Research Report

Electroacupuncture (EA) modulates the expression of NMDA receptors in primary sensory neurons in relation to hyperalgesia in rats

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptor on the central terminals of the dorsal root ganglion (DRG) appears to be playing an important role in the development of central sensitization related to persistent inflammatory pain. Acupuncture analgesia has been confirmed by numerous clinical observations and experimental studies to be a useful treatment to release different kinds of pains, including inflammatory pain and hyperalgesia. However, the underlying mechanisms of the analgesic effect of acupuncture are not fully understood. In the present study, using a rat model of inflammatory pain induced by complete Freund’s adjuvant (CFA), we observed the effect of electroacupuncture (EA) on animal behavior with regard to pain and the expression of a subunit of NMDA receptor (NR1) and isolectin B4 (IB4) in the neurons of the lumbar DRG. Intraplantar injection of 50 μl CFA resulted in considerable changes in thermal hyperalgesia, edema of the hind paw and “foot-bend” score, beginning 5 h post-injection and persisting for a few days, after which a gradual recovery occurred. The changes were attenuated by EA treatment received on the ipsilateral “Huan Tiao” and “Yang Ling Quan” once a day from the first day post-injection of CFA. Using an immunofluorescence double staining, we found that the number of double-labeled cells to the total number of the IB4 and NR1-labeled neurons increased significantly on days 3 and 7 after CFA injection. The change was attenuated by EA treatment. These results suggest that EA affects the progress of experimental inflammatory pain by modulating the expression of NMDA receptors in primary sensory neurons, in particular, IB4-positive small neurons.

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1. Introduction

There is considerable evidence that N-methyl-D-aspartate (NMDA) receptors in the central nervous system contribute to the development and maintenance of sensitization, which is an important component of inflammatory pain (Coderre and Melzack, 1992). Electrophysiological studies have demonstrated that NMDA receptor agonists activate nociceptive neurons in the spinal dorsal horn (Dougherty and Willis, 1991), and behavioral studies have also indicated that intrathecal injection of NMDA receptor agonists induces biting and scratching behavior and hyperalgesia in the tail-flick and hot-plate tests in mice (Aanonsen and Wilcox, 1987) and rats (Raiogorsky and Urca, 1987). The presence of NMDA receptors in the unmyelinated cutaneous sensory axons (Carlton et al., 1995), dorsal root ganglion (DRG) (Marvizon et al., 2002) and
primary afferent terminals in the spinal cord (Liu et al., 1994) indicates that peripheral NMDA receptors may also play an important role in modulating the responses of nociceptors and contribute to the mechanical sensitivity and heat sensitization that accompanies by persistent inflammation (Du et al., 2003).

A variety of stimuli ranging from intense pressure and extremes of temperature to inflammation are conducted by the primary sensory neurons located in the DRG. The DRG consists of different types of neurons, and various markers have been found to correlate with cell size, and thus putatively to correspond to cells with nociceptive and non-nociceptive function. Many investigators have divided the DRG cell population into small dark and large light cells on the basis of their appearance in Nissl-stained sections. But more recently, the primary sensory neurons were categorized into three groups: small (under 30 μm in diameter), medium (from 30 to 45 μm) and large (over 45 μm). Small cells of DRG have been proposed to be nociceptive and a number of biochemical labels, such as Bandeiraea Simplicifolia Isolectin B4 (IB4), are present only in the small cells. NMDA receptors are distributed densely in the superficial dorsal horn of the spinal cord where primary nociceptive afferents terminate (Liu et al., 1994; Aanonsen et al., 1990).

It has also been considered that NMDA receptors are important targets for the treatment of pain, especially those resulting from inflammation or nerve injury. NMDA receptor antagonists attenuate inflammation-induced responses in the spinal dorsal horn (Haley et al., 1990; Ren et al., 1992a,b). Injection of NMDA receptor antagonists suppresses a variety of nociceptive responses produced by formalin (Nishiyama, 2000; Hamada et al., 2001), nerve injury (Chaplan et al., 1997), peripheral inflammation (Ren et al., 1992a,b; Sakurada et al., 1998), C-fiber stimulation or cutaneous mustard oil application (Woolf, 1991).

Acupuncture is an ancient therapeutic technique and is an important part of “Traditional Chinese Medicine” (TCM). Acupuncture analgesia is rapidly gaining interest and has been confirmed by numerous clinical observations and experimental studies (Ulett et al., 1998). Electroacupuncture (EA), a modified therapy method based on the theory of traditional manual acupuncture has proved to be more effective. However, the underlying mechanisms of the analgesic effect of acupuncture are not fully understood. In the present study, we wanted to explore whether the inflammatory pain affects the expression of NMDA receptor in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the present study. We wanted to explore whether the inflammatory pain affects the expression of NMDA receptor in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the superficial dorsal horn of the spinal cord and DRG by using a widely accepted animal model of inflammatory pain in the superficial dorsal horn of the spinalcord and DRG by using a widely accepted animal model of inflammatory pain. The distribution of the IB4-like (IB4-LI) and NR1-like immunoreactive (NR1-LI) neurons in the lumbar DRG (L4–5) of normal rats is illustrated in Fig. 2. The fluorescence signals for IB4 (Fig. 1A) and NR1 (Fig. 1C) were localized in the cytoplasm and did not enter the nucleus. IB4-LI neurons are all small sizes. NR1-LI neurons are different sizes, but most are small. IB4/NR1 double-labeled neurons are small sizes (Fig. 2E). In the dorsal horn of the lumbar spinal cord, the overlapping area of IB4 and NR1-labeled fibers or terminals was mainly located in lamina I and II (Fig. 2F). The percentage of the number of double-labeled neurons to the total number of the IB4 and NR1-positive neurons in the NS control group was 45.95 ± 2.56% and 45.83 ± 1.28%, respectively on day 3 (Table 1). In the left lumbar DRG (L4–5) corresponding to the CFA injection paw, the percentage of the number of double-labeled neurons to the total number of the IB4-labeled neurons on days 3 and 7 post-injection was 65.9 ± 4.30% and 60.57 ± 6.01%, respectively, and increased significantly compared to the NS control group (P < 0.01, Table 1). The percentage of the number of double-labeled neurons to the total number of the NR1-positive neurons increased on day 3 (62.8 ± 2.04%) and day 7 (59.86 ±

2. Results

2.1. Behavioral studies

In general, intraplantar injection of 50 μl CFA resulted in considerable changes in the thermal hyperalgesia, edema of the hind paw and “foot-bend” score by 5 h post-injection compared with baseline (Fig. 1). The changes persisted until day 6 post-injection, and could be significantly attenuated by EA treatment.

2.1.1. Thermal hyperalgesia

The baseline of the pain threshold of four groups was similar. After CFA injection, the thermal threshold of the control side of four groups showed no obvious change when compared with the baseline (P > 0.05). There was no significant difference in the thermal threshold between two hind paws of the NS control group (P > 0.05). From 5 h post-injection, the thermal threshold of the left hind paw (injection side) significantly decreased and persisted at low level until day 3 (Fig. 1A). However, after EA treatment, the thermal threshold gradually increased and showed obvious differences beginning on day 5, when compared with that of the CFA group without EA treatment and the EA control group (P < 0.01, Fig. 1A). In addition, there was no difference between the CFA group and the EA control group (P > 0.05).

2.1.2. Edema of the hind paw

CFA injected rats exhibited a marked increase in thickness and width of the left hind paw compared to the ipsilateral side of the NS control group, and peaked at 2 days post-injection (Figs. 1B, C). Only a little decrease of thickness and width of injection hind paw can be seen by EA treatment during the acute phase (1–3 days). However, the significant changes were found in the thickness and width on days 3 and 8, respectively, when EA treatment persisted (Figs. 1B, C).

2.1.3. “Foot-bend” scores

Flexion and extension scores increased quickly after CFA injection and reached peak values on day 2 (Figs. 1D, E). After EA treatment, the “foot-bend” scores gradually decreased and showed obvious differences compared with those of the CFA group without EA treatment and the EA control group (P < 0.01).

2.2. Immunofluorescence staining

The distribution of the IB4-like (IB4-LI) and NR1-like immunoreactive (NR1-LI) neurons in the lumbar DRG (L4–5) of normal rats is illustrated in Fig. 2. The fluorescence signals for IB4 (Fig. 1A) and NR1 (Fig. 1C) were localized in the cytoplasm and did not enter the nucleus. IB4-LI neurons are all small sizes. NR1-LI neurons are different sizes, but most are small. IB4/NR1 double-labeled neurons are small sizes (Fig. 2E). In the dorsal horn of the lumbar spinal cord, the overlapping area of IB4 and NR1-labeled fibers or terminals was mainly located in lamina I and II (Fig. 2F). The percentage of the number of double-labeled neurons to the total number of the IB4 and NR1-positive neurons in the NS control group was 45.95 ± 2.56% and 45.83 ± 1.28%, respectively on day 3 (Table 1). In the left lumbar DRG (L4–5) corresponding to the CFA injection paw, the percentage of the number of double-labeled neurons to the total number of the IB4-labeled neurons on days 3 and 7 post-injection was 65.9 ± 4.30% and 60.57 ± 6.01%, respectively, and increased significantly compared to the NS control group (P < 0.01, Table 1). The percentage of the number of double-labeled neurons to the total number of the NR1-positive neurons increased on day 3 (62.8 ± 2.04%) and day 7 (59.86 ±
3.33%) post-injection in CFA group and showed significant differences as compared to controls ($P<0.01$, Table 1). It was also found that the effects of CFA injection on the change of double-labeled cells were more obvious on day 3 than that on day 7. Interestingly, EA treatment had an attenuating effect: the percentage of the number of double-labeled neurons to the total number of the NR1/IB4-positive neurons decreased significantly on days 3 and 7 post-injection compared to the CFA group, although it did not reach the levels of the control group (Table 1). No effect (66.02±1.92% and 63.79±2.35%, respectively, Table 1) was found in the EA control group on day 3. In addition, the percentage of the number of double-labeled neurons to the total number of the NR1/IB4-positive neurons on day 3 was larger than that on day 7 in both the CFA and EA treatment groups ($P<0.05$, Table 1). With double immunofluorescence staining employed here, it was difficult to compare the NR1/IB4 double-labeled overlapping area in the spinal dorsal horn among each group.

3. Discussion

In the present study, we found numerous DRG neurons of all sizes expressing NR1 by using double immunofluorescence staining, which is in agreement with a previous report (Wang et al., 1999). Our double staining of IB4/NR1 also confirmed the
presence of NMDA receptor in the terminals of primary afferent fibers, since IB4-labeled positive fibers in the dorsal horn originate only from primary afferent DRG neurons (Plenderleith and Snow, 1993).

The number of double-labeled neurons to the total number of the IB4 and NR1-positive DRG neurons in the NS control group was 45.95±2.56% and 45.83±1.28%, respectively. The percentage could reflect the fact that about 46% of NR1-positive neurons are small size and may be related to nociceptive sensory system since the previous findings have demonstrated that IB4-positive neurons are almost small population of DRG cells, giving off unmyelinated axons (Plenderleith and Snow, 1993). In addition, in our preliminary observation, we found that the number of IB4-labeled DRG neurons is stable even after CFA injection and EA treatment (data not shown). Therefore, the increased number of IB4/NR1 double-labeled neurons mainly reflects the increased expression of NR1 in small neurons.

An earlier study demonstrated that the number of NR1-immunostained neurons in the DRG of CFA-inflamed animals decreased for at least 20 days following the initial inflammation, and the decrease in the proportion of NR1 neurons could reach its peak 3 days post-CFA injection (Wang et al., 1999). However, along with the overall regression of inflammation following injection of CFA in the hind paw, a significant increase in the proportion of NR1-labeled unmyelinated axons occurred on days 2 and 7 in a recent study (Du et al., 2003). We found that CFA injection resulted in the increase of the percentage of the number of double-labeled cells to the total number of the IB4-labeled neurons on days 3 and 7 post-injection. Our data seemed to be in agreement with this recent study, but disagreed with earlier observations. A possible

Fig. 2 – IB4 and NR1-labeled or IB4/NR1-double labeled neurons and fibers at the lumbar dorsal root ganglion (DRG) and dorsal horn of spinal cord, respectively. (A) IB4-labeled neurons at the lumbar DRG, showing small size; (B) IB4-labeled fibers and terminals at the neurons at the dorsal horn of spinal cord; (C) NR1-labeled neurons at the lumbar DRG. Most are small neurons and a few are median and large ones; (D) NR1-labeled fibers and terminals at the neurons at the dorsal horn of spinal cord; (E) IB4/NR1 double-labeled neurons at the lumbar DRG, all showing small size. (F) IB4/NR1 double-labeled fibers and terminals at the neurons at the dorsal horn of spinal cord. The overlapping area of IB4-labeled fibers and terminals with NR1-immunoactive products was mainly located in laminae I and II. Arrows in panels A, C and D indicate a same small neuron. Scale bar = 50 μm for panels A–F.
investigated. It is possible that EA signals are integrated in the neurons after CFA injection is still unclear and remains to be depressed effects of EA on the functional change of DRG of acupuncture analgesia. The detailed mechanism of sensory neurons might be involved in the primary integration in inflammatory reaction induced by CFA and that the primary et al., 2002), suggesting that EA treatment could attenuate the mRNA expression in the dorsal horn was significantly weaker are in agreement with the previous study that the NMDAR-2 B NR1 in the DRG small neurons after CFA injection. Our results were not found in the EA control group. EA treatment could the rats received electroacupuncture (EA) once a day, which were significant changes in all pain-related parameters after injection compared with the baseline, which was more obvious from day 1 to 3. In addition, we found that there were significant changes in all pain-related parameters after the rats received electroacupuncture (EA) once a day, which were not found in the EA control group. EA treatment could significantly reduce the heat hyperalgesia in CFA-induced inflammatory rats and had an accumulative effect. Clinical investigation has demonstrated that EA treatment is effective against chronic pain in human. For example, some earlier studies (Tukmachi et al., 2004; Sangdee et al., 2002) examined the effectiveness of manual and EA on symptom relief for patients with osteoarthritis of the knee. After five weeks’ treatment, a significant improvement was seen using a visual analogue pain scale (VAS) and the Western Ontario McMaster (WOMAC) questionnaire for osteoarthritis of the knee.

We found that EA depressed the increased expression of NR1 in the DRG small neurons after CFA injection. Our results are in agreement with the previous study that the NMDAR-2 B mRNA expression in the dorsal horn was significantly weaker in the formalin-induced inflammation after EA treatment (Li et al., 2002), suggesting that EA treatment could attenuate the inflammatory reaction induced by CFA and that the primary sensory neurons might be involved in the primary integration of acupuncture analgesia. The detailed mechanism of depressed effects of EA on the functional change of DRG neurons after CFA injection is still unclear and remains to be investigated. It is possible that EA signals are integrated in the central nervous system and in their turn affect the functions of DRG neurons via the change of stress hormone secretion and outputs of the sympathetic and parasympathetic system.

It has been demonstrated that glucocorticoids, one of the important stress hormones, has wide effects on the expression of neurotransmitters and their receptors and the expression of high level glucocorticoid receptors were found in the DRG (Donaldson et al., 1994; Smith et al., 1991). In addition, the innervations of rich autonomic terminals have been found in the DRG (Li and Chen, 1996). The depressed effect of EA treatment on the increased expression of NR1 in the DRG neurons after CFA injection that is associated with the improvement of behavioral changes indicates that the effect may take place at the DRG level. Although this study only investigated the effect of EA treatment on the expression of NR1 in the DRG neurons, our findings are interesting in the light of the proposed mechanisms in relation to hyperalgesia.

| Table 1 – The effects of EA on the change of IB4/NR1 double-labeled neurons on days 3 and 7 post-injection of CFA |
| V (v=n−1) | Day 3 post-injection | Day 7 post-injection |
| A (%) | B (%) | A (%) | B (%) |
| CFA group | 5 | 65.9 ± 4.30*** | 62.8 ± 2.04** | 60.57 ± 6.01** | 59.86 ± 3.33** |
| EA group | 5 | 54.65 ± 3.37*** | 52.43 ± 2.12** | 51.98 ± 4.35*** | 49.40 ± 3.030*** |
| EA control group | 3 | 66.02 ± 1.92*** | 63.79 ± 2.35** | 60.10 ± 1.77*** | 60.07 ± 3.01*** |
| NS control group | 3 | 45.95 ± 2.56 | 45.83 ± 1.28 | 45.70 ± 2.51 | 45.23 ± 1.42 |
| F | 221.08 | 120.21 | 134.70 | 100.14 |

Note: A: The percentage of number of double-labeled cells to the total number of the NR1-LI cells. B: The percentage of number of double-labeled cells to the total number of the IB4-labeled cells. * or ** as compared with NS control group (P<0.05, **P<0.01); ***: as compared with EA control group (P<0.01); #: as compared with the group of day 7 post-injection (P<0.05). n=6 for days 3 or day 7 for CFA and EA; n=4 for day 3 or day 7 for EA and NS control group.

4. Experimental procedures

4.1. Experimental animals

The experiments were carried out on male adult Sprague-Dawley rats (SD, 180–200 g), purchased from the Experimental Animals Center of Tongji Medical College of Huazhong University of Science and Technology. All animal experiments were approved by the Animal Care Committee of Huazhong University of Science and Technology and conformed to the ethical guidelines of the International Association for the Study of Pain (IASP). SD rats were individually housed in cages with a 12 h light/dark cycle and had free access to food and water for a week before being treated. Rats were randomly divided into four groups: (1) Complete Freund’s Adjuvant (CFA, Sigma) induced inflammatory pain group (CFA). The rats were pretreated with an intra-articular injection of 50 μl CFA into the left tibio-tarsal joint as described before (Butler et al., 1992); (2) Adjuvant induced inflammatory pain + EA treatment group (EA); (3) Adjuvant induced inflammatory pain + EA control group (EA control). Two acupuncture needles were inserted into two acupoints, respectively, without electrical stimulation in EA control group; (4) Normal saline control group (NS control). The rats were pretreated with an intra-articular injection of 50 μl physiological saline (0.9%) into the left (ipsilateral) tibio-tarsal joint. The injections were carried out...
under light anesthesia with 10% chloral hydrate (300 mg/kg) by abdominal injection.

4.2. Animal behavioral studies

The rats from each group were tested before the injection to obtain baseline pain parameters. Thermal threshold, edema of the hind paw (preclinical studies) and “foot-bend” score were tracked for 14 days following the injection of CFA or saline. The rats of the EA treatment group were tested 3 h after EA treatment every time.

4.2.1. Thermal hyperalgesia

The method of Hargreaves et al. (1988) was used to assess pain withdrawal latency (PWL) following a thermal nociceptive stimulus. Rats were allowed to acclimatize within a plexiglass enclosure on a clear glass plate maintained at 30 °C. A radiant heat source (i.e. high-intensity projector lamp bulb) was activated with a timer and focused onto the plantar surface of the hind paw. Thermal threshold was determined by a photocell that halted both lamp and timer when the paw was withdrawn. Three trials at 5 min intervals were conducted on each of the rat’s hind paws. The voltage was adjusted to derive an average baseline thermal threshold of approximately 20 s, and a maximal cut-off of 40 s was employed to prevent tissue damage.

4.2.2. Edema of the hind paw

A vernier caliper was used to measure the thickness of the hind paw from the dorsum of the foot to the planta pedis. Width of the hind paw was calculated by taking the surface of the external malleolus and malleolus medialis as two detecting points of the vernier caliper.

4.2.3. “Foot-bend” score

Hyperalgesia elicited by manipulating the hind paw was measured using 2 tests in the following sequence (Calvino et al., 1994; Li et al., 2005), and the animal was allowed to rest for 15 min in the cage before the second test was performed. (1) Flexion score: The total scores of squeaking and leg-withdrawal ranged from 0 to 10 were obtained according to previous study (Li et al., 2005). Five stimuli were administered at 5 s intervals; a rating of 1 or 0 was given, respectively, depending on whether the animal emitted a squeak or withdrew its leg; Total scores were evaluated by a mean of five flexions; (2) Extension score: Total score was evaluated by extension of the left hind paw (identical rating used as for flexion score).

4.3. Electroacupuncture (EA) treatment

In the EA treatment group, the rats received EA on the ipsilateral “Huantiao”(GB30) and “Yanglingquan”(GB34) once a day, starting at the first day after injection of CFA by using the DM-A Electroacupuncture Apparatus (Model DM-A, Haidian Medical Electronic Instrument, Beijing, China). Two acupuncture needles were inserted in two acupoints, respectively, which correspond to “Huantiao”(GB30) and Yanglingquan”(GB34) in human: “Huantiao”(GB30) is located at the junction of the lateral 1/3 and medial 2/3 of the distance between the greater trochanter and the hiatus of the sacrum, and “Yanglingquan”(GB34) lies on the lateral aspect of the leg, in the depression anterior and inferior to the head of the fibula in rats (Fig. 3). Alternating trains of dense-sparse frequencies (dense 16 Hz for 1.5 s and sparse 4 Hz for 1.5 s, respectively) were selected. The intensity of stimulation was increased according to a preset schedule of 0.5–1–1.5 V, with the procedure lasting for 10 min, respectively, which added up to 30 min. No electrical stimulation was delivered to rats in the EA control group.

4.4. Double immunofluorescence staining

On days 3 and 7 after injection, animals were deeply anesthetized with chloral hydrate (300 mg/kg) and perfused through the ascending aorta with 100 ml physiological saline

Fig. 3 – Illustration of the EA procedures and acupoints (a, b) in rats, which correspond to “Huantiao”(GB30) and Yanglingquan”(GB34) in human (A, B).
(0.9%) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4, 4 °C). After the perfusion, the L4–L5 DRG and corresponding segments L4–L5 of the spinal cord were removed and kept in the same fixative for 6 hr and then cryoprotected overnight in 20% sucrose in 0.1 M PB. DRG and transverse spinal sections (10 μm) were cut on a cryostat. Sections were collected in 0.01 M phosphate buffer saline (PBS, pH 7.4). Free-floating sections were processed for double immunofluorescence staining of NR1 and IB4. Briefly, the sections were blocked with 2% normal goat serum (NRS, Vector) in 0.3% Triton X-100 for 1 h at room temperature (RT) and incubated overnight at 4 °C in a mixture of the two primary antibodies (Goat anti-NR1, 1:600, Santa Cruz; and BS-Isoclect B4 FITC Conjugate, 1:900, Sigma). Sections were rinsed and incubated in 2% NRS for 30 min and in fluorochrome-conjugated secondary antibodies for 3 h. The secondary antibody was Cy3-conjugated rabbit anti-goat (Sigma, 1:200). The sections were washed and mounted on slides with mounting medium (Vector). To test the specificity of the immunofluorescence staining, the primary or secondary antibodies were omitted during the staining and resulted in the lack of specific staining. To control for possible cross-reaction between the first primary antibody and the second secondary antibody, the second primary antibody was omitted. In this condition, no staining was observed on the fluorescent channel corresponding to the second antibody. Moreover, we obtained identical results when the order of the two primary antibodies was reversed. The staining sections were analyzed and examined by Laser scanning confocal microscope (Leica, German) and fluorescent microscope (Olympus, Japan), respectively. When only the standard fluorescence filters (first the FITC and then the Cy3 filter) were available, the separately digitized images of a specific field were taken by automatic mode exposure to obtain a pair of two different color images for NR1 and IB4-labeled DRG neurons, respectively. For the quantification, the number of immunofluorescence-stained NR1/IB4-positive neurons in each image was counted with a computer-assisted image analyzer (Image Pro-plus Kodak, USA).

4.5. Data analysis

Digital images were captured in 5 sections per animal and 3 squares (250×250 μm each) per section of immunofluorescence staining using an Olympus microscope under the ×200 magnification. The number of NR1 and IB4-positive neurons and NR1/IB4 double immunofluorescence staining neurons was counted with a computer-assisted image analyzer (Image Pro-plus Kodak, USA). The diameter of labeled neurons was measured and categorized into small (under 30 μm in diameter), medium (from 30 to 45 μm) and large (over 45 μm). For the sections of double immunofluorescence staining, the percentage of the number of double-labeled ganglion cells to the total number of the IB4 and NR1-positive cells was calculated, respectively. SPSS software was used for data analysis. The data were expressed as mean ± SEM. Paired t-test was used to test the difference between left and right side of DRG in the same group, and the analysis of variance (ANOVA) was used to compare the difference among different groups and at different time points. P<0.05 was considered statistically significant.

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