

GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR CONTRIBUTES TO DELAYED INFLAMMATORY HYPERALGESIA IN ADJUVANT RAT PAIN MODEL

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Abstract—Neurotrophic factors, such as nerve growth factor and brain-derived neurotrophic factor, are members of the structurally related neurotrophin family that play important roles in pain modulation. Although there are also indications for the involvement of glial cell line-derived neurotrophic factor (GDNF), it is unclear whether and how GDNF is involved in inflammatory pain. In the present study, we studied the expression pattern of GDNF in dorsal root ganglia (DRG) and spinal cord, using confocal microscopy. We demonstrate that GDNF is well associated with nonpeptidergic pain pathway and that GDNF could possibly be anterogradely transported from DRG neurons to superficial spinal cord dorsal horn. We also studied the dynamic changes of GDNF expression in rats during chronic inflammation using injection of complete Freund's adjuvant as a model of chronic pain. We found that GDNF was down-regulated in both dorsal root ganglia and spinal cords 2 weeks after arthritis induction. To assess the impact of this down-regulation on pain transmission, we used a function-blocking antibody against GDNF delivered intrathecally in the same chronic-pain animal models. Injection of this antibody to GDNF produced no immediate effect, but decreased the delayed, bilateral hyperalgesia induced from a unilateral injection of complete Freund's adjuvant. The effect of this antibody coincided with the down-regulation of GDNF immunoreactivity in response to inflammation, suggesting that GDNF supports biochemical changes that contribute to hyperalgesia. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: GDNF, pain pathway, nociception, arthritis, IB4.

Neurotrophic factors have recently been shown to play important roles in the pain process, beyond their survival promoting actions in mammalian nervous system (Bennett, 2001; Mendell et al., 1999; Millan, 1999). Neurotrophins, a group of structure-related polypeptides, are the best char-

acterized among them. It has been shown that nerve growth factor (NGF) is responsible for the production of inflammation-induced hyperalgesia, and brain-derived neurotrophic factor (BDNF) may function as a neurotransmitter for pain perception (Mannion et al., 1999; Mendell et al., 1999; Thompson et al., 1999). Interestingly, NGF and BDNF exert their pain modulatory function exclusively in the peptidergic pathway. However, it is not clear whether other neurotrophic factors might have such modulatory actions for sensation of pain. In addition, it is not clear whether neurotrophic factors might exert the modulatory functions in the nonpeptidergic pathway.

Glial cell line-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor- β superfamily (Lin et al., 1993). GDNF has been demonstrated to have trophic effects on several types of neurons in the CNS (Arenas et al., 1995; Beck et al., 1995; Bowenkamp et al., 1995; Krieglstein et al., 1995; Oppenheim et al., 1995; Yan et al., 1995). In the peripheral nervous system, it has been suggested that a proportion of small-sized dorsal root ganglion (DRG) neurons shift their dependence from NGF to GDNF in the postnatal period by expressing GDNF receptor components instead of TrkA, a high-affinity receptor for NGF (Molliver et al., 1997). GDNF has been shown to protect these neurons after nerve injury (Bennett et al., 1998; Akkina et al., 2001). It is interesting that these cells possess the binding sites for lectin isolectin B4 from *Griffonia simplicifolia* (IB4), a characteristic that distinguishes these neurons from the subpopulation of small DRG neurons that express TrkA receptor (Stucky and Lewin, 1999; Snider and McMahon, 1998). Immunohistochemical studies have revealed the existence of GDNF in the superficial layers of spinal cord, an area closely related to pain transmission (Holstege et al., 1998; Jongen et al., 1999; Kawamoto et al., 2000). GDNF has also appeared to have pain-relief effect in neuropathic pain model, possibly through the regulation of sodium-channel subunits expression in DRG (Boucher et al., 2000). It is thus believed that GDNF is involved in the pain process. However, it is not clear whether and how endogenous GDNF could be involved in the inflammatory pain model.

The aim of the present study was to (1) study the expression pattern of GDNF in DRG and spinal cord in detail, and to specifically clarify whether or not the distribution is associated with the pain pathway; (2) look at the possible dynamic changes of GDNF expression in intra-articular complete Freund's adjuvant (CFA) injection-induced arthritic rats serving as a chronic model; (3) observe the influence of intrathecally administered GDNF antibody

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Abbreviations: BDNF, brain derived neurotrophic factor; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; FITC, fluorescein isothiocyanate; GDNF, glial cell line-derived neurotrophic factor; GDNF-ir, glial cell line-derived neurotrophic factor immunoreactivity; IB4, isolectin B4; NGF, nerve growth factor; SG, substantia gelatinosa; TRITC, tetramethyl-rhodamine isothiocyanate; TSA, tyramide signal amplification.

on mechanical allodynia in the intraplantar CFA-injected chronic pain model.

EXPERIMENTAL PROCEDURES

Animal experiment

Adult female Wistar rats weighting 150–200 g were used throughout the experiment. The animal protocols were approved by the Animal Use Committee of Peking University Health Science Center, including a special note to minimize the number of animals used and their suffering. CFA was prepared as described (Liu et al., 1996) by thoroughly mixing incomplete adjuvant (Gibco BRL) with a suspension of killed *Mycobacterium tuberculosis* (Human strain, 20 mg/ml, China Institute of Biological Materials, Beijing) in equal volume. Special care was taken in the preparation to ensure CFA was water-in-oil type.

Arthritic rats for morphological and biochemical studies were prepared by an intra-articular injection of 30 μ l CFA into the right (ipsilateral) tibio-tarsal joint under light anesthesia with 10% chlorohydrate (300 mg/kg, i.p.) (Butler et al., 1992; Liu et al., 1996). Injected animals were then randomly divided into four groups of seven to eight rats, which survived for 1, 2, 4 and 8 weeks respectively. Pain tests were performed in the day following the CFA injection and the day before killing as described (Liu et al., 1998). Briefly, one ankle joint was gently flexed dorsally for five times with an inter-test interval of 5 s. In each test, the occurrences of squeaking (scored 1) and/or leg-withdrawal (scored 1) responses were recorded, so that a total score between 0 and 10 was obtained for each test session composed of five consecutive tests. Since previous study indicated that the pain scores obtained from the contralateral side were very low and remained unaffected after CFA injection, pain tests were only performed in the ipsilateral side. The performance was done by persons not informed about the experimental treatments. Naive rats survived for the same periods were used as controls.

Rats for behavioral study were anesthetized and prepared with a lumbar catheterization of the spinal subarachnoid space as described (Storkson et al., 1996). Two days later, the viabilities of catheterizations were examined, and the rats with signs of neurological impairment were discarded. The rats were then weighed, and measurements of tactile threshold were made for both hind paws. The rat was placed in a testing cage with wire mesh floor and left to acclimatize for 30 min prior to testing. Calibrated von Frey nylon filaments (Stoelting, Wood Dale, IL, USA) with stiffness corresponding to 0.17, 0.41, 0.69, 1.2, 1.5, 2.0, 3.6, 5.5, 8.5, 12, or 15 \times g bending force were applied to the midplantar surface of the hind paw in an ascending order of stiffness. A brisk withdrawal of the hind limb was considered a positive response. Each filament was applied five times, thus 50% threshold value was considered equal to the force of the filament that induced two or more positive responses. The filament corresponding to 15 [time]g was selected as cutoff, since forces more than 15 \times g tended to lift hind paw (Chaplan et al., 1994). In the following day, 16 of the rats were randomly assigned to receive an intrathecal administration of previously characterized (Hashino et al., 2001) function-blocking anti-GDNF IgG (10 μ l, 50 μ g/ml, R&D Systems). Twelve rats received normal saline of the same volume as the control group. The intrathecal administrations were repeated once a day for the next 4 days. All rats received an intraplantar injection of 50 μ l CFA in the right (ipsilateral) hind paw immediately after the first intrathecal administration. The tactile thresholds were measured about 3 h after intraplantar injection of CFA, and once a day for the next 10 days and every other day the following days.

Staining procedure

Tissue preparation. Rats were perfused transcathetically under deep anesthesia with approximately 50 ml 37 °C normal

saline followed by approximately 250 ml 4 °C 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.2. The L4/5 DRGs and L4–L5 segments of spinal cord were dissected out and postfixed for 4–6 h in the same fixative, and cryoprotected for 48 h in 30% sucrose in 0.1-M phosphate buffer, pH 7.2 (all solutions at 4 °C). Tissues were embedded with OCT compound (Tissue Tek, Miles Laboratories, Elkhart, IN, USA).

Staining and quantification. Tissues were frozen and cut coronally in a cryostat at 10- μ m thickness and mounted onto alum-gelatin-coated slides. Sections of different rats from the same observation and the corresponding control group were mounted onto the same slide, so that the staining procedures of these sections were under exactly the same condition.

For semi-quantitative analysis of GDNF immunoreactivity, the spinal cord sections were incubated with rabbit anti-GDNF (1:100, Santa Cruz, CA, USA), raised against residues 186–205 within the C-terminal domain of the precursor form of human GDNF, for 48 h at 4 °C, with secondary antibody (instant working solution of biotinylated goat anti-rabbit; Zymed Laboratories) for 50 min at 37 °C, and with streptavidin–peroxidase conjugate (Zymed Laboratories) for 30 min at 37 °C. The sections were then reacted with a solution containing 0.05% diaminobenzidine tetrahydrochloride, 0.03% H₂O₂ in 0.05-M Tris–HCl buffer, pH 7.6 for 10 min, serially dehydrated, and resin coverslipped. We chose this GDNF antibody because it has been extensively used for immunohistochemical and blot study of GDNF in both rat and human tissues (Hashino et al., 2001; Quartu et al., 1999; Holstege et al., 1998; Kawamoto et al., 2000; Jongen et al., 1999; Russell et al., 2000; Meng et al., 2000). The images were captured directly off the microscope at 5 \times objective magnification using a digital camera (Imaging Research Inc., Canada). The relative grayscale level of the labeling was determined restrictive to the lamina I and II region using the white-matter region as background levels. At least 3 sections from the same rat, with distances of more than 1 mm from each other, were performed for staining and grayscale analysis.

For analyses of colocalization of GDNF immunoreactivity with other products, dual-labeling immuno-fluorescence was used. Sections were incubated with anti-GDNF (1:1000, Santa Cruz), and visualized by indirect tyromide signal amplification (TSA, New England Nuclear) with dichlorotriazinyl amino fluorescein or tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated streptavidin (1:400, Jackson Laboratories). The sections were then reacted with rabbit anti-substance P (1:100, Zymed Laboratories) or monoclonal mouse anti-neurofilament (1:400, Zymed Laboratories) and visualized with proper TRITC-conjugated second antibody, or reacted with fluorescein isothiocyanate (FITC)-labeled IB4 (10 μ g/ml, Sigma). The lack of cross-reactivity of the two rabbit antisera was ascribed to the highly diluted primary antibody for TSA compared with the followed indirect immunofluorescence, and therefore the second-series reactions could not detect it (Michael et al., 1997). This was confirmed by a control single-labeled preparation. The slides were coverslipped with buffered glycerol, and the images were obtained by a laser scanning confocal microscopy (Leica TCS NT). No cross-contamination of two channels was confirmed by blocking one of the exciting laser sources.

To determine the percentage of positive neuron profiles in L4 DRG in arthritic and naive rats, counts of GDNF- and IB4-positive neuron profiles were done on the ipsilateral sections. Totally 24 sections, 12 from three arthritic rats (2 weeks after arthritis induction) and 12 from three rats of the corresponding control group, were included in the analysis. The sections were selected distant enough from each other to make sure no cell was counted more than once. Counts were done in images obtained from independent red channel (TRITC, for GDNF) or green channel (FITC, for IB4) of confocal microscopy.

Table 1. Glial cell line-derived neurotrophic factor (GDNF)- and isolectin B4 (IB4) positive cell numbers in L4 dorsal root ganglia after arthritis induction

	GDNF (+)			Total IB4 (+)
	Total	IB4 (+)	IB4 (-)	
Naïve ^a	147	119 ^c	28 ^c	260
	100%	81%	19%	
Arthritis ^b	125	85 ^c	40 ^c	269
	100%	68%	32%	

^a Cell counts in 12 slides of L4 dorsal root ganglia from three naïve rats.

^b Twelve slides of the ipsilateral L4 dorsal root ganglia from three arthritic rats.

^c $P=0.022$ as analyzed by Fisher's exact test.

Western blot

The ipsilateral or the contralateral L4 and L5 DRGs from three to four rats of the same group were collected as one sample, and the protein extractions were prepared with Trizol reagent (Gibco BRL) according to the manufacturer's instruction. The protein concentrations were adjusted and 50 μg of total protein was loaded for 18% SDS-polyacrylamide gel electrophoresis. Western blot was done on polyvinylidenedifluoride membranes. GDNF was detected with rabbit polyclonal antibody to human GDNF (Santa Cruz), followed by horseradish peroxidase-conjugated antibody (Zymed Laboratories) and ECL chemiluminescence (Amersham).

RESULTS

Distribution of glial cell line-derived neurotrophic factor in dorsal root ganglion and spinal cord

Expression of GDNF in isolectin B4-positive small-sized DRG neurons. GDNF labeling was found in some of the L4/5 DRG cells. Virtually all these cells were of small to intermediate size, up to 75% of them were less than 27 μm in diameter. To identify the cell types that were GDNF labeled, GDNF staining was combined with staining for IB4, a widely used maker to characterize the subpopulation of nonpeptidergic small DRG neurons (Averill et al., 1995; Molliver et al., 1995; Bennett et al., 1998). The result showed that most GDNF-positive cells (approximately 81% of total GDNF-positive cells) were at the same time IB4 labeled (Table 1, Fig. 1). On the other hand, these GDNF-positive cells make an approximately 40% proportion of the total IB4-positive cells. Therefore GDNF was

mainly expressed by IB4-positive and small-sized DRG neurons, suggesting the localization of GDNF in nonpeptidergic nociceptive DRG neurons.

Expression of GDNF in lumbar spinal cord. Light microscopic examination of the sections processed for GDNF immunocytochemistry revealed dense reaction product mainly restricted to the superficial layer of the lumbar spinal cord (Fig. 2a, b). A similar pattern of distribution of GDNF immunoactivity (GDNF-ir) was observed in immunofluorescent stained sections using a laser scanning confocal microscopy (Fig. 2c). To observe further the localization of GDNF-ir in spinal cord, GDNF immunofluorescence was combined with staining of substance P or neurofilament. We found that GDNF-ir displayed in a pattern very similar to that of substance P (Fig. 2d–f). In addition, GDNF-ir existed just about the area where neurofilament signal was relatively weak (Fig. 2g–i). It is well known that substance P is distributed in lamina I, II. On the other hand, substantia gelatinosa (SG) is composed of very densely arranged small neurons; the nerve fiber proportion in this area was relatively low and so caused the weak staining of neurofilament. Based on these two reasons, GDNF-ir was mainly localized in SG as well as lamina I in rat lumbar spinal cord. This was coincident with others' finding (Holstege et al., 1998; Jongen et al., 1999).

When examined at a higher magnification, GDNF-ir displayed chiefly as punctate profiles (Fig. 2j), part of which was colocalized with neurofilament (Fig. 2j–l), indicating its existence in the nerve fibers. To clarify whether these fibers projected from DRG neurons, dual staining for GDNF and IB4 was performed in these sections (Caterina et al., 2000). Strikingly, most of the GDNF-ir was observed in IB4 fibers (Fig. 2m–o). The coexistence of GDNF and IB4 can be seen more clearly in computer-generated optical sections. The fiber traces revealed by GDNF-ir or IB4 were often coincident (data not shown). We have noticed that the overall distribution pattern of GDNF-ir (i.e. throughout the lamina I and II) is different from that of IB4 (more restricted to II_c) (Fig. 4d). However, this does not exclude the possibility that some of GDNF in the spinal cord may codistribute with IB4. Taken together, most of the GDNF-ir might exist in spinal nerve fibers projected from nonpeptidergic DRG neurons.

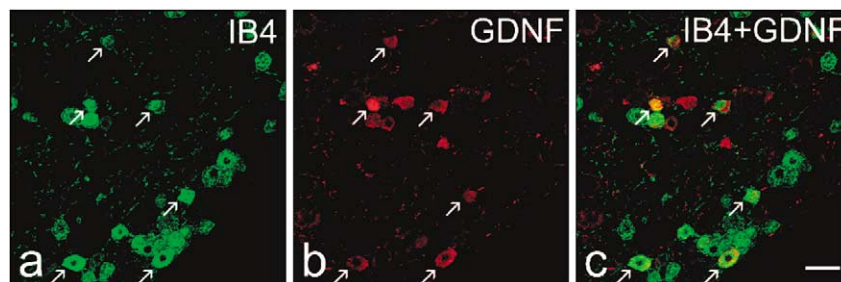


Fig. 1. Immunofluorescence micrographs of L5 DRG of naive rat double-stained with isolectin B4 (IB4, a) and glial cell line-derived neurotrophic factor (GDNF, b). The image with both signals was presented in (c). Arrows point to neurons that are both IB4- and GDNF-positive. (a, b) IB4- and GDNF-positive neurons were of small to medium size. (c) Most of the GDNF-positive neurons were also IB4-positive. Scale bar=50 μm .

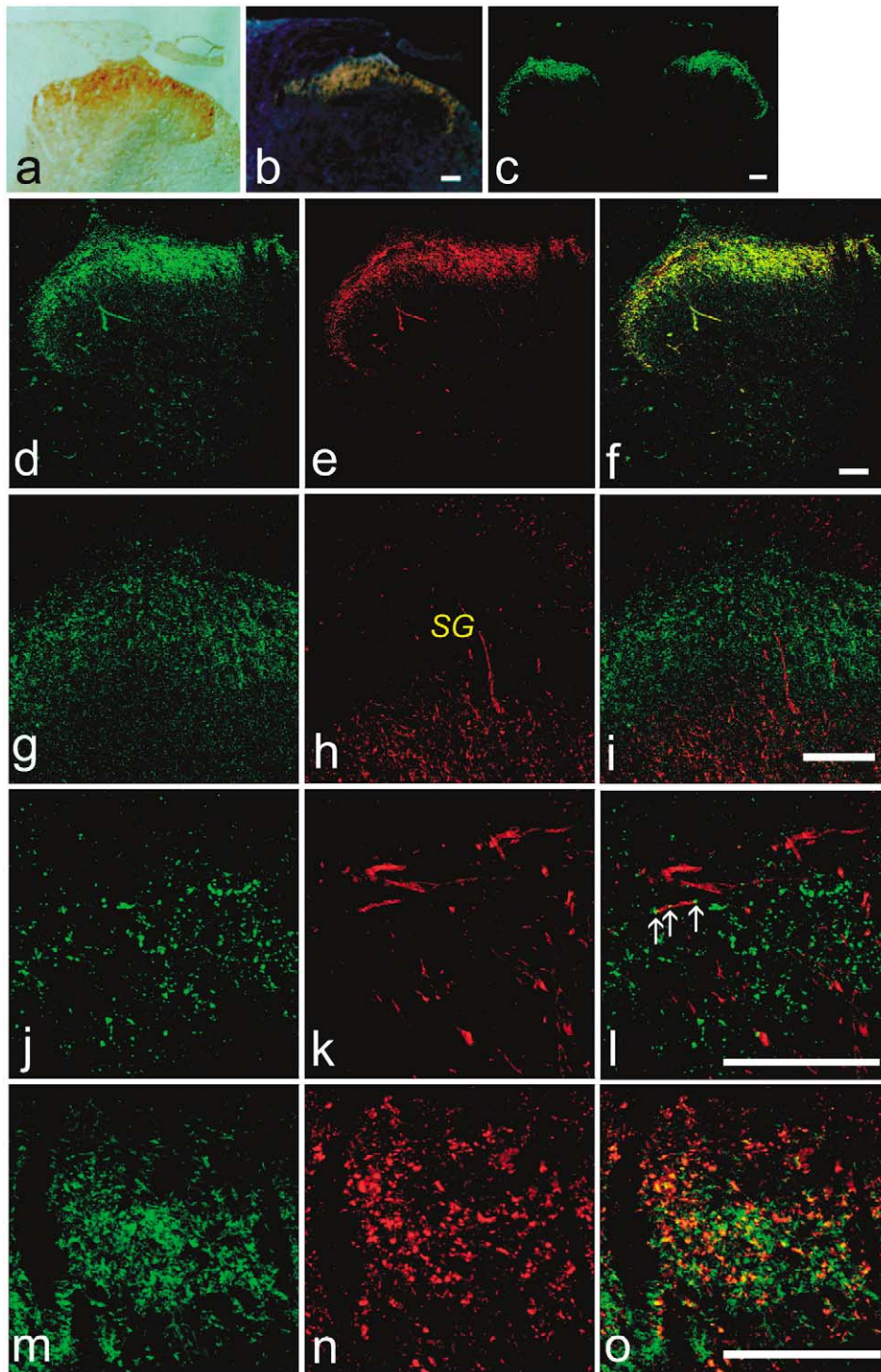


Fig. 2. Immunostaining of GDNF in the lumbar spinal cord tissue of naïve rats. (a–c) GDNF immunocytochemical localization was restricted to superficial layer of spinal cord. Same distribution patterns were obtained by DAB pigmentation observed under bright (a) or dark (b) fields, and by tyramide signal-amplified immunofluorescent visualization (c). (d–f) Dual labeling with GDNF (green) and substance P (red) revealed similar pattern (yellow). (g–i) Dual labeling with GDNF (green) and neurofilament (red) indicated that GDNF mainly existed in substantia gelatinosa (SG). (j–l) GDNF immunoreactivity (GDNF-ir) (green) displayed mostly as punctate profiles in the spinal cord (j), and was colocalized (arrows in l) with neurofilament (red) indicating its existing in nerve fibers. (m–o) Colocalization of isolectin B4 (IB4) (green) and GDNF (red) showed GDNF's existence in IB4-positive DRG neuron-projected nerve fibers. Scale bars=100 μ m.

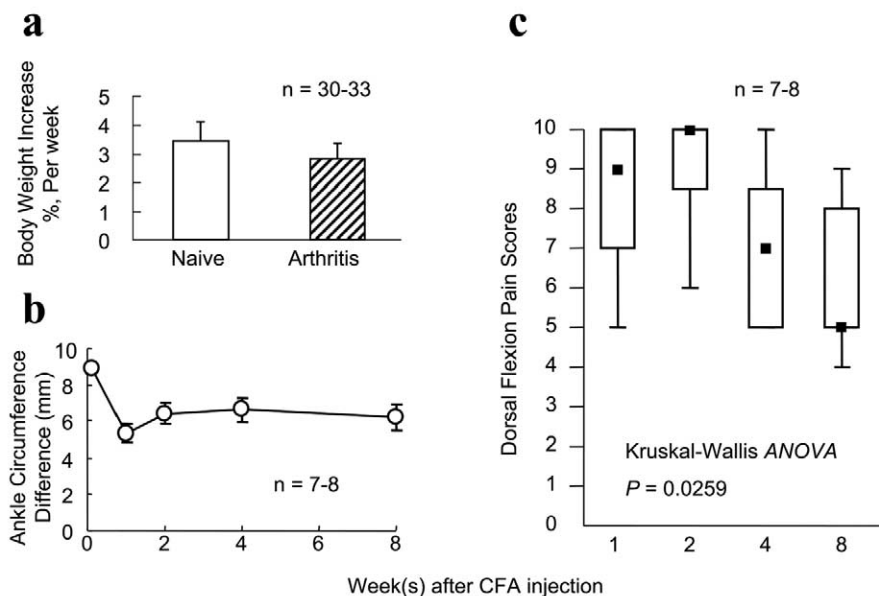


Fig. 3. Behavioral observation after induction of arthritis by unilateral intra-articular injection of complete Freund's adjuvant (CFA). (a) There was no difference of body weight increase observed between CFA-injected rat group and naive control group for 8 weeks after CFA injection, indicating that the overall condition of arthritic rats was quite well. (b) Differences of circumferences of ankle joint of the ipsilateral side to the contralateral side show the persistent swelling around the injection site. (c) Dorsal flexion pain scores showed the long-lasting pain status after induction of arthritis, with tendency of decrease after 4 weeks. Data were represented as means \pm S.E.M. in (a) and (b), or maximum, 75 percentile, median, 25 percentile and minimum (box and whisker plot) in (c).

Characteristic of pain hypersensitivity in intra-articular CFA injection-induced arthritic rats

In as long as 8 weeks after CFA injection, arthritic rats gained body weight steadily and did not differ from naive rats (Fig. 3a), suggesting that the overall condition of arthritic rats was fine. However, arthritic rats showed a persistent swelling in the ankle joint of injected side (Fig. 3b), which reflected the long-accompanying inflammatory status. Also, tactile hypersensitivity was observed in the dorsal flexion pain tests (Fig. 3c), although there was a tendency for the pain scores to become lower in the fourth and the eighth week after CFA injection.

Down-regulation of GDNF in DRG of the arthritic rats

Western blot analysis revealed a down-regulation of GDNF expression in both the ipsilateral and the contralateral L4/5 DRGs 2 weeks after CFA injection (Fig. 4a). In addition, the number of GDNF-positive neurons indicated a significant lessened proportion in the ipsilateral L4 DRG of arthritic rats compared with naive rats ($P < 0.05$, Fisher's exact test). It is likely that the reduction took place mainly in IB4 neurons since the proportion remained unchanged in IB4-negative neurons (Table 1).

Down-regulation of GDNF in spinal cord of the arthritic rats

A semi-quantitatively histochemical study indicated a dramatically diminished GDNF-ir in lumbar spinal cord 2 weeks after CFA injection (Fig. 4b). Semi-quantitative grayscale analysis showed significant decrease ($P < 0.05$, compared with the first-week group) of GDNF signal in

both the ipsilateral and the contralateral sides (Fig. 4c). This was confirmed by dual immunofluorescent staining of GDNF and IB4 (Fig. 4d). While the dorsal horn intensities of GDNF-ir in the arthritic rats were markedly lowered compared with the naive preparation, the intensities of IB4 staining remained little affected.

Pain-relief effect of intrathecally administered GDNF antibody in intraplantar CFA-injected rat model

Unilateral intraplantar injection of CFA caused a marked edema in the injected paw in several hours. In 2 to 4 days, edema could also be observed in the contralateral hind limb, even forelimbs for some rats. This was a typical symptom of polyarthritis. Tactile threshold assay showed gradually aggravated mechanical allodynia in the ipsilateral hind paw, and also in the contralateral hind paw but with a delay of 2 to 3 days. The allodynia was most severe in about 6 to 7 days after CFA injection, and lasted for at least 15 days (Fig. 5).

Intrathecal administration of GDNF antibody did not stop the development of tactile allodynia following injection of intraplantar CFA. However, it showed a clear alleviation of the pain hypersensitivity for both the ipsilateral and the contralateral hind paw, especially in the sixth day and thereafter (Fig. 5).

DISCUSSION

Anterograde transport of GDNF from nonpeptidergic DRG neurons to spinal cord

According to the classical neurotrophic hypothesis (Levi-Montalcini, 1987; Oppenheim, 1991), a neurotrophic factor

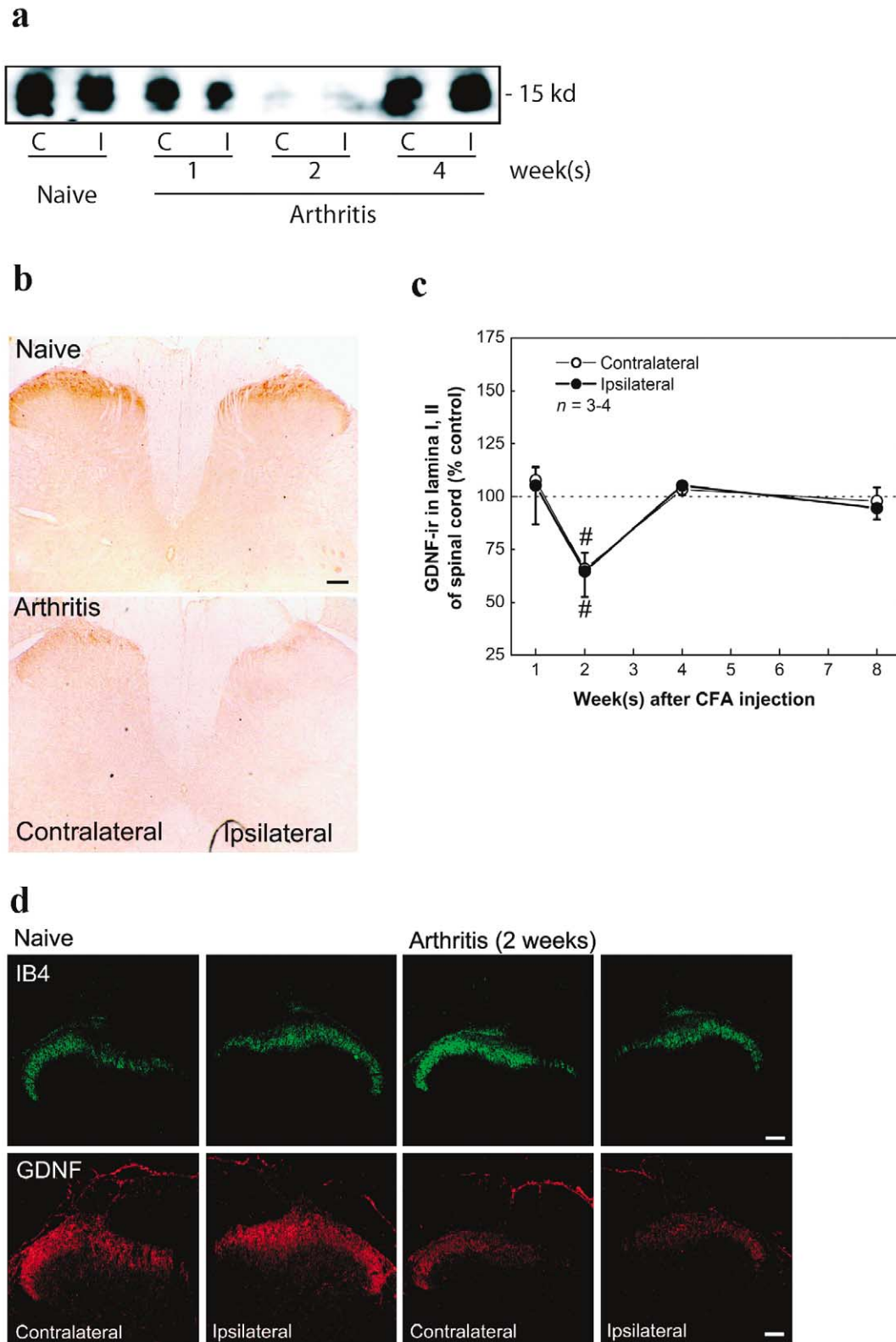


Fig. 4. Down regulation of GDNF in DRG and spinal cord after arthritic induction. (a) Western blot analysis of GDNF in L4/5 DRGs revealed a decrease of GDNF content in both the contralateral (C) and the ipsilateral (I) sides 2 weeks after arthritis induction. (b) Immunostaining of GDNF in lumbar spinal cord showed the GDNF signal was diminished in arthritic rats 2 weeks after complete Freund's adjuvant injection compared with naive rats. (c) Semi-quantitative grayscale analysis showed that GDNF was down-regulated exclusively in the second week after arthritis induction. Data represent mean percent (\pm S.E.M) of control GDNF labeling intensity. * $P < 0.05$ compared with the first week as analyzed by ANOVA followed by Newman-Keuls test. (d) Dual labeling of GDNF and isolectin B4 (IB4) confirmed the down-regulation of GDNF. Notice that the IB4 signals remained unaffected in arthritic rats. Scale bar = 100 μ m

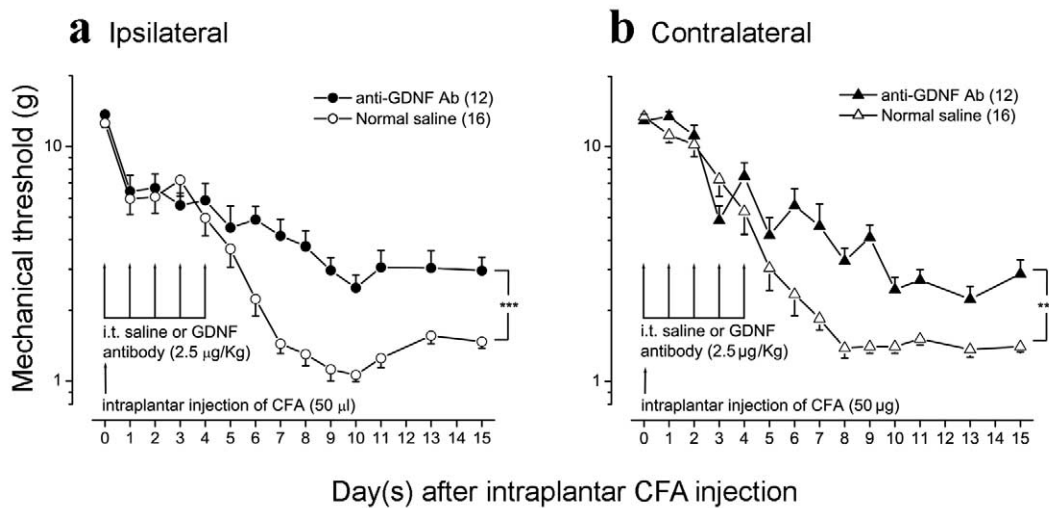


Fig. 5. Effects of intrathecal administration of anti-GDNF antibody on the mechanical allodynia in intraplantar CFA-injected rat pain model. Data were shown as means and the S.E.M. *** $P < 0.001$, analyzed by ANOVA with a repeated measurement (day), followed by post hoc Student-Newman-Keuls test for comparison of the two groups. In both the ipsilateral (a) and the contralateral sides (b), a lesser extent of mechanical pain hypersensitivity was observed in GDNF antibody-injected group (filled symbols) than in normal saline-injected group (open symbols), which reflects a pain-relief effect of anti-GDNF antibody in the experimentally induced arthritis.

is a target-derived survival factor transported from the terminals to neuronal cell bodies through retrograde trafficking. NGF, the first member of neurotrophin family, fits the hypothesis perfectly. However, recent studies suggest that neurotrophic factors may act in manners other than retrograde trafficking, such as in an autocrine fashion or via anterograde transport. BDNF, for example, is anterogradely transported in both the central (Altar et al., 1997; Conner et al., 1997; Fawcett et al., 1998; Kokaia et al., 1998; Smith et al., 1997) and peripheral nervous systems (Caleo et al., 2000; Tonra et al., 1998; von Bartheld et al., 1996; Zhou and Rush, 1996). Moreover, BDNF meets every requirement to work as a neurotransmitter, including presynaptic synthesis and vesicular storage, depolarization-evoked release, postsynaptic receptor localization and so on (for reviews see Altar and DiStefano, 1998; Tonra, 1999). Indeed, a role of BDNF as a neuron modulator was recently documented (Mannion et al., 1999), suggesting a specific mediator of tactile stimulus-induced inflammatory pain hypersensitivity. Neurotrophin-3 has also appeared to be trafficked anterogradely (von Bartheld and Butowt, 2000; von Bartheld et al., 1996). Therefore, it could be true that neurotrophic factors work as signaling molecules via a direction different from the traditional one, i.e. a neurotransmitter-like manner from neuronal somata to its nerve ending. This expansion of the possible functions of neurotrophic factors opened a new angle for neurotrophic research field.

GDNF is another neurotrophic factor being suggested in anterograde trafficking. Immunohistochemical studies revealed the spinal cord localization of GDNF in both rat (Holstege et al., 1998) and human (Kawamoto et al., 2000). In both species, GDNF labeling was most intense in fibers or terminals of the superficial layers (I, II) of spinal cord. A dorsal-root transection diminished the GDNF labeling in rat spinal cord, and GDNF accumulated in only

proximal end after a dorsal-root ligation (Holstege et al., 1998) or a hypoglossal nerve ligation (Russell et al., 2000). Ultrastructural studies not only support the anterograde transportation of GDNF from DRG neurons to spinal cord, they even suggest the possible localization of GDNF in dense-cored vesicles within the axons (Ohta et al., 2001).

The present work revealed colocalization of GDNF with IB4 in both spinal cord fibers and small-sized DRG neurons (Fig. 1 and Fig. 2m–o), besides confirmation of the distribution pattern of GDNF in rat spinal cord. Since IB4 is a widely used marker for non-peptidergic DRG neurons, and IB4-fibers in spinal cord are presumably projected from IB4 DRG neurons, this may suggest that GDNF in spinal cord fibers is possibly anterogradely transported from DRG neurons and therefore GDNF may localize mainly along non-peptidergic DRG–spinal cord pain pathway.

The involvement of GDNF in chronic pain model by a down-regulation

There is evidence that a population of DRG neurons shifted its dependence from NGF to GDNF in the postnatal period by expressing GDNF receptor component Ret instead of TrkA, the high-affinity receptor of NGF (Molliver et al., 1997). Most of these neurons are of small size, and therefore presumed to have nociceptive function. Interestingly, their dependence on GDNF is maintained throughout adulthood and GDNF protect these neurons from several axotomy-induced changes (Bennett et al., 1998). Recent work showed the potent analgesic effects of GDNF in neuropathic pain states, GDNF both prevented and reversed sensory abnormalities that developed in neuropathic pain models, without affecting pain-related behavior in normal animals (Boucher et al., 2000). These effects suggest a possible therapeutic effect of GDNF on pain

disorder. However, the distribution of GDNF in spinal cord implies the possibility of GDNF's nociceptive function: GDNF was mainly revealed in the superficial layer of spinal cord which was believed an area closely related to pain transmission (Holstege et al., 1998; Kawamoto et al., 2000; Jongen et al., 1999).

In our previous work we found a persistent down-regulation of GDNF mRNA in arthritic rat spinal cord dorsal half and such down-regulation correlated negatively with pain-test scores in a time window of 8 weeks, indicating a link between GDNF and chronic pain status (Fang et al., 2000). Interestingly, the present work also showed a down-regulation of GDNF in both the DRG and spinal cord levels in the arthritic rats serving as a chronic pain model (Fig. 4). In addition, the time courses were consistent in these two levels. The changes of expression occurred exclusively in 2 weeks after arthritis induction, a time regarded as the critical period for the transformation of inflammation from acute phase to persist one. This may suggest a functional involvement of GDNF in the development of chronic pain. This time course of GDNF protein, however, is inconsistent with that of GDNF mRNA, possibly reflecting the existence of two pools of GDNF in spinal cord. Since the change in protein concentration of GDNF did not reflect the dramatic change in GDNF at mRNA level, it could be inferred that the majority of spinal cord GDNF protein might be from DRG neuron, whereas a minority of GDNF may synthesize *in situ* in spinal cord. In support of this, although some overlap of GDNF and IB4 is observed, the overall staining pattern of GDNF is not identical to that of IB4 in spinal cord. While GDNF is rather diffused in the spinal cord sensory terminals throughout laminae I and II (Holstege et al., 1998), IB4 is more restricted to inner part of laminae II (Caterina et al., 2000; Bennett et al., 1998). Our data also reflect such a difference (Fig. 4d). It is therefore possible that GDNF may have two functional roles in spinal cord, one as long-distance modulatory signaling and the other working locally.

GDNF might work as pain modulator in the spinal cord

There are several possible mechanism by which down-regulation of GDNF could be involved in chronic pain and the modulation of nociception. *In vitro* experiments have suggested GDNF's regulatory effect on the expression of substance P (SP), a well-known pain substance (Humpel et al., 1996; Ogun-Muyiwa et al., 1999). Although NGF appears to be a more powerful SP regulator (Wong and Oblinger, 1991; Lindsay and Harmar, 1989), GDNF could still have an auxiliary effect on SP plasticity. GDNF has also been shown to be able to increase the expression of other pain-related substances, including vallinoid receptor subtype 1 (Ogun-Muyiwa et al., 1999), P2X₃ (Bradbury et al., 1998) and SNS and NaN sodium channels (Cummins et al., 2000). In addition, GDNF could enhance the neurotransmitter release (Ribchester et al., 1998) and promote the sodium-channel currents, therefore strengthening the functional activity of these pain substances. The down-regulation of GDNF in chronic-pain model shown by the

present work might thus be a compensation to reduce the inflammatory pain. The suppression of GDNF causes the reduced expressions or weakened activities of some of the pain substances and that would in turn offer a relief and/or reduction of chronic pain. GDNF therefore could contribute indirectly to the inflammatory pain process by regulating pain-related substances. On the other hand, the anterograde trafficking of GDNF gives the possibility that GDNF may work directly as a pain transmitter. Such role has been suggested for BDNF (Mannion et al., 1999), which meets many of the criteria necessary to establish it as a neurotransmitter/neuromodulator in small-diameter nociceptive neurons (Bennett, 2001; for reviews see Thompson et al., 1999). If this is true for GDNF, then the down-regulation of GDNF may reflect the excessive release of GDNF in more severe conditions of chronic pain, where there is a heavier neurotransmitter signaling. Such conditions might cause temporary depletion of GDNF. Therefore, GDNF might contribute directly to the chronic-pain process. In either case, it is likely that GDNF plays a nociceptive role in the development of inflammatory pain.

Since changes of GDNF in arthritic rats were found at both DRG and spinal cord levels, we favor a central action of GDNF on pain transmission. This was supported by our findings that intrathecal administration of a function-blocking GDNF antibody alleviated pain behavior (Fig. 5), although a more concise and convincing way to test if a signaling protein molecule participates in a given physiological activity is to apply the specific antagonist to its receptor. However, as the specific antagonist for GDNF has not been available, spinal antibody delivery, an approach successfully used for many years (Han, 1987), could be a reliable alternative. Nevertheless, while this approach provides a direct link between GDNF and pain transmission, it is difficult to interpret the result in a more comprehensive way, since the underlying mechanism as to how the antibody may affect the process is present unknown. On the other hand, since this study focused on the central role of GDNF in pain process, we cannot rule out the possibility that GDNF may also play a role peripherally. It is worthwhile to look at the possible expressional changes of GDNF in inflammatory tissue, and to test if anti-GDNF antibody affects the pain behavior when applied topically.

In conclusion, the codistribution of GDNF with IB4 in both DRG and spinal cord levels and the down-regulation of GDNF in these two levels in chronic-pain model suggest that GDNF might be working as a pain signal via anterograde trafficking mainly associated with nonpeptidergic pain pathway and contribute to the development of chronic-pain condition. GDNF may serve as a new target for the treatment of chronic inflammatory pain and be important in the development of neurogenic, widespread pain.

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REFERENCES

- Akkin SK, Patterson CL, Wright DE (2001) GDNF rescues nonpeptidergic unmyelinated primary afferents in streptozotocin-treated diabetic mice. *Exp Neurol* 167:173–182.
- Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* 389:856–860.
- Altar CA, DiStefano PS (1998) Neurotrophin trafficking by anterograde transport. *Trends Neurosci* 21:433–437.
- Arenas E, Trupp M, Akerud P, Ibanez CF (1995) GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron* 15:1465–1473.
- Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci* 7:1484–1494.
- Beck KD, Valverde J, Alexi T, Poulsen K, Moffat B, Vandlen RA, Rosenthal A, Hefti F (1995) Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* 373:339–341.
- Bennett DL (2001) Neurotrophic factors: important regulators of nociceptive function. *Neuroscientist* 7:13–17.
- Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci* 18:3059–3072.
- Boucher TJ, Okuse K, Bennett DL, Munson JB, Wood JN, McMahon SB (2000) Potent analgesic effects of GDNF in neuropathic pain states. *Science* 290:124–127.
- Bowenkamp KE, Hoffman AF, Gerhardt GA, Henry MA, Biddle PT, Hoffer BJ, Granholm AC (1995) Glial cell line-derived neurotrophic factor supports survival of injured midbrain dopaminergic neurons. *J Comp Neurol* 355:479–489.
- Bradbury EJ, Burnstock G, McMahon SB (1998) The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol Cell Neurosci* 12:256–268.
- Butler SH, Godefroy F, Besson JM, Weil-Fugazza J (1992) A limited arthritic model for chronic pain studies in the rat. *Pain* 48:73–81.
- Caleo M, Menna E, Chierzi S, Cenni MC, Maffei L (2000) Brain-derived neurotrophic factor is an anterograde survival factor in the rat visual system. *Curr Biol* 10:1155–1161.
- Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trifunovic J, Petersen-Zeitl KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288:306–313.
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53:55–63.
- Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. *J Neurosci* 17:2295–2313.
- Fang M, Wang Y, Liu HX, Liu XS, Han JS (2000) Decreased GDNF mRNA expression in dorsal spinal cord of unilateral arthritic rat. *Neuroreport* 11:737–741.
- Fawcett JP, Bamji SX, Causing CG, Aloyz R, Ase AR, Reader TA, McLean JH, Miller FD (1998) Functional evidence that BDNF is an anterograde neuronal trophic factor in the CNS. *J Neurosci* 18:2808–2821.
- Han JS (1987) Antibody microinjection technique as a tool to clarify the role of opioid peptides in acupuncture analgesia. *Chin Med J (Engl)* 100:459–464.
- Hashino E, Shero M, Junghans D, Rohrer H, Milbrandt J, Johnson EM Jr (2001) GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development. *Development* 128:3773–3782.
- Holstege JC, Jongen JL, Kennis JH, van Rooyen-Boot AA, Vecht CJ (1998) Immunocytochemical localization of GDNF in primary afferents of the lumbar dorsal horn. *Neuroreport* 9:2893–2897.
- Humpel C, Marksteiner J, Saria A (1996) Glial-cell-line-derived neurotrophic factor enhances biosynthesis of substance P in striatal neurons in vitro. *Cell Tissue Res* 286:249–255.
- Jongen JL, Dalm E, Vecht CJ, Holstege JC (1999) Depletion of GDNF from primary afferents in adult rat dorsal horn following peripheral axotomy. *Neuroreport* 10:867–871.
- Kawamoto Y, Nakamura S, Matsuo A, Akiguchi I, Shibasaki H (2000) Immunohistochemical localization of glial cell line-derived neurotrophic factor in the human central nervous system. *Neuroscience* 100:701–712.
- Kokaia Z, Andsberg G, Yan Q, Lindvall O (1998) Rapid alterations of BDNF protein levels in the rat brain after focal ischemia: evidence for increased synthesis and anterograde axonal transport. *Exp Neurol* 154:289–301.
- Kriegstein K, Suter-Crazzolara C, Fischer WH, Unsicker K (1995) TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. *EMBO J* 14:736–742.
- Levi-Montalcini R (1987) The nerve growth factor 35 years later. *Science* 237:1154–1162.
- Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260:1130–1132.
- Lindsay RM, Harnmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* 337:362–364.
- Liu HX, Xiong L, Luo F, Han JS (1998) Improved scoring method for arthritic flexion pain test in Freund's adjuvant monoarthritic model in the rat. *Chinese J Pain Med* 4:173–178.
- Liu HX, Xu W, Luo F, Wang JS, Han JS (1996) A limited arthritic model for the chronic pain in rats. *Chinese J Pain Med* 2:223–228.
- Mannion RJ, Costigan M, Decosterd I, Amaya F, Ma QP, Holstege JC, Ji RR, Acheson A, Lindsay RM, Wilkinson GA, Woolf CJ (1999) Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. *Proc Natl Acad Sci USA* 96:9385–9390.
- Mendell LM, Albers KM, Davis BM (1999) Neurotrophins, nociceptors, and pain. *Microsc Res Tech* 45:252–261.
- Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 287:1489–1493.
- Michael GJ, Averill S, Nitkunan A, Rattray M, Bennett DL, Yan Q, Priestley JV (1997) Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. *J Neurosci* 17:8476–8490.
- Millan MJ (1999) The induction of pain: an integrative review. *Prog Neurobiol* 57:1–164.
- Molliver DC, Radeke MJ, Feinstein SC, Snider WD (1995) Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *J Comp Neurol* 361:404–416.
- Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* 19:849–861.
- Ogun-Muyiwa P, Helliwell R, McIntyre P, Winter J (1999) Glial cell line derived neurotrophic factor (GDNF) regulates VR1 and substance P in cultured sensory neurons. *Neuroreport* 10:2107–2111.
- Ohta K, Inokuchi T, Gen E, Chang J (2001) Ultrastructural study of anterograde transport of glial cell line-derived neurotrophic factor from dorsal root ganglion neurons of rats towards the nerve terminal. *Cells Tissues Organs* 169:410–421.

- Oppenheim RW (1991) Cell death during development of the nervous system. *Annu Rev Neurosci* 14:453–501.
- Oppenheim RW, Houenou LJ, Johnson JE, Lin LF, Li L, Lo AC, Newsome AL, Prevette DM, Wang S (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* 373:344–346.
- Quartu M, Serra MP, Bachis A, Lai ML, Ambu R, Del Fiacco M (1999) Glial cell line-derived neurotrophic factor-like immunoreactivity in human trigeminal ganglion and nucleus. *Brain Res* 847:196–202.
- Ribchester RR, Thomson D, Haddow LJ, Ushkaryov YA (1998) Enhancement of spontaneous transmitter release at neonatal mouse neuromuscular junctions by the glial cell line-derived neurotrophic factor (GDNF). *J Physiol* 512 pt 3635–641.
- Russell FD, Koishi K, Jiang Y, McLennan IS (2000) Anterograde axonal transport of glial cell line-derived neurotrophic factor and its receptors in rat hypoglossal nerve. *Neuroscience* 97:575–580.
- Smith MA, Zhang LX, Lyons WE, Mamounas LA (1997) Anterograde transport of endogenous brain-derived neurotrophic factor in hippocampal mossy fibers. *Neuroreport* 8:1829–1834.
- Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron* 20:629–632.
- Storkson RV, Kjorsvik A, Tjolsen A, Hole K (1996) Lumbar catheterization of the spinal subarachnoid space in the rat. *J Neurosci Methods* 65:167–172.
- Stucky CL, Lewin GR (1999) Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *J Neurosci* 19:6497–6505.
- Thompson SW, Bennett DL, Kerr BJ, Bradbury EJ, McMahon SB (1999) Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. *Proc Natl Acad Sci USA* 96:7714–7718.
- Tonra JR (1999) Classical and novel directions in neurotrophin transport and research: anterograde transport of brain-derived neurotrophic factor by sensory neurons. *Microsc Res Tech* 45:225–232.
- Tonra JR, Curtis R, Wong V, Cliffer KD, Park JS, Timmes A, Nguyen T, Lindsay RM, Acheson A, DiStefano PS (1998) Axotomy upregulates the anterograde transport and expression of brain-derived neurotrophic factor by sensory neurons. *J Neurosci* 18:4374–4383.
- von Bartheld CS, Butowt R (2000) Expression of neurotrophin-3 (NT-3) and anterograde axonal transport of endogenous NT-3 by retinal ganglion cells in chick embryos. *J Neurosci* 20:736–748.
- von Bartheld CS, Byers MR, Williams R, Bothwell M (1996) Anterograde transport of neurotrophins and axodendritic transfer in the developing visual system. *Nature* 379:830–833.
- Wong J, Oblinger MM (1991) NGF rescues substance P expression but not neurofilament or tubulin gene expression in axotomized sensory neurons. *J Neurosci* 11:543–552.
- Yan Q, Matheson C, Lopez OT (1995) In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* 373:341–344.
- Zhou XF, Rush RA (1996) Endogenous brain-derived neurotrophic factor is anterogradely transported in primary sensory neurons. *Neuroscience* 74:945–953.

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