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TrkB Expression and Phospho-ERK Activation by Brain-Derived Neurotrophic Factor in Rat Spinothalamic Tract Neurons

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a neurotrophin implicated in the phenomena of synaptic plasticity in the adult. It is found in terminals of nociceptive primary afferents. Following a pain-related stimulus, it is released in the spinal cord, where it activates its high-affinity receptor TrkB, leading to the phosphorylation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK). A large body of evidence suggests that BDNF has a positive neuromodulatory effect on glutamate transmission in the spinal cord. However, none of these studies examined anatomically whether projection neurons known to be involved in transmission of nociceptive inputs express BDNF's receptor. Because the spinothalamic tract (STT) is a well-characterized pathway for its role in the transfer and integration of sensory and nociceptive informations, this study in rats aimed to 1) determine whether neurons of the STT pathway express the TrkB receptor, 2) establish the rostrocaudal and laminar distribution of STT-TrkB neurons in the whole spinal cord, and 3) test the potential functionality of TrkB expression in these cells by investigating the ability of BDNF to activate the MAP kinase ERK. Using tract tracing coupled to immunofluorescent labeling for TrkB, we observed that in all levels of the spinal cord most STT neurons were immunoreactive for TrkB. Furthermore, microinjections of BDNF into the spinal cord or release of endogenous BDNF by intraplantar injection of capsaicin activated ERK phosphorylation in TrkB-containing STT neurons. These data suggest an important role for BDNF in nociception as an activator of spinothalamic projection neurons. J. Comp. Neurol. 489:59–68, 2005.

Indexing terms: thalamus; supraspinal; plasticity; pain; nociception

Noxious information is encoded in peripheral tissues by specialized peripheral neurons, termed nociceptors. The central terminals of these primary afferent neurons terminate in the dorsal horn of the spinal cord. Increased activity in nociception due to the application of an acute noxious stimulus or following a peripheral inflammation leads to the arrival of volleys of action potentials in the spinal dorsal horn. This pain-related information is transmitted and integrated by second-order projection neurons in the spinal cord that convey the information to different parts of the brain (for review, see Besson and Chaouch, 1987; Guilbaud et al., 1994; Craig and Dostrovsky, 1999). The projection pathways implicated in the process of nociceptive informations are the spinosolitary tract (Menetrey and Basbaum, 1987), the spinoreticular tract (Menetrey et al., 1980, 1983), the spinomesencephalic tract (Menetrey et al., 1982), the spino(trigemino)pontoamygdaloid pathway (Cechetto et al., 1985), the spinohypothalamic pathway (Burstein et al., 1990a), and the spinothalamic tract (STT), which terminates in various nuclei of

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the thalamus (Peschanski et al., 1980; Guilbaud et al., 1980).

The thalamus has classically been regarded as the key relay structure for the supraspinal integration and transfer of nociceptive information (Guilbaud et al., 1987ab). Spinothalamic projection neurons that relay nociceptive information from the spinal cord are located in laminae I and II outer, the dorsal horn deep layers (IV–VI), and intermediate parts of the spinal gray matter (base of the dorsal horn and laminae VII and VIII). Axons of the spinothalamic neurons decussate in the spinal cord to ascend to the thalamic nuclei in the lateral part, consisting of the ventrobasal zone (VB; the ventroposterolateral [VPL] and ventroposteromedial [VPM] regions), the posterior nuclei (Po), the nucleus centralis lateralis, and the nucleus submedius, where they terminate by synapsing on third-order neurons that project to various forebrain areas (for review, see Besson and Chaouch, 1987).

The neurotrophin brain-derived neurotrophic factor (BDNF) is a trophic factor implicated in the survival of certain neuronal populations during development. In the adult, it is an important mediator of synaptic plasticity in the hippocampus and the spinal cord (Pezet et al., 2002b; Malcangio and Lessmann, 2003). In naïve animals, BDNF is synthesized by a population of sensory neurons that contain the nerve growth factor (NGF) receptor TrkA (Zhou and Rush, 1996). Its expression is increased in the cell body of these neurons in the dorsal root ganglion (DRG), following NGF treatment (Michael et al., 1997), peripheral inflammation (Mannion et al., 1999), or a peripheral nerve injury (Ernfors et al., 1993). At the terminals of these neurons, i.e., in the spinal dorsal horn, BDNF is localized in varicosities in the superficial and deep laminae of the spinal cord (Ernfors et al., 1990; Zhou and Rush, 1996 ; Yan et al., 1997b; Walker et al., 2001), copackaged with calcitonin gene-related peptide (CGRP) in dense-core vesicles. BDNF is released from the primary afferent fibers following electrical stimulation at C fiber strength or noxious stimulation with capsaicin (Lever et al., 2001). BDNF's high-affinity receptor TrkB is located on neurons in the spinal dorsal horn (Zhou et al., 1993; Yan et al., 1997a), where it is also upregulated following chronic inflammation (Mannion et al., 1999).

Several behavioral studies have revealed a pronociceptive role for BDNF in the spinal cord. Local delivery of BDNF to the L5 DRG induced mechanical allodynia (Zhou et al., 2000). Intrathecally delivered BDNF or TrkB antisense oligonucleotides attenuated carageenan-induced hyperalgesia (Groth and Aanonsen, 2002). Intrathecal delivery of the BDNF-sequestering molecule TrkB-IgG reduced formalin-, carrageenin-, and nerve injury-induced hyperalgesia (Kerr et al., 1999). In addition, the use of a BDNF-sequestering antibody reduced the foot-withdrawal responses induced by mechanical and thermal stimulations in models of neuropathic pain (Zhou et al., 2000; Yajima et al., 2002).

Application of a peripheral stimulus has been shown to induce the release of BDNF in the cord and the subsequent activation of TrkB (Pezet et al., 2002a). The activation of TrkB leads to activation of the downstream mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) in the dorsal horn (Pezet et al., 2002a), an event known to be important in the development of hyperalgesia (Ji et al., 1999). BDNF is thought to induce its neuromodulatory role via activation of ERK, which subsequently modulates glutamatergic neurotransmission (Kerr et al., 1999; Slack and Thompson, 2002; Garraway et al., 2003; Slack et al., 2004). As TrkB-immunoreactive neurons have been poorly characterized, our study aimed to 1) determine whether neurons of the spinothalamic pathway express the TrkB receptor, 2) determine the rostrocaudal and laminar distribution of these neurons, and 3) investigate the potential activation of ERK by BDNF in these STT cells.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. All experiments were performed in accordance with institutional and Home Office regulations.

Stereotaxic injections of FG in the thalamus: tracing of STT neurons

Wistar rats (225–250 g, Harlan, Bicester, UK, n = 18) were anesthetized with medetomidine (250 mg/kg) and ketamine (Parke-Davis, Gwent, UK, 60 mg/kg i.p.) and placed in a stereotaxic frame. Access to the left thalamus was acquired through small incisions in the skull. Twelve injections of 2% Fluoro-Gold (FG) (0.1 μl each) were performed unilaterally into the left ventrobasal complex of the thalamus using a 1-μl Hamilton syringe (Carnforth, UK) mounted on a micromanipulator. Three anteroposterior coordinates were used, with each having two lateral points and two heights, according to Burstein et al. (1990b) and using coordinates from the atlas of Paxinos and Watson (1986) as follows: Bregma: AP −3.8 mm, −3.0 mm, −2.3 mm; H: 6.5 mm, 5.5 mm; L: 2.5 mm and 1.5 mm from Bregma 0. Following placement of the syringe in each coordinate, the animal was left for 5 minutes to allow equilibration, and then the injection was made over a 5-minute period to allow slow penetration of the tissue. The syringe was removed slowly after a 5-minute period to minimize leakage of FG along the tract. The wounds were then surgically closed and the animals were left to recover.

Intraspinal injection of BDNF

One week after the injection of FG into the left thalamus, 14 adult rats were anesthetized with urethane (1.5 g/kg, i.p), and a 1-cm laminectomy was performed at the lumbar level. Animals received either 1 μl of saline (n = 7) or BDNF (100 ng/μl, n = 7), injected from a glass micropipette (50-μm tip diameter) with the tip located at a depth of 500 μm into the right dorsal horn of the cord (laminae I–V). Animals were then perfused with paraformaldehyde/picric acid (4% w/v paraformaldehyde, 15% saturated picric acid in 0.1 M. phosphate buffer pH 7.4) 10 minutes after the injections.

Induction of ERK by intraplantar capsaicin

Eight adult rats received stereotaxic injections of FG (see above) in the right thalamus in order to trace STT neurons. Ten days later, animals were anesthetized with urethane (1.5 g/kg, i.p) and then received 50 μg capsaicin (8-methyl-*N*-vanillyl-6-noneamide; Sigma, injected in 50 μl into the plantar surface of paws, dissolved in 10% ethanol, 10% Tween 80 in 0.9 % NaCl) either in the left hindpaw (n = 4) or forepaw (n = 4). Two minutes after the injection, animals were transcardially perfused with 100

ml heparinized saline (0.9% w/v NaCl) followed by 400 ml of 4% w/v paraformaldehyde in 0.1 M. phosphate buffer (PB), pH 7.4 (PFA), 15% of a saturated solution of picric acid. The spinal cords were postfixed in the same fixative overnight and cryoprotected overnight in 20%w/v sucrose in 0.1 M PB at 4°C.

Immunohistochemistry

Rats were deeply anesthetized with pentobarbital (140 mg/kg) and transcardially perfused with 100 ml heparinized saline (0.9% w/v NaCl) followed by 400 ml of 4% w/v paraformaldehyde in 0.1 M. phosphate buffer, pH 7.4 (PFA). p-ERK staining required the addition of picric acid with the PFA (see above). The brains and whole spinal cords dissected were cryoprotected in 20%w/v sucrose in 0.1 M PB for 24 hours at 4°C. The spinal cords were cut into five portions, embedded in OCT (BDH, Poole, UK), and then frozen over liquid nitrogen; the brains were frozen in isopentane at −40°C. Transverse sections of spinal cord and brain were cut serially (20 μm thickness) on a cryostat, and every third section was kept in PBS solution (free-floating method) or mounted onto Superfrost slides (BDH; every second section for the lumbar portion of cords). A small cut was made to identify the right side of the ventral cord segment and the left side of the brain to permit orientation.

One in six of all sections was processed for TrkB immunostaining. Sections were washed in phosphate-buffered saline solution 0.01 M (PBS), and then endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS (10 minutes). Following several washes in PBS, sections were blocked with 10% v/v normal goat serum made in PBS-Triton 0.3% v/v (PBST) for 1 hour. They were then stained for TrkB protein by incubation overnight with rabbit anti-TrkB antibody 1:3,000 (gift from Prof. R.A. Rush, Flinders University, Australia). The following morning, after several PBS washes, the sections were incubated with biotinylated goat anti rabbit-IgG 1:400 (Jackson ImmunoResearch, West Grove, PA) for 1 hour. After washing with PBS, the sections were incubated in streptavidin-horseradish peroxidase (HRP; 1:400 in PBS, 45 minutes), washed in PBS, and incubated in fluorescein tyramide reagent for 10 minutes (1:400 in amplification buffer provided by the manufacturer; TSA Fluorescein System, PerkinElmer, Oak Brook, IL). Finally, free-floating sections were mounted onto Superfrost plus slides (BDH). In terms of the specificity of the anti-TrkB antibody used, a previous study had established that preabsorption with immunizing peptide did not produce any staining. In addition, Western blot analysis showed that this antibody recognizes specifically the full-length and truncated forms of TrkB (Zhou et al., 1993).

For TrkB-NeuN double labeling, sections were immunostained for TrkB as described above, then washed in PBS, and incubated overnight with mouse anti-NeuN (Ref# MAB 377, Chemicon, Hampshire, UK; 1:500, in PBST 0.3% v/v). This antibody is raised against neuronal nuclei (clone A60) and recognizes two to three bands in molecular weights 46–48 kDa. The following day they were washed in PBS, incubated in goat anti-mouse Cy3-conjugated antibody (Molecular Probes, Eugene, OR; 2 hours, 1:500), and then washed and mounted onto Superfrost slides in Vectashield medium (Vector, Burlingame, CA).

For p-ERK-TrkB double immunolabeling, following the TrkB immunolabeling (described as above), slides were washed in PBS, blocked in normal goat serum 10% v/v for 1 hour, and incubated overnight in mouse anti-phosphorylated-ERK1/ERK2 (recognizing sites Thr 202 and Tyr 204 of ERK1/2, Ref # 9106, E10 monoclonal antibody, New England Biolabs, Beverly, MA, 1:400). After several washes in PBS, sections were incubated in goat anti-mouse antibody conjugated to Cy3 (Molecular Probes; 1:1,000) for 2 hours. Finally slides were washed and mounted with Vectashield medium (Vector). The single p-ERK staining (Fig. 7) was performed in the same way, omitting the steps for TrkB staining. The anti-p-ERK antibody recognizes the phosphorylation site only of ERK 1/2, recognizing on Western blots two bands at 42 and 44 kDa (Pezet et al., 2002a). Control experiments showed that omitting the primary antibody did not produce any staining. In addition, pretreatment of sections or PVDF membranes of Western blots with calf intestinal phosphatase induce a lack of p-ERK staining, suggesting that this antibody only recognizes the phosphorylated form of the protein (Pezet et al., 2002a).

For FG-BDNF double labeling, free-floating sections were incubated in 0.3% hydrogen peroxide in PBST for 10 minutes, washed extensively, and then incubated for 1 hour in normal goat serum 10% v/v. Sections were then incubated in rabbit anti-BDNF (a gift from Amgen, Thousand Oaks, CA; 1:2,000, 38 hours). Sections were then washed and incubated in biotinylated goat anti-rabbit (1:400, 90 minutes; Jackson ImmunoResearch), washed in PBS followed by incubation in avidin-biotin complex (Vector; 1:5 in PBS, 30 minutes), and incubated in biotinylated tyramide (1:75, 8 minutes in amplification buffer; Perkin Elmer). Finally, after several PBS washes, sections were incubated in extra-avidin-fluorescein isothiocyanate (FITC; Sigma; 2 hours, 1:500) and mounted onto Superfrost Plus slides (BDH) in Vectashield medium (Vector).

To maintain consistency, control and treated sections were immunostained simultaneously. Omission of the primary antibody or omission of any stage in the protocol did not result in labeling. All antibodies and sera were diluted in 0.01 M PBS, 0.1% w/v sodium azide, and 0.2% v/v Triton X-100, and incubation of slides was performed at room temperature.

Sections were viewed under an Axioplan 2 Imaging microscope (Imaging Associates) fitted with 10×, 20×, and 40× Plan-Neofluor objectives (Zeiss, Germany) and images of the dorsal horn were taken by using an AxioCam Hrm digital camera (Zeiss, Welwyn Garden City, UK) and AxioVision software (Imaging Associates, Bicester, UK).

Quantification

Proportion of STT neurons that contain TrkB. Sections were first examined by using darkfield microscopy to determine the segmental level according to Molander et al. (1984, 1989).

Twenty sections of each segment of cord were chosen at random, and the number of FG-labeled profiles with and without TrkB double labeling was counted. For each animal, data from sections of adjacent levels (C1–C4, C5–C8, T1–T9, T10–T13, L1–L6, and S1–S4) were pooled. Each count was sorted into laminae groups, as follows: I–II (for simplicity as very few lamina II STT cells were observed), III–IV, V–VI, VII–VIII, IX–X, and dorsolateral funiculus. The percentage of double-labeled neurons over total Fluoro-Gold-labeled profiles was calculated. (The total

population of FG-containing profiles is 100%.) Labeled neurons in the lateral cervical nucleus (LCN) were counted as STT profiles, as they project to the thalamus and have been counted as STT neurons in previous studies of the STT (Burstein et al., 1990b).

Proportion of STT neurons that are p-ERK positive and contain TrkB. At least 40 sections were counted per animal to provide a good proportion of STT cells. In each section the left (saline treated) and right side (BDNF treated) were quantified separately as follows: 1) the number of phospho-ERK-positive only neurons (which had no TrkB or FG labeling), 2) the number of phospho-ERK-positive and TrkB-positive (but no FG) neurons, and 3) the number of FG- and phospho-ERK-positive neurons (but no TrkB) neurons.

Statistical analysis

A Mann-Whitney rank sum test was used, employing Sigma stat software. A value of $P < 0.05$ was considered significant and a value of $P < 0.001$ highly significant.

RESULTS

As previously reported, TrkB immunostaining was observed throughout the spinal cord, in all spinal (cervical, thoracic, lumbar, and sacral) segments analyzed. In all segments, strong labeling was observed in motorneurons (Fig. 1B), whereas the immunolabeling was weaker in the dorsal horn (Fig. 1A,C). In the dorsal horn, TrkB immunolabeling was observed in the superficial dorsal horn (laminar I and II) and in the neck of the dorsal horn (laminae V and VI; Fig. 1A,C), areas that contain large populations of nociceptive neurons. Labeling was also recorded in the nucleus proprius (laminae III and IV), which is considered to be non-nociceptive. Immunoreactivity was also detected in the ventromedial gray matter, including laminae VII, VIII, and X, as well as in lamina IX (Fig. 1B). Labeling was confined to neuronal cells, as revealed by the colocalization of TrkB and the neuronal marker NeuN (Fig. 1C–E). TrkB labeling was found on cell bodies, axons, and dendrites, as described previously in other parts of the rat central nervous system (CNS; Zhou et al., 1993; Yan et al., 1997a).

The whole thalamus was labeled with FG in all the animals included in this experiment. The ventrobasal zone (VB; ventroposterolateral [VPL] and ventroposteromedial [VPM] regions; Fig. 2A,B) was labeled more strongly than other parts of the thalamus. FG was only observed in the thalamus and in a part of the hippocampus (due to the spread of the tracer during removal of syringe).

In most sections of cervical, thoracic, lumbar, and sacral cord, spinothalamic projection neurons (Fluoro-Gold labeled) were observed. Grains of retrogradely transported Fluoro-Gold accumulated in cell bodies as well as in dendrites (Fig. 3). Many of the labeled cells could be typed as pyramidal, multipolar, or fusiform (Fig. 3B). However, some cells had atypical morphology. A large proportion of FG-labeled neurons was found in the superficial dorsal horn (mostly in lamina I and very rarely in lamina II), particularly in the upper cervical regions of the cord. STT cells were also located deeper in the dorsal horn in laminae V, VII, and VIII in all segments of the cord, especially in the lumbar enlargement (Figs. 4, 5A). Finally, in half of the animals a small proportion of STT cells was found in area X and in the lateral spinal nucleus of the dorsal funiculus (Figs. 4, solid and open circles, 5A). Most of the retrogradely labeled neurons were found contralateral to the injection site; small but significant numbers were also located ipsilaterally, especially in upper cervical segments (Fig. 4). The overall distribution of labeled neurons in the gray matter was thus similar to that previously described (Burstein et al., 1990b; Marshall et al., 1996).

Figure 5A shows the rostrocaudal distribution of the mean number (± SEM) of STT labeled cells per laminae (in laminae I–II, V–VI, VII–IX, and X). Figure 5B presents the average percentage of STT cells positive for TrkB. It shows that at all levels of the spinal cord (cervical, thoracic, lumbar, and sacral), a large proportion of STT neurons (50–100%) in laminae I–II, V–VI, and VII–IX were TrkB immunopositive. As mentioned in the section above, many of the dually labeled cells could be typed as pyramidal, multipolar, or fusiform, but some cells had atypical morphology, and hence quantification of STT neurons immunopositive for TrkB pooled all three types. In contrast, in area X, almost no STT cells were TrkB positive in all cord level examined, except in T1–T9, (Fig. 5B). In T1–T9, where the average number of STT cells in lamine X was less than 5 for 10 sections, all these neurons were positive for TrkB (Fig. 5A,B).

These results suggest that (with the exception of area X) most neurons of the spinothalamic tract express BDNF's high-affinity receptor TrkB. Immunofluorescent staining for BDNF showed that in the superficial laminae of the spinal cord, FG-positive neurons are located in the vicinity of BDNF-immunoreactive terminals, suggesting that not only do STT neurons express BDNF's receptor but also that these neurons are located in the vicinity of the trophic factor. This suggests that physiological BDNF release from terminals could act on STT neurons in superficial laminae (Fig. 6A).

ERK has previously been shown to be activated in spinal neurons specifically in relation to noxious stimulations (Ji et al., 1999), and we have shown that such activation is partly due to TrkB activation by BDNF (Pezet et al., 2002b; Lever et al., 2004), *N*-methyl-D-aspartate (NMDA) receptor activation, and metabolic glutamatergic receptor activation, but not via AMPA or NK1 receptors (Lever et al., 2004). In order to show that TrkB receptors expressed by spinothalamic neurons could be functionally activated by BDNF, we microinjected BDNF into the spinal cord of rats that had received prior STT tracing with FG. We microinjected the molecule rather than applying a noxious stimulus (which would induce its release) due to the topographical discrepancy between TrkB expression and the distribution of BDNF-immunoreactive terminals.

The BDNF injection significantly induced ERK phosphorylation in numerous neurons of the dorsal horn (Fig. 6B): 116 ± 30 phospho-ERK neurons were labeled per 10 sections in BDNF-treated cords compared with 48 ± 4 phospho-ERK-labeled neurons per 10 sections in saline-treated cords ($n = 6–8$, $P < 0.01$). In BDNF-injected animals the majority (99.6 ± 0.2%) of phospho-ERK neurons contained TrkB, as revealed by double labeling, whereas approximately 4.5% of phospho-ERK cells contained both TrkB and FG.

Injection of capsaicin (50 μg) into the plantar surface of the left hindpaw or forepaw induced activation of ERK in many neurons of the dorsal horn in spinal cord levels L3–L5 and C5–C8, respectively. The distribution and the amplitude of p-ERK activation (up to 15–20 neurons per

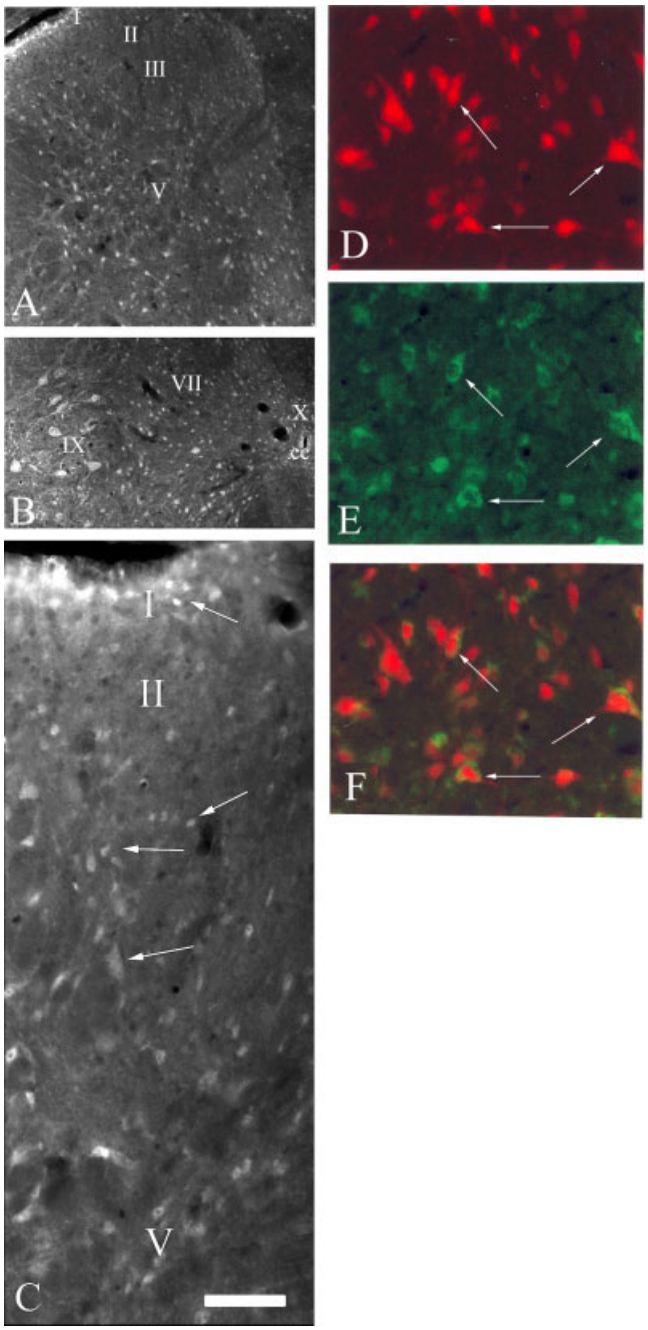


Fig. 1. TrkB immunostaining in the rat spinal cord and its expression in neuronal cells. **A,B:** Immunostaining for the BDNF high-affinity receptor TrkB in the dorsal (A,C) and ventral (B) horn of the rat spinal cord. TrkB immunoreactivity was observed in the superficial dorsal horn (laminae I and II), in the neck of the dorsal horn (laminae V and VI), and in the nucleus proprius (laminae III and IV). In the ventral horn TrkB labeling was observed in the ventromedial gray (including laminae VII, VIII, and X) as well as in lamina IX (in motorneurons). **C:** Higher magnification of TrkB staining in the dorsal horn. Arrows indicate examples of TrkB-positive cells. **D,E:** Double labeling for TrkB (D) and a neuronal marker (E). **F:** Merge of D and E. Note that not all neurons are TrkB positive. In contrast, all TrkB-positive cells are NeuN positive, suggesting that TrkB is expressed by neurons. Arrows indicate examples of double staining TrkB/NeuN. **E:** Merge between C and D. Numbers indicate Rexed's laminae. cc, central canal. Scale bar = 38 μm in C; 125 μm for A; 150 μm for B; 62 μm for D–F.

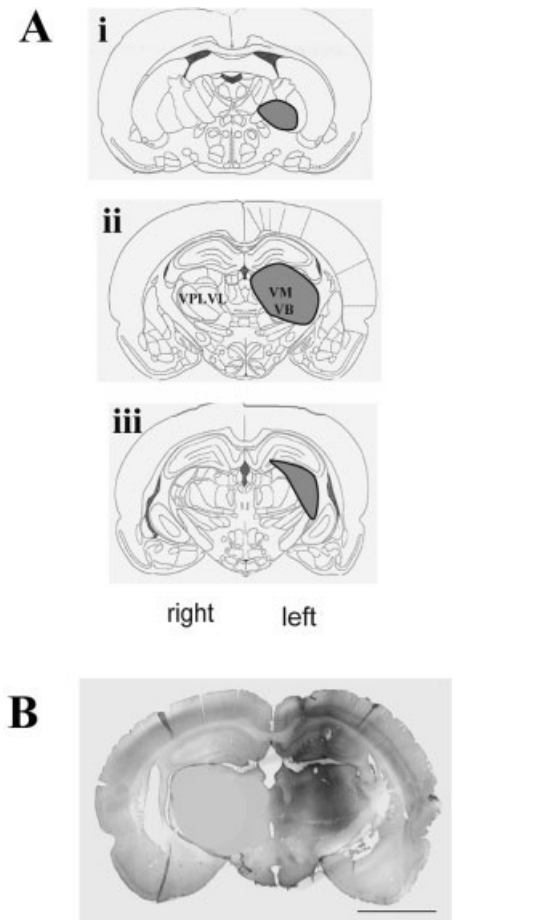
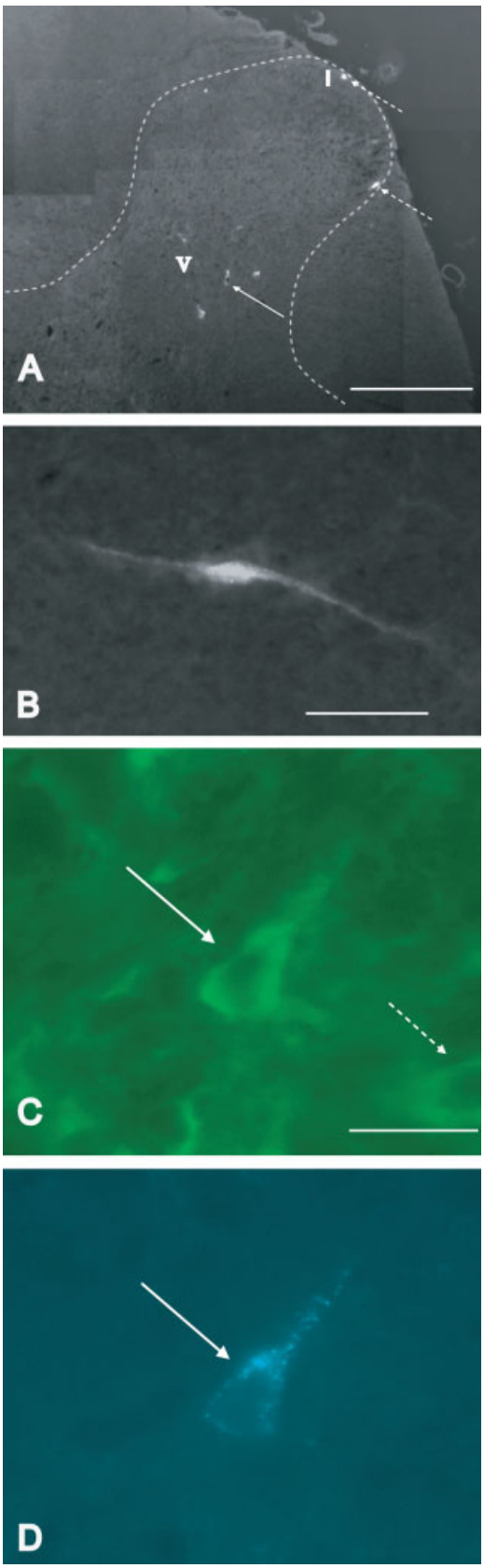


Fig. 2. Example of injection site of FG in the thalamus. **A:** Representative example of rostrocaudal distribution of FG in the thalamus of a rat. Drawings are based on those by Paxinos and Watson (1986). VL, ventrolateral thalamic nucleus; VPL, ventroposterior thalamic nucleus; VM, ventromedial thalamic nucleus; VB, ventrobasal nucleus. **B:** Example of section with DAB-immunostained FG in the left thalamus. Scale bar = 3 mm in B; 4 mm for A.

sections) was similar for both sites of stimulation. As previously described, most of these neurons were located in superficial laminae of the cord. Some colocalization between STT and p-ERK was observed in some horizontal lamina I neurons, but they were not very frequent (approximately 1 neuron in every 10 sections). In contrast, most of the STT neurons in deeper laminae (laminae III–VI were p-ERK positive (Fig. 7). As p-ERK was never found in laminae deeper than lamina V–VI, colocalization between p-ERK and STT cells in these layers was never found. Figure 7 shows an example of STT cells positive for p-ERK in the cervical cord C8. Four STT neurons are traced in this hemicord. Two are located in the cervical lateral nucleus and two in lamina V, and they are all p-ERK positive. The distribution of STT p-ERK-positive neurons was similar in the cervical and lumbar segments after their respective stimulation with capsaicin.

DISCUSSION

This study aimed to establish whether spinothalamic neurons express the BDNF receptor TrkB and whether



this population of neurons could be activated by BDNF. We observed that the majority of neurons of the STT pathway indeed express TrkB, and that this receptor is functional, as it could be activated by exogenous BDNF, which via phosphorylation mechanisms activated MAPK ERK. These results support growing evidence demonstrating that BDNF has a pronociceptive neuromodulatory role in the spinal cord and suggest that it may act on neurons of the STT pathway.

TrkB is present in most spinothalamic tract cells

Previous studies have highlighted the role of STT cells in the transmission of nociceptive information to the thalamus in rats and cats (Willis and Coggeshall, 1991; Guilbaud et al., 1994). Cell bodies of these neurons are located in laminae I and IV–VI and are somatotopically organized in rat and monkey (Willis and Coggeshall, 1991). Neurons in lamina I usually have extremely small receptive fields (RFs), in contrast to neurons of laminae V–VII, which have expanded and complex RFs that receive additional proprioceptive inputs. Lamina I STT neurons are high-threshold nociceptive-specific neurons that respond to heat/pinch/cold. However, STT neurons located in lamina IV–V or VII–VIII are wide dynamic range (WDR) neurons that respond to nociceptive and innocuous stimuli.

By using combined retrograde tracing and immunofluorescent staining, this study analyzed the proportion of STT pathways that express TrkB. The distribution of STT cells in this study (traced with FG) was consistent with that described by Burstein et al (1990b). Colocalization of FG with TrkB showed that BDNF's receptor is found in all types of STT neurons: in fusiform, multipolar lamina I STT cells (nociceptive), in pyramidal STT cells (non-nociceptive), and in STT cells with no distinguishable morphology (possibly a mixture of WDR nociceptive STT cells and lamina IV–V and VII–VIII non-nociceptive STT cells). Increasing evidence suggests that when BDNF is endogenously released from primary afferents, it acts as a pronociceptive neuromodulator in the spinal cord (Pezet et al., 2002b). TrkB expression by STT neurons suggests that BDNF may activate these neurons. Our observation of ERK activation in STT neurons induced by injection of BDNF or natural release of BDNF following noxious stimulation supports this hypothesis and suggests that pathophysiologically released BDNF may activate this subpopulation of neurons, therefore having a pro-nociceptive role on TrkB-expressing STT cells.

Fig. 3. Spinothalamic projection neurons in the spinal cord and their colocalization with TrkB. A,B: Photomicrographs showing typical examples of FG-traced spinothalamic neurons in the spinal dorsal horn. **A:** STT neuron located in lamina I (dashed arrows) and lamina V (solid arrow). The dotted line shows the border between gray and white matter. **B:** Enlarged photomicrograph of a lamina I neuron showing the typical morphology of arborization in a horizontal plane with dendrites spreading in laminae I and IIo. **C,D:** Example of STT FG traced neuron immunopositive for TrkB. The solid arrow indicates an STT traced neuron positive for TrkB, and the dashed arrow indicates a non-STT TrkB-positive neuron. Scale bar = 100 μm in A; 50 μm in B,C (applies to C,D).

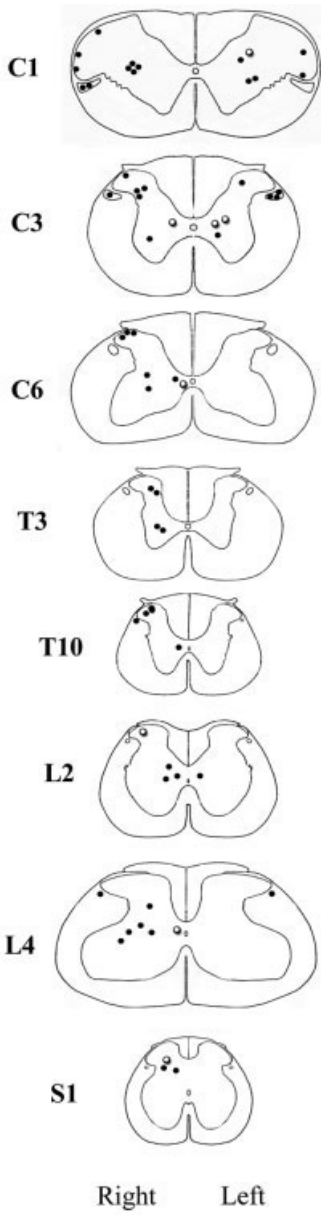


Fig. 4. Example of rostrocaudal distribution of STT neurons that were TrkB immunopositive (black circles) or TrkB immunonegative (open circles) in a representative animal. Note that animals received FG injections in the left thalamus and that traced STT cells are located contralateral to the injection site due to the decussation of the tract. Most of these neurons are TrkB immunopositive. Each symbol represents one neuronal cell body. Spinal cord levels are indicated to the left of each diagram.

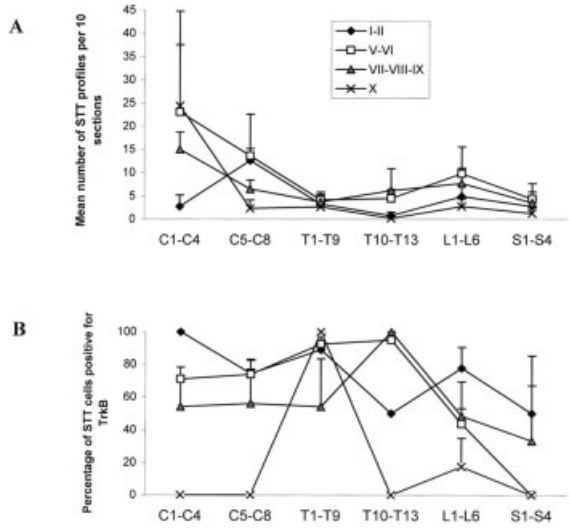


Fig. 5. Graphs representing the rostrocaudal variations of the mean number of STT neurons per 10 sections (**A**) and the percentage of STT profiles containing TrkB (**B**), in different laminae of the dorsal horn. Data are mean ± SEM (n = 4).

TrkB may be expressed by other projection neurons of the spinal cord

BDNF and TrkB are widely expressed in the CNS. In the spinal cord, TrkB is expressed in a large number of neurons in both the ventral and the dorsal horn. STT neurons, in contrast, represent only a small proportion of this population (less than 10%). TrkB is expressed by neurons of other origins, i.e., by interneurons and potentially by other projection neurons involved in nociceptive transmission. Candidate pathways that have their cell bodies neighboring BDNF-containing afferent terminals include the spinobulbar projections (spinoreticular and spinomesencephalic tracts), the spinohypothalamic tract, and indirect ascending pathways such as the postsynaptic dorsal column system, the spinocervicothalamic pathway, and the spinoparabrachial pathway (Fields et al., 1974; Pompeiano, 1975; Menetrey et al., 1982; Cechetto et al., 1985; Menetrey and Basbaum, 1987; Burstein et al., 1990a).

Indeed, spinoreticular tract projection (SRT) neurons can respond to both nociceptive and non-nociceptive information: those located in laminae III–IV (where TrkB was found on numerous neurons) have properties similar to STT cells of the WDR type responding to both non-noxious and noxious stimulation (Menetrey et al., 1980). In contrast, cells in the dorsolateral funiculus (DLF) nucleus project bilaterally to the cuneiformis area and were shown to be activated by stimulation of subcutaneous and/or deep structures but not by noxious stimulation (Menetrey et al., 1980). It is likely that a population of SRT cells (as well as STT cells) receives input from BDNF-containing nociceptors and conveys nociceptive information from the cord to the brainstem.

A majority of lamina I projection neurons have also been found to project to the caudal ventrolateral medulla (CVLM), parabrachial area, dorsal reticular nucleus, and periaqueductal gray matter (PAG; Marshall et al., 1996; Todd et al., 2000). Spinomedullary neurons are as numerous as STT cells and have a similar distribution (Andrew et al., 2003), as do spinosolitary tract projection neurons, which are thought to integrate somatic and visceral afferent inputs (Menetrey and Basbaum, 1987). Therefore, because these projection neurons are distributed in the same laminae as STT cells and have similar morphology to them, they are likely to contain TrkB and respond to BDNF released from primary afferents following nocicep-

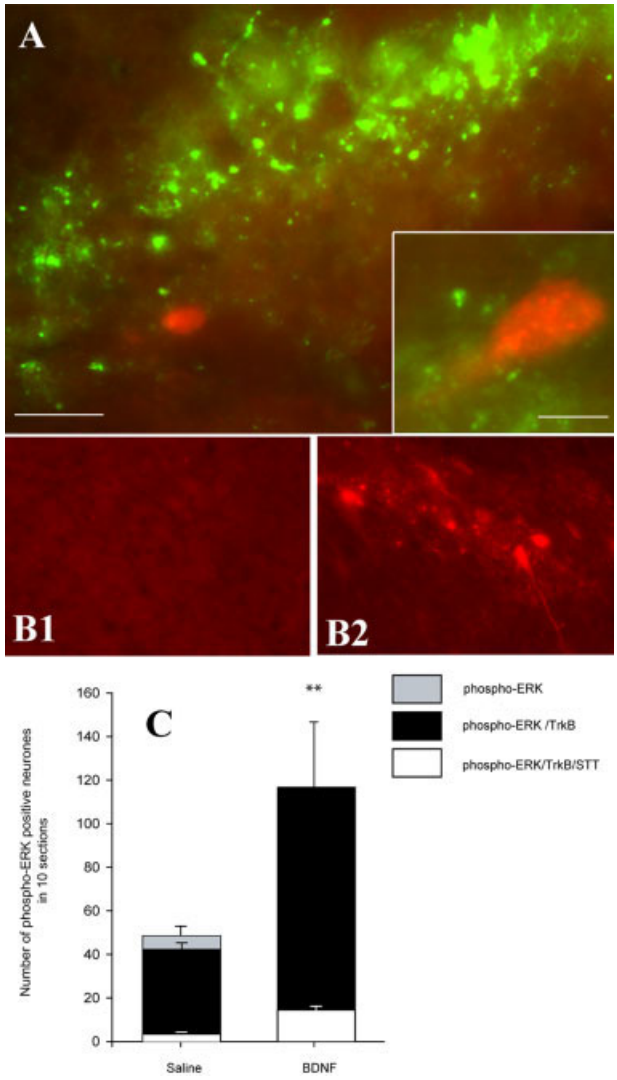


Fig. 6. Intraspinal injections of BDNF induced ERK phosphorylation in STT neurons. **A:** Double-immunofluorescent labeling for BDNF (green) and STT neurons (FG staining: red) in superficial laminae of the cord. Endogenous BDNF is found in the superficial laminae of the dorsal horn (in green), where numerous STT cells (in red) are found. **Inset:** Enlargement of an STT neuron (red) surrounded by BDNF-containing primary afferent terminals (green). B: Exogenously applied BDNF (B2) induced ERK phosphorylation in the rat dorsal horn. **B1:** Example of phospho-ERK staining in saline-injected cords. **B2:** Example of phospho-ERK staining in BDNF-injected cords. Note the cytoplasmic staining of phospho-ERK. **C:** Mean number of neuronal profiles (± SEM) in 10 sections containing phospho-ERK only (gray), phospho-ERK with TrkB (black), or phospho-ERK with TrkB and FG (white). BDNF injection induced ERK phosphorylation in many neurons of the dorsal horn (n = 7 per group; **, $P < 0.01$). A high proportion of phospho-ERK neurons contained TrkB, and 4–6% were triple labeled. Scale bar = 60 μm in A; 10 μm in inset A; 85 μm for B.

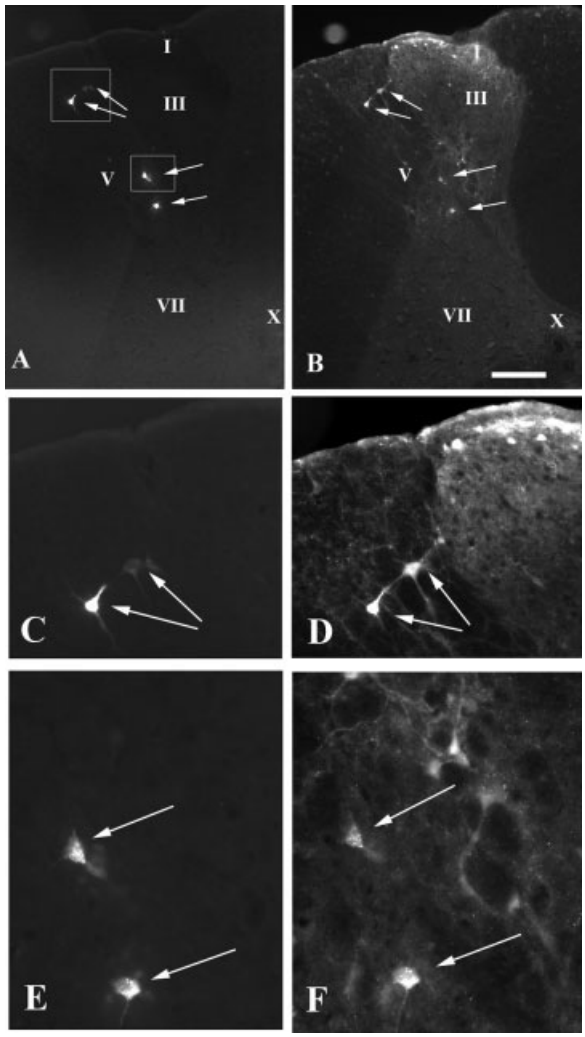


Fig. 7. Intraplantar injection of capsaicin induced ERK activation in STT neurons. Representative example of p-ERK immunostaining (**B,D,F**) and Fluoro-Gold traced neurons (**A,C,E**) in the same sections in the cervical spinal cord 2 minutes after peripheral injection of 50 μg capsaicin in the plantar surface of the forepaw. ERK activation is observed in non-STT neurons (superficial laminae) and in STT traced neurons (arrows). C,D and E,F are high-power magnification of A,B at the level of the LCN (C,D) or in lamina V (E,F), respectively. In A and B, numbers indicate Rexed's laminae. Scale bar = 200 μm in B (applies to A,B); 88 μm for C,D; 60 μm for E,F.

tive stimulation of various origins (cutaneous, muscular, and/or visceral).

We can hypothesize that if BDNF was able to act on projection neurons of different tracts, BDNF might be able to contribute modulation of several facets of pain, i.e., activation of spinothalamic projection neurons will transmit information of a discriminative nature to the thalamus, whereas activation of spinoparabrachial projection neurons (for instance) may drive more affective and emotional aspects of pain.

Topographical mismatch between areas of BDNF release and TrkB expression

As mentioned above, TrkB was found throughout all laminae of the cord, from the marginal zone to the ventral horn laminae. BDNF immunostaining, however, is present in the terminals of primary sensory neurons in the superficial laminae and to a lesser extent in deeper laminae of the cord. There is thus a topographical mismatch between the expression of the ligand and the receptor.

This mismatch may be functionally overcome in several ways: 1) BDNF may diffuse to deeper laminae (a theory proposed in the case of SP and the NK1 receptor; Liu et al., 1994), 2) dendrites of neurons of deep laminae may reach the superficial laminae and hence respond to BDNF released in the dorsal horn, which is likely to be the case for lamina V neurons (of a WDR nature); and 3) For certain neuronal populations, such as motorneurons, BDNF is retrogradely transported from their peripheral targets (Thoenen, 1995).

Functional significance of TrkB expression on STT neurons

Our data, together with previous reports, suggest that following a peripheral noxious stimulus BDNF, contained in terminals of a subset of DRG neurons (Michael et al., 1997; Luo et al., 2001), will be released in the spinal cord in an activity-dependent manner (Lever et al., 2001) and will activate TrkB (Pezet et al., 2002a) on postsynaptic STT neurons. Following the binding of BDNF to TrkB on those neurons, the receptor is activated by autophosphorylation mechanisms, allowing an intracellular signaling cascade that leads to a positive modulation of the glutamate receptor NMDA, through phosphorylation of its NR1 subunit (Di Luca et al., 2001; Slack and Thompson, 2002; Slack et al., 2004) and possibly NR2 subunit, hence exacerbating the glutamatergic transmission (Kerr et al., 1999; Heppenstall and Lewin, 2001; Arvanian and Mendell, 2001; Groth and Aanonsen, 2002), thereby increasing the response of postsynaptic neurons. The ultimate functional consequence of this biochemical activation is increased neuronal activation of the spinothalamic neurons and hence increased transmission of pain-related signals. Our observations are consistent with previous observations reporting an increased activation of NR1 subunit in neurons of the spinothalamic pathway following acute noxious stimulation (Zou et al., 2000).

We report that exogenous application of BDNF leads to TrkB and ERK activation by phosphorylation in spinothalamic neurons. It is likely that downstream effects of this activation is the activation by phosphorylation of stimulus-inducible transcription factors such as calcium/cyclic AMP response element binding protein (CREB) and/or the induction of expression of immediate early genes, such as c-fos. Their activation following noxious stimulation or BDNF application is well documented (Hunt et al., 1987; Abbadie and Besson, 1993, 1994; Soyguder et al., 1994; Abbadie et al., 1994; Honore et al., 1995; Presley et al., 1990; Kerr et al., 1999). Consistent with this hypothesis, c-fos-containing ascending tract neurons make up 6–8% of spinal neurons that express c-fos following noxious stimulation (Menetrey et al., 1989). These changes are likely to lead to phenotypic changes and long-term functional changes in these projection STT neurons.

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