That is, more sensitive measures of pain and sensory transmission may actually demonstrate that there is more pain in the younger animals that lasts longer instead of shorter, as we have interpreted in our study. The data from the inflammatory model suggest that injury at 2 weeks of age does not produce the same long-term nociceptive processing as injury in the neonatal period.<sup>28</sup> However, the inflammatory model may resemble the nerve injury model in some respects, and in other respects is vastly different.<sup>30</sup> Therefore, further study of age of nerve injury will be of value to establish direct similarities between the two models.

Many peripheral and central changes are occurring during maturation. Differences in immune function

which may alter the inflammatory response to tissue trauma, development differences in opioid, *N*-methyl-D-aspartate, and other receptors systems, as well as neuro-anatomical changes are all occurring.<sup>20,31–36</sup> The observed differences in pain duration could be from direct inhibition on nerve fibers. This could be from anatomical development and modulation of the spinal signals,<sup>31</sup> differences in responses to sensory input as a function of age,<sup>35</sup> or developmental differences in other inhibitory neurotransmitters.<sup>36</sup> Shortened pain duration may also reflect a more rapid change in the way connections are formed in the central nervous system during development. This may be a result of differentiation and development whereby establishment of more permanent connections in the dorsal horn from the peripheral nerve fibers is altered or accelerated in the injured animal, reducing hyperalgesia in the young more rapidly.

Neuropathic pain in children clearly exists. The best documentation of neuropathic pain is in children with cancer.<sup>37</sup> Other types of neuropathic pain exist in children, but there is a paucity of documentation of the prevalence of these problems. Two clear examples are postthoracotomy pain syndrome and postherniorrhaphy pain syndrome. Both problems exist in adults, with a prevalence as high as 30–50% of cases, but are of unknown prevalence in children undergoing similar surgery.<sup>38,39</sup> In another study looking at overall incidence of complex regional pain syndrome, there were no patients identified between 0 and 9 yr of age and few from 10 to 19 yr of age, the majority being adults.<sup>40</sup> Although these pain syndromes clearly exist and are treated in children, no study documents their prevalence. One attempt to define postoperative neuropathic pain in children with cerebral palsy exists, but this may be a unique problem with underlying neurologic problems contributing to the development and maintenance of pain.<sup>41</sup> This may be related to the pain being of shorter duration than in adults, consistent with the findings in this study; or failure of the medical establishment to acknowledge its presence; or both. Further studies to characterize the prevalence and natural history of neuropathic pain in children are needed for better understanding of this clinical entity.

Although our results help to define the role of development beyond the neonatal period in responses to neuropathic pain, much is still unknown. The establishment of these two models will allow further studies of the anatomical and biologic mechanisms responsible for the observed behavioral differences. Further studies may help us to understand the differences in chronic pain responses during development beyond the neonatal period, allow improved treatment, and possibly lead to prevention or reduction in duration of hyperalgesia and allodynia in adults.

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