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Neuropathic Pain Behaviors and the Change of Spinal Neuropeptides following Peripheral Nerve Injury in Neonatal Rats

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Objective : It has been suggested that the occurrence of persistent pain signal during the early postnatal period may alter an individual's response to pain later in life. The aim of this study is to assess whether neonatal nerve injury resulted in long-lasting consequences on nociceptive system in the rat.
Methods : We examined whether neuropathic pain behaviors and the changes of spinal neuropeptides (SP, CGRP, VIP and VIP) induced by peripheral nerve injury within 1 day after birth (Neonate group) were different from those at 8 weeks after birth (Mature group).
Results : The Neonate group showed more robust and long-lasting pain behaviors than the Mature group. Immunohistochemical findings demonstrated that spinal SP- & CGRP-immunoreactivities(ir) of the ipsilateral to the contralateral side increased in the Neonate group, whereas those decreased in the Mature group. In addition, increase in spinal VIP- & NPY-ir of the ipsilateral to the contralateral side was more robust in the Mature group than in the Neonate group.
Conclusion : These results suggest that peripheral nerve injury in the early postnatal period may result in long-lasting and potentially detrimental alterations in nociceptive pathways.

KEY WORDS : Peripheral nerve injury · Neuropathic pain · Neonatal · Allodynia · Spinal neuropeptides.

Introduction

It has been known that the nervous system for pain transmission during neonatal period is not developed fully and thus pain is not felt properly. Hence, various invasive manipulations in neonatal intensive care units and simple surgeries are performed without anesthesia. Recently, however, it has been reported that if neonates experience severe pain, their nervous system for pain transmission would undergo a structural and neurochemical change resulting in abnormal pain patterns after growth[2,6,20,21]. Animal studies supporting it have been reported. Neonatal tissue injury causes long-standing changes in spinal sensory connections[1,22], but does not induce the depletion of spinal excitatory neuropeptides normally observed after nerve section in the adult[17]. In addition, the increase in skin innervation density in the wounded area is more robust when wounds are performed at neonatal period than in the adult[16]. Furthermore, peripheral inflammation experienced during the neonatal period induces long-lasting consequences on nociceptive behaviors and neuronal circuitry development[18].

Several successful experimental animal models for neuropathic pain, produced by a partial injury of the nerves supplying the rat hind paw, were developed in recent years by Bennett and Xie[3], Seltzer, Dubner and Shir[19] and Kim and Chung[8]. Although these models display clear signs of neuropathic pain, there are some inherent limitations to perform the behavioral tests due to foot deformity. To avoid these problems we have developed a rat model[12], by partial injury of the nerves innervating the rat tail. This model, similar to the previously developed ones, displays chronic neuropathic symptoms like mechanical and thermal (cold and warm) allo-

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dynia. Furthermore, surgical procedures for this model are very simple that even neonates can be used.

In the present study, using this animal model, we compared the neuropathic pain behaviors, and spinal excitatory [substance P(SP) and calcitonin gene-related peptide(CGRP)] and inhibitory [vasoactive intestinal peptide(VIP) and neuropeptide Y(NPY)] neuropeptides following peripheral nerve injury between the Neonate (within 1 day after birth) and the Mature (8 weeks after birth) groups.

Materials and Methods

Experimental animals and neuropathic surgery

Forty eight neonatal (within 1 day after birth) and 52 mature (8 weeks after birth) Sprague-Dawley rats were used. The neonatal rats were anesthetized by low temperature of keeping them in a freezer for 15~20 minutes, and the mature rats were anesthetized by using 0.5~2% enflurane. Nerve injury was performed according the method developed by Na et al.[12]. Briefly, under enflurane anesthesia (0.5~2%), the left inferior and superior caudal trunks were exposed, freed carefully from the surrounding tissues and transected at the level between the S1 and S2 spinal nerves. To prevent the possible rejoining of the proximal and distal ends of the severed trunk, about 2-mm piece of the trunk was removed from the proximal end. This surgery eliminated the S1 spinal nerve innervation of the tail via the left and superior inferior caudal trunks.

Behavioral test for mechanical, cold and warm allodynia

To examine the generation of neuropathic pain, the behavioral tests for mechanical, cold and warm allodynia were performed in the Neonate and the Mature groups 6, 7, 8, 10, 14, 18 weeks after the surgery. As previous reports[9,12], mechanical allodynia was assessed by the tail withdrawal response following poking the tail with von Frey hairs (bending force : 0.5 and 2.0g). The most sensitive spot of the tail was first determined by rubbing or poking various areas with the von Frey hair. Then, this spot was challenged 10 times with 5~10 sec intervals. The occurrence of tail withdrawal in response to the stimulation was expressed as a percentage of trials. The tests for cold and warm allodynia were performed by immersing the tail into cold (4˚C) or warm (40˚C) water, respectively. Following the tail immersion, the latency of tail withdrawal or twitch was measured within a cut-off time of 15 sec. The tests for cold and warm allodynia were repeated five times with 5 min intervals. The average latency of tail response was calculated.

Immunohistochemical test

Six weeks after the nerve injury, we compared the two groups of rats (Neonate and Mature) with respect to the spinal levels of CGRP, SP, VIP and NPY neuropeptides. The rats were perfused with 4% paraformaldehyde in 0.1M phosphate buffer(PB) containing 0.1% picric acid. The S1 spinal segment (injured level) excised was post-fixed for 6~8 hour in the same fixative and placed overnight in 0.1M PB (pH 7.4) containing 30% sucrose at 4˚C. The excised segment was sectioned at 14㎛ interval on a freezing microtome. Sections were reacted with anti-CGRP, anti-SP, anti-VIP and anti-NPY antibodies (Peninsula Lab, Belmont, CA, USA) according to the ABC method (Vector Elite Kit, Vector, Burlingame, CA, USA). Sections were then rinsed with 1% bovine serum albumin and 10% normal goat serum(NGS) for 1 hour and incubated in SP (1 : 60,000), CGRP (1 : 80,000), VIP (1 : 8,000) or NPY (1 : 8,000) antisera for 48 hours at 4˚C. Sections were rinsed sequentially in 0.05M phosphate buffer saline(PBS) and 3% NGS (30 min each). Then, they were incubated in diluted biotinylated goat anti-rabbit IgG PBS solution for 1 hour,

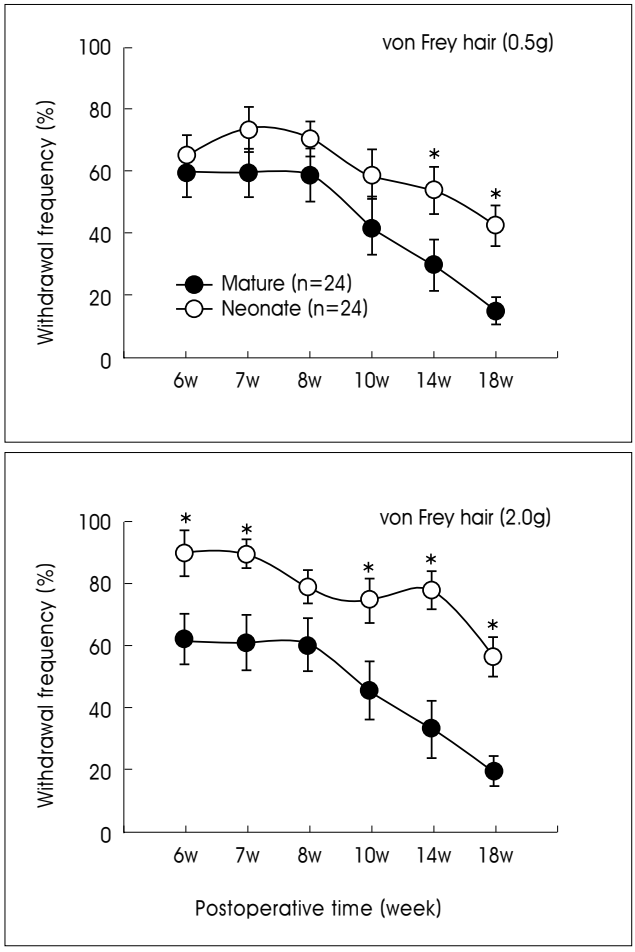

Fig. 1. Tail withdrawal responses to mechanical stimulation in the rats subjected to nerve injury at postnatal day 0 (Neonate) and 8 week (Mature). The mean (±SEM) response frequencies to mechanical stimulation (von Frey hairs, 0.5 & 2.0g) are plotted against the posto-perative time (weeks). Asterisks represent the scores significantly different from corresponding scores of the Mature rats (Mann-Whitney *U*-test, *, $P<0.05$).

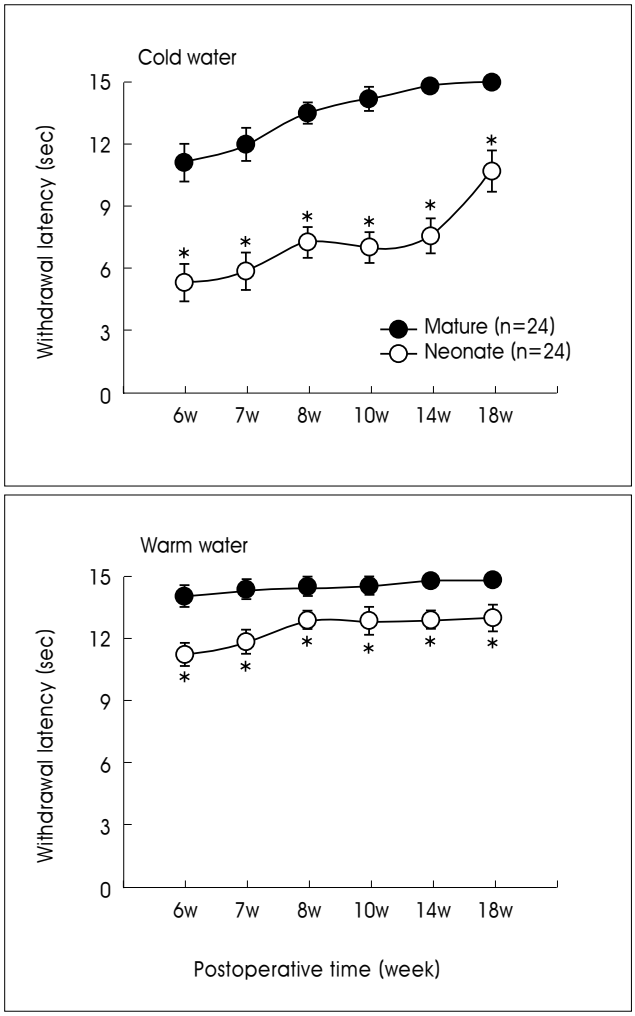

Fig. 2. Tail withdrawal responses to cold and warm water stimulation in the rats subjected to nerve injury at postnatal day 0 (Neonate) and 8 week (Mature). The mean (±SEM) response latencies to cold (4°C) and warm (40°C) water immersion are plotted against the postoperative time (weeks). Asterisks represent the scores significantly different from corresponding scores of the Mature group (Mann–Whitney *U*–test, *, $P<0.05$).

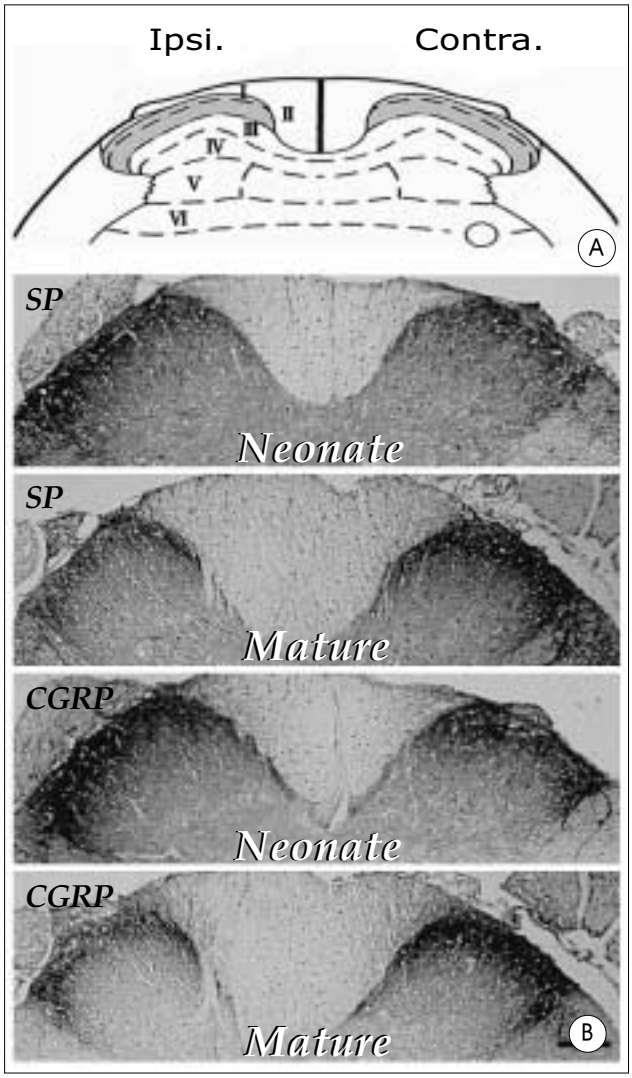

Fig. 3. A : Schematic diagram illustrating the method for the quantification of immunoreactivities (ir) of the superficial dorsal horn. The mean gray scales (range from 0 to 255) of pixels in the ipsilateral (Ipsi) and contralateral (Contra) superficial dorsal horn (gray area, lamina I and II) were calculated. The mean gray scales were used as the density of substance P(SP)– and calcitonin gene–related peptide(CGRP)–ir. Circled area was used for background density. B : These microphotographs illustrate that SP–and CGRP–ir in the S1 ipsilateral superficial dorsal horn are increased and decreased in the Neonate and Mature groups, respectively, 6 weeks after neuropathic surgery. Scale bar= 150㎛.

rinsed sequentially in 1% NGS and 3% NGS and incubated in an avidin-biotinylated horseradish peroxidase complex for 1 hour. Following three 10-min washes in phosphate buffer, the sections were then incubated in a solution of diaminobenzidine (0.05%) containing 0.01% hydrogen peroxide for approximately 5~10 min. Reacted sections were dehydrated, cleared and coverslipped. The tissues of the Neonate and Mature groups were processed in parallel.

Quantification of immunoreactivity

We measured the density of CGRP, SP, VIP and NPY immunoreactivities (ir) in 4 to 10 spinal cord sections from each rat. To quantify the density of labeling, we used a computer-assisted image analysis system (NIH Image Software). Images of the spinal cord sections were captured with a × 4 objective and a CCD camera and converted to digital images with a gray value ranging from 0 to 255. Then, we counted the mean density of the lamina I & II (SP, CGRP, VIP and NPY) and the lamina III & IV (NPY). For each spinal cord section, the ratio of the density of the injured to the intact side was calculated using the following formula :

Ratio = (IMD-BD) / (CMD-BD)

IMD : mean density of ipsilateral lamina I & II or III & IV (gray area in Fig. 3A). CMD : mean density of contralateral lamina I & II or III & IV (gray area in Fig. 3A). BD : background density (circled area of lamina VI and VII in Fig. 3A)

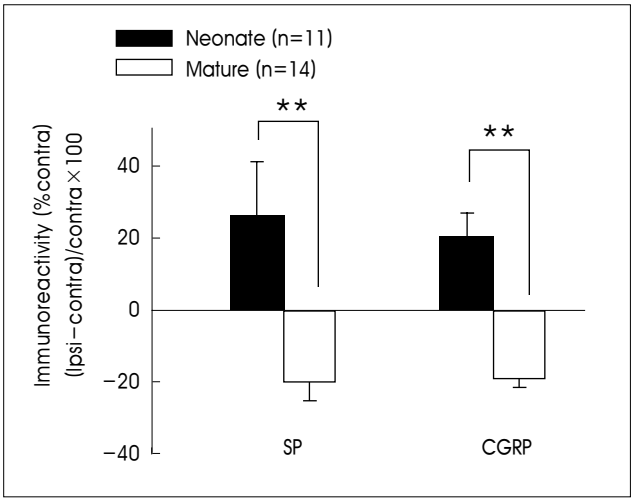

Fig. 4. Ratios of substance P− and calcitonin gene−related peptide−immunoreactivities of the ipsilateral to the contralateral dorsal horn. Data are mean (±SEM) percentage of the density on the ipsilateral over the contralateral side. There are significant differences between the Neonate (n=11) and Mature (n=14) groups (Mann−Whitney *U*−test, ** $P<0.001$).

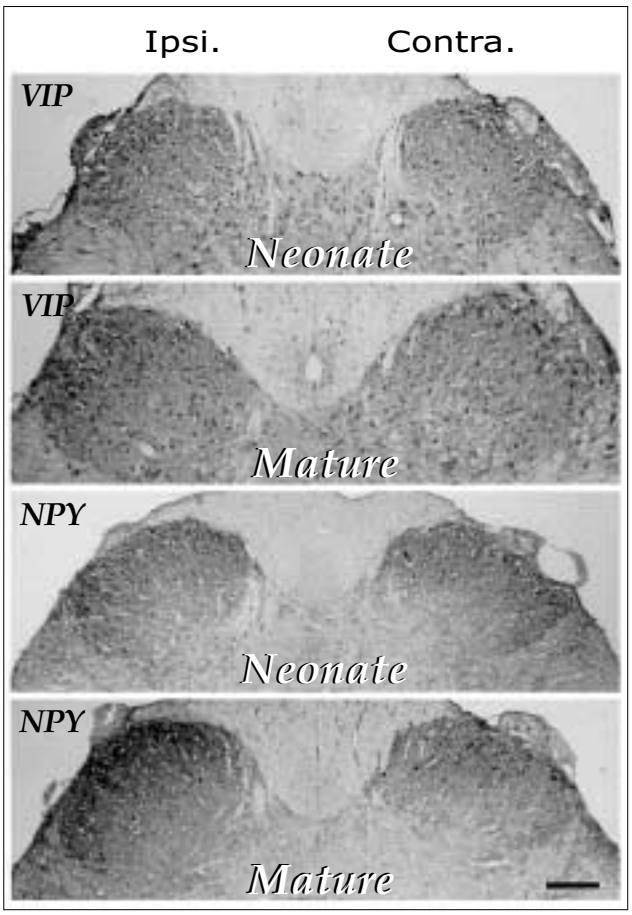

Fig. 5. These microphotographs illustrating the increases of vasoactive intestinal polypeptide(VIP)− and neuropeptide Y(NPY)−immunoreactivities in the S1 ipsilateral dorsal horn in both the Neonate and Mature groups 6 weeks after neuropathic surgery. The increases in VIP− and NPY−ir are more robust in the Mature group than in the Neonate group. Scale bar=150㎛.

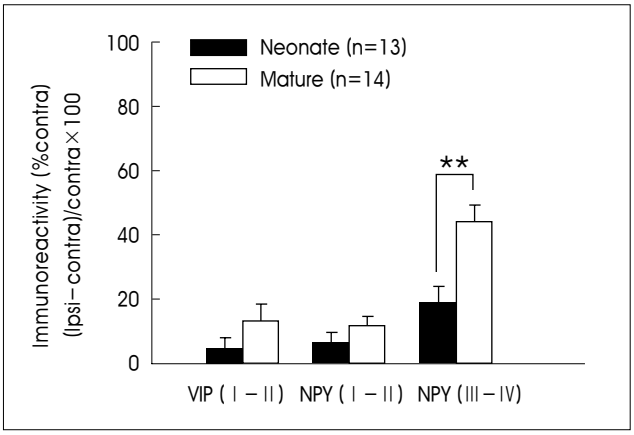

Fig. 6. Ratios of vasoactive intestinal polypeptide(VIP)− and neuropeptide Y(NPY)−immunoreactivities of the ipsilateral to the contralateral dorsal horn. Data are mean (±SEM) extent of the change in the density on the ipsilateral over the contralateral side. Statistical analysis indicated that the increase of spinal NPY−ir in the lamina Ⅲ & Ⅳ, but not VIP− and NPY−ir in the lamina Ⅰ & Ⅱ, was more predominant in the Mature (n=14) group than in the Neonate (n=13) group (Mann−Whitney *U*−test, ** $P<0.001$).

Statistical treatments

Data are expressed as the mean±S.E.M. Mann-Whitney *U*-test was used to determine whether behavioral test scores and the spinal levels of neuropetidesir were significantly different between the Neonate and Mature groups. $P<0.05$ was considered significant.

Results

Mechanical allodynia

As shown in Fig. 1, the magnitude of mechanical allodynia in the Neonate group (n=24) was greater and longer-lasting than the Mature group (n=24). In the case of 0.5g von Frey hair, the frequency of tail withdrawal showed the tendency to be higher in the Neonate group than the Mature group from 6 to 10 weeks after the surgery, and significant differences were detected at 14 and 18 weeks after the surgery (Fig. 1A, Mann-Whitney *U*-test, *, $P<0.05$). In the case of 2 g von Frey hair, the frequency of tail withdrawal was significantly higher in the Neonate group than the Mature group from 6 to 18, except 8, weeks after the surgery, (Fig. 1B, Mann-Whitney *U*-test, *, $P<0.05$).

Cold and warm allodynia

As shown in Fig. 2, the Neonate group (n=24) showed more severe cold and warm allodynia than the Mature group (n=24). Cold allodynia began to disappear from 8 weeks after the surgery in the Mature group, whereas it was persistent up to 18 weeks in the Neonate group. Comparing the two groups, the Neonate group showed more severe response than the Mature group from 6 weeks after the nerve injury, and this difference

persisted up to 18 weeks (Fig. 2A, Mann-Whitney *U*-test, *, $P<0.05$). Warm allodynia also persisted up to 18 weeks after the surgery in the Neonate group, unlike the Mature group. Comparing the two groups, the Neonate group also showed more severe response than the Mature group from 6 to 18 weeks (Fig. 2B, Mann-Whitney *U*-test, *, $P<0.05$).

SP and CGRP immunoreactivity

As illustrated in Fig 3B, spinal SP-CGRP-ir of superficial dorsal horn (lamina I & II) of the injured side were higher than those of the opposite side 6 weeks after the nerve injury in the Neonate group, whereas both neuropeptides were decreased in the injured side in the Mature group. Statistical analysis indicated that the ratios of SP-and CGRP-ir of the ipsilateral to the contralateral dorsal horn are significantly different between the Neonate (n=11) and Mature (n=14) groups (Fig. 4. Mann-Whitney *U*-test, $P<0.001$).

VIP and NPY immunoreactivity

As illustrated in Fig. 5, the spinal VIP- (lamina I & II) and NPY-ir (lamina I-IV) were increased in the ipsilateral to the contralateral side in the both groups. However, statistical analysis indicated that the increase of spinal NPY-ir in the lamina III & IV, but not VIP- and NPY-ir in the lamina I & II, was more predominant in the Mature (n=14) group than in the Neonate (n=13) group (Fig. 6, Mann-Whitney *U*-test, $P<0.001$).

Discussion

In the present study, neonatal rats showed more severe and longer duration of neuropathic pain behaviors following peripheral nerve injury than mature rats.

Our results are in line with the previous reports that local skin damage in neonatal period resulted in a profound sensory nerve sprouting of the wounded area[16], which was accompanied by long-lasting hypersensitivity and lowered mechanical threshold in the injured region[4,5]. Neonatal peripheral inflammation also induced dynamic alterations of small diameter primary afferent spinal circuits, thus might cause a permanent facilitated response to noxious stimulation[18]. Furthermore, neonatal nerve damage resulted in abnormal connectivity from primary nociceptive afferents to higher levels of the nervous system, including the cerebral cortex[5]. To our knowledge, present report is the first one that peripheral nerve injury within 1 day after birth induces pronounced neuropathic pain.

Several types of prolonged structural and functional alterations in pain pathways following nerve damage in neonatal period, that are not observed when the same injury is performed in an adult, are proposed to be the causes of profound long-term sensory hypersensitivity. Consistent with previous reports[7,13,17], present results show that the increase or absence of reduction of spinal excitatory neuropeptides (SP and CGRP) following neonatal nerve injury, whereas nerve injury in the adult leads to decrease in spinal SP and CGRP. Due to that SP and CGRP potentiate the release of glutamate and its actions on the NMDA receptors, an in-crease in spinal SP and CGRP following neonatal peripheral nerve injury may cause severe neuropathic pain signs.

Here we also showed that spinal VIP and NPY are increased more evidently in mature rats than in neonatal rats. VIP and NPY are believed to exert antinociceptive actions by inhibiting the release of excitatory neuropeptides such as SP in the spinal cord dorsal horn[14]. Thus, the less increase of VIP and NPY in neonatal rats than in mature rats may also lead to severe neuropathic pain signs.

Clinical reports have also suggested that neonatal exposure to excessive pain has some effect on future pain responses[2,6]. For example, the infants underwent circumcision without anesthesia showed more severe pain reactions to vaccination than the infants underwent circumcision under anesthesia[20,21], and the premature babies treated in intensive care units reacted more strongly to pain stimulation after maturation[15]. In addition, the neuropathic pain induced by peripheral nerve injury and reflex sympathetic dystrophy occurred more frequently in children than adults[11].

Limitation of the present study was that we could not examine the developmental time course of neuropathic pain behaviors in neonatal rats until 6 weeks following the nerve injury. This is due to the immature mechanisms underlying neuropathic pain behaviors including motor function until 3~4 weeks and the gradually increasing sensory threshold of the skin until 5~6 weeks.

Conclusion

Neonatal rats (within 1 day after birth) showed more severe and long-lasting neuropathic pain behaviors following peripheral nerve injury than mature rats (8 weeks after birth). In addition, excitatory neuropeptides (SP and CGRP) in the superficial dorsal horn were increased in neonatal rats, whereas they were decreased in mature rats. Furthermore, inhibitory neuropeptides (VIP and NPY) were increased more evidently in mature rats than in neonatal rats. These results suggest that peripheral nerve injury in the early postnatal period can result in alterations in nociceptive circuitry, and thus long-lasting abnormal pains.

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Commentary

The authors investigated whether neonatal nerve injury resulted in long lasting and potentially detrimental alterations in nociceptive pathways. They compared two experimental models which were treated the S1 spinal nerve and evaluated the pain behaviors and the change of excitatory and inhibitory neuropeptides. They concluded that peripheral nerve injury in early postnatal period in rat can result in alterations in nociceptive circuitry and thus long-lasting pains.

Central sensitization after peripheral nerve injury is the main pathophysiologic illustration for chronic neuropathic pain. In general, it has been studied in adult animal models. Unfortunately, the change of central nervous system to chronic pain in adult can not fully explain the difference of the change in childhood. Although it is difficult to see a patient who suffers from chronic neuropathic pain in infant or child in clinical field, several articles revealed that the pain induced by peripheral injury and reflex sympathetic dystrophy occurred more frequently in children than adults. This article showed an excellent results about the explanation of the more vulnerable response of neural plasticty than in case of adult.

The change of central nervous system after peripheral nerve injury has been explained the change of neuron in dorsal horn, biochemical events, neurotansmitters, neuropeptieds, dorsal ganglion cells, and abnormal signal from injured peripheral nerve and disinihibition of descending pathway.

In this article, authors considered the change of spinal neuropetides. But it is insufficient to explain the complicated change in dorsal horn of spinal cord. In the future the cause of difference of change of neuropeptides between infant and adult rat after peripheral nerve injury will be studied.

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