Modulation of Neuropathic Pain by a Glial-Derived Factor

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ABSTRACT

Introduction. An unfortunate consequence of incomplete nerve injury is development of neuropathic pain and associated allodynia, a feeling of discomfort in response to benign stimuli. Nerve injuries induce alterations in cytokine expression by Schwann cells, locally at the site of injury, and by glia, remotely in the spinal cord. Although most cytokines are pronociceptive, one, glial cell line-derived neurotrophic factor (GDNF), appears to be antinociceptive.

Methods. We have developed a novel method of delivering GDNF to the site of nerve injury. Using a model of partial nerve injury that reduces withdrawal thresholds to innocuous stimuli, we have found that local application of GDNF abolishes this allodynic behavior.

Results. Intrathecal application of GDNF stimulates expression of somatostatin, a neuropeptide with potential analgesic properties. Moreover, stimulation of dorsal root sensory neurons with GDNF in vitro increases cell content of somatostatin, which, in turn, leads to increased secretion in response to depolarizing stimuli.

Conclusion. We suggest that GDNF exerts its anti-allodynic effect via somatostatinergic mechanisms. Our observations suggest new approaches for treating nerve injury that may prove useful in preventing delayed complications that contribute to long-term debility.

Key Words. Allodynia; Neuropathic Pain; GDNF; Somatostatin

Peripheral nerve injury triggers a cascade of processes both locally and centrally that ultimately results in neurologic dysfunction. With complete nerve transection, both sensory and motor functions are completely lost as the disruption of neural transmission is total. More commonly, however, nerves are damaged during trauma, without complete transection, and the resultant functional consequences are far less predictable. One unfortunate consequence of such nerve injury is development of neuropathic pain and associated allodynia, a feeling of discomfort in response to benign stimuli. The pathophysiology of allodynia is incompletely understood. It cannot be explained solely on the basis of peripheral nerve activation [1]. Rather, there appears to be increased or inappropriate responsiveness of central neurons to relatively mild peripheral stimuli in allodynic states [2].

Nerve injuries induce alterations in cytokine expression by Schwann cells, locally at the site of injury, and by glia, remotely in the spinal cord [3]. A number of these polypeptides are associated with nociception and most are pronociceptive. For example, expression of interleukin-1α and β is increased in dorsal spinal cord after experimental nerve injury. We have recently reported that both cytokines, especially interleukin-1α, increase expression and release of the nociceptive peptide, substance P, an action that is mediated by the neurotrophin, nerve growth factor ([4]; NGF). NGF is also expressed by Schwann cells and support glial in damaged nerves [5–7] and can...
independently induce pain-associated behaviors in animals [8–10]. One glial factor, however, glial cell line-derived neurotrophic factor (GDNF) appears to be antinociceptive. Thus, intrathecally administered GDNF eliminates the allodynic response in several animal models of neuropathic pain [11]. Our long-range goal has been elucidation of the mechanisms underlying neuropathic pain syndromes and the development of novel methods for treating them. Using both in vivo and in vitro studies we have investigated the roles of nociceptive peptides in these pain states. We have found that partial injury of sciatic nerve in rats leads to a marked reduction in withdrawal threshold to benign stimuli. We now present a series of studies describing the effects of GDNF after nerve injury and potential underlying somatostatinergic mechanisms. Our observations may have implications in treatment of nerve injury that may prove useful in preventing delayed complications.

Methods

Animals, Surgery, and Withdrawal Testing

All animal protocols used in these studies were approved by the John D. Dingell VA Medical Center and Wayne State University Institutional Animal Care and Use Committee and conform to NIH guidelines. Female Sprague-Dawley rats (>3 months) were used for all experiments. Rats were subjected to a modification of the spared nerve mode of Decosterd and Woolf, as described in the discussion [12]. For experiments using peripheral application of GDNF, 1 week after injury, the nerve was re-exposed at the previous operative site. A pledget composed of chitosan bound to GDNF was placed in contact with the nerve and the wound was reclosed. Controls rats received only the chitosan pledget. After injury, the nerve was re-exposed at the previous operative site. A pledget composed of chitosan bound to GDNF was placed in contact with the nerve and the wound was reclosed. Controls rats received only the chitosan pledget. GDNF was covalently attached to chitosan through a chemical blending method. The GDNF blend ratio was 1:4 (GDNF : chitosan) by weight. Volumes were adjusted so that there was approximately 4 µg of GDNF per cm² of surface area. Withdrawal threshold was determined in all experiments using Von Frey hairs according to the up–down method of Dixon [13] as described by Chaplan et al. [14]. Intrathecal catheters were inserted according to the method of Pogatzki [15]. Placement was checked 1 day after insertion by injection of 5 µL of lidocaine 2%. Only those rats that developed transient paraplegia were used. Rats received either GDNF 125 µg dissolved in 5 µL of artificial CSF for three consecutive days or a similar amount of fluid devoid of GDNF. Dorsal root ganglia (DRG) were analyzed for somatostatin expression by real-time polymerase chain reaction (RT-PCR) 7 days after the last injection.

Dissected Cell Culture

DRG were dissociated and plated as described previously [4]. Medium was supplemented with 5% heat-inactivated rat serum. Neurons were seeded onto 12-well culture plates (24 mm/well) coated with poly D-lysine (100 µg/mL) and laminin (1 µg/cm²). Ganglia from one rat provided sufficient cells for one plate. When more plates were needed for an experiment, the DRG from two or more rats were pooled before dissociation and distributed into wells at the ratio of one rat per plate. Cultures were neuron-enriched by including of cytosine arabinoside within the culture medium. However, approximately 25% of cells present at a week after plating were Schwann cells or satellite glia.

RNA Isolation and Amplification, cDNA Synthesis and Quantitative RT-PCR

The program, “RealTime PCR,” was used for primer design. It is available at http://www. idtdna.com/Scitools/Applications/RealTimePCR. The primers were synthesized by Integrated DNA Technologies (Coralville, IA). To avoid amplification of contaminating genomic DNA and to assure transcript-specific amplification, exon-spanning primers were used. Primer pairs for rat prosomatostatin amplified a 141-bp fragment. The sequences are: 5′-AACCAGACAGAGAA CGATGCCCT-3′ (forward) and 5′-GTTCCT TT GCAGGCCAGC T7 T7 GCG T7 (reverse). The sequences for rat CGRP release of 206 bp in length. qRT-PCR experiments were performed on individual samples run in triplicate and was performed on Mx4000 instrument (Stratagene, La Jolla, CA). 0.2 µL of each RT product was diluted in 50 µL of PCR mix, containing 25 µL of Quantitect SYBR GREEN PCR (Qiagen, Valencia, CA), and 0.3 µM primers. Following a 15-minute denaturation at 95°C, 40 amplification cycles were performed. Each cycle included a 15-second dena-
turation at 94°C, a 30-second hybridization at 55°C and a 30-second elongation at 72°C. Fluorescence was measured on line after each cycle. After full amplification, the temperature was slowly raised above the melting temperature of the PCR product to establish the dissociation curve. Nonspecific amplification products such as primer dimers could be readily distinguished by their lower melting points. The presence of a single PCR product of the expected size was checked by electrophoresis. Negative controls without RT were also included. The amount of PCR product corresponding to a given mRNA was determined from the crossing point value and was expressed relative to the amount of GAPDH product used as a housekeeping gene for the same sample. Fold change values of mRNA levels represent the mean of six samples divided by the mean of six control samples, and each sample was run in triplicate.

**Peptide Extraction and Radioimmunoassay**

Somatostatin was extracted and assayed according to the method of Arimura [16] with minor modifications [17].

**Statistics**

Statistical analyses utilized a Student’s *t*-test for comparison of paired samples and one-way analysis of variance with Newman-Keuls posttest for comparison of multiple groups.

**Results**

Complete transection of the sciatic nerve abolishes sensation in the distal hind limb and results in severe reduction in levels of sensory neuropeptides [18]. By contrast, incomplete transection frequently leads to development of neuropathic pain and allodynia. In rats, this is manifested by a reduction in withdrawal threshold to various stimuli such as nylon filaments (Von Frey hairs), which, under normal circumstances, would elicit little response. Initially we employed the partial transection model of Lindenlaub and Sommer [19], but switched to a modification of the spared nerve model of Decosterd and Woolf [12] because it produced more consistent allodynia and provided an opportunity for a better control. Rats underwent section of the common peroneal and sural branches of that nerve, leaving the tibial branch intact. As a control, rats underwent section of the tibial and sural branches, leaving the common peroneal branch intact. Lesioned rats have a characteristic gait with an inverted foot, the result of damage to motor fibers within the nerve, but do not display any evidence of spontaneous discomfort. Cage behavior and grooming are normal. However, by as little as 24 hours, some rats have begun to show a reduction in withdrawal thresholds and by 4 days this behavior is well established (Figure 1). Thresholds are reduced by 60–70% in virtually every rat. Moreover, the reduction is prolonged, lasting for well over 4 weeks. Control rats do not display any change in withdrawal threshold.

To determine whether glial-derived factors could be used to modify this abnormal behavioral response, we exposed rats to GDNF. GDNF eliminates allodynic behavior in rats when it is infused continuously intrathecally. This, however, is not a practical therapeutic approach. Moreover, GDNF appears to provoke sufficient adverse effects when applied directly to the central nervous system in humans that it has been deemed unsuitable as treatment of neurodegenerative conditions such as Parkinson’s disease. To investigate whether other methods of application might prove useful, we bound GDNF within a bioengineered matrix of chitosan. One week after the nerve injury, when allodynia was firmly established and withdrawal thresholds had been reduced, we applied a piece of chitosan to injured nerve (Figure 2). When rats were tested 1 and 2 weeks later, withdrawal thresholds in GDNF-treated rats had returned to normal. By contrast, control rats that had not

**Figure 1** Spared tibial nerve injury reduces withdrawal threshold. After the determination of baseline thresholds, rats were subjected to left-sided peroneal and sural branch transections, leaving the tibial branch intact, or tibial and sural branch transections, leaving the peroneal branch intact. They were retested periodically thereafter. By convention, results are expressed as the 50% withdrawal threshold ± standard error of the mean. *P < 0.001 when compared with the spared peroneal injury.
received chitosan, and rats that had received chitosan without GDNF still had reduced withdrawal thresholds. It appears then that GDNF may prove to be a useful agent when applied peripherally, well after the time of injury.

What might be a potential mechanism underlying the anti-allodynic activity of GDNF? It has been reported that the factor promotes survival of the so-called nonpeptidergic, isolectin B4 (IB4)-binding population of nociceptive C-fiber neurons in the DRG [20]. A subpopulation of these neurons expresses the neuropeptide somatostatin. To investigate whether somatostatinergic mechanisms might play a role in reversing allodynia, rats were infused intrathecally for 3 days by bolus injection with the factor. Two weeks later, prosomatostatin RNA expression was determined by RT-PCR in the L4 and L5 lumbar DRG (Figure 3). Prosomatostatin levels were significantly increased over those of controls in ganglia treated with GDNF, an effect that was not seen for other peptides such as calcitonin gene-related peptide (CGRP).

We further investigated the action of GDNF by exposing DRG neurons directly to the factor in vitro. Neuron-enriched cultures of dissociated adult spinal sensory ganglia were grown for 5 days, after which cell content of somatostatin was measured. Exogenous GDNF or (NGF) was added 24 hours after plating (Figure 4). NGF had no effect on somatostatin content. By contrast, somatostatin was nearly threefold higher in GDNF-treated neurons.

Is the increase in somatostatin physiologically relevant? For the increase in neuronal somatosta-

**Figure 2** Glial cell line-derived neurotrophic factor (GDNF) reverses the effect of nerve injury on withdrawal threshold. Groups of rats were treated as in Figure 1. They were retested after 1 week, at which time two groups received chitosan implants placed in proximity to the injury nerve. One group of implants contained GDNF bound to chitosan. Threshold tested was repeated 1 and 2 weeks later. *P < 0.05 when compared with rats who received no implant or a plain chitosan implant.

**Figure 3** Intrathecal glial cell line-derived neurotrophic factor (GDNF) increase somatostatin expression. Groups of rats were implanted with lumbar intrathecal catheters. One group received artificial cerebrospinal fluid (CSF). A second group received GDNF 125 μg dissolved in the same volume of artificial CSF. After 2 weeks, expression of prosomatostatin and procalcitonin gene-related peptide (CGRP)α were determined by real-time polymerase chain reaction. Results are normalized to expression of GADPH, a housekeeping gene. *P < 0.01 when compared with control (artificial CSF).

**Figure 4** Glial cell line-derived neurotrophic factor (GDNF) increases somatostatin content of sensory neurons. Dissociated adult dorsal root ganglia neurons were grown for 5 days in the absence of exogenous factors or in the presence of GDNF (10 ng/mL) or nerve growth factor (NGF; 10 ng/mL). Peptide content is expressed as pg/well ± standard error of the mean. *P < 0.001 when compared with control.

Potassium-induced somatostatin release was then determined from both sets of cultures (Figure 5). Depolarization resulted in a multifold increase in somatostatin release over that from control cultures, which then returned to normal when the
stimulus was removed. Thus, increased somatostatin content leads to increased secretion of the peptide.

**Discussion**

Treatment of chronic pain remains a major failure of modern medicine. Although great advances have been made in providing relief from acute traumatic and postoperative pain, treatment of chronic, unremitting pain, whether from nerve injury or diabetic neuropathy, has met with relatively little success. In spite of expanding knowledge about neurotransmitters and neuromodulators that convey nociceptive information and the existence of several animal models that mimic the characteristics of human chronic pain syndromes, the pathophysiology underlying development of chronic pain after nerve injury has yet to be elucidated. We now demonstrate a potential novel method for treating pain after nerve injury by delivering the glial-derived factor, GDNF, to the site of the damage without having to invade the central nervous system. Moreover, our data suggest that GDNF may exert its effects through somatostatin as the peptide increases dramatically in response to stimulation.

As with most neuropeptides, the role of somatostatin in nociception is somewhat confusing. It is released from the dorsal spinal cord in response to noxious stimuli [21,22], and intrathecal injection of somatostatin can lead to nociceptive behavior [23,24]. However, there is a substantial body of data suggesting somatostatin is antinociceptive or analgesic. At doses lower than those used to induce nociceptive reaction, intrathecal somatostatin evokes no behavioral response [25] and may even raise the pain threshold [26]. As early as 1984, intrathecal or epidural somatostatin injections were used to provide analgesia from cancer or postoperative pain [27,28]. This effect has been mimicked by stable somatostatin analogues in humans [29,30], as well as in animals [31–34]. The analgesic role of somatostatin is consistent with its primary activity as an inhibitory peptide in the central nervous system [35,36]. It inhibits secretion of multiple transmitters and hormones, including the pronociceptive peptide, substance P [37–40]. To date, GDNF is the only neurotrophic factor that has been found to stimulate somatostatin expression. Previous studies have applied the factor to injured neurons in vivo and in vitro. Accordingly, it has not been possible to determine whether the stimulatory effect is due solely to improved survival or whether it enhances expression of the peptide. We have now demonstrated that intrathecal GDNF can also increase somatostatin expression in intact, uninjured DRG suggesting that its role may be regulatory rather than just trophic for sensory neurons, much as NGF is both trophic and regulatory for substance P and CGRP-expressing neurons. This presents the opportunity to use an endogenous analgesic peptide through therapeutic application of a naturally occurring, locally synthesized factor. Somatostatin expression was stimulated in our experiments by injecting GDNF intrathecally whereas the behavioral effect was demonstrated by the local application. We are currently investigating whether distal application of GDNF to injured nerves duplicates its stimulatory effect on the peptide. However, GDNF is transported proximally in a retrograde fashion in neurons so there is reason to expect that it will have a similar effect.

Somatostatin has a distribution that is relatively distinct from other neuropeptides. In the rat, there is virtually no colocalization with substance P or CGRP whereas in higher order mammals there is still only minor overlap [41]. It is contained in a subpopulation of small, C-fiber neurons in the DRG that stain with isolecitin-B4, which does not label most nociceptors. Neurons within this population do not express trkA as demonstrated by the
lack of response to NGF. They do, however, express GFRα1 and c-RET, a membrane-bound tyrosine kinase that transduces the effects of GDNF [42–47]. They do not appear to be segregated within the DRG separate within the dorsal horn of the spinal cord where they terminate in the inner layer of lamina II, as opposed to most nociceptors that terminate in lamina I and the outer layer of lamina II. Their specific function, as well as the neurotransmitters expressed by the somatostatin-negative populations remains undetermined. However, if the action of somatostatin is prototypical, they may also play an antinociceptive, modulatory role.

Increased neuronal content of somatostatin is reflected by a small increase in basal release but a marked increase in stimulated release, suggesting the possibility of a physiological effect after treatment. It was necessary to depolarize neuronal cultures with KCl to achieve release as nothing is known about the receptors expressed on somatostatinergic neurons. They appear not to express TRPV1 receptors as capsaicin exposure does not induce somatostatin release as it does for substance P and CGRP (data not shown).

We previously reported that GDNF, a member of the transforming growth factor-β superfamily, regulates somatostatin in adult sensory neurons in vitro [17]. Complete sciatic nerve section depletes GDNF from the dorsal horn of the spinal cord [48] and increases expression of GFRα1, in DRG neurons [44]. Sciatic nerve section also decreases expression of somatostatin in DRG neurons [49], which is reversed by intrathecal application of GDNF [20]. Study of expression of GDNF in models of neuropathic pain has yielded somewhat contradictory results. Thus, Nagano et al. [50] report decreased GDNF in lumbar DRG in the chronic constriction injury model. However, Dong et al. report an increase in both GDNF and GFR-α1 in the same model [51]. Interestingly, Dong et al. also report that down-regulation of GFR-α1 by intrathecal injection of an antisense oligonucleotide to the receptor increases alldynia at the same time that it decreased expression of somatostatin. Boucher et al. [11] have demonstrated that intrathecal GDNF can reverse the alldynia induced by a number of methods suggesting a novel potential therapeutic option for treatment of chronic pain. Moreover, Hao et al. [52] have reported that peripheral injection of a replication-deficient herpes viral vector that can express GDNF decreases alldynia in the spinal nerve transaction model of chronic pain.

Although both of these observations indicate that GDNF might prove useful for treating neuropathic pain, neither presents a practical method of delivery. Our observation that application of GDNF to an injured nerve suggests just such a method. By inserting a reservoir that slowly releases the factor in proximity to the site of injury later complications could be avoided. Much further work is necessary to elucidate how GDNF provides analgesia. However, its action on somatostatinergic neurons suggests one potential mechanism.

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Disclosures

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References


