J Formos Med Assoc 2010;109(6):438–445

Contents lists available at ScienceDirect

Journal of the Formosan Medical Association

Journal homepage: http://www.jfma-online.com

Original Article

Norepinephrine can Act via α_2-Adrenoceptors to Reduce the Hyper-excitability of Spinal Dorsal Horn Neurons Following Chronic Nerve Injury

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Background/Purpose: Rats display behavioral signs of neuropathic pain lasting for months in the chronic constriction injury (CCI) model. During intrathecal anesthesia, the administered drugs mainly diffuse directly into the superficial neurons in the spinal dorsal horn. This study aimed to investigate the effect of bath application of norepinephrine on whole cell patch clamp recordings from spinal cord slices of CCI rats with allodynia.

Methods: An assessment of paw withdrawal threshold in response to mechanical stimulation was performed on the operated side on the day before surgery and was repeated after recovery from anesthesia and on the 7th and 14th days after surgery. Spinal cord slice preparations containing dorsal horn neurons were obtained from both sham-operated rats and CCI rats (after the 14th postoperative day behavior test).

Results: Compared with normal controls, CCI rats had significantly lower levels of both hyperpolarization and spike threshold in single action potentials recorded from lamina I and II neurons of the spinal dorsal horn. In contrast, a series of action potential recordings showed that the percentage of spiking neurons of the spinal dorsal horn of CCI rats were significantly higher than those of normal controls. The CCI-induced reduction in hyperpolarization, as well as the increased numbers of spinal dorsal horn spiking neurons could be significantly reduced by norepinephrine application. The norepinephrine-induced increased hyperpolarization and input resistance could be abolished by the application of an α_2-adrenoceptor antagonist (idazoxan; 200 nM).

Conclusion: The results suggest that chronic nerve injury may induce neuropathic pain by increasing the excitability of spinal dorsal horn neurons. This excitability can be reduced by norepinephrine.

Key Words: α_2-adrenoceptors, allodynia, neuropathic pain, norepinephrine, spinal dorsal horn

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Received: May 6, 2009
Revised: July 8, 2009
Accepted: August 11, 2009

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In the chronic constriction injury (CCI) model, four ligatures of chromic gut sutures, with 1 mm spacing, are loosely tied around the common sciatic nerve.[1] Fourteen days after CCI, animals display behavioral signs of neuropathic pain that last for months. During intrathecal anesthesia, the administered drugs diffuse directly into the superficial neurons in lamina I and II of the spinal dorsal horn.[2] In fact, most unmyelinated C- and myelinated Aδ-fibers terminate within lamina I and II. Therefore, these areas are considered to be key components in the spinal dorsal horn acting during the processing of nociception.[3,4] However, data showing that CCI can cause an increase in spinal dorsal horn neuronal excitability are not available.

Evidence now suggests that perineural clonidine acts on α_2-adrenoceptors to reduce hypersensitivity in established nerve injury[5] and to suppress the generation of action potentials in tonic-firing spinal dorsal horn neurons in rats.[6] In addition, recent findings reveal that epinephrine itself induces an unexpected transient, partial block of the cutaneous trunci muscle reflex in response to a local dorsal cutaneous noxious pinprick.[7,8] This observation raises the possibility that norepinephrine may act via α_2-adrenoceptors to reduce the excitability of spinal dorsal horn neurons following CCI.

Therefore, the purpose of the present study was twofold: to investigate the action of CCI on the membrane properties of spinal dorsal horn neurons and to assess the effect of application of norepinephrine alone or norepinephrine plus idazoxan (an α_2-adrenoceptor antagonist) on whole cell patch clamp recordings from spinal cord slices of CCI rats with allodynia.

Methods

Animals

Male Wistar rats, aged 6 weeks and weighing 150–180 g at the time of surgery, were obtained from the Animal Resource Center, Warwick Medical School, University of Warwick, Coventry, UK. They were housed in Perspex cages in groups of four in a controlled environment of constant temperature and moisture (temperature = 21 ± 1°C; a light and dark cycle of 12:12 hours) and fed *ad libitum*. All procedures in this study were approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act, 1986.

CCI model

The CCI model was performed as described previously.[1] Briefly, rats were anesthetized with a 5% isoflurane/95% oxygen gas mixture for induction followed by an intraperitoneal injection of sodium pentobarbitone (50 mg/kg). The lateral side of the left thigh was shaved and disinfected with 75% ethanol. A small incision of about 1 cm was made parallel to the femur. The muscle was carefully separated to expose the sciatic nerve. Four loose ligatures were placed on the sciatic nerve with 4-0 suture chromic gut at 1 mm intervals. The wound was closed in layers with suture chromic gut and the animals were placed in a recovery chamber with the temperature controlled at 30°C. The sham-operated controlled animals received the same operation, but no ligation of the sciatic nerve with chromic gut was performed. Each injured and sham-injured animal for the CCI model was placed back in the home cage after complete return of consciousness and free movement. A dose of amoxicillin (0.1 mL, 15 mg) was routinely injected intraperitoneally after surgery to prevent infection.

Assessment of paw withdrawal threshold

The assessment of paw withdrawal threshold (PWT) in response to mechanical stimulation was carried out on the operated side on the day before surgery and was repeated after recovery from anesthesia, and on the 7th and 14th day after surgery. The PWT was measured using a series of graduated von Frey hairs (Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL, USA). The animals were placed in individual Perspex boxes on a raised metal mesh for at least 30 minutes before the test. A Von Frey filament (20.9 g), to which the cut end of an 18-gauge needle was affixed, was used to standardize the stimulus intensity. Starting from the filament of lowest force,

each filament was applied perpendicularly to the center of the ventral surface of the paw until slightly bending for 6 seconds. If the animal withdrew or lifted the paw upon stimulation, then a hair with force immediately lower than that tested would be used. If no response was observed, then a hair with force immediately higher would be tested. The lowest amount of force required to induce reliable response (positive in 3/5 trials) was recorded as the value of PWT. Only animals showing significant mechanical allodynia (PWT ≤ 3.5 g) were used for further electrophysiological experiments. Six pinpricks per test were sufficient to obtain reproducible results among the different rats within study groups. Six pinpricks also enabled injury of the skin (redness, swelling) to be avoided during repeated testing of the studied skin patches.

Spinal cord slice preparation

Spinal cord slice preparations containing dorsal horn neurons were obtained from both sham-operated rats and CCI rats (after the 14th post-operative day behavior test). The methods of preparation were similar to those described previously.[9] Briefly, animals were deeply anaesthetized with isoflurane and were rapidly decapitated. The spinal cords were carefully removed and cut out in freshly prepared, pre-oxygenated (95% O_2 and 5% CO_2), ice-cold (0–5°C) artificial cerebrospinal fluid (ACSF). After de-sheathing the spinal cord of pia-arachnoid matters with fine forceps, the lumbar enlargement segments were transversely sectioned and hemi-dissected along the midline. The lumbar enlargement from the operated side of both sham-operated rats and CCI rats were employed in our research. The lumbar enlargement was then removed into the dissection chamber and glutted on the stage of the slicing chamber, and cut into longitudinal 300–350 μm thick slices using a Leica VT1000S vibrating blade microtome (Leica Microsystems UK, Milton Keynes, UK). The slices were maintained in ACSF at room temperature for 1 hour after slicing before electrophysiological recording.

Preparation solution contained: NaCl 115 mM, KCl 5.6 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, glucose 11 mM, NaH_2PO_4 1 mM, and $NaHCO_3$ 25 mM (pH 7.4 when bubbled with 95% O_2/5% CO_2). In the experimental chamber, the slices were superfused by low-Ca^{2+}-solution in order to reduce spontaneous synaptic activity and to prevent activation of Ca^{2+} currents and Ca^{2+}-dependent K^+ currents. The following drugs with discrete concentrations were directly added to control solutions: norepinephrine (a non-specific α- and β-adrenoceptor agonist, 0.5, 1, 10, 40 μM) and/or idazoxan (an α-2 adrenoceptor antagonist, 200 nM). The experimental chamber, with a volume of 0.4 mL, was continuously perfused with external solution at a rate of 2–3 mL/min. The pH of drug solutions was tested and corrected to eliminate potential pH-induced effects. All drugs were dissolved in deionized water to make the stock solution. The final dilution was made with ACSF for electrophysiological studies. All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Action potential recordings

High K^+-1-solution used for action potential recordings from intact neurons contained: NaCl 5 μM, KCl 144.4 μM, NaCl 1 μM, EGTA 3 μM, and HEPES 10 μM, pH 7.3 (adjusted to 1 MKOH). KCl was then added to a final K^+ concentration of 155 mM.

Lamina II [containing the substantia gelatinosa (SG) and dorsal horn neurons] has a distinct translucent appearance and can be easily distinguished under a binocular microscope placed in an illuminated recording chamber (Olympus SZ40 Stereo zoom Microscope; Axon Instruments, Union City, CA, USA).[10,11] The relatively translucent band across the dorsal horn is clearly discernible. Lamina I is the outer zone of the translucent band and is located in the most superficial aspect of the grey matter.[12]

Slices were transferred to a custom-made recording chamber and continuously perfused at room temperature with 4–10 mL/min ACSF for recording. Whole cell recordings were performed with an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA). Recordings with series resistances in the range of 8–20 MΩ were obtained,

using the "blind" methods described previously.[10] Patch pipettes were pulled using a horizontal puller (Sutter Instrument Co, Novato, CA, USA) from thin-walled borosilicate glass capillaries (GC150-TF10, Harvard Apparatus Edenbridge, Kent, UK), which had resistances of 3–7 MΩ when filled with recording solution. Correction of the liquid junction potential was applied to whole cell recordings. Neuronal input resistance was measured by injecting small rectangular-wave hyperpolarizing current pulses on constant amplitude (10–100 pA) and measuring the mean amplitude of a minimum of five resulting electronic potentials (in control conditions and in the presence of the test compound). The experimental trace was viewed online with a digital oscilloscope (Gould DSO 1602; Axon Instruments, Union City, CA, USA), filtered at 5 kHz and stored on computer. For subsequent analysis, the signal was digitized at 2–10 kHz and processed on a personal computer running the pClamp 8.2 software package (Axon Instruments, Union City, CA, USA).

Statistical analysis

All data are expressed as mean ± standard deviation. Paired *t* test was used for intragroup comparison. Independent *t* test was used to analyze the difference between groups. A *p* value less than 0.05 was considered statistically significant.

Results

CCI decreases PWT

Table 1 summarizes the PWT values for diseased legs in 26 sham-operated rats and 26 CCI rats the day before and days after operation (0,7, and 14). The diseased legs of CCI rats displayed lower PWT values as compared with those of sham-operated rats, at least when evaluated at day 7 and 14 after CCI ($p < 0.01$; $n = 26$). These animals were euthanized 14 days after CCI or sham operation for whole cell patch clamp recordings in the following experiments.

Action potentials

Current clamp experiments were performed in intact neurons in the spinal cord slice using external solution in the bath and pipettes filled with high K^+-1-solution. Single action potentials were elicited using 1-ms depolarizing current. In all cells, the resting membrane potential (mV), input resistance (MΩ), membrane time constant (τ), spike duration (ms), spike amplitude (mV), and spike threshold (mV) of single action potentials were measured and summarized in Table 2. In addition, a "series" of action potentials were elicited using a depolarizing current pulse (500 ms) of increasing amplitude. The percentages of spiking neurons over total neurons encountered were determined.

CCI increases excitability of spinal dorsal horn neurons

Table 2 summarizes the membrane properties of spinal dorsal horn neurons obtained from 26 CCI rats and 26 sham rats. The values of resting membrane potential, spike threshold, and fractional sparking neurons (or percentage of spiking neurons) in CCI rats were higher than those of sham rats. In contrast, the values of spike altitude in CCI rats were significantly lower than those of sham rats (Table 2).

Table 1. Paw withdrawal threshold values obtained from the ipsilateral leg in sham-operated and chronic constriction injury rats the day before and days after surgery*

Groups	Paw withdrawal threshold (g)			
	1 Day before surgery	After surgery		
		Day 0	Day 7	Day 14
Sham rats ($n = 26$)	9.9 ± 1.6	9.8 ± 1.7	8.1 ± 1.8	9.0 ± 1.9
CCI rats ($n = 26$)	10.1 ± 1.9	9.9 ± 1.8	2.1 ± 0.9†	1.3 ± 0.7†

**Data presented as mean ± standard deviation; †$p < 0.01$ compared with sham-operated rats. CCI = Chronic constriction injury.*

Table 2. Membrane properties of spinal dorsal horn neurons obtained from sham-operated and chronic constriction injury rats with peripheral neuropathic injury*

Membrane properties	Sham-operated rats ($n=26$)	CCI rats ($n=26$)	p
Resting membrane potential (mV)	–52 ± 11 (63)	–47 ± 9 (66)†	0.04
Input resistance (MΩ)	798 ± 419 (63)	789 ± 422 (66)	0.90
Membrane time constant (τ)	56 ± 30 (63)	58 ± 31 (66)	0.76
Spike duration (ms)	2.7 ± 0.7 (63)	2.9 ± 0.7 (66)	0.17
Spike amplitude (mV)	67 ± 17 (63)	58 ± 16 (66)†	0.01
Spike threshold (mV)	–52 ± 7 (63)	–22 ± 7 (66)†	0.03
Percentage of spiking neurons/total neurons	9% (63)	52% (66)†	0.001

**Data presented as mean ± standard deviation or %, followed by numbers of neurons studied in parentheses; †$p<0.05$ compared with sham-operated rats. CCI = Chronic constriction injury.*

Table 3. Membrane potential, input resistance change and number of hyperpolarized spinal dorsal horn neurons in response to bath application of 40 μM norepinephrine in sham-operated and chronic constriction injury rats*

	Sham-operated rats	CCI rats
Membrane potential (mV)	–0.55 ± 0.38 (49)	–9.86 ± 0.42 (57)†
Input resistance change (%)	97.5 ± 10.8 (49)	74.1 ± 11.7 (57)†
Fraction of hyperpolarized neurons/total neurons	33/49 (67%)	52/57 (91%)†

**Data presented as mean ± standard deviation followed by the number of neurons studied in parentheses or fraction (%); †$p<0.05$ compared with sham rats. CCI = Chronic constriction injury.*

Table 4. Effects of bath application of norepinephrine and/or idazoxan on both membrane potential and input resistance changes of spinal dorsal horn neurons obtained from 10 rats with chronic constriction injury*

Treatment	Membrane potential (mV)	Input resistance (%) change
Norepinephrine only		
0.5 μM	–0.52 ± 0.41 (10)	94.7 ± 11.6 (10)
1 μM	–1.70 ± 0.76 (10)	83.1 ± 8.0 (10)
10 μM	–5.88 ± 0.44 (10)†	87.2 ± 14.2 (10)
40 μM	–10.66 ± 0.57 (10)†	73.7 ± 18.9 (10)†
40 μM norepinephrine + 200 nM Idazoxan	2.42 ± 0.89 (10)‡	94 ± 11 (10)‡

**Data presented as mean ± standard deviation followed by numbers of neurons studied in parentheses; †$p<0.05$ compared with 0.5 μM norepinephrine; or ‡$p<0.05$ compared with 40 μM norepinephrine.*

Norepinephrine decreases CCI-induced excitability of spinal dorsal horn neurons

Table 3 summarizes the values of mean hyperpolarization, input resistance change, and fraction of hyperpolarized neurons over total neurons from CCI rats and sham rats in response to bath application of 40 μM norepinephrine. The spinal dorsal horn neurons of CCI rats had significantly higher membrane potential, as well as fraction of hyperpolarized neurons/total neurons than those of sham rats. In contrast, the spinal dorsal horn neurons of CCI rats displayed significantly lower input resistance change (74.1% *vs.* 97.5%) than those of sham controls.

Norepinephrine may act via α_2-adrenoceptors to decrease CCI-induced excitability of spinal dorsal horn neurons

Table 4 summarizes the values of membrane potential and input resistance of spinal dorsal horn neurons from 10 CCI rats in response to bath application of norepinephrine alone or

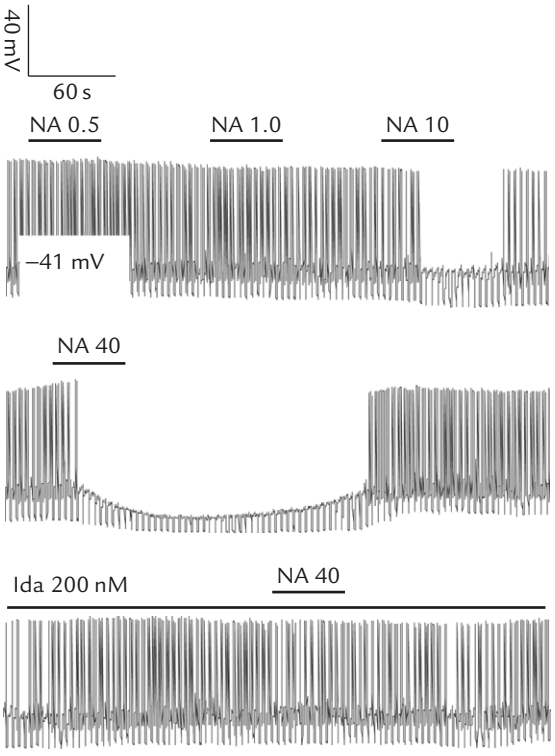

Figure. Effects of bath application of 0.5–40 μM norepinephrine and/or 200 nM idazoxan on both membrane potential and input resistance of a spinal dorsal horn neuron obtained from a rat with allodynia. NA = Norepinephrine; Ida = idazoxan.

norepinephrine plus idazoxan. Bath application of norepinephrine causes a dose-dependent hyperpolarization and a decrease in input resistance change over a dose range of 0.5–40 μM in spinal dorsal horn neurons of CCI rats. The norepinephrine-induced hyperpolarization and decreased input resistance change in spinal dorsal horn neurons were significantly reversed by idazoxan application. A representative example is depicted in the Figure. The figure also shows that firing frequencies of trains of action potentials in spinal cord neurons were reduced at high concentration of norepinephrine (10–40 μM), and could be abolished by idazoxan.

Discussion

At least five different sites of pathophysiological changes have been proposed for a spinal nerve injury, leading to alterations at many sites along the neural axis for pain.[13] These alterations include: (1) spontaneous neural activity to mechanical stimuli develops at the site of nerve injury; (2) spontaneous neural activity develops in the dorsal root ganglia; (3) augmentation of dorsal horn neurons to cutaneous stimuli; (4) activation of microglial cells to the development of this spinal dorsal horn sensitization; and (5) the enhanced responsiveness of spinal dorsal horn neurons to changes in descending modulation. The present results tend to support the hypothesis that CCI may induce mechanical allodynia by augmenting the excitability of spinal dorsal horn neurons in CCI rats. As demonstrated in the present results, whole cell patch clamp recordings revealed that spinal dorsal horn neurons of CCI rats displayed less hyperpolarization (or more depolarization). This observation was characterized by higher values of resting membrane potential and spike threshold of single action potentials compared with those of normal controls. In addition, the spinal cord horn of CCI rats had higher amounts of spiking neurons when compared with those of normal controls. It is likely, therefore, that the firing frequency of spiking neurons in CCI rats may be significantly higher than those of normal controls. However, it is not known whether peripheral nerve constriction alters the membrane properties of spinal dorsal horn neurons by acting on enhancement of excitatory synaptic transmission and/or attenuation of inhibitory modulation.[14]

Intrathecal administration of the α_2-adrenoceptor agonist, clonidine, significantly attenuates hyperalgesia and tactile allodynia associated with chronic nerve injury in the rat spinal nerve ligation model.[15,16] Recent findings[7,8] have also shown that the epinephrine itself induces an unexpected block of the cutaneous trunci muscle reflex, which is characterized by reflex movement of the skin over the back produced by twitches of lateral thoracic-spinal muscles in response to local dorsal cutaneous noxious pinprick. In addition, dexmedetomidine (a specific α_{2A}-adrenoceptor agonist) enhances the local anesthetic action of lidocaine in guinea pigs.[17] These observations prompted us

to speculate that clonidine, epinephrine, and dexmedetomidine may act via α_{2A}-adrenoceptors to exert their local anesthetic action. This hypothesis is partially supported by the present results. Our results demonstrate that bath application of norepinephrine can act via α_2-adrenoceptors to reduce the spinal dorsal horn neuronal excitability obtained from CCI rats with tactile allodynia.

During spinal anesthesia, the superficial neurons in laminae I and II of the dorsal horn can be particularly affected by the applied drugs diffusing directly into the spinal cord.[2] Laminae I and II contain most fine-caliber C-and Aδ-fibers; these are considered to be a key element in the pain processing system. At least, three classes of superficial dorsal horn neurons have been distinguished.[18] Tonically-firing neurons were described as exhibiting little spike frequency adaptation during sustained depolarization. Adapting-firing neurons generate a short burst of spikes at the beginning of depolarization. Single-spike neurons generate only one or seldom two spikes in the beginning of a depolarizing pulse. Both tonically-firing and adapting-firing neurons are classified as nociceptive neurons,[18] whereas single-spike neurons could act as coincidence detectors.[19] According to a recent report by Wolff et al,[6] firing frequency of trains of action potentials in tonic-firing neurons in the spinal dorsal horn are reduced by clonidine, but not in adapting or single-spike-firing neurons. These results are supported by several investigators. For example, α_2-adrenoceptor agonists are thought to produce analgesia primarily by actions in the spinal cord by hyperpolarizing dorsal horn neurons.[6,20] Spinal nerve ligation neuropathy increases α_2-adrenoceptor G-protein coupling in the spinal cord; this is associated with the enhanced potency of clonidine treatment in neuropathic pain.[21] The intrathecal antihyperalgesic/antiallodynic effect of clonidine on neuropathic pain is associated with a significant reduction in spinal NMDA receptor phosphorylation and suggests a spinal dorsal horn neuronal mechanism of action for clonidine.[22] However, a spinal dorsal horn neuronal mechanism of clonidine action cannot be extrapolated to reflect the norepinephrine effect.

It has been suggested that the majority of the SG consists of local interneurons that do not project to the thalamus.[23–26] The main projections of SG neurons are to lamina I and to the deep dorsal horn (lamina IV–V). It has been shown that α_1-receptor activation inhibits nociceptive input to deep dorsal horn neurons.[24] In the majority of SG neurons tested, norepinephrine (10–100 μM) increased the frequency of γ-aminobutyric acid and glycinergic miniature postsynaptic currents.[23] This augmentation was mimicked by an α_1-receptor agonist, phenylephrine, and inhibited by α_1-receptor antagonists prazosin and 2-(2,6-dimethoxylphenoxyethyl) amino-methyl-1,4-benzodioxane.[23] Primary-afferent-evoked polysynaptic excitatory postsynaptic potentials or excitatory postsynaptic currents in wide-dynamic-range neurons of the deep dorsal horn were also attenuated by phenylephrine.[24] These observations strongly suggest that norepinephrine facilitates inhibitory transmission in the SG of adult rat spinal cord through activation of presynaptic α_1-receptors located in the deep dorsal horn (lamina IV–V). Presently, it is thought that norepinephrine may also act via facilitating this inhibitory transmission in the SG to reduce hyperexcitability of spinal dorsal horn neurons following chronic nerve injury.

In summary, our results suggest that chronic nerve injury may induce neuropathic pain by increasing the excitability of spinal dorsal horn neurons. The data also indicate that norepinephrine can act via α_2-adrenoceptors in spinal dorsal horn neurons to reduce their hyperexcitability following chronic nerve injury. In addition, CCI-induced increase in number of spiking spinal dorsal horn neurons could be reduced by topical application of norepinephrine.

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