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Growth Cone Collapsing Effect of Lidocaine on DRG Neurons Is Partially Reversed by Several Neurotrophic Factors

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Background: **Local anesthetics were suggested to have a potential for neurotoxicity in both clinical reports and laboratory experiments. Growing neurons have been shown to be susceptible to the toxic effects of local anesthetics in culture. These findings have generated the interest in factors that would rescue the neurons affected by the neurotoxicity of local anesthetics.**

Methods: **Primary cultured dorsal root ganglia were isolated from age-matched chick embryos and exposed to lidocaine. After 60 min of exposure, the culture media were replaced to wash out the lidocaine. Neurotrophic factors (NTFs)—brain-derived neurotrophic factor, glial-derived neurotrophic factor, or neurotrophin 3—were added to the replacement media to examine the capacity of these NTFs to support the reversibility of the lidocaine-induced growth cone collapse. The growth cone collapse assay was applied a quantitative method of assessment.**

Results: **When any of the three NTFs was added to the replacement media at a minimum concentration of 10 ng/ml, significantly high reversibility of the lidocaine-induced growth cone collapse was observed, especially at 48 h after washout ($P < 0.05$). At that time point, there was no significant difference between the values of growth cone collapse percentage in the cells that were exposed to lidocaine and supported by the NTFs after the washout, and the control cells (not exposed to lidocaine) ($P > 0.05$).**

Conclusion: **The NTFs—brain-derived neurotrophic factor, glial-derived neurotrophic factor, and neurotrophin 3—were demonstrated to support the reversibility of lidocaine-induced growth cone collapse in primary cultured sensory neurons, an effect that was concentration- and time-dependent. Because similar effects were observed after tetracaine washout, the supporting effects of NTFs may not be specific to lidocaine.**

GROWING neurons have been shown to be susceptible to the potential toxic effects of local anesthetics in culture. Short-term exposure to local anesthetics produced irreversible changes in growing sensory neurons and the growth cone was the area most quickly affected as shown in our previous study.[1,2] Since local anesthetics are sometimes applied to sites where peripheral nerves may be growing or regenerating after injury, *e.g.*, after exposure to chemical injury, mechanical injury, or neurodegenerative disease, their effects on growing neurons are not to be ignored in the clinical practice. This has generated the interest in factors that would rescue the neurons affected by the potential neurotoxicity of local anesthetics.

Neurotrophic factors (NTFs) are a family of proteins that have a broad spectrum of biologic functions in several tissues, but their effects are best studied in the developing nervous system where they help neutralization of the naturally occurring neuronal cell death program.[3] The observations that different populations of sensory neurons are located in the same embryonic ganglia and have neurotrophic dependence on different neurotrophins have led to the idea that neurotrophic factors act as cues in the navigation of growing axons.[4] Recently, it has become apparent that neurotrophic factors can also protect neurons against excitotoxic, ischemic, and oxidative insults.[5,6] The role of NTFs in supporting the growth of developing neurons exposed to the potential toxic effects of local anesthetics has never been studied.

The objective of the present work was to examine the possibility that NTFs can reverse the local anesthetic–induced effects on growing sensory neurons. For this study, we examined the morphological changes induced in the growing primary sensory neurons after exposure to the local anesthetic, lidocaine, and the reversibility of these changes after washing out the local anesthetic-containing media. To test the effects of neurotrophic factors on the reversibility of these changes, different NTFs were added to the culture media after the washout. We have compared the effects of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and neurotrophin 3 (NT-3). For these experiments, embryonic chick embryo dorsal root ganglia (DRGs) were isolated for primary explant culture, and the growth cone collapse assay was used as a quantitative method for assessment. The growth cone collapse assay is the established quantifying method of examining the effects of substances on developing neurites.[7]

Methods

With Institutional Animal Care Committee approval (Gunma, Japan), DRGs were isolated from lumbar paravertebral sites of chick embryos at the seventh embryonal day. DRGs were plated on laminin-coated coverslips

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and cultured in F-12 medium supplemented as in the method of Bottenstein,[8] containing 100 μg/ml bovine pituitary extract, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml mouse 7S NGF. Cultures were maintained at 37°C and at 5% carbon dioxide. After 20 h in culture, lidocaine hydrochloride (Sigma Co. Ltd., St. Louis, MO) in prewarmed fresh culture media was gently added to the culture media. The volume of the added lidocaine solution was 1/100 of the total volume of the culture media to produce a final concentration of 4 mM.

NGF 7S, human recombinant BDNF, and human recombinant GDNF were purchased from CibcoBRL (Rockville, MD). Human recombinant NT-3 was purchased from Sigma Co. Ltd. (St. Louis, MO). To examine the washout effect with the different NTFs, the tissues were kept in the incubator for 60 min after the addition of lidocaine that produced 85–90% growth cone collapse. Then, the media were gently replaced twice with the fresh prewarmed media that was free from the local anesthetic, and NGF was not added to this replacement media in order to investigate the effect of each of the other NTFs individually. In time course studies, the tissues were kept in the incubator a further 48 h after the exchange of the media. The replacement media either contained no NTF, or one of the three NTFs (BDNF, GDNF, or NT-3) was added to the replacement media, each in a separate experiment. All NTFs were tested at concentrations of 1, 5, 10, and 20 ng/ml. A negative control was included in every experiment, in which the media was exchanged though these cells were not exposed to the local anesthetic, to detect any time effect during the experiments or mechanical disturbances potentially associated with the washout.

The tissues were fixed with 4% paraformaldehyde in PBS, pH 7.4, containing 10% sucrose as described previously,[9] and viewed with a 40× phase objective using a phase-contrast microscope (Axiovert; Zeiss, Germany).

Growth cones at the periphery of the explants were scored for the growth cone collapse assay, providing that they were not in contact or close proximity to the other growth cones or neurite. The microscope stage was moved manually. One hundred growth cones were randomly chosen and viewed on a coverslip for scoring. The assessments were done by an assessor who was blinded to the experimental conditions. A dissociated assay was applied such that the chosen regions were marked and repeatedly assessed at each time point throughout the experiments in order to differentiate a true reversibility from *de novo* neurite growth. Growth cones without filopodia or lamellipodia were counted as collapsed.[7]

Statistical Analyses

Data are presented as mean and SD of six independent measurements. One-way analysis of variance for repeated measurements was used to determine statistically significant differences between the curves of growth cone collapse. Each result of the growth cone collapse assays was statistically analyzed by two-way analysis of variance with the Scheffé method using Stat View 5.0 (SAS Institute Inc., Cary, NC).

Results

Growth cones with lamellipodia and filopodia were observed at the leading edges of most neurites after 20 h in culture. Exposure to 4 mM lidocaine for 60 min induced 87.1 ± 1.9% growth cone collapse (fig. 1α). The growth cone collapse percentage was assessed after the washout of the lidocaine-containing media. For all NTFs, at 6 h after washout, the number of growth cones with filopodia or lamellipodia was significantly higher than that observed before the washout at all concentrations and not different from the preexposure values ($P < 0.05$). There were no statistically significant differences between growth cone collapse values at 6 h after washout and those obtained at 24 h after the washout ($P > 0.05$). However, the number of growth cones with filopodia or lamellipodia at 48 h after washout was significantly decreased at all concentrations. However, the number of these growth cones was significantly higher than the prewash values at 10- and 20-ng/ml NTFs ($P < 0.05$). When the media was exchanged after 60 min exposure without the application of any NTF, the growth of the neurites was much diminished or even stopped, blebs were formed at the leading edges and alongside the neurites, and the neurites' shafts were narrowed and ultimately destroyed (fig. 1β). On the other hand, with the support of NTFs, the neurites showed greater outgrowth with intact shafts and healthy growth cones (fig. 1α). In the absence of NTFs, the number of growth cones with filopodia or lamellipodia was significantly lower than the preexposure values at all time points ($P < 0.05$), and was not statistically different from the prewash values at 48 h after the washout ($P > 0.05$; fig. 2).

When the concentration–response was studied 48-h after the washout, the number of growth cones with filopodia or lamellipodia observed after the application of either 10 or 20 ng/ml was significantly higher than those measured after the washout using 1 or 5 ng/ml of either the BDNF or the GDNF ($P < 0.05$). For the NT-3, there was no statistically significant difference between the 5- and 10-ng/ml concentrations ($P > 0.05$), but values of growth cone collapse with 20 ng/ml NT-3 were significantly lower than those with the 5-ng/ml concentration ($P < 0.05$; fig. 3). The number of growth cones with filopodia or lamellipodia scored after the washout using 10- or 20-ng/ml concentration were not statistically different from the control values (cells not exposed to lidocaine; figs. 2 and 3A).

Studying the concentration–response of NTFs 48 h after the washout of tetracaine, a significantly higher

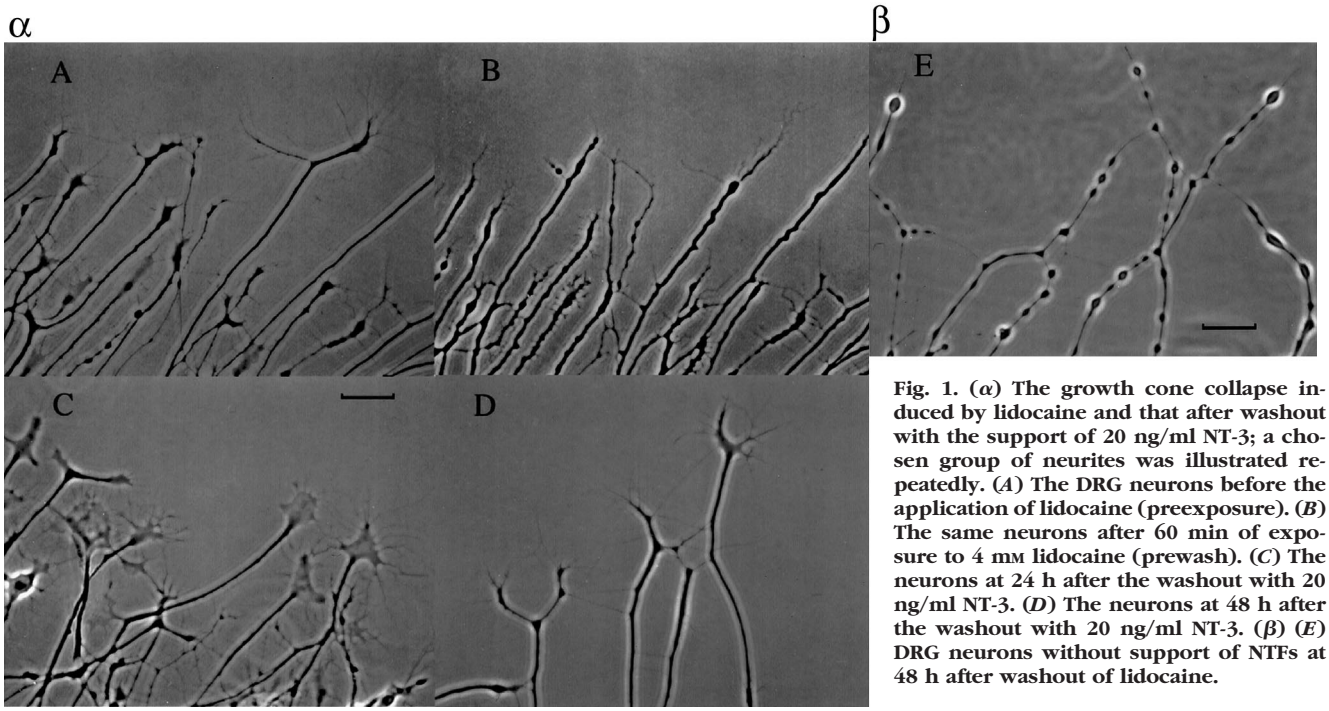

Fig. 1. (*α*) The growth cone collapse induced by lidocaine and that after washout with the support of 20 ng/ml NT-3; a chosen group of neurites was illustrated repeatedly. (*A*) The DRG neurons before the application of lidocaine (preexposure). (*B*) The same neurons after 60 min of exposure to 4 mM lidocaine (prewash). (*C*) The neurons at 24 h after the washout with 20 ng/ml NT-3. (*D*) The neurons at 48 h after the washout with 20 ng/ml NT-3. (*β*) (*E*) DRG neurons without support of NTFs at 48 h after washout of lidocaine.

number of growth cones with filopodia or lamellipodia was obtained after with 10 or 20 ng/ml than those with 1 or 5 ng/ml of either the BDNF or the NT-3 ($P < 0.05$). There was no statistically significant difference between 5 and 10 ng/ml of the GDNF ($P > 0.05$), but values of growth cone collapse with 20 ng/ml GDNF were significantly lower than those with the 5-ng/ml concentration ($P < 0.05$; fig. 3B). Also, the number of growth cones with filopodia or lamellipodia scored after the tetracaine washout using 10- or 20-ng/ml concentration were not statistically different from the control values (cells not exposed to tetracaine).

Discussion

We previously showed that exposure to lidocaine induced growth cone collapse in developing neurons.[2] A general destruction of growth cones in growing or regenerating nervous tissues by externally applied substances, like that induced by local anesthetics, could lead to the disturbance of the normal establishment of cytoarchitecture in the developing nervous system.[9–11] When lidocaine was applied to the embryonic DRGs for 60 min, most of their dendrites showed growth cone collapse. Significant reversibility of this collapse was observed during the first 24 h after the washout, when the cells where no longer exposed to the collapsing effect of lidocaine, even without the application of any NTF. However, this reversibility was significantly dropped in the next 24 h; the growth cone collapse values scored at 48 h after the washout exceeded the prewash values when the media were not supported by the NTFs. On the other hand, when the replacement media were supported with NTFs at 10- or 20-ng/ml concentrations, the collapsing activity was significantly low that growth cone collapse values showed no statistically significant differences in comparison with the control cells at that time point. As similar positive effects of the NTFs were observed after the washout of tetracaine, these effects may not be specific to lidocaine. From these results, we suggest that these three NTFs can support the reversibility of the local anesthetic–induced growth cone collapse in the cultured growing neurons and this effect is concentration-dependent.

It is suggested that the cellular processes involved in local anesthetic–induced neuronal toxicity are initiated by exposure and proceed further even after stoppage of this exposure. Although the role of NTFs in protecting neurons against the local anesthetics neurotoxicity has never been studied, it has been proposed that neurotrophic factors may protect neurons from excitotoxic/metabolic insults. For example, both BDNF and GDNF protected embryonic chick spinal cord motor neurons from ethanol neurotoxicity[12] and protected against motor neuron death following axotomy in rats.[13] GDNF promoted the recovery of dopamine neurons damaged by 6-hydroxydopamine[14] and reduced apoptosis in human embryonic dopaminergic neurons *in vitro*.[15] BDNF protected cultured hippocampal neurons from ethanol as well as ethanol combined with hypoxic conditions.[16] In addition, NT-3 and BDNF protected CNS neurons against metabolic/excitotoxic insults[17] and promoted survival of cultured vestibular ganglion neurons and protected them against neurotoxicity of ototoxins.[18]

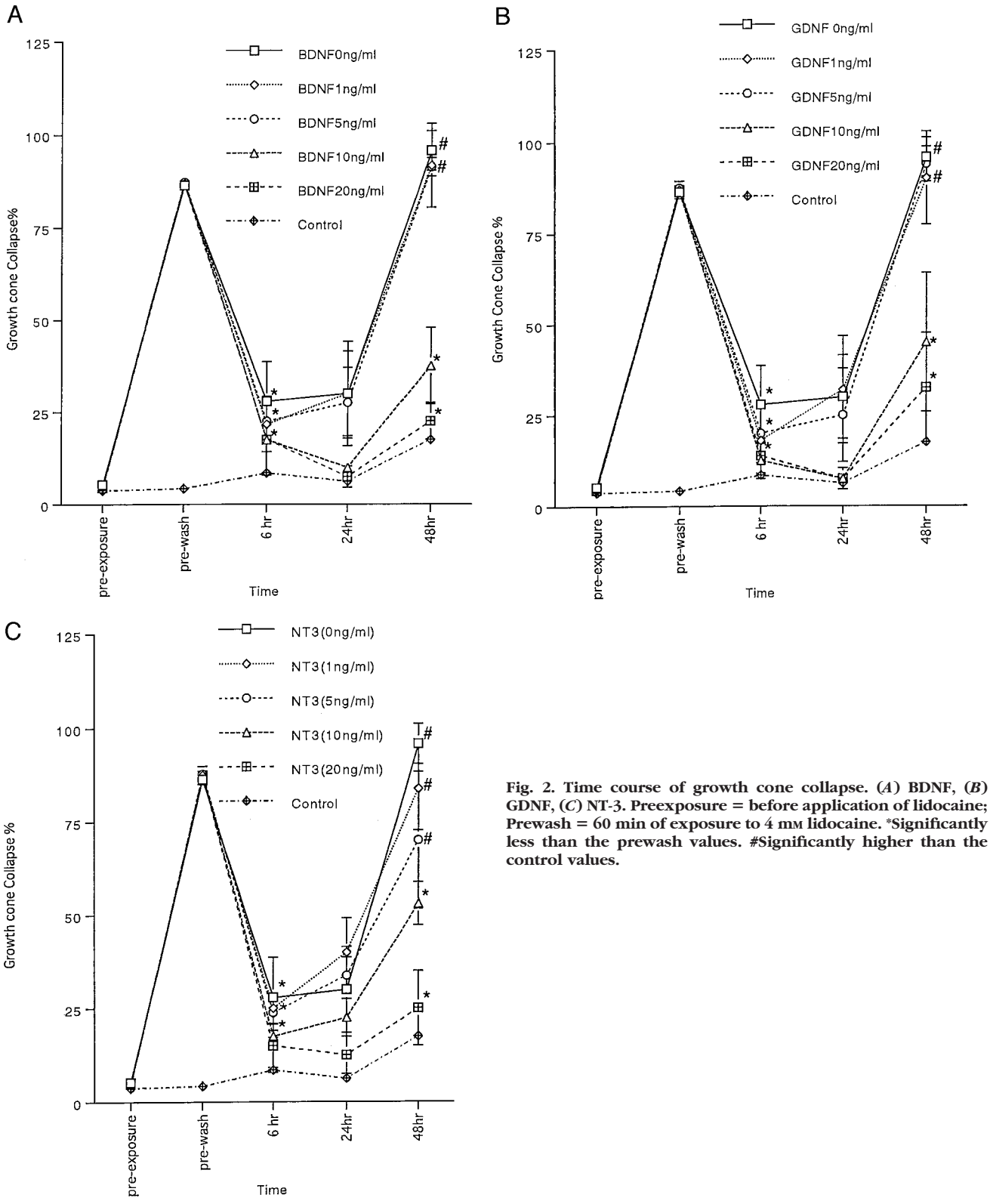

Fig. 2. Time course of growth cone collapse. (*A*) BDNF, (*B*) GDNF, (*C*) NT-3. Preexposure = before application of lidocaine; Prewash = 60 min of exposure to 4 mM lidocaine. *Significantly less than the prewash values. #Significantly higher than the control values.

The three NTFs—BDNF, GDNF, and NT-3—almost equally supported the reversibility of growth cone collapse induced by the local anesthetic exposure. However, it is difficult to assess the comparative effectiveness of these factors depending on one parameter, the growth cone collapse.

Neurotrophic factors are known to bind and activate receptor tyrosine kinase (trk), and their interaction is

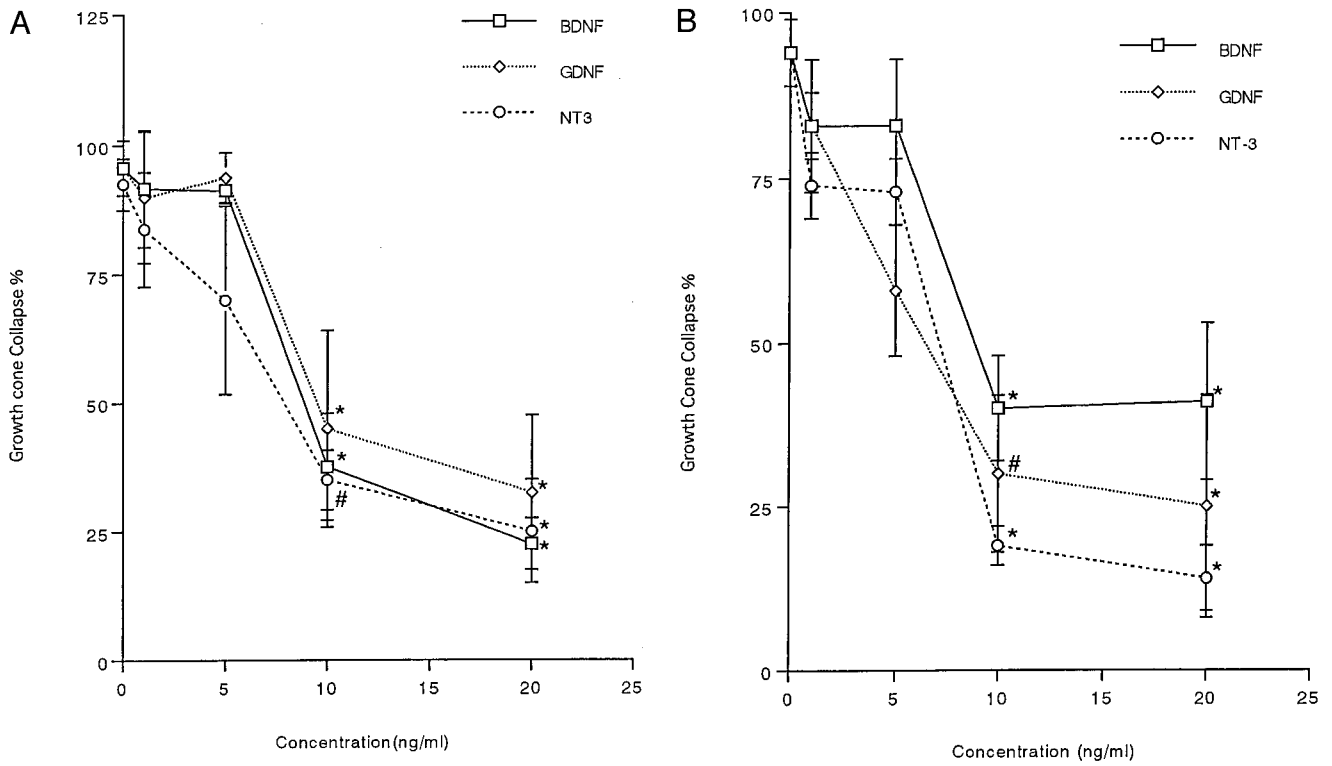

Fig. 3. Concentration–response for the three NTFs at 48 h after the washout of lidocaine (*A*), tetracaine (*B*). *Significantly different from growth cone collapse values with 0, 1, or 5 ng/ml. #Significantly different from growth cone collapse values with 0 or 1 ng/ml.

crucial for the internalization of neurotrophins within the neuronal terminal.[19] Of the trk receptor family, trk B is the signal transducing receptor of BDNF,[20] while NT-3 binds preferentially to trk C.[21] All trk receptors are distributed to discrete but partially overlapping subpopulations of primary sensory neurons.[22,23] Consistently, the target tissue innervated by the afferents of the different trk neurons express mRNA for the relevant neurotrophin, and this expression starts in the embryonic life.[24,25] Primary sensory neurons have been also found to express Ret mRNA, the signal transduction component of the receptor for the GDNF, which also has a trophic effect on dorsal root ganglion cells.[26,27] The RET is the product of the c-ret proto-oncogene, an orphan receptor tyrosine kinase.[28]

A number of mechanisms and events resulting from local anesthetic exposure have been postulated to be involved in the subsequent neurotoxicity.[29] Increase in intracellular Ca^{2+} ions has been reported as an underlying mechanism of lidocaine-induced toxicity.[30] The increase in intracellular Ca^{2+} ions as short as 5 min may be sufficient to induce delayed neuronal death.[31] It has been proposed that NTFs may protect neurons by enhancement of calcium hemostatic mechanisms reducing the elevation of Ca^{2+} ions.[5,32] These reports may provide a mechanistic explanation for the "positive" role of NTFs in the reversibility of local anesthetic induced neurotoxicity observed in this study. However, future studies which focus on the mechanism of this effect may yield important information about the possibility of rescuing neurons from the local anesthetic–induced neurotoxicity.

In previous studies, we demonstrated that increasing the concentration of the nerve growth factor (NGF) up to 100 ng/ml in the culture media did not influence the growth cone collapsing activity after the washout of the local anesthetic–containing media.[1,2] Although (trkA), the receptors responsible for the signal transduction of NGF, were demonstrated to be expressed in DRGs,[22–24] they may not necessarily have the same function. Mattson *et al.*[33] showed that NGF was less effective than BDNF in suppressing Ca^{2+} ions responses to glutamate. Hory-Lee *et al.*[34] showed that NT-3 is capable of supporting survival and neurite outgrowth of muscle sensory neurons from developing chicken lumbar DRGs better than NGF. However, it is difficult to compare these studies due to the numerous differences in the culture and exposure conditions and quantification.

Although the results of this *in vitro* study could not be directly applied in clinical settings, the positive role of NTFs in supporting the reversibility of the local anesthetic–induced changes in growing neurons should be considered, and further studies investigating this role *in vivo* would be beneficial.

In conclusion, the NTFs—BDNF, GDNF, and NT-3—were demonstrated to support the reversibility of lidocaine-induced growth cone collapse in primary cultured sensory neurons, an effect that was concentration- and time-dependent.

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