Decreased GDNF mRNA expression in dorsal spinal cord of unilateral arthritic rat

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It is now well established that nerve growth factor (NGF) plays a key role in inflammation-induced hyperalgesia. It was also reported that brain derived neurotrophic factor (BDNF), another member of neurotrophins, contributed to the pain pathway as a neurotransmitter in the CNS. The present work demonstrated a down-regulation of glial cell line-derived neurotrophic factor (GDNF) mRNA expression in dorsal spinal cord in complete Freund's adjuvant-induced unilateral arthritic rats serving as a chronic pain model. The fast occurring and long lasting down-regulations suggest that GDNF might contribute to pain pathway in a way different from neurotrophins and might play a role in the maintenance of chronic pain status. *NeuroReport* 11:737–741 © 2000 Lippincott Williams & Wilkins.

Key words: Arthritis; Chronic pain; GDNF; Neurotrophin; Rat; Spinal cord

INTRODUCTION

It is well known that small type dorsal root ganglion (DRG) neurons use glutamate and substance P (SP) as chemical mediators of nociception [1]. Recent studies have shown that neurotrophic factors are also involved in pain mediation or modulation [2]. Thus, nerve growth factor (NGF) is characterized to play a key role in the production of inflammation-induced hyperalgesia [3], and brain-derived neurotrophic factor (BDNF) functions as a neurotransmitter for pain perception [4]. In contrast to NGF and BDNF, which have a 40% homology in chemical structure, glial cell line-derived neurotrophic factor (GDNF) possesses a distinct chemical structure. It was interesting to find a subgroup of small DRG cells expressing the GDNF receptor, and GDNF is protective for these neurons after nerve injury [5]. These cells possess isolectin B_4 (IB_4) binding sites but not Trk binding sites, and are distinct from that subpopulation of small DRG cells equipped with the TrkA receptor and unresponsive to IB_4 [6,7]. The possible implication of GDNF in pain transmission and modulation remains unclear.

The aim of the present study was to explore the possible difference in mRNA expression of NGF and BDNF on one hand and GDNF on the other in the dorsal spinal cord of the rats subject to experimentally induced monoarthritis.

MATERIALS AND METHODS

Animal experiments: Female Wistar rats weighing 150–180 g, obtained from the center for animal breeding of Beijing Medical University, were used throughout the experiment. Animals were given food and water *ad lib* and kept in an animal house with a natural light:dark cycle. Complete Freund's adjuvant (CFA) was prepared by thoroughly mixing an incomplete adjuvant (Gibco BRL) and a suspension of killed *Mycobacterium tuberculosis* (human strain, 20 mg/ml) in equal volume. Special care was taken in the preparation to ensure the CFA was of the water-in-oil type. Arthritic rats were prepared by an intra-articular injection of 30 μl CFA into the right tibio-tarsal joint under light anesthesia with 10% chlorohydrate (300 mg/kg, i.p.). Injected animals were then randomly divided into four groups of 3–4 rats, which survived for 1, 2, 4 and 8 weeks respectively. Pain tests were performed on the day following the CFA injection and the day before sacrifice [8]. Briefly, one ankle joint was gently flexed dorsally 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. Pain tests were performed by persons without information on the experimental treatments. Naive rats were used as controls.

HPLC coupled quantitative RT-PCR: Since growth factors are often expressed at a very low level, we developed an HPLC coupled RT-PCR method which measured overall changes of expression in dorsal spinal cord samples with high sensitivity.

Rats were sacrificed by decapitation, the lumbar spinal cord was isolated as quickly as possible and divided into left and right hemicords which were further cut along the middle line into the ventral and dorsal parts. The left and right dorsal spinal cords (weighing about 10 mg) were immediately homogenized in 0.6 ml pre-cooled Trizol reagent (Gibco BRL) and the total RNA was extracted according to the manufacturer's instruction. The total RNAs were then isolated by electrophoresis in 1.5% agarose gel for confirmation of their structural integrity. Their absorbances at 260 nm were measured for estimation of the RNA concentration. About 2 μg total RNA was reverse transcribed (RT) with oligo-dT_{12-18} primers using the MMLV reverse transcription system (Promega) in a total volume of 25 μl. PCRs were performed in a 15 μl reaction volume containing 10 mM Tris, pH 8.3, 50 mM KCl, 100 μM of each dNTP, 0.75 U Taq polymerase (Promega), 15 pmol of primer pairs, an optimal amount of $MgCl_2$ and appropriate RT products (Table 1). The amplification were then performed on a thermo-cycler (Robocycler, Stratagene), beginning with a 3 min pre-incubation at 94°C, followed by optimal cycles of 45 s at 94°C, 60 s at optimal annealing temperature and 80 s at 72°C, ending with a 5 min incubation at 72°C (Table 1). The amplified products were electrophoresed in 3.0% agarose gel, visualized with ethidium bromide for fragment size estimation. Aliquots of 10 μl of the products were directly loaded onto high performance liquid chromatography (HPLC) system and the peak areas were measured as indices for quantification. The HPLC system consisted of two solvent delivery pumps, an autoinjector and a UV/Vis detector (LC-6A, Schimazu), the analysis was performed on a TSK-gel column (DNA-NPR, 4.6 × 75 mm, 2.5 μm, Tosoh, Japan) eluted with a linear gradient of 0.4–0.7 M NaCl in 20 mM Tris-acetate buffer (pH 9.0) within 10 min.

Data manipulation: To correct for subtle sample-to-sample differences in the amounts of starting material, all values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) peak areas, which served as internal controls. Differences between groups were analyzed by ANOVA with a repeated measurement (ipsilateral *vs* contralateral), followed by *post hoc* Student–Newman–Keul's test for multiple comparisons if needed. α level was set to 0.05.

RESULTS

Electrophoresis of RT-PCR products on agarose gel showed that amplification with the specific primers yielded the products of expected sizes (Fig. 1a). The chromatograms (Fig. 1b) showed that the amplified products could be separated well and the peak areas were in good linear relationship with the starting material under our optimized PCR conditions (data not shown). Thus we could measure the alterations of mRNA expression reliably and precisely. One advantage of the method was the comparability of data from batch to batch, and this was often difficult for methods using imaging techniques.

Down-regulation of GDNF mRNA in the dorsal spinal cord after CFA injection: In the first week after CFA injection, a dramatic decrease of GDNF mRNA in the ipsilateral dorsal spinal cord occurred ($-29.3 \pm 3.3\%$, $p < 0.01$, $n = 3$).

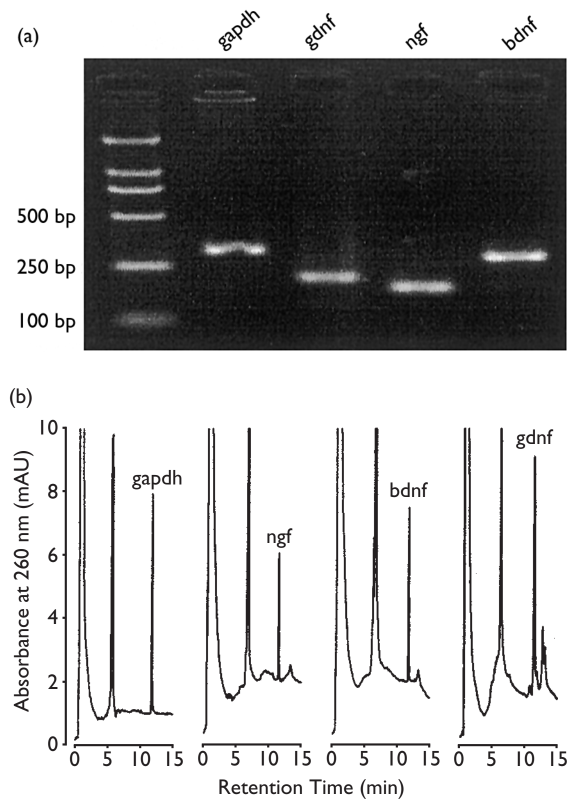

Fig. 1. RT-PCR analysis of neurotrophic factor mRNA expression in dorsal spinal cord of rats with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. (**a**) Electrophoresis in 3.0% agarose gel. (**b**) Chromatograms of HPLC separations.

Table 1. Sequences of primers and optimized conditions for PCR amplification.

cDNA		Sequence of primers	Fragment size (bp)	RT products input (μl)	$[Mg^{2+}]$ (mM)	Annealing temperature (°C)	Cycles
GDNF	S	ctgtctgcctggtgttgctc	216	1.0	0.5	63	40
	AS	ctggtgaacttttcagtcttttga					
NGF	S	ccaaggacgcagctttctatactgg	188	0.2	1.5	61	30
	AS	ggctgtgtcaagggaatgctgaagt					
BDNF	S	agcctcctctgctctttctgctgga	198	0.2	1.5	61	25
	AS	cttttgtctatgcccctgcagcctt					
GAPDH	S	tccctcaagattgtcagcaa	309	0.2	1.5	57	20
	AS	agatccacaacggatacatt					

S, sense; AS, anti-sense; RT, reverse transcription.

The decreases lasted for at least 8 weeks (Fig. 2a–d) and tended to recover slowly to normal level with time. The level of mRNA expression in the eighth week was obviously higher than that of the first week, but was still significantly lower than that of the naive control group (Fig. 3). On the contralateral side, the reduction of GDNF mRNA level did not take place until the weeks 2–4, and was of a lesser extent than that on the ipsilateral side (Fig. 2a–d). In the same observation period, the GDNF mRNA level on the contralateral side was also markedly lower than that in the first week (Fig. 3). In the eighth week, GDNF mRNA level was not significantly different from the naive controls (Fig. 2a–d), but was still statistically lower than that of the first week. Thus, after CFA injection, the dorsal spinal cord of the inflamed side displayed a rapid and prolonged down-regulation of GDNF mRNA expression, whereas the contralateral spinal cord showed only a delayed and milder down-regulation accompanied by a quicker recovery.

To observe whether such downregulation of GDNF mRNA in dorsal spinal cord related to pain transmission, a correlative analysis was performed between the relative GDNF mRNA levels and pain test scores. The results (Fig. 4) showed that the ipsilateral but not the contralateral GDNF mRNA levels correlated negatively with pain test scores ($r = -0.424$, $p = 0.01$, $n = 36$), which indicated that a link might exist between GDNF expression and pain modulation.

Neurotrophins mRNA expression in the dorsal spinal cord after CFA injection: Slight but significant decreases of NGF mRNA expression were observed in the first week of CFA injected rats in both sides of dorsal spinal cord (Fig. 2e–h). The mRNA levels then returned to normal in 1–2 weeks. No obvious changes of the abundance of BDNF mRNA occurred during the observation period of 8 weeks (Fig. 2i–l). No correlation was found in the ipsilateral mRNA levels of either NGF or BDNF with the pain test scores (Fig. 4).

DISCUSSION

The implication that GDNF might contribute to pain pathway came from the distribution of the GDNF receptor component Ret in small-diameter DRG neurons with IB_4 binding activity. The distribution of IB_4 binding activity showed little overlap with that of TrkA. In addition, Ret positive neurons innervate the inner parts (lamina II_i) of the dorsal spinal cord, which is distinct from the TrkA-positive neurons innervating the outer parts of lamina II. These findings suggest that this subgroup of DRG neurons

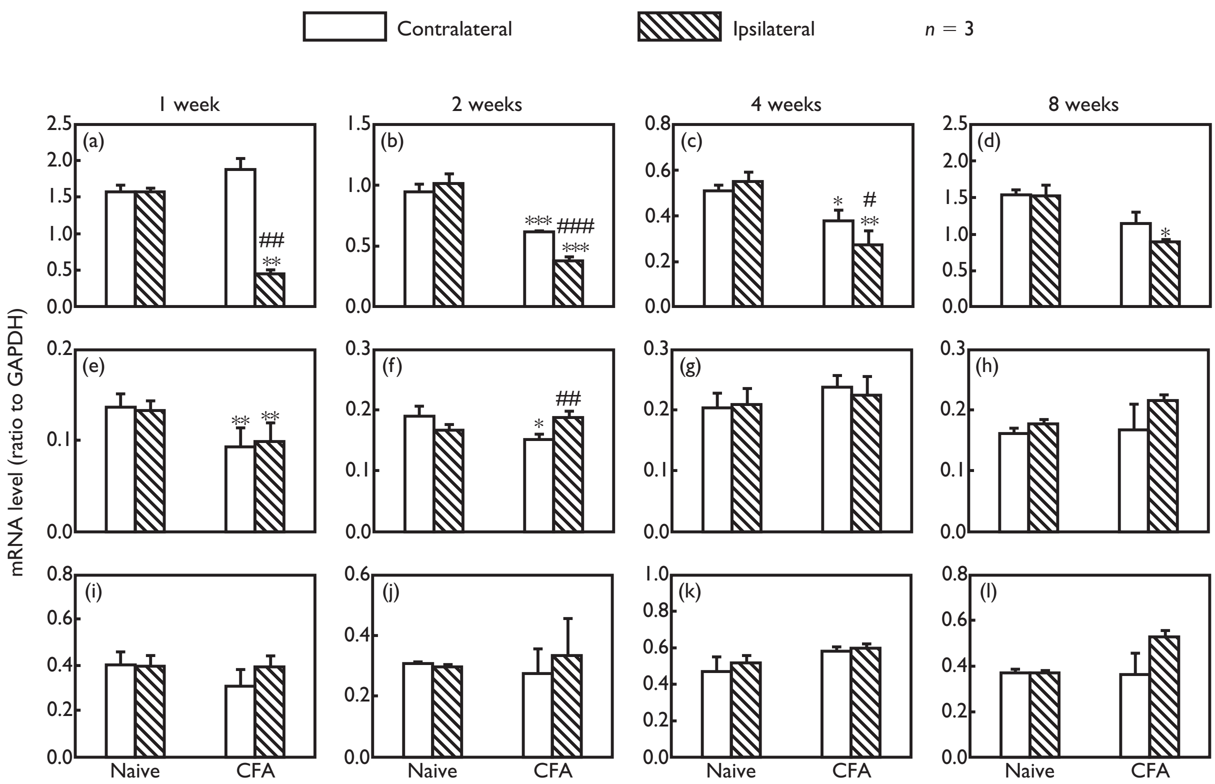

Fig. 2. Quantification of GDNF, NGF and BDNF mRNA levels by HPLC analysis of RT-PCR products as adjusted by GAPDH mRNA levels derived from the same samples. Columns represented the mean values of ratios to GAPDH mRNA levels. Error bars show s.e.m. (**a**–**d**) GDNF mRNAs showed dramatically decreased expressions in the ipsilateral dorsal spinal cord in the whole observation period of 8 weeks after unilateral intra-articular injection of complete Freund's adjuvant into one ankle joint. They were significantly lower than that of the contralateral side in the first, second and fourth week. In the second and fourth weeks GDNF mRNAs levels of the contralateral dorsal cord were lower than that of the naive controls ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* the relevant normal controls; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs* the contralateral sides). (**e**–**h**) NGF mRNA of both sides of the cord showed slight but significant decreases in the first week and went back to normal level in the second or fourth week. (**i**–**l**) No marked differences were observed in dorsal cord BDNF mRNA levels in the observation period.

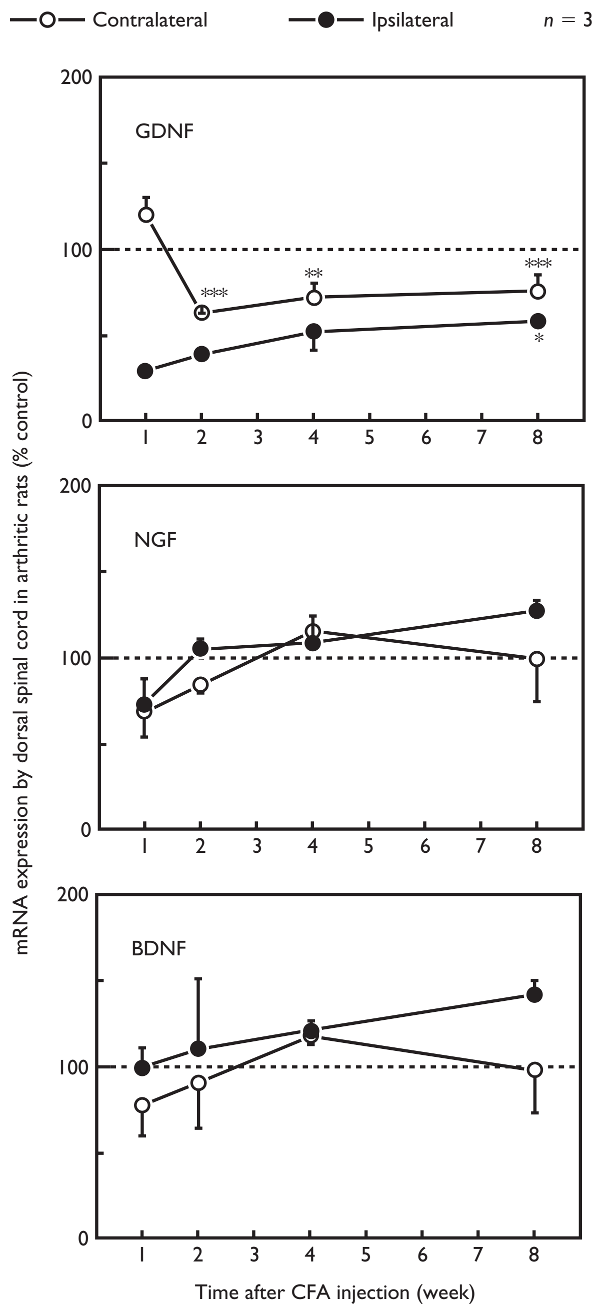

Fig. 3. Time courses of relative mRNA levels of GDNF, NGF and BDNF, normalized to the naive control groups and shown as the mean percentiles of the control groups. Error bars show s.e.m. GDNF mRNA levels in the ipsilateral dorsal cords decreased significantly in the first week and went up slowly in the course of 8 weeks. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* the first week). No statistically significant alterations of the abundance of NGF and BDNF mRNAs were observed.

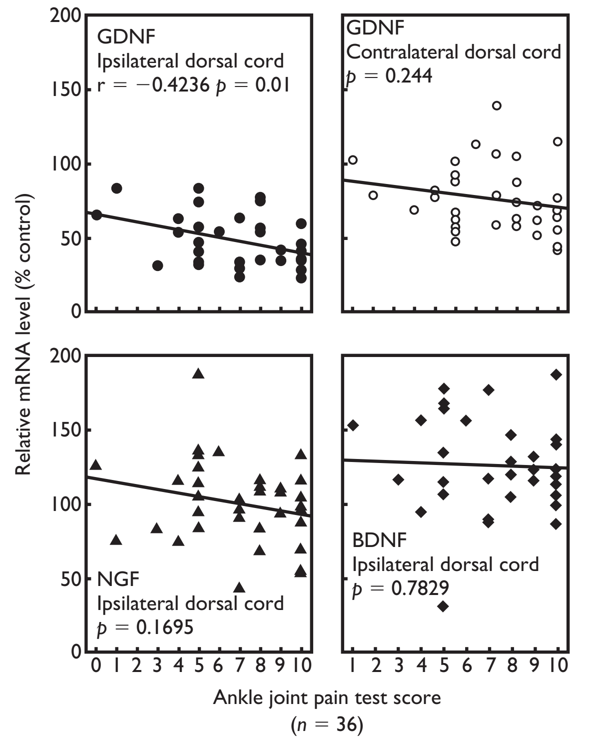

Fig. 4. Correlative analysis between the mRNA levels and dorsal flexion pain test scores. A statistically significant negative correlation was found between the pain scores and the levels of GDNF mRNA in the ipsilateral dorsal cord. No such correlation was found for the contralateral GDNF mRNA, or the ipsilateral NGF or BDNF mRNA, respectively.

might be involved in nociceptive transmission, and functionally regulated by GDNF family in a way different from the already known NGF-dependent peptidergic pathway [5,7]. Recent histochemical study revealed that GDNF immunoreactivity (GDNF-ir) existed selectively in the DRG afferents projecting to the superficial lamina (I, II_o) of dorsal spinal cord. Such GDNF-ir diminished markedly after the ligation of dorsal root. The authors inferred that GDNF protein might be synthesized in DRG neurons and anterogradely transported to their central projection [9]. However, the same laboratory demonstrated that sciatic nerve section also caused a rapid and persistent down-regulation of GDNF-ir similar to that of dorsal root ligation [10]. The rapid onset indicated that such decrease could be the result of an active down-regulation rather than a degenerative process of the DRG neurons. Therefore, the origin of GDNF-ir in DRG neurons was so far not clear. Nevertheless, the preferential localization of GDNF in nociceptive fibers presumed a nociceptive role of GDNF. The latest *in vitro* study demonstrated that GDNF could greatly induce the expression of substance P and vallinoid receptor subtype 1 (VR1), two well-known molecules with nociceptive properties [11]. These data strengthened the possibility of GDNF's nociceptive role.

To our knowledge, the present study reports for the first time the evidence of an involvement of GDNF in a chronic pain model. In the inflammatory pain status, GDNF mRNA expression demonstrated a rapid and persistent down-regulation in the dorsal spinal cord of the inflamed side. The extent of GDNF mRNA reduction showed a significant correlation with the severity of the pain. These results suggest that GDNF may play an active role in pain pathway. As to how GDNF exerted its nociceptive function, it was rather difficult to infer according to the present data. This is in sharp contrast to NGF and BDNF, whose precise roles in nociception are quite clear [3,4]. NGF is produced in inflamed peripheral tissue, and is retrogradely transported to the cell body of DRG neuron where it promotes the synthesis of BDNF; BDNF is NGF-dependently up-regulated in TrkA-carrying small-sized DRG neurons, and anterogradely transported to superficial lamina of dorsal spinal cord where it works as a nociceptive transmitter.

Accordingly, there are at least three possible working hypotheses for GDNF's nociceptive function: (1) NGF manner: it is true that there is abundant expression of GDNF in peripheral tissue, particularly the muscle [12], however it is not known how GDNF expression alters under inflammation conditions; (2) BDNF manner: this is not likely to occur since DRG neurons express GDNF at very low level [12]; (3) a novel manner different from that of NGF or BDNF.

Theoretically, the down-regulation of GDNF mRNA in dorsal spinal cord might have at least three possible functional meanings. One is that GDNF may play a nociceptive role. The active suppression of GDNF synthesis causes the reduction of GDNF content, which inevitably weakens the role of GDNF and eases the chronic pain, i.e. a compensation to reduce the pain of inflammation. The second is that GDNF might serve an inhibitory role in nociception, such that a suppression of its expression leads to an augmentation of pain transmission. The third is that owing to the increased input of GDNF protein from peripheral tissue, the synthesis of GDNF is inhibited due to a negative feedback mechanism. The latter is less likely since we have simultaneously detected the mRNA expression levels of NGF and BDNF in the same preparations but did not find negative feedback changes in either of them.

In any event, since GDNF mRNA in dorsal spinal cord was inhibited to such a great extent and lasted for such a long period of time in chronic pain status, the role of GDNF in chronic pain is worth deeper investigation.

CONCLUSION

Rapid and persistent down-regulation of GDNF mRNA expression occurred in dorsal spinal cord after intra-articular CFA injection, without obvious alteration in expression of either NGF or BDNF. The results suggest that GDNF might contribute to pain pathway in a way different from neurotrophins and might play a role in the maintenance of chronic pain status.

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