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Peripheral Nerve Injury Alters Excitatory and Inhibitory Synaptic Transmission in Rat Spinal Cord Substantia Gelatinosa

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Following peripheral nerve injury, excessive nociceptive inputs result in diverse physiological alterations in the spinal cord substantia gelatinosa (SG), lamina II of the dorsal horn. Here, I report the alterations of excitatory or inhibitory transmission in the SG of a rat model for neuropathic pain ("spared nerve injury"). Results from whole-cell recordings of SG neurons show that the number of distinct primary afferent fibers, identified by graded intensity of stimulation, is increased at 2 weeks after spared nerve injury. In addition, short-term depression, recognized by paired-pulse ratio of excitatory postsynaptic currents, is significantly increased, indicating the increase of glutamate release probability at primary afferent terminals. The peripheral nerve injury also increases the amplitude, but not the frequency, of spontaneous inhibitory postsynaptic currents. These data support the hypothesis that peripheral nerve injury modifies spinal pain conduction and modulation systems to develop neuropathic pain.

Key Words: Spinal substantia gelatinosa, Synaptic transmission, Neuropathic pain, Spared nerve injury

INTRODUCTION

Many chemical synapses in the central nervous system use glutamate as an excitatory neurotransmitter, and γ-aminobutyric acid (GABA) and glycine as inhibitory neurotransmitters. At such synapses, sequential events such as arrival of action potentials at presynaptic terminals, opening of voltage-gated calcium channels and calcium influx into the presynaptic terminals trigger rapid release of quanta of the neurotransmitters stored as vesicles in the nerve terminals. The released neurotransmitters immediately detected by their ionotropic receptors. When a quantum of neurotransmitter is released from the presynaptic terminal, there is a probability that an arrival of action potential at a synapse will result in neurotransmitter release, namely "release probability". In the central nervous system, the change in the release probability is the main mechanism underlying short-term synaptic plasticity, an activity-dependent decrease (depression) or increase (facilitation) of synaptic transmission occurring within several hundreds of milliseconds (Zucker & Regehr, 2002).

In the spinal cord substantia gelatinosa (SG), lamina II of the dorsal horn, the activation of primary afferent fibers, particularly Aδ and C, or excitatory interneurons, releases glutamate from the nerve terminals. Three ionotropic glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA), which exist on postsynaptic neurons, respond to the released glutamate to mediate fast synaptic transmission. Recent studies indicate that the fast synaptic transmission in the spinal SG region experiences the short-term plasticity, particularly short-term depression (Gerber et al, 2000; Wan & Hu, 2003; Youn & Randic, 2004). The role of short-term synaptic plasticity is known to contribute to the computational process of sensory information via low- pass temporal filtering (Fortune & Ross, 2001).

Peripheral nerve injury causes excessive nociceptive inputs into the SG (Woolf & Salter, 2000) via irregular burst discharge of primary sensory neurons (Amir et al, 2002), that lead to diverse physiological alterations in the excitatory and inhibitory synaptic transmission systems in the spinal cord SG region. One of the excessive nociceptive inputs is typically represented by an excessive release of glutamate, over a limit of scavenging processes at synaptic cleft, from the nerve terminals of damaged or undamaged primary afferents and presumably also excitatory interneurons. Although many studies have suggested the increased level of glutamate in the spinal SG region following peripheral nerve injury (Woolf & Salter, 2000; Somers & Clemente, 2002; Sung et al, 2003), no study has demonstrated the increased release of glutamate from primary afferent terminals after peripheral nerve injury. Therefore, to demonstrate the feasible increase of the glutamate release from primary afferent terminals following peripheral nerve injury, I investigated the short-term synaptic plasticity of primary afferent-mediated transmission in the SG of the spinal cord slices obtained from a rat model for

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ABBREVIATIONS: SG, substantia gelatinosa; GABA, γ-aminobutyric acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NMDA, N-methyl-D-aspartate; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; D-AP5, D-(-)-2-amino-5-phosphonopentanoic acid.

neuropathic pain (Decosterd & Woolf, 2000). In addition, changes of excitatory synaptic inputs from primary afferent fibers into the SG neurons and spontaneous inhibitory synaptic transmission between spinal interneurons were investigated.

METHODS

Spared nerve injury model

All experiments followed the ethical guideline of the International Association for the Study of Pain. Six week-old Sprague-Dawley male rats were subjected to a surgical procedure to produce neuropathic pain, as previously described as "spared nerve injury" (Decosterd & Woolf, 2000). Briefly, under 2.5% isoflurane anesthesia, the tibial and common peroneal branches of sciatic nerve were tightigated with 5-0 silk and transected distal to the ligature. The characteristic of this procedure was to leave the sural nerve, another branch of the sciatic nerve, intact. Therefore, a great care was required to avoid any contact with or stretching of the intact sural nerve.

Spinal cord slice preparation

Two weeks after the surgical procedure for the neuropathic pain, lumbosacral spinal cords from neuropathic or age-matched naïve rats under urethane anesthesia (1.5 g/kg, i.p.) were dissected out by laminectomy and placed in preoxygenated ice-cold Krebs' solution (composition in mM: NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11). From the lumbosacral segment (L2-S1) of the spinal cord, dura, pia and arachnoid membranes as well as all ventral and dorsal roots, except the L4 dorsal root ipsilateral to the injury, were removed before cutting a 650-μm-thick transverse slice with the L4 dorsal root (15~20 mm) intact (Moore et al, 2002). The cut slice was placed on a nylon mesh in the recording chamber and perfused with Krebs' solution for at least 30min before recording (perfusion rate: about 10 ml/min).

Whole-cell patch clamp recording

For whole-cell recording, the resistance of patch pipettes was usually 8~12 MΩ, when filled with internal solution (composition in mM: Cs_2SO_4 110, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, TEA 5, ATP-Mg salt 5). Electrodes were positioned in the SG, which was recognized as a distinct translucent band across the superficial dorsal horn under a dissecting microscope with transmitted illumination, of the spinal cord dorsal horn. Placing the electrode in such a manner targets a heterogeneous group of intrinsic stalk and islet neurons in inner and outer lamina II. Graded intensity of dorsal root stimulation sufficient to sequentially recruit Aβ, Aδ and C-fibers was applied with a suction electrode linked to a constant current stimulator, and evoked excitatory postsynaptic currents (EPSCs) in lamina II neurons. Membrane holding potential was clamped to -70 mV which is the reversal potential of evoked inhibitory postsynaptic currents (IPSCs) in this recording condition. Aδ fiber-evoked EPSCs were typically recorded at the stimulus intensity/duration of above 20 μA/0.05 ms or 10 μA/0.5 ms, and their conduction velocities were above 1.0 m/sec. If EPSCs had the conduction velocity of less than 1.0 m/sec at the stimulus intensity of >200 μA/0.5 ms, they were considered as C fiber-evoked EPSCs (Kohno et al, 2003). Aδ fiber-evoked EPSCs were classified as monosynaptic if the latency remained constant at a repetitive stimulation of 20 Hz. To calculate paired-pulse ratio (ratio of the amplitude of 2nd EPSC to the amplitude of 1st EPSC), only Aδ fiber-evoked monosynaptic EPSCs were used, and the inter-stimulus intervals were 50 or 100 ms. To record spontaneous IPSCs, neurons were voltage-clamped at 0 mV which is the reversal potential of spontaneous EPSCs in this recording condition. In this condition, spontaneous IPSCs were typically blocked by bicuculline, the $GABA_A$ receptor antagonist, and strychnine, the glycine receptor antagonist. Because the conductance of $GABA_A$ receptor is sensitive to "run-down" that usually occurs during whole-cell recordings, records of spontaneous IPSCs recorded within 5 min were included for analysis. A record at least for 1 min and with more than 50 events was analyzed in the aspect of frequency and amplitude. Synaptic responses, including evoked EPSCs and spontaneous IPSCs, were amplified using an Axopatch 200A amplifier (Axon Instruments, Union City, CA). Signals were filtered at 2 kHz and digitized at 5kHz. Data were collected and analyzed using pCLAMP software (version 8). D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5; Tocris) or/and cyclothiazide (Tocris) were included in the external solution (the final concentration: 50 μM and 10 μM, respectively) in experiments indicated. Data are expressed as mean$\pm$SEM. Student's t and χ^2 tests were used to evaluate statistical significance in means or distributions, respectively (*$P<0.05$ or **$P<0.01$).

RESULTS

Under whole-cell configurations of spinal SG neurons, graded intensity of dorsal root stimulation in the range of Aδ fiber stimulation evoked EPSCs with the same latency in a case (Fig. 1Aa) or with quite different latencies in the other (Fig. 1Ab). Since different latencies of synaptic responses represent different fibers in the aspect of impulse conduction, the latter case could be determined by being evoked by three distinct Aδ fibers. Therefore, similar experiments were carried out in naïve and 2-week spared nerve injury rats. As shown in Fig 1B, the number of SG neurons with more than two distinct fibers was significantly increased in 2-week spared nerve injury rats ($P<0.05$, χ^2 tests).

In naïve SG two pulses of stimuli at a 50 ms inter-stimulus interval (*see* Methods) evoked two successive Aδ fiber-activated EPSCs recorded in normal Krebs' solution and also in the solution containing 50 μM D-AP5, the NMDA receptor antagonist. The amplitude of the second EPSC was typically smaller than that of the first EPSC (Fig. 2A), producing the paired-pulse ratio of below 1 (0.73 ± 0.05 and 0.76 ± 0.06, respectively; Fig. 2B). This form of short-term synaptic plasticity is known as paired-pulse depression (Zucker & Regehr, 2002). The paired-pulse depression of synaptic transmission indicates that the initial release probability of glutamate is relatively high in the spinal SG. On the other hand, further addition of 10 μM cyclothiazide, the inhibitor of AMPA receptor desensitization, to the D-AP5-containing solution did not affect the paired- pulse ratio (0.75 ± 0.09; Fig. 2B), indicating a minimal contribution of AMPA receptor desensitization to

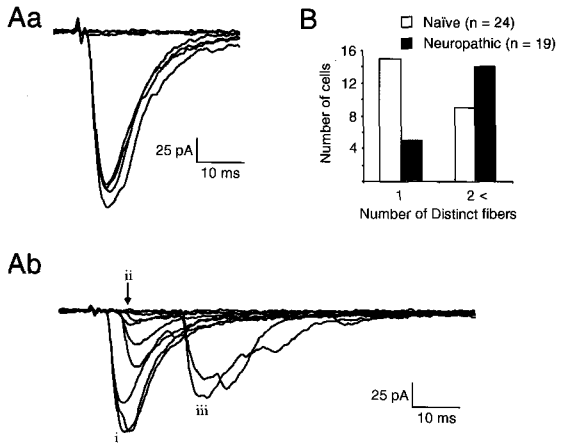

Fig. 1. The increased number of distinct fibers in the neuropathic rat induced by spared nerve injury. EPSC traces (A) demonstrate two different examples in SG neurons recorded in naïve rats. Graded intensity of dorsal root stimulation evokes EPSCs with the same latency (Aa), indicating only one kind of monosynaptic A δ fiber. However, three distinguishable latencies of EPSCs are evoked in the other example (Ab) (stimulus intensities: 12~40 μA, 0.5 ms), indicating three distinct A δ fibers (calculated conduction velocities: *i*, 9.5 m/sec, monosynaptic; *ii*, 7.1 m/sec, polysynaptic; *iii*, 1.1 m/sec, monosynaptic). In summary, the number of neurons with more than two distinct fibers (A δ only, or A δ and C), based on the conduction velocity, is increased in spared nerve injury rats (B).

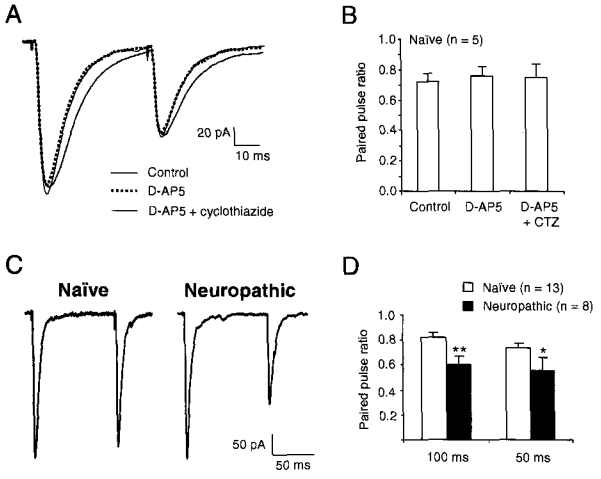

Fig. 2. The increase of glutamate release probability in neuropathic rat. Perfusing Krebs' solution containing D-AP5 (50 μM), the NMDA receptor antagonist, or D-AP5 and cyclothiazide (10 μM), the inhibitor of AMPA receptor desensitization, does not have any effect on paired pulse ratio of A δ fiber-evoked EPSCs at 50 ms inter-stimulus interval in a naïve rat (A). A histogram indicates no significant change among normal Krebs', D-AP5, and D-AP5 and cyclothiazide solutions (n = 5 naïve SG neurons; B). In EPSCs evoked by two successive stimuli at an interval of 100 ms, the calculated paired-pulse ratio is reduced in a neuropathic rat, compared to naïve rat (C), indicating the increase of paired-pulse depression and glutamate release probability. A histogram (D) indicates that the paired pulse ratio of EPSCs at intervals of 100 ms and 50 ms is significantly reduced in the neuropathic rat.

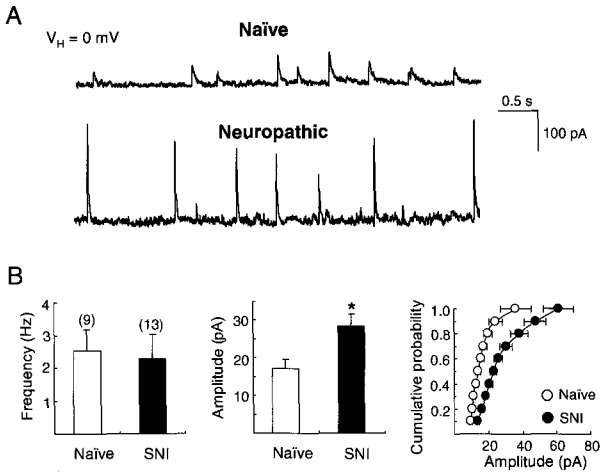

Fig. 3. The alteration of spontaneous inhibitory synaptic transmission in the neuropathic rat. In sampled traces, spontaneous IPSCs recorded in a SG neuron obtained from a neuropathic rat are bigger in amplitude than those from a naïve rat (A). In summary, the mean (B, *middle*) and the cumulative probability (B, *right*) of spontaneous EPSC amplitude from thirteen neuropathic rats are significantly increased, when compared to those from nine naïve rats (*$P<0.05$). However, the mean frequency of spontaneous IPSCs is not changed after spared nerve injury (B, *left*).

the paired-pulse depression in the spinal SG.

To further investigate the change of glutamate release probability in the spinal SG following peripheral nerve injury, the paired-pulse ratio was measured from A δ fiber-activated EPSCs evoked in SG neurons obtained from 2-week spared nerve injury rats. As shown in Fig. 2C and D, the paired-pulse ratios of EPSCs at intervals of 100 ms and 50 ms were significantly reduced in 2-weeks spared nerve injury rats (0.6 ± 0.07, $P<0.01$ vs. naïve, and 0.55 ± 0.1, $P=0.05$ vs. naïve, respectively), when compared to naïve rats (0.81 ± 0.04 at 100 ms and 0.73 ± 0.04 at 50 ms). This result indicates that the initial probability of glutamate release at central terminals of A δ fibers is higher in the neuropathic rat.

To find out a change in inhibitory transmission following peripheral nerve injury, spontaneous IPSCs were recorded in SG neurons at holding potential of 0 mV, where no spontaneous EPSC appears. Two weeks after spared nerve injury, mean amplitude of spontaneous IPSCs was significantly increased (28.45 ± 3.13 pA; $P<0.05$ vs. naïve rat, 17.19 ± 2.4 pA; Fig. 3), whereas mean frequency was not changed significantly (2.29 ± 0.74 Hz; $P>0.05$ vs. naïve rat, 2.53 ± 0.65 Hz; Fig. 3).

DISCUSSION

The present study demonstrates that, following peripheral nerve injury, the number of distinct primary afferent fibers and the release probability of glutamate at the terminals of primary afferent fibers are increased. In addition, the alteration in the spinal inhibitory synaptic transmission resulting from the peripheral nerve injury is demonstrated. These new findings indicate that peripheral nerve injury can trans-synaptically produce various functio-

nal alterations in the pain conduction and modulation systems existing in the spinal cord.

As observed in the experiment with graded intensity of primary afferent fiber stimulation, EPSCs with a single latency were evoked in most SG neurons recorded from naïve rats, whereas EPSCs with more than two different latencies predominantly in spared nerve injury rats. Based on the assumption that different latencies of synaptic responses are evoked by different fibers in conducting action potentials, this result can be interpreted as the increase in the number of fibers evoking synaptic responses in the SG neurons with injury. On the other hand, the present data, in some aspects, are in agreement with a previous study showing the increase in the percentage of spinal SG neurons with polysynaptic A δ fiber-evoked EPSCs after spared nerve injury (Kohno et al, 2003). Both studies suggest that peripheral nerve injury may result in anatomical changes in the spinal cord SG, such as sprouting of A β fiber from deep dorsal horn or A δ fibers from lamina I into the SG region (Woolf et al, 1992; Nakamura & Myers, 1999; Ma & Tian, 2001).

It has been shown that spontaneous EPSCs recorded in the spinal SG are not changed in frequency and amplitude following peripheral nerve injury (Moore et al, 2002; Kohno et al, 2003), indicating that the function of presynaptic terminals to spontaneously release glutamate is intact. However, a characteristic change of glutamate release upon electrical stimulation of primary afferent fibers has not benn known. Moreover, because spontaneous EPSCs are the mixed events that are made by the glutamate released from the terminals of both primary afferent fibers and excitatory interneurons, the result from the recordings of spontaneous EPSCs cannot be interpreted as any change in the ability of primary afferent fibers to release glutamate, once electrically stimulated. Therefore, to investigate the nerve injury-induced change in the function of primary afferent fibers to release glutamate upon electrical stimuli, paired-pulse ratio of primary afferent-activated EPSCs is measured in the present study; the method estimates the probability of glutamate release from activated primary afferent fiber terminals. In this way, the increase of short-term synaptic depression, i.e., the increase of glutamate release probability, following peripheral nerve injury could be revealed. Although the short-term depression has been known to exist in the SG of naïve rat and mouse spinal cord SG (Gerber et al, 2000; Wan & Hu, 2003; Youn & Randic, 2004), it has not been studied in the animal under various pathological states, particularly in relation to neuropathic pain. Thus, this result is the first study demonstrating the change of glutamate release probability from primary afferent terminals under pathological condition. On the other hand, the short-term depression can be mediated by postsynaptic mechanisms, for instance, AMPA receptor desensitization (Zucker & Regehr, 2002). Since AMPA receptors require at least a second to fully recover from their desensitization after being activated (Donevan & Rogawski, 1998), the desensitization can contribute to the smaller amplitude of the second EPSC. In the present study, I also excluded the possibility of postsynaptic mechanism in short-term depression by using cyclothiazide, the inhibitor of AMPA receptor desensitization (Voitenko et al, 2004). Interestingly, the short-term depression was not affected by bath application of cyclothiazide, indicating a minimal contribution of AMPA receptor desensitization in the spinal SG under normal naïve condition.

On the contrary to the previous report which showed no change in frequency and amplitude of spontaneous IPSC following peripheral nerve injury (Moore et al, 2002), the present study shows the significant increase in the amplitude, but not the frequency, of spontaneous IPSCs following nerve injury, indicating no change in excitability of inhibitory interneurons but a postsynaptic change of recorded neurons. Although similar number of SG neurons has been investigated in both experiments, there exists a possibility of variation among cell types or recording conditions. On the other hand, $GABA_A$ receptors mediating spontaneous IPSCs can easily be 'run-down' in their function due to a decreased level of intracellular ATP (Stelzer et al, 1988; Kapur et al, 1999), and the faster run-down process may occur in pathological conditions (Harata et al, 1997). In this context, the amplitudes of spontaneous IPSCs may be different, depending on the recording time after membrane rupture even in the internal patch pipette solution containing ATP. Thus, only records within 5 min from the beginning of each recording were analyzed in the present study. However, this kind of information was not available in the previous study (Moore et al, 2002). Moore et al (2002) has demonstrated no change in the cell profile of $GABA_A$ receptor expression in the spinal dorsal horn following peripheral nerve injury, supporting the result that there was no significant change in the amplitude of spontaneous IPSCs. However, a finding, that $GABA_A$ receptor function was potentiated by glutamate at concentration below that required for its excitatory action (Stelzer & Wong, 1989), may provide a possible mechanism for the significant increase of spontaneous IPSC amplitude observed in the present study. This possibility needs to be investigated further.

A picture for changes of the synaptic transmission in the spinal SG after peripheral nerve injury keeps still evolving. Here, I suggest one of possible scenarios for that (Fig. 4). At the start, ectopic discharge of primary afferents increases the amount of glutamate released from primary afferent terminals (Amir et al, 2002; the present study), thus leading to an overflow of glutamate out of the synaptic cleft ("spill-over"). An increased release of glutamate, a decrease in expression of glutamate transporter (Sung et al, 2003) and releases of other soluble factors, such as interleukin-1 and tumor necrosis factor α (Zimmermann, 2001), all together contribute to various excitotoxic effects on the dorsal horn neurons, thereby leading to the death of dorsal horn neurons, particularly inhibitory interneurons (Azkue et al, 1998; Whiteside & Munglani, 2001; Coggeshall et al,

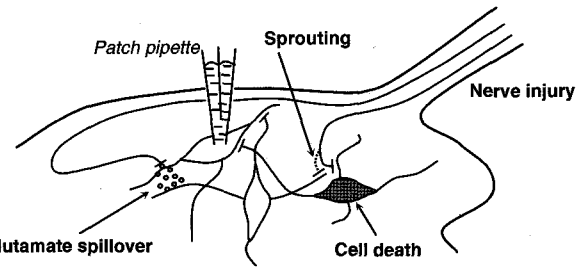

Fig. 4. A diagram demonstrating physiological changes in the spinal cord dorsal horn following peripheral nerve injury (*see* Discussion for the detail).

2001; Moore et al, 2002). The death of inhibitory neurons in the spinal cord dorsal horn may produce the 'disinhibited' state, a phenomenon known to be induced by peripheral (Moore et al, 2002) and central (Farkas et al, 2003) injuries. After the death of dorsal horn neurons, primary afferent terminals that previously synapsed with the dead cells may sprout to another neighboring neuron rather than stay there. The sprouted fibers may result in the increase of the polysynaptic connection and also of the number of distinct fibers evoking synaptic responses in the spinal SG neurons. These anatomical and physiological alterations in the spinal SG following peripheral nerve injury may contribute to the increase of gain in the neuropathic pain state (Woolf & Salter, 2000).

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