Actions of brain-derived neurotrophic factor on spinal nociceptive transmission during inflammation in the rat

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The aim of the current study was to investigate whether, and if so how, brain-derived neurotrophic factor (BDNF) acts to develop the spinal sensitization underlying inflammation-induced hyperalgesia. In spinal cord slice preparations from rats with inflammation induced by complete Freund's adjuvant (CFA), BDNF, but not nerve growth factor (NGF) or neurotrophin-3 (NT-3), acted presynaptically to increase the frequency of excitatory miniature EPSCs in substantia gelatinosa (SG) neurones of the CFA-treated, but not untreated rats, through activation of lidocaine (lignocaine)-sensitive, TTX-resistant Na^+ channels. This effect was observed in the spinal cord slices of the CFA-treated rat only 2–4 days after the CFA injection. On the other hand, the number of monosynaptic Aβ afferent inputs to the SG significantly increased 1 week after the onset of the inflammation, and this increase was significantly suppressed by treatment with anti-BDNF antiserum administered 1 day before and just after the CFA injection. In addition, the treatment with anti-BDNF antiserum significantly attenuated the CFA-induced hyperalgesia and/or allodynia. These findings, taken together, suggest that BDNF, which is considered to be released from the sensitized primary afferents, increases the excitability of SG neurones through its action on the presynaptic terminals. BDNF may thereafter induce monosynaptic Aβ afferents to the SG, thereby developing hyperalgesia and/or allodynia during inflammation.

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In addition to modulatory effects on hippocampal synapses involved in learning and memory (Kang *et al.* 1997), brain-derived neurotrophic factor (BDNF) is known to act at the synapses between primary sensory and spinal dorsal horn neurones to affect pain transmission during inflammation (Malcangio & Lessmann, 2003). It has been suggested that the primary afferent is the only source of BDNF in the spinal cord (Michael *et al.* 1997; Lever *et al.* 2001). BDNF is upregulated in the dorsal root ganglion (DRG) by nerve growth factor (NGF) produced peripherally for only a few days after inflammation, and is transported and released into the superficial dorsal horn in an activity-dependent manner (Cho *et al.* 1997; Michael *et al.* 1997; Mannion *et al.* 1999; Woolf & Salter, 2000; Malcangio & Lessmann, 2003). In addition, an increased expression of trkB, a high-affinity receptor for BDNF, in the dorsal horn continued also for a few days after inflammation (Mannion *et al.* 1999). These findings suggest that BDNF may act during the restricted period in the early phase of inflammation.

Recent electrophysiological studies have revealed that large primary Aβ afferents from low-threshold mechanoreceptors, which terminate preferentially in laminae III to VI in the spinal dorsal horn in normal conditions, make polysynaptic/monosynaptic connections with substantia gelatinosa (SG; lamina II of Rexed (1952) neurones in rats receiving plantar injection of complete Freund's adjuvant (CFA) (Baba *et al.* 1999; Nakatsuka *et al.* 1999). Since neurotrophic factors including BDNF are known to promote the neurite outgrowth and survival of sensory neurones, it is possible that BDNF may have a pathophysiological significance during inflammation. However, it is poorly understood whether, and if so how, BDNF acts on SG neurones, which are known to

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receive noxious information mainly from primary Aδ and C afferent fibres (Yoshimura & Jessell, 1989), to induce hyperalgesia/allodynia during inflammation (Mannion *et al.* 1999).

In the present study we first identified neurones activated by CFA injection in the SG, then the site- and time-dependent effects of BDNF on synaptic transmission in the SG neurones were examined using spinal cord slice preparations from rats with inflammation induced by CFA. Furthermore, an involvement of BDNF in the inflammation-induced changes in Aβ afferent inputs to the SG neurones, which would be one of the underlying mechanisms of the allodynia, and in the nociceptive behaviour, was examined using rats treated with anti-BDNF antiserum just before and after CFA injection.

Methods

All experimental procedures involving the use of animals were approved by the Committee of the Ethics on Animal Experiments, Kyushu University, and were in accordance with the Guidelines of the Japanese Physiological Society. All efforts were made to minimize animal suffering and the number of animals used for the studies.

Inflammation model

Male Sprague-Dawley rats (8–9 weeks old, 270–320 g) were housed in groups of two in plastic boxes. Under anaesthesia with pentobarbital sodium (50 mg kg^{-1}, I.P.), the animals received a subcutaneous injection of 300 μl CFA (Mycobacterium; Sigma, St Louis, MO, USA), which was suspended in an oil/saline (1 : 1) emulsion at a concentration of 0.5 mg ml^{-1}, into the plantar surface of the left hindpaws, as reported previously (Nakatsuka *et al.* 1999). This procedure produced persistent peripheral inflammation in the injected hindpaw that was characterized by mechanical hyperalgesia/allodynia. Vehicle (300 μl oil/saline emulsion)-injected as well as untreated rats served as controls. The vehicle group showed no hyperalgesic behaviour.

Immunocytochemistry

In order to confirm the projection field from the inflammatory site in the SG, neurones activated by subcutaneous injection of CFA were visualized by immunocytochemical staining of c-FOS protein. Two hours after the injections of CFA (300 μl) into the left hindpaw and the same volume of vehicle into the contralateral side, rats were deeply anaesthetized with pentobarbital sodium and transcardially perfused with 100 ml heparinized saline, followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). The spinal cord was then removed, postfixed in fixative for 1 h, cryoprotected in 30% sucrose overnight, frozen and sectioned. Coronal sections (50 μm) were washed in PBS twice, and incubated with 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS for 30 min. After washing with PBS twice, sections were incubated with anti-Fos polyclonal antibody (Santa Cruz Biotechnology, Inc. CA, USA, 1 : 5000 dilution) overnight. Signals were visualized using the avidin–biotin–peroxidase technique (ABC Kit, Vector Laboratories, Burlingame, CA, USA) and standard diaminobenzidine reaction.

Preparation of spinal cord slices

Transverse slices of the spinal cord were made as reported previously (Yoshimura & Jessell, 1989). Briefly, the rats were anaesthetized with urethane (1.2 g kg^{-1}, I.P.), and a lumbosacral laminectomy was performed. The lumbosacral segments of the spinal cord (L1–S1) were removed with ventral and dorsal roots intact and placed in ice-cold Krebs solution equilibrated with 95% O_2–5% CO_2. The rats were killed by exsanguination. Slices (thickness, 650 μm) that retained an attached L4 or L5 dorsal root were cut using a vibratome. The slice was placed on a nylon mesh in the recording chamber, and perfused at a drip rate of 10–20 ml min^{-1} with Krebs solution equilibrated with 95% O_2–5% CO_2 at 36 ± 1°C.

Whole-cell patch-clamp recordings from SG neurones

The SG was easily discernible as a relatively translucent band across the dorsal horn in transverse slice preparation under a binocular microscope with transmitted light. Blind whole-cell voltage-clamp recordings were made from SG neurones with patch pipettes that had a tip resistance of 8–10 MΩ. Typical seal resistances of the electrodes were 10–20 GΩ. To examine the time-dependency of the effects of BDNF, slices were made from rats in the early phase of inflammation (day 1–4) and 1 week after the CFA injection.

Spontaneous and miniature EPSCs were recorded at a holding potential of −70 mV. The program AXOGRAPH v.4.6 used for analysing EPSCs detects spontaneous events if the difference between the baseline and a following current value exceeds a given threshold of 5 pA and separating valleys are less than 50% of adjacent peaks. The validity of this method was confirmed by measuring visually the individual EPSCs on a fast time scale in several cases.

Orthodromic stimuli (duration, 100 μs) to elicit EPSCs were given to the dorsal root at a frequency of 0.2 Hz (unless otherwise mentioned) through a suction electrode. The intensity was 1.2–1.5 times the

threshold required to elicit an EPSC in the most excitable Aδ or C afferents. The Aβ-, Aδ- or C-afferent evoked EPSCs were distinguished on the basis of the conduction velocity ($C < 0.9\ m\ s^{-1}$; $2 < A\delta < 13\ m\ s^{-1}$; $A\beta > 14\ m\ s^{-1}$) and stimulus threshold ($C > 160\ \mu A$; $10 < A\delta < 60\ \mu A$; $A\beta < 9\ \mu A$). As reported previously (Okamoto *et al.* 2001), the monosynaptic nature of Aβ, Aδ and C afferent EPSCs was determined by the constant latency and lack of failures when the dorsal root was stimulated at 50, 20 and 2 Hz, respectively. It is critical to determine the accurate conduction velocity to confirm the sprouting of Aβ afferent fibres electrophysiologically. In addition, it has been reported that the conduction velocity of the primary afferents decreased in the spinal cord (Yoshimura & Jessell, 1989). Therefore the exact conduction velocities were further calculated from the time difference between the onset of EPSCs evoked by focal stimulation using a monopolar silver wire electrode, positioned at the proximal and distal portions of the dorsal root (see Fig. 5).

Application of drugs

Drugs were dissolved in Krebs solution and applied by perfusing via a three-way stopcock without any alteration in the perfusion rate and temperature. The drugs used in the present study were BDNF, NGF (Sigma), NT-3 (Calbiochem, San Diego, CA, USA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; non-*N*-methyl-D-aspartic acid (NMDA) receptor antagonist, Tocris Cookson, St Louis, MO, USA), tetrodotoxin (TTX, WAKO, Osaka, Japan), K252a (receptor tyrosine kinase inhibitor, Sigma), lidocaine (non-selective sodium channel blocker, WAKO), and U73122 (phospholipase C inhibitor, Calbiochem). All drugs were first dissolved in distilled water or 0.1% DMSO (K252a and U73122) at 1000 times the concentration required, and then diluted in Krebs solution immediately before use.

Behavioural test

Mechanical hyperalgesia/allodynia was determined using a set of von Frey hairs (0.0045–447 g, Stoelting, Keil, WI, USA). The minimum force-reproducible flexion-withdrawal reflex on each of three applications of von Frey hairs to both sides of the hindpaw was measured as a mechanical nociceptive threshold before, 1, 2, 3, 4, 5, 7 and 14 days after the CFA injection. To examine any involvement of BDNF in the CFA-induced hyperalgesia/allodynia, antiserum against BDNF ($5\ \mu l\ g^{-1}$, approximately 1.5 ml of antiserum per rat) or vehicle was injected intraperitoneally on each of two successive days from 1 day before CFA injection; this was based on a previous study using the same antiserum (Theodosiou *et al.* 1999). Saline-injected groups also received antiserum or vehicle.

Statistical analysis

All results were presented as means ± S.E.M. Statistical significance of the relative frequency of mEPSCs was determined as $P < 0.05$ using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test and indicated by asterisks in the figures. In all cases, *n* refers to the number of neurones studied. Cumulative distributions, effects of BDNF on the evoked EPSCs, and behavioural data were analysed by the Kolmogorov-Smirnov test and χ^2 test, respectively. Differences in behavioural data among groups were first tested using the Kruskal-Wallis test on each day, followed by Dunn's multiple comparison *post hoc* test.

Results

BDNF increases EPSC frequency in CFA-treated rats

As shown in Fig. 1*A*, Fos-positive neurones were detected in the SG of the ipsilateral lumbar spinal cord ranging from L3 to L5, but most prominently in the L4 segment, 2 h after injection of CFA into the plantar surface of the left hindpaws (arrows), while there was no significant Fos staining in the contralateral side following vehicle injection into the right hindpaws. The higher magnification micrograph clearly indicates that neurones expressing Fos protein were mainly located in the medial part of the SG from the entry zone of the dorsal root (Fig. 1*B*, arrowhead) in all of the animals examined ($n = 6$).

In the present study, one to three neurones were recorded from each rat. We first recorded from neurones in the medial part of the SG. Although bath applied BDNF ($200\ ng\ ml^{-1}$) did not cause any inward or outward currents, BDNF applied for 2 min increased spontaneous EPSCs (sEPSCs) for 1–5 min that were completely blocked by a non-NMDA receptor antagonist, CNQX (10 μM), in all of the ipsilateral SG neurones tested ($n = 10$) 2–4 days after the CFA injection (Fig. 1*C*). On the other hand, no effect was observed on day 1 ($n = 4$) or days 7–14 (Fig. 1*D*, $n = 5$) after injection. BDNF was also without effect in the untreated rats ($n = 6$, summarized in Fig. 2*C*). Furthermore, sEPSCs were not affected by the application of BDNF in neurones located in the lateral part of the SG (Fig. 1*E*, $n = 4$). Therefore, in the following experiments, recordings were performed exclusively from neurones in the medial part of the SG.

The enhancement of the frequency by BDNF on day 2–4 was well preserved in the presence of TTX (1 μM, $n = 4$; Fig. 2*A*), which blocked sEPSCs. The duration of the BDNF effect on miniature EPSCs (mEPSCs, maximum, about 5 min) with TTX was not different from

the effect on sEPSCs. Normalized cumulative curves of mEPSCs demonstrated that BDNF significantly shifted the frequency curve to the left and this recovered after washout (Fig. 2*B*, upper panel, Kolmogorov-Smirnov test, $P < 0.01$), without affecting the amplitude (lower panel, $P > 0.05$), suggesting a presynaptic action of BDNF. The effects of BDNF are summarized in Fig. 2*C*. The absence of effect of BDNF on mEPSCs on day 1 might be due to the slower upregulation of trkB receptors (Mannion *et al.* 1999), while no effect later than day 7 (Fig. 1*E*) correlated well with the decrease in trkB expression (Mannion *et al.* 1999). As summarized in Fig. 2*C*, the transient increase in frequency of mEPSCs was not observed by application of NGF (200 ng nl^{-1}, $n = 3$) or NT-3 (200 ng ml^{-1}, $n = 4$) in

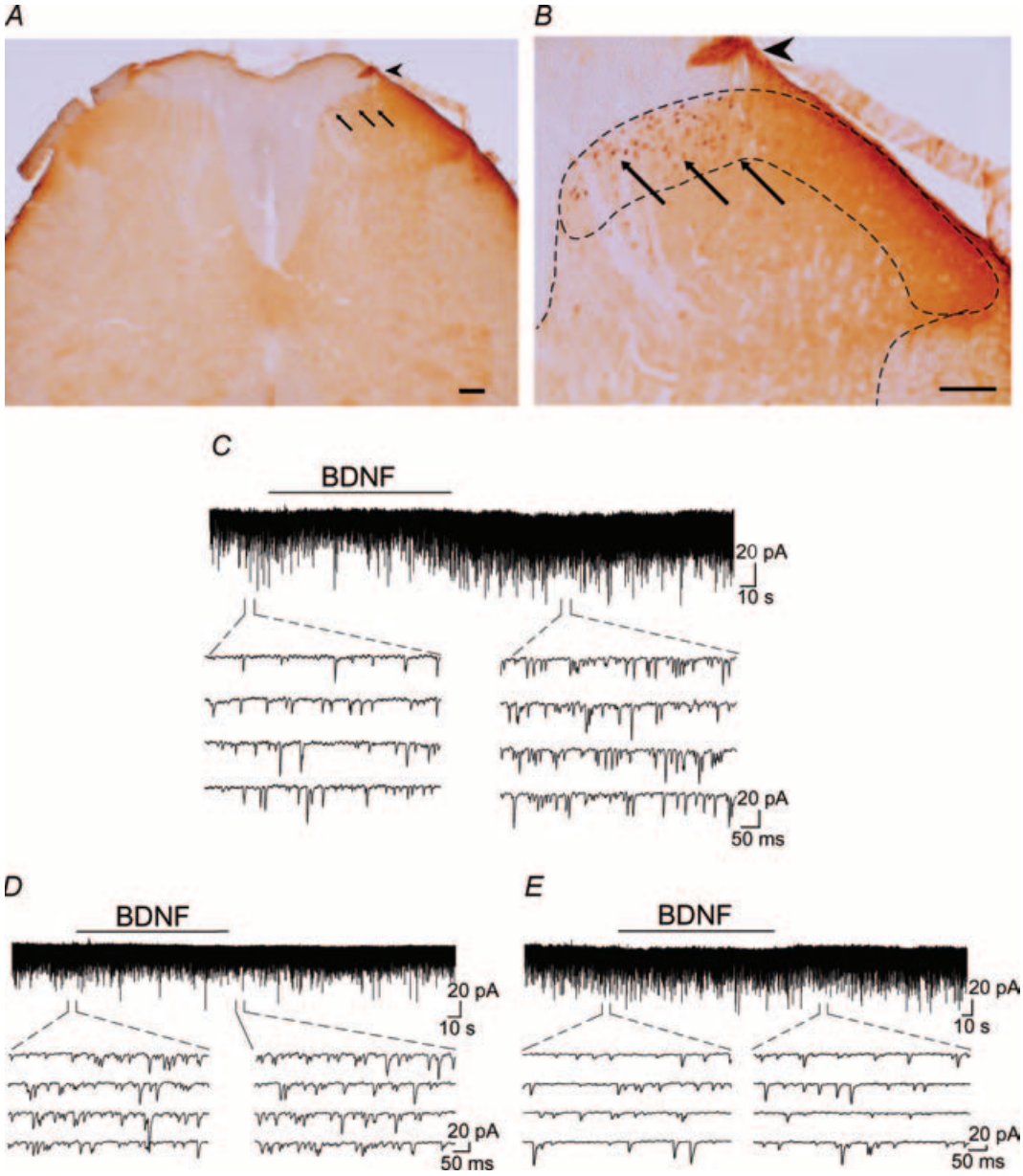

Figure 1. Fos expression in the spinal cord following CFA injection and presynaptic actions of BDNF on excitatory transmission in SG neurones
A, most Fos-positive neurones (arrows) were observed only in the ipsilateral SG 2 h after CFA injection. Scale bar, 100 μm. *B*, higher magnification clearly demonstrated that Fos-positive neurones (arrows) were mainly located in the medial part of SG. Arrowhead indicates the entry zone of the dorsal root. Scale bar, 100 μm. *C*, BDNF (200 ng ml^{-1}) increased sEPSCs in rats 2–4 days after induction of inflammation with CFA. *D*, BDNF did not affect sEPSCs in rats 7 days after CFA injection. *E*, BDNF was also without effect on sEPSCs in neurones located in the lateral side of SG.

the CFA-treated (day 2–4) rats, or by application of NGF in untreated rats (nNGF), suggesting that the effects were specific to BDNF. BDNF was without effect on mIPSCs recorded at the holding potential of 0 mV in the presence of TTX ($n = 8$, data not shown).

BDNF activates lidocaine-sensitive Na^+ channels through trkB receptors

As shown in Fig. 3*A*, the enhancement by BDNF of mEPSCs was completely blocked by K252a (200 nM, $n = 4$), an inhibitor of receptor tyrosine kinase, in all neurones tested using slice preparations, suggesting BDNF-induced activation of trkB receptors. However, we cannot completely exclude the possibility that the blocking effects of K252a were due to an inhibition of other kinases such as protein kinase C (PKC) and cAMP-dependent protein kinase. The trkB receptor has been shown to associate with two distinct channels: (1) transient receptor potential canonicals (TRPC3), which produces a slow response by opening of a non-selective cation channel through activation of a phospholipase C (PLC)/inositol trisphosphate pathway (Li *et al.* 1999); and (2) Na^+ channel, which mediates a rapid depolarization in the hippocampus (Barde, 2002). Although the effect of BDNF was not blocked by TTX (Fig. 2*A*), the enhancement was blocked by pretreatment with lidocaine (10 mM), a non-selective Na^+ channel blocker (Fig. 3*B*, $n = 4$). Application of lidocaine itself had no effect on mEPSCs. On the other hand, a PLC inhibitor, U73122 (2 μM), was without effect on the actions of BDNF (Fig. 3*C*). Figure 3*D* summarizes the effects of the drugs.

No effect of BDNF on the dorsal root-evoked EPSCs

In addition to mEPSCs, the effects of BDNF on the dorsal root-evoked EPSCs (eEPSCs) were examined in the CFA-treated rats on days 2–4. Stimulation of the dorsal root attached to the spinal cord slices evoked short and/or long latency eEPSCs, which were confirmed as monosynaptic in nature (see Methods). The average conduction velocity of the short latency eEPSCs was 4.3 ± 0.4 m s^{-1} (2.6–5.8 m s^{-1}, $n = 7$), which was within the Aδ-afferent range, while stimuli with an intensity larger than 160 μA elicited monosynaptic eEPSCs, which had an estimated conduction velocity of 0.5 ± 0.05 m s^{-1} (0.4–0.9 m s^{-1}, $n = 8$). These values are comparable to those of C-fibres based upon experiments in rat DRG (Nakatsuka *et al.*

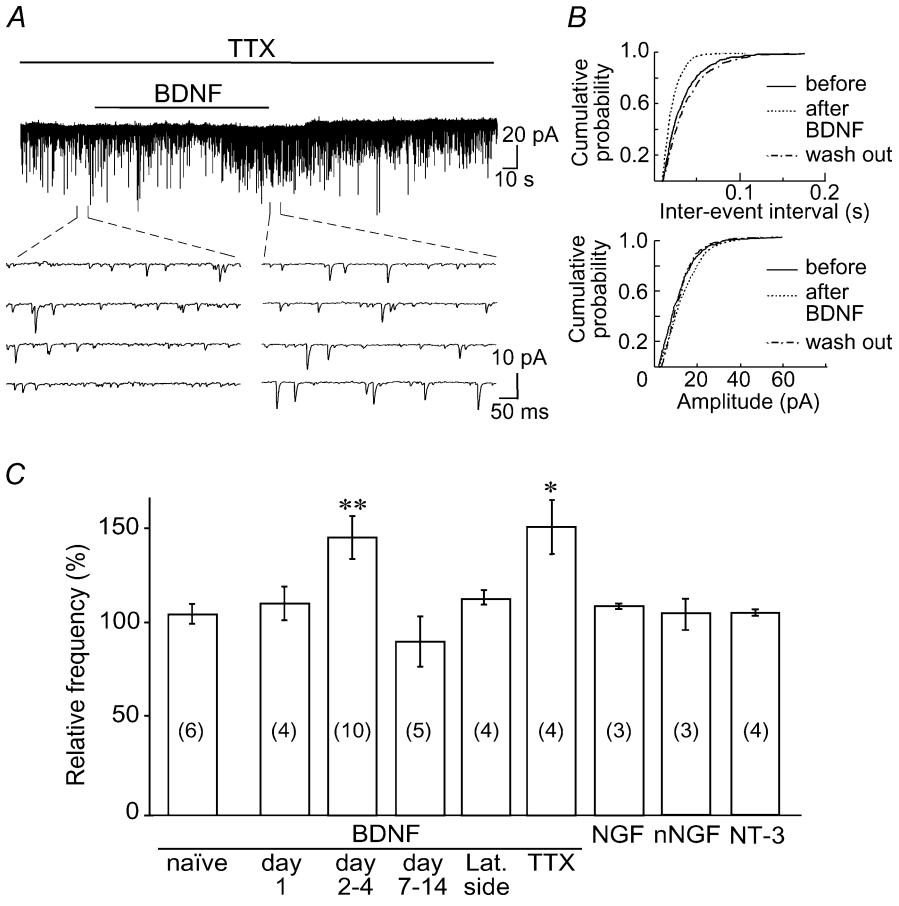

Figure 2. BDNF transiently increases mEPSC frequency of neurones in the medial part of SG
A, the increased mEPSC frequency by BDNF was preserved in the presence of TTX (1 μM). *B*, cumulative probability histograms show an increase in frequency (upper graph) but not amplitude (lower graph) of mEPSCs after addition of BDNF. *C*, relative frequency of EPSCs after application of BDNF was calculated as the percentage of that before BDNF in untreated (naive) and CFA-treated rats on different days after CFA injection. Effects of BDNF on neurones located in the lateral part of SG (Lat. side) and in the presence of TTX were both from days 2–4. Effects of NGF, NT-3 in CFA-treated rats and NGF in untreated rats (nNGF) were also from days 2–4. Numbers in parentheses indicate number of neurones tested.

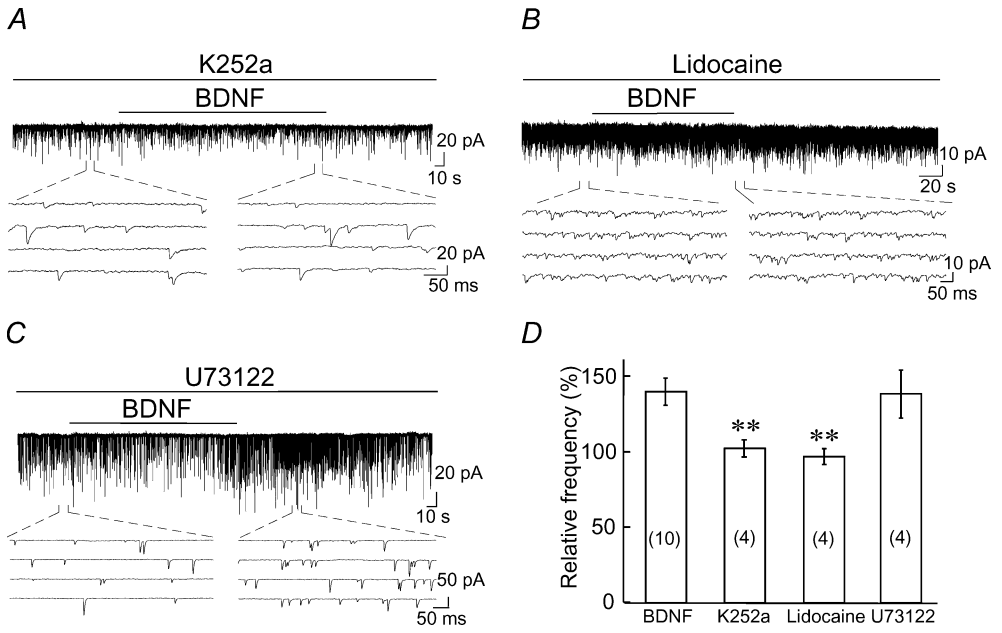

Figure 3. Effects of inhibitors for receptor tyrosine kinase, Na^+ channels and PLC on the BDNF-induced increase in mEPSC

A, prior administration of a receptor tyrosine kinase inhibitor, K252a (200 nM) blocked the effect of BDNF in rats 2 days after induction of inflammation. *B*, non-selective Na^+ channel blocker lidocaine (10 mM) also blocked the effect of BDNF. *C*, a PLC inhibitor, U73122 (2 μM) did not change the effect of BDNF. *D*, summary of effects of inhibitors. Numbers in parentheses indicate number of neurones tested. $^{**}P < 0.01$, one-way ANOVA followed by Fisher's PLSD test.

1999). As shown in Fig. 4, the amplitudes of the monosynaptic Aδ- (Fig. 4*A*) and C-afferent (Fig. 4*B*) eEPSCs were not affected by bath application of BDNF in the CFA-treated rats on days 2–4.

BDNF-dependent increase in monosynaptic Aβ inputs in CFA-treated rats

Hypersensitivity induced by inflammation is considered to involve not only the sensitization of the peripheral

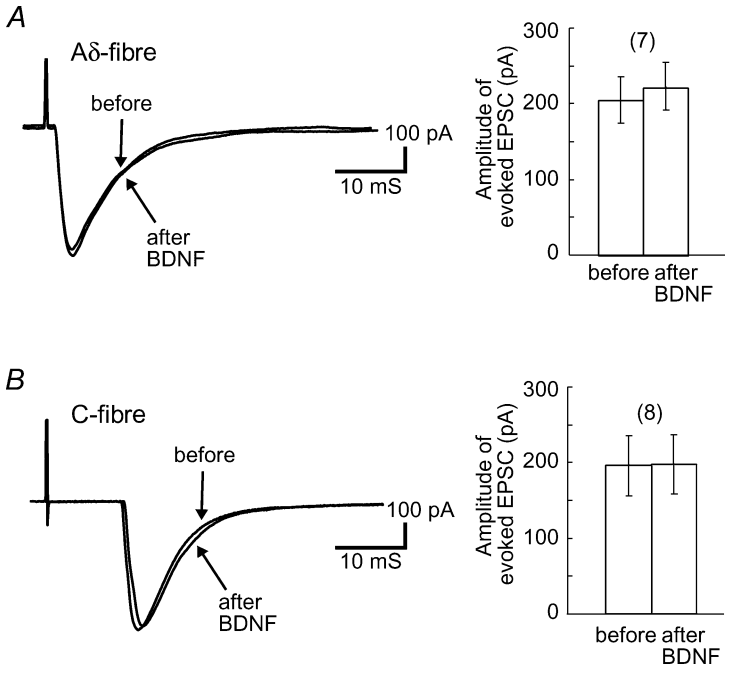

Figure 4. Effect of BDNF on eEPSCs

A, BDNF did not affect the time course and amplitude of Aδ-fibre eEPSC. *B*, C-fibre eEPSC was also not affected by BDNF. Each trace in *A* and *B* was obtained by averaging five consecutive EPSCs. Right-hand panel of *A* and *B* shows summary of effects on amplitude. Numbers in parentheses are number of neurones tested.

sensory receptors by chemical mediators, but also plastic changes of sensory pathways in the spinal dorsal horn (i.e. central sensitization). Neuroanatomical studies have shown that Aβ afferents from low-threshold mechanoreceptors sprout into the SG after nerve injury (Woolf *et al.* 1992; Koerber *et al.* 1994). In addition, electrophysiological studies have shown that monosynaptic transmission from Aβ afferents to the SG becomes evident more than 7 days after the CFA injection (Nakatsuka *et al.* 1999). To examine the role of BDNF in the increase in Aβ afferents during inflammation, the primary afferent inputs to the SG were examined in slices from rats 7–10 days after CFA and anti-BDNF antiserum treatment. As reported previously (Yoshimura & Jessell, 1989), the majority of SG neurones receive polysynaptic or monosynaptic inputs from Aδ (Fig. 5*A*) and/or C afferents, and only a few neurones have inputs from Aβ afferents in untreated rats. In contrast, the number of SG neurones receiving Aβ monosynaptic inputs (Fig. 5*B*) significantly increased in CFA-treated rats (Table 1; χ^2-test, $P < 0.01$). However, as shown in Table 1, the treatment with anti-BDNF antiserum significantly decreased the number of monosynaptic Aβ inputs, while a substantial number of Aδ eEPSCs (Fig. 5*C*) was recorded. Figure 5*D* shows a two-point stimulation method, in which the distance between the stimulating electrodes was 6–10 mm. Figure 5*E*–*G* shows examples of two traces evoked by two-point stimulation of Aδ-eEPSCs in untreated (Fig. 5*E*), Aβ-eEPSCs in CFA-treated (Fig. 5*F*), and Aδ-eEPSCs in rats treated with CFA and antiserum (Fig. 5*G*). The conduction velocities of Aβ and Aδ afferents measured by the two-point stimulation method were 14.1–29.8 m s^{-1} ($n = 12$) and 3.5–13.0 m s^{-1} ($n = 8$), respectively, which were consistent with those obtained from a DRG study (Nakatsuka *et al.* 1999).

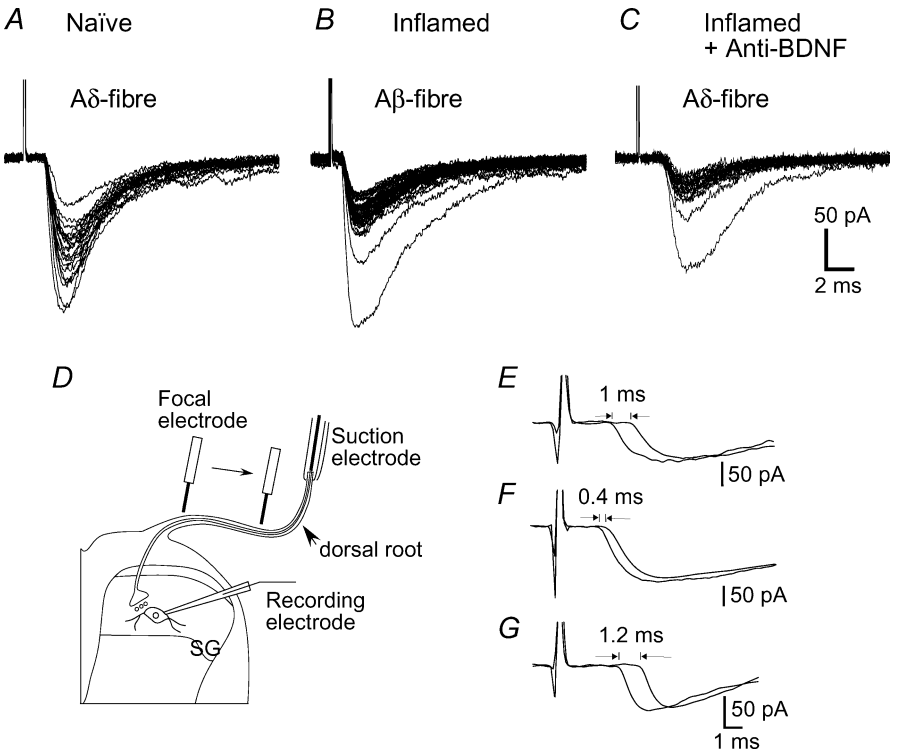

Figure 5. Anti-BDNF effect on eEPSCs and examples of eEPSCs by two-point stimuli
A, Aδ-evoked monosynaptic EPSCs following one-point stimulus recorded from an SG neurone in untreated rats. *B*, Aβ-evoked monosynaptic EPSCs in CFA-treated rats. *C*, Aδ-evoked monosynaptic EPSCs in rats treated with CFA and anti-BDNF antiserum. No change in latencies was observed even though the amplitude of the responses was attenuated by repetitive stimulation at 20 Hz for Aδ (*A* and *C*) or 50 Hz for Aβ (*B*). *D*, schematic illustration of two-point stimulation. *E*, superimposed traces of Aδ-evoked monosynaptic eEPSCs by two-point stimulation recorded from an SG neurone from an untreated rat. *F*, Aβ-evoked monosynaptic EPSCs in CFA-treated rat. *G*, Aδ-evoked monosynaptic EPSCs in rat treated with CFA and anti-BDNF antiserum. The distance of the stimulating electrodes was 7.5 (*E*), 10 (*F*) and 9 mm (*G*), and the conduction velocities calculated by two-point stimulation were 7.5 (*E*), 25 (*F*), and 7.5 m s^{-1} (*G*), while those calculated by one-point stimulation were 6.3, 18.3 and 6.9 m s^{-1}, respectively.

Table 1. Number of neurones receiving monosynaptic Aβ, Aδ or C afferent eEPSCs in untreated, CFA-treated and CFA- and anti-BDNF-treated rats on days 7–10

	Aβ afferent	Aδ afferent	C afferent
Untreated ($n = 138$)	5 (3.6%)	86 (62.3%)	62 (44.9%)
CFA-treated ($n = 57$)	16 (28.1%)	14 (24.6%)	30 (52.6%)
CFA + antiserum-treated ($n = 42$)	1 (2.4%)	16 (38.1%)	29 (69.1%)

Neurones receiving only polysynaptic inputs are not included. The χ^2-test revealed a significant difference between groups ($P < 0.01$). Both Aδ and C afferent eEPSCs were observed in the same neurone in untreated ($n = 15$), CFA-treated ($n = 3$) and CFA + antiserum-treated ($n = 4$) groups; therefore, the total percentage of each group (in parentheses) becomes more than 100%. The number of rats used was 42 (untreated), 26 (CFA), and 11 (CFA + antiserum group).

Attenuation of hyperalgesia/allodynia by anti-BDNF antiserum

In a previous study, the dose of antiserum injected in the same way has been shown to be effective in neutralizing the actions of BDNF to attenuate the mechanical and thermal hyperalgesia (Theodosiou *et al.* 1999). A Kruskal-Wallis test revealed that there are significant differences among groups from day 1 to day 10. As shown in Fig. 6, an injection of CFA into the plantar surface coupled with vehicle (I.P.) induced a sustained decrease in the withdrawal threshold in the von Frey hair test that lasted more than 1 week (CFA/vehicle group, ●, $n = 10$) compared with the saline/vehicle group (○, $n = 10$, $\#\#P < 0.01$, Dunn's *post hoc* test). However, there was a significant increase in the withdrawal threshold in CFA-treated rats treated with the antiserum (CFA/anti-BDNF, ■, $n = 10$) compared with the CFA/vehicle group ($^*P < 0.05$ and $^{**}P < 0.01$) on days 2–4 and 7–8. Antiserum treatment did not restore the threshold to the control levels ($\dagger P < 0.05$ *versus* saline/vehicle, ○, $n = 10$; $\dagger\dagger P < 0.01$ *versus* saline/anti-BDNF, □, $n = 10$). Since the swelling of the injection site was not affected by antiserum, the residual component might be due to peripheral sensitization.

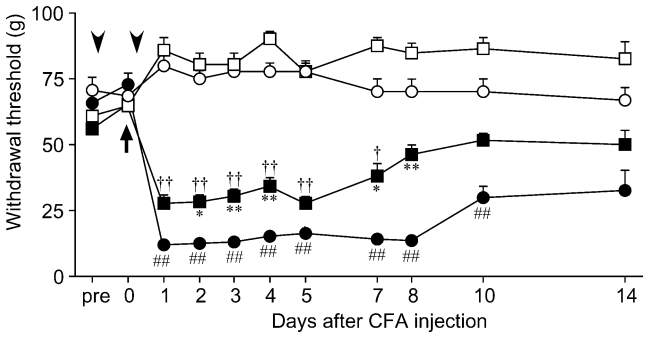

Figure 6. Reduction of hyperalgesia by anti-BDNF antiserum treatment

Mechanical hyperalgesia (von Frey hair test) in CFA/vehicle-treated rats (●, $n = 10$) was significantly reduced by treatment with anti-BDNF antiserum (CFA/anti-BDNF, ■, $n = 10$) performed 1 day before and just after (arrowheads) CFA injection (arrow), and it did not return to the control level. Saline/vehicle-treated (○, $n = 10$), and saline/anti-BDNF-treated rats (□, $n = 10$). $^*P < 0.05$, and $^{**}P < 0.01$; CFA/anti-BDNF *versus* CFA/vehicle. $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$, CFA/anti-BDNF *versus* saline/vehicle. $\#\#P < 0.01$, CFA/vehicle *versus* saline/vehicle (Dunn's *post hoc* test).

Discussion

Our slice experiments revealed that BDNF acted on presynaptic terminals and increased the frequency of mEPSCs of SG neurones in the CFA-treated rats during days 2–4 after CFA injection through activation of receptor tyrosine kinase and lidocaine-sensitive, but TTX-resistant Na^+ channels. These neurones sensitive to BDNF were exclusively located in the medial, but not lateral, part of the SG, where Fos-positive neurones were observed after the CFA injection into the hindpaw, suggesting that BDNF modulated excitability of neurones that received inputs from inflammatory regions. It is well known that Fos protein is transiently induced by trans-synaptic stimulation and declines to baseline levels within a few hours after the stimulation. However, an enhancement of nociceptive inputs from the inflammatory sites is considered to continue for more than 1 week, since the swelling of the hindpaw is still present, thereby producing the peripheral sensitization. In fact, it has been shown that Fos expression in the superficial layers of the rat spinal dorsal horn increases for 2 weeks following subcutaneous injection of CFA into the plantar foot, although deep layers showed more predominant expression of Fos protein (LantÈri-Minet *et al.* 1993). Nevertheless, the effects of BDNF were observed only during days 2–4 after the CFA injection in the present study. It has been shown that the BDNF-immunoreactive terminals in the spinal cord become most evident 2–4 days after the CFA-induced peripheral inflammation and return to normal by day 10 (Cho *et al.* 1997). Furthermore, trkB also increases in the DRG and SG within 1 week after induction of inflammation (Mannion *et al.* 1999). Therefore, it is reasonable that the effects of BDNF on mEPSCs are restricted to the first week after injection of CFA. Although BDNF may be washed out in slice preparations, the trkB

levels in the dorsal horn are considered to be high during this period.

Mechanisms of synaptic modulation by BDNF have been extensively studied in the hippocampus (Poo, 2001) including activation of NMDA receptors (Levine *et al.* 1998), direct membrane depolarization (Kafitz *et al.* 1999) and reduction of mIPSCs by rapid downregulation of $GABA_A$ receptor expression (Brunig *et al.* 2001) at the postsynaptic sites. The presynaptic effect of BDNF on mEPSC in the SG has been demonstrated in the spinal dorsal horn for the first time in the present study, although the BDNF-induced increase in mEPSC frequency has been reported in the hippocampus (Li *et al.* 1998). Our results indicated that the actions of BDNF require activation of trkB receptors that link to the TTX-resistant Na^+ channels. It has been shown that TTX-resistant Na^+ channels such as $Na_v1.8$ and $Na_v1.9$ are expressed close to the sensory terminals of small DRG neurones, but not neurones and glial cells in the spinal cord, and upregulated in pathological condition (Baker & Wood, 2001; Dib-Hajj *et al.* 2002). In addition, the primary afferent axons are the only source of BDNF (Michael *et al.* 1997; Lever, 2001), and the release of BDNF is activity dependent (Malcangio & Lessmann, 2003). Therefore, it is possible that BDNF released from the primary afferents acts on primary afferents in the SG, resulting in a positive feedback excitation in the early phase of inflammation.

In spite of an increase in mEPSC frequency by BDNF, the amplitudes of the monosynaptic Aδ- and C-afferent eEPSCs were not affected by BDNF in the CFA-treated rats in the present study (Fig. 4). This may be because there are differences in mechanisms between miniature and evoked transmitter release. On the other hand, it has been shown that BDNF enhanced the dorsal root-evoked EPSCs by potentiating postsynaptic NMDA responses, which are blocked by PLC/protein kinase C inhibitors in neonatal rats (Garraway *et al.* 2003). One of the reasons for this discrepancy may be due to the difference in age (neonatal *versus* adult in our study). Another possibility is that NMDA receptor-mediated currents were not fully detected in our dorsal root-evoked EPSCs at the holding potential of -70 mV, thereby resulting in no observable facilitation following application of BDNF. Further studies will be needed to clarify the effects of BDNF on NMDA receptor-mediated EPSCs in the spinal dorsal neurones.

Concerning the plastic changes underlying allodynia associated with neuropathic condition, it has been recently reported that only a few Aβ afferents sprout into the lamina II after sciatic nerve transection (Hughes *et al.* 2003), which does not convincingly support the Aβ sprouting hypothesis (Woolf *et al.* 1992). Consistent with the anatomical study (Hughes *et al.* 2003), we have found that spinal cord slices obtained from rats whose sciatic nerve had been transected 2–4 weeks beforehand show an increase in Aβ afferent inputs to the SG, but monosynaptic Aβ afferent inputs are only about 10%, while most of them are polysynaptic in nature (81%) (Okamoto *et al.* 2001). On the contrary, in CFA-induced inflammation, although only polysynaptic Aβ inputs increased 48 h after CFA injection (Baba *et al.* 1999), a substantial number of monosynaptic Aβ afferent-evoked EPSCs were observed 7–10 days after CFA injection (Nakatsuka *et al.* 1999).

To confirm electrophysiologically the direct inputs of Aβ afferents into the SG following inflammation, it is essential to identify the nature of the primary afferents that evoke monosynaptic EPSCs in SG neurones. For this purpose, in addition to one-point stimulation, two-point stimulation was performed in the present study for the rigorous determination of conduction velocity. This method compensates for errors in calculation due to decrement in conduction velocity of afferents within the tract of Lissauer and dorsal horn, and avoids the need to estimate the intraspinal length of afferents. As shown in Table 1, monosynaptic Aβ afferent inputs to the SG were significantly increased following inflammation, and the increase was blocked by anti-BDNF treatment. Nevertheless, if the conduction velocity of primary afferent fibres was accelerated due to a change in excitability of the fibres during inflammation, it might be difficult to discriminate electrophysiologically the Aβ monosynaptic inputs from the accelerated Aδ-fibres. However, intracellular recordings from rat DRG neurones revealed that the DRG neurones were classified into three subgroups (Aβ, Aδ and C-type) on the basis of axonal conduction velocity, action potential duration and threshold stimulus intensity, and these were not affected by inflammation (Nakatsuka *et al.* 1999). It is thus quite conceivable that, in contrast to nerve transection (Okamoto *et al.* 2001), the monosynaptic inputs of Aβ afferents into the SG are induced following inflammation, and that BDNF plays a crucial role in the plastic changes in the sensory pathway underlying the mechanisms for the development of hyperalgesia and/or allodynia. A previous study using trkB-Fc fusion protein that neutralizes BDNF activity has also suggested an involvement of Aβ-fibres in the BDNF-mediated tactile stimulus-induced inflammatory hypersensitivity (allodynia) (Mannion *et al.* 1999).

It is still unclear whether the increase in mEPSC frequency by BDNF observed in the early phase of inflammation (days 2–4) is responsible for the increase in monosynaptic Aβ inputs to the SG 1 week after the onset of inflammation. We found that no such augmentation of mEPSCs by BDNF was observed in untreated rats, and that BDNF was without effect 7 days after inflammation. On the other hand, our behavioural study demonstrated that administration of anti-BDNF antiserum at the time of the CFA injection effectively attenuated the inflammation-induced hyperalgesia/allodynia until days 7–8. Therefore, it is suggested that the critical period of BDNF actions may be the early stage of inflammation,

and that the BDNF-induced increase in EPSCs is highly associated with the following induction of monosynaptic Aβ inputs to the SG that causes hyperalgesia and/or allodynia. In support of this, EPSCs enhanced by BDNF are glutamatergic, as they were completely blocked by CNQX in the present study, and BDNF has been demonstrated to sensitize nociceptive transmission in the spinal dorsal horn through NMDA receptors (Kerr *et al.* 1999). In addition, BDNF, as well as other molecules such as netrin-1, has been reported to play a role in pathfinding by growing axons working as the guidance factors (Ming *et al.* 2002). Further studies on the possible association between the increase in monosynaptic Aβ afferents to the SG and the enhanced releases of BDNF might disclose the underlying mechanisms for induction of plastic changes in the spinal sensory pathways. These studies may also lead to a development of new chemicals that block or reduce the BDNF–trkB signalling as a novel therapeutic target for chronic pain states.

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