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Direct Neurotoxicity of Tetracaine on Growth Cones and Neurites of Growing Neurons In Vitro

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Background: **Local anesthetics have direct neurotoxicity on neurons. However, precise morphologic changes induced by the direct application of local anesthetics to neurons have not yet been fully understood. Also, despite the fact that local anesthetics are sometimes applied to the sites where peripheral nerves may be regenerating after injury, the effects of local anesthetics on growing or regenerating neurons have never been studied.**

Methods: **Three different neuronal tissues (dorsal root ganglion, retinal ganglion cell layer, and sympathetic ganglion chain) were isolated from an age-matched chick embryo and cultured for 20 h. Effects of tetracaine were examined microscopically and by a quantitative morphologic assay, growth cone collapse assay.**

Results: **Tetracaine induced growth cone collapse and neurite destruction. Three neuronal tissues showed significantly different dose–response, both at 60 min and at 24 h after the application of tetracaine ($P < 0.01$). The ED_{50} values (mean ± SD) at 60 min were 1.53 ± 1.05 mM in dorsal root ganglion, 0.15 ± 0.05 mM in retinal, and 0.06 ± 0.02 mM in sympathetic ganglion chain cultures. The ED_{50} values at 24 h were 0.43 ± 0.15 mM in dorsal root ganglion, 0.07 ± 0.03 mM in retinal, and 0.02 ± 0.01 mM in sympathetic ganglion chain cultures. Concentration of nerve growth factor in the culture media did not influence the ED_{50} values. The growth cone collapsing effect was partially reversible in dorsal root ganglion and retinal neurons. However, in the sympathetic ganglion culture, no reversibility was observed after exposure to 1 mM tetracaine for 10 or for 60 min. Bupivacaine had similar neurotoxicity to the three types of growing neurons. (The ED_{50} values at 60 min were 2.32 ± 0.50 mM in dorsal root ganglion, 0.96 ± 0.16 mM in retinal, and 0.18 ± 0.05 mM in sympathetic ganglion chain cultures. The ED_{50} values at 24 h were 0.34 ± 0.09 mM in dorsal root ganglion, 0.21 ± 0.06 mM in retinal, and 0.45 ± 0.10 mM in sympathetic ganglion chain cultures.)**

Conclusions: **Short-term exposure to tetracaine produced irreversible changes in growing neurons. Growth cones were quickly affected, and neurites degenerated subsequently. Sensitivity varied with neuronal type and was not influenced by the concentration of nerve growth factor. Because a similar phenomenon was observed after exposure to bupivacaine, the toxicity to growing neurons may not be unique to tetracaine.**

LOCAL anesthetics are widely used in clinical settings to interrupt neuronal conduction reversibly by blocking the sodium channels on neurite membrane. However, both clinical experiences and laboratory examinations have raised the possibility that local anesthetics have permanent neurotoxic effects when applied to peripheral neurons at a high concentration or for long duration.[1-3] This neurotoxicity could be a factor in an undesirable side effect of continuous subdural nerve block.[4,5] In other clinical settings, this toxicity has been used to bring about semipermanent nerve blocks in the periphery of patients with chronic pain.[6]

Several *in vivo* studies have demonstrated that local circulation around neurites is affected by highly concentrated local anesthetics and that the resultant poor perfusion to neurites induces neuronal destruction.[3,7] However, other *in vivo* and *in vitro* studies have demonstrated that local anesthetics have a direct neurotoxicity. Recently, Takenami *et al.*[8] showed that tetracaine most often induced neuronal destruction at sites where glial sheaths were interrupted, and suggested that neurotoxicity could be derived from direct toxicity to neurites. In one *in vitro* study, Gold *et al.*[9] demonstrated that lidocaine had direct neurotoxicity when applied to primary neurons isolated from murine ganglions.

Although previous studies implied that local anesthetics have direct neurotoxicity to neurons, precise morphologic changes induced by the direct application of local anesthetics to neurons have not yet been fully understood. Also, despite the fact that local anesthetics are sometimes applied to the sites where peripheral nerves may be regenerating after injury, the effects of local anesthetics on growing or regenerating neurons have never been studied. In the present study, we examined morphologic changes of growing neurons exposed to a local anesthetic, tetracaine. For quantitative assessment, we used a growth cone collapse assay that has been established as a method of examining the biologic effects of repellent molecules and toxic substances.[10,11] To compare the sensitivities of neuronal subtypes, three different neuronal tissues (dorsal root ganglion [DRG], retinal ganglion cell layer, and sympathetic ganglion chain) were isolated for primary explant culture. To isolate the neuronal tissues from an age-matched embryo and culture them under identical conditions for the collapse assay, avian embryo was used as the tissue source. Vigorously regenerating avian primary neurons are often used for cell biologic assessment of biologic and toxic substances.[12] Also, to examine whether the effect of tetracaine is unique to the drug or common among local anesthetics, the ED_{50} values of tetracaine were compared with those of bupivacaine.

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Materials and Methods

After approval by the Institutional Animal Care Committee, chick neural tissues were isolated from day-7 embryos. As a preparation of peripheral neurons, dorsal root ganglia were dissected from lumbar paravertebral sites. As a preparation of central neurons, temporal halves of retinas were prepared from bilateral eyeballs and cut into sections of approximately 0.5 mm^2. Sympathetic ganglia were dissected from lumbar sympathetic chains. After cutting off the original neurites, the tissues were plated to laminin-coated coverslips and cultured in F-12 medium supplemented as in the method of Bottenstein *et al.*,[13] containing 100 μg/ml bovine pituitary extract, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml mouse 7S nerve growth factor (NGF). In the experiments in which effect of NGF was examined, concentration of NGF was increased to 40 or 60 ng/ml. Cultures were maintained at 37°C and at 5% CO_2.

Tetracaine hydrochloride was purchased from Sigma Company Ltd., St. Louis, MO). Tetracaine was prepared in prewarmed, fresh culture media and was gently added to the culture media after 20 h in culture. The volume of the added tetracaine solution was 1/100 of the total volume of the culture media. Dose–responses of the cultured neurons were examined after 60 min and after 24 h of exposure to tetracaine (0.01, 0.03, 0.10, 0.32, and 1.00 mM). Cell viability was examined by exposing the cells to the vehicle solution for the identical durations. In time-course studies, the tissues were kept in an incubator for 98 h after the addition of tetracaine. In the experiment in which effect of washout was examined, the tissues were kept in the incubator for 10 or 60 min after the addition of tetracaine; then the media were gently replaced twice with the fresh, prewarmed media that did not contain tetracaine.

The tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, containing 10% sucrose, as described previously,[14,15] and viewed with a 40× phase objective using a phase-contrast microscope (Axiovert; Zeiss, Germany). Growth cones at the periphery of explants were scored for collapse assay, if they were not in contact or close proximity to other growth cones or neurite. Fifty growth cones were viewed and scored on a coverslip. Growth cones without filopodia and lamellipodia were counted as collapsed.[10] The counting was performed by a trained assayist who was blinded to the experimental protocol.

The ED_{50} values in the growth cone collapse assays were determined by fitting the data to the following equation:

$$Y = 100 \times X/(X + ED_{50}) \quad (1)$$

In the formula, Y represents a growth cone collapse percentage induced by tetracaine and X represents the corresponding dose of tetracaine. To examine the ED_{50} value of another anesthetic, the identical ED_{50} determination was conducted with bupivacaine (Sigma Company Ltd.).

The data are expressed as the mean ± SD of six independent measurements. The dose–response curve of growth cone collapse, time-course, and washout effect study were analyzed by one-way analysis of variance for repeated measurements. Each result of the growth cone collapse assays was statistically analyzed by two-way analysis of variance with the Scheffé test using StatView 5.0 software (Abacus Coop., Berkeley, CA). *P* values less than 0.05 were considered significant.

Results

Morphologic Observations

Most of the intact neurites had growth cones with lamellipodia and filopodia at their leading edges before the application of tetracaine. However, the shapes of the growth cones of the three different neurons examined were not exactly the same, a finding consistent with the results of previous studies.[15,16] Generally, the area of lamellipodia was larger in DRG neurons than in retinal and sympathetic neurons. With the application of tetracaine to the culture media, filopodia of growth cones retracted and lamellipodia were diminished (growth cone collapse; fig. 1). In some instances, blebs were formed at growth cones and alongside the neurites. After growth cone collapse, neurite shafts narrowed and ultimately were destroyed. After the destruction of the neurites, the clusters of cell bodies lost their force of adhesion to the culture plate and detached from the bottom of the well. Although the time courses were different for each of the three neuronal types examined, the sequences of the morphologic changes were all similar.

Dose–Response Study of Tetracaine

Three neuronal tissues showed significantly different dose–response, both at 60 min and at 24 h of exposure to tetracaine (fig. 2; $P < 0.01$). Statistically significant growth cone collapse was observed when tetracaine concentration exceeded 0.1 mM in DRG, 0.03 mM in retinal, and 0.01 mM in sympathetic ganglion cultures at 60 min after application. When the assay was performed at 24 h after tetracaine application, the minimum concentrations of tetracaine that were necessary to induce statistically significant growth cone collapse were 0.03 mM in DRG, 0.03 mM in retinal, and 0.01 mM in sympathetic ganglion cultures. The ED_{50} values at 60 min were 1.53 ± 1.05 in DRG, 0.15 ± 0.05 in retinal, and 0.06 ± 0.02 mM in sympathetic ganglion cultures. The ED_{50} values at 24 h were 0.42 ± 0.15 mM in DRG, 0.07 ± 0.03 mM in retinal, and 0.02 ± 0.01 mM in sympathetic ganglion cultures. Both at 60 min and at

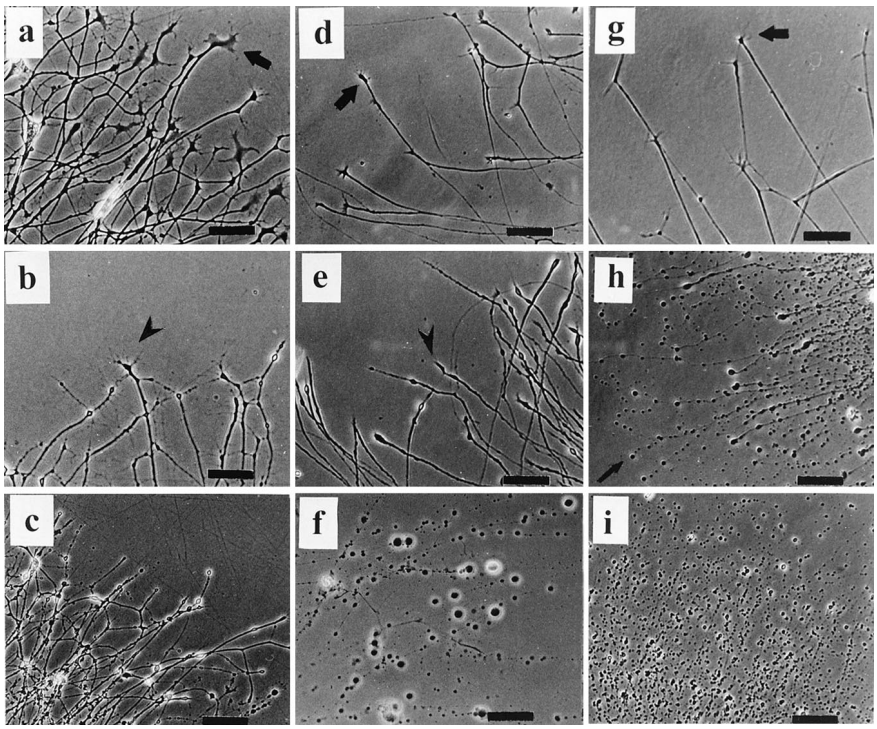

Fig. 1. Typical growth cone collapse and neurite retraction induced by 1 mM tetracaine. (*A–C*) Dorsal root ganglion neurons, (*D–F*) retinal ganglion cell layer neurons, and (*G–I*) lumbar sympathetic chain neurons. (*A*, *D*, and *G*) Neurons cultured for 20 h, immediately before exposure to tetracaine; bold arrows indicate intact growth cones. (*B* and *E*) Arrowheads indicate collapsed growth cones. (*H*) An arrow indicates a retracted and shrinking neurite of a sympathetic ganglion neuron (60 min after exposure to 1 mM tetracaine). (*C*, *F*, and *I*) Photographs were taken 24 h after the exposure. Size bars in *A–C* and *D–G* = 30 μm; bars in *H* and *I* = 60 μm.

24 h after the application of tetracaine, ED_{50} values were significantly different for each of the three neuronal tissue types. Concentration of NGF in the culture media did not influence the ED_{50} values (table 1).

Time Course of Growth Cone Collapse

Growth cone collapse percentage was assessed after the application of 0.1 and 1 mM tetracaine. In the DRG and retinal cultures, the time courses after the 0.1-mM application and the 1-mM application were significantly different ($P < 0.01$). In the sympathetic ganglion culture, there was no significant difference between the growth cone collapse time course after the 0.1-mM application and after the 1-mM application. In DRG culture, growth cone collapse percentage was significantly increased at 30 min after the application of 1 mM tetracaine

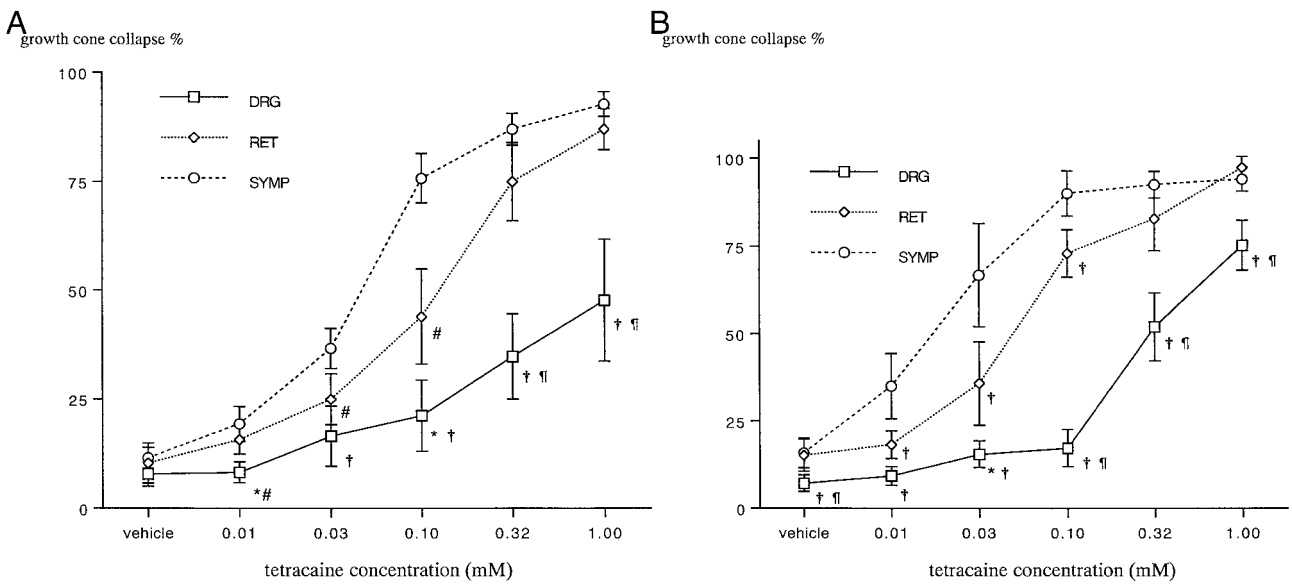

Fig. 2. Dose–response relations of tetracaine-induced growth cone collapse. Growth cone collapse percentage was examined at 60 min after tetracaine exposure (*A*) and at 24 h after the exposure (*B*). One-way analysis of variance for repeated measurements indicated that three dose–response curves at each time point were significantly different from each other ($P < 0.01$). *¶Significantly different from the corresponding value of retinal neurons (*$P < 0.05$, ¶$P < 0.01$). #†Significantly different from the corresponding value of sympathetic neurons (#$P < 0.05$, †$P < 0.01$). Values are mean ± SD. DRG = dorsal root ganglion neurons; RET = retinal ganglion cell layer neurons; SYMP = lumbar sympathetic chain neurons.

Table 1. ED_{50} (mM) Values of Growth Cone Collapsing Effects of Tetracaine

	DRG Neurons	Retinal Neurons	Sympathetic Neurons
60 min after exposure			
20 ng/ml NGF	1.53 ± 1.05	0.15 ± 0.05	0.06 ± 0.02
40 ng/ml NGF	1.58 ± 1.00	0.13 ± 0.07	0.05 ± 0.04
60 ng/ml NGF	1.26 ± 0.79	0.20 ± 0.13	0.08 ± 0.05
24 h after exposure			
20 ng/ml NGF	0.43 ± 0.15	0.07 ± 0.03	0.02 ± 0.01
40 ng/ml NGF	0.63 ± 0.32	0.10 ± 0.05	0.03 ± 0.02
60 ng/ml NGF	0.79 ± 0.40	0.10 ± 0.06	0.03 ± 0.02

At 60 min and 24 h after the application of tetracaine, ED_{50} values were significantly different among three neuronal tissue types ($P < 0.05$). The concentration of nerve growth factor (NGF) in culture media did not influence the ED_{50} values. Values are mean ± SD.

DRG = dorsal root ganglia.

and at 24 h after the application of 0.1 mM tetracaine. In the retinal and sympathetic ganglion cultures, the growth cone collapse percentage was significantly increased at 10 min both after the application of 0.1 mM and the application of 1 mM tetracaine (fig. 3).

Reversibility of Growth Cone Collapse

In the DRG culture, time course of growth cone collapse after 60-min exposure to 1 mM tetracaine was significantly different from that observed after the 60-min exposure to vehicle solution and the 10-min exposure to 1 mM tetracaine. Partial reversibility (reduction of growth cone collapse percentage from the maximum value of 51.8 ± 10.9%, immediately after the exposure, to 26.8 ± 5.1% at 48 h after the exposure) was observed after the 60-min exposure. Ten-minute exposure to 1 mM tetracaine did not induce significant growth cone collapse in the DRG culture, immediately after the application. However, at 98 h after the 10-min exposure to tetracaine, significant growth cone collapse was observed, despite the fact that tetracaine had been washed out from the culture media (fig. 4A).

Ten-minute exposure to 1 mM tetracaine induced significant growth cone collapse in retinal neurons immediately after exposure. When tetracaine was not present in the culture media, collapsing activity ceased and the growth cone collapse percentages at 12–72 h after the 10-min exposure were not different from those observed after 10-min exposure to vehicle solution. However, at 98 h after the 10-min exposure to tetracaine, significant growth cone collapse was observed. No reversibility was observed after the exposure to 1 mM tetracaine for 60 min (fig. 4B).

In the sympathetic ganglion culture, no reversibility was observed, both after exposure to 1 mM tetracaine for

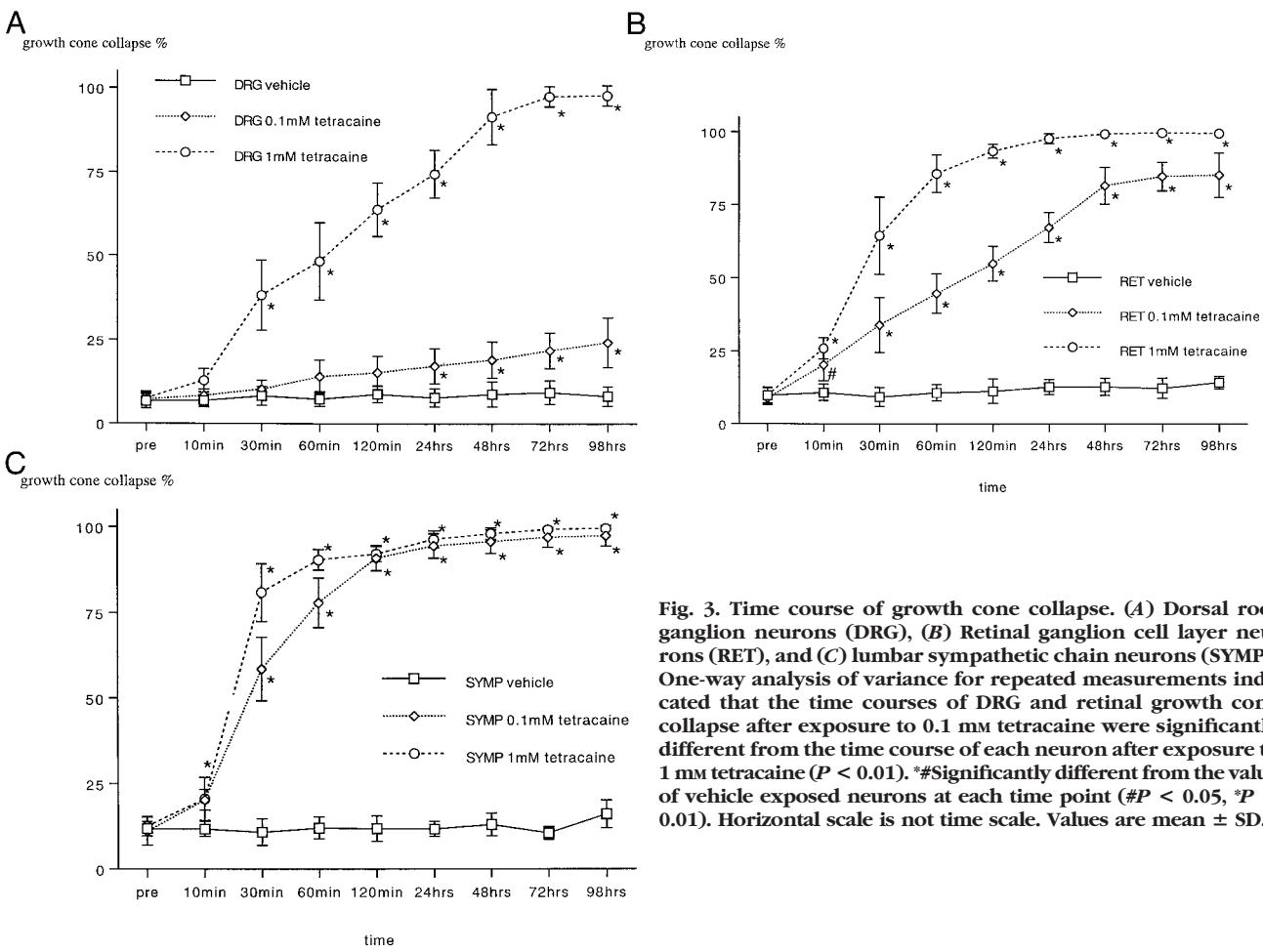

Fig. 3. Time course of growth cone collapse. (*A*) Dorsal root ganglion neurons (DRG), (*B*) Retinal ganglion cell layer neurons (RET), and (*C*) lumbar sympathetic chain neurons (SYMP). One-way analysis of variance for repeated measurements indicated that the time courses of DRG and retinal growth cone collapse after exposure to 0.1 mM tetracaine were significantly different from the time course of each neuron after exposure to 1 mM tetracaine ($P < 0.01$). *#Significantly different from the value of vehicle exposed neurons at each time point (#$P < 0.05$, *$P < 0.01$). Horizontal scale is not time scale. Values are mean ± SD.

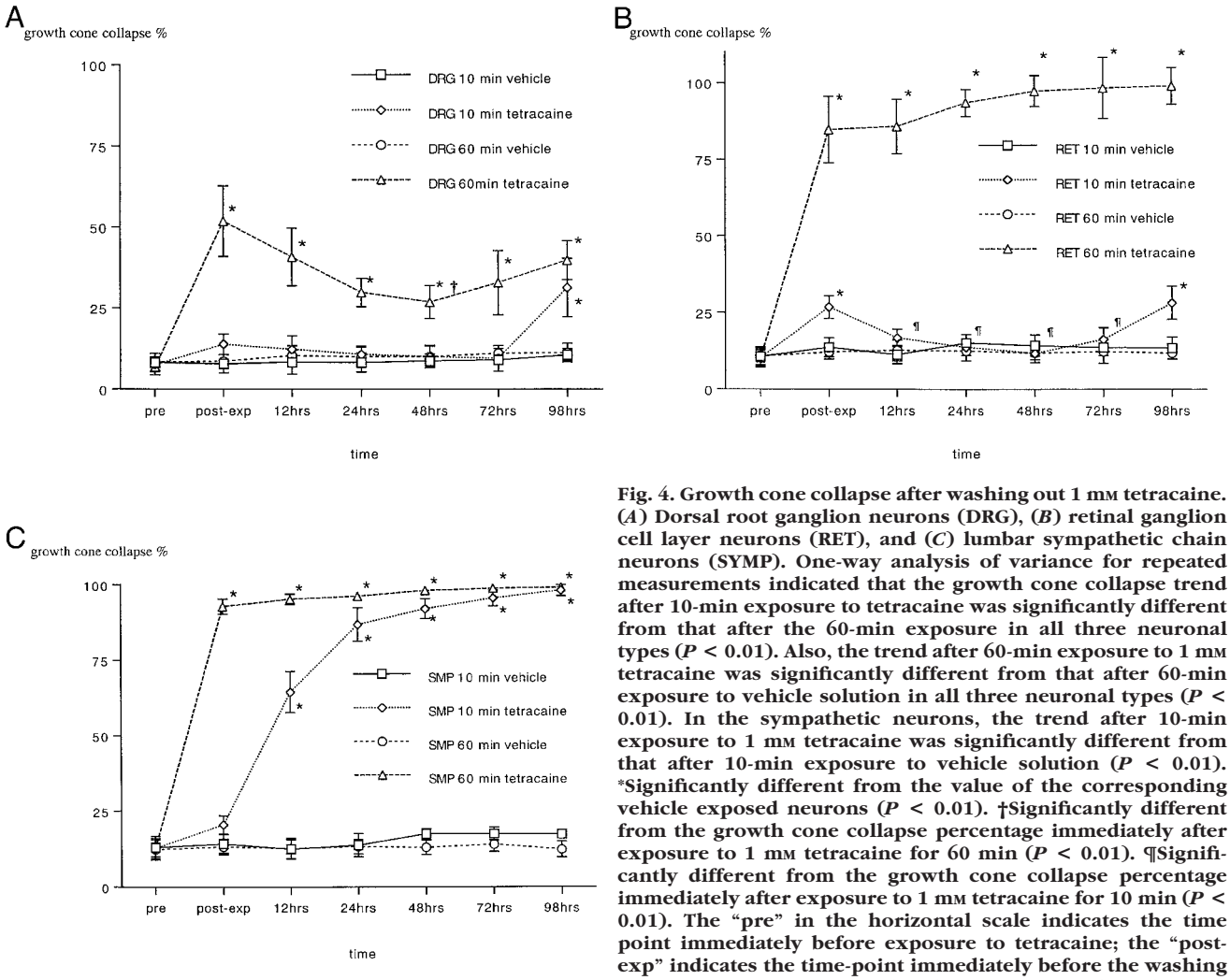

Fig. 4. Growth cone collapse after washing out 1 mM tetracaine. (*A*) Dorsal root ganglion neurons (DRG), (*B*) retinal ganglion cell layer neurons (RET), and (*C*) lumbar sympathetic chain neurons (SYMP). One-way analysis of variance for repeated measurements indicated that the growth cone collapse trend after 10-min exposure to tetracaine was significantly different from that after the 60-min exposure in all three neuronal types ($P < 0.01$). Also, the trend after 60-min exposure to 1 mM tetracaine was significantly different from that after 60-min exposure to vehicle solution in all three neuronal types ($P < 0.01$). In the sympathetic neurons, the trend after 10-min exposure to 1 mM tetracaine was significantly different from that after 10-min exposure to vehicle solution ($P < 0.01$). *Significantly different from the value of the corresponding vehicle exposed neurons ($P < 0.01$). †Significantly different from the growth cone collapse percentage immediately after exposure to 1 mM tetracaine for 60 min ($P < 0.01$). ¶Significantly different from the growth cone collapse percentage immediately after exposure to 1 mM tetracaine for 10 min ($P < 0.01$). The "pre" in the horizontal scale indicates the time point immediately before exposure to tetracaine; the "post-exp" indicates the time-point immediately before the washing out of tetracaine. Horizontal scale is not time scale. Values are mean ± SD.

10 min and after exposure for 60 min. However, the time courses of growth cone collapse after 10-min exposure and 60-min exposure were significantly different ($P < 0.01$). The washing out of tetracaine after 10-min exposure delayed growth cone collapse compared with that which was observed with washing out after 60-min exposure (fig. 4C). Reversibility was not observed in any of the three neuronal types after the morphologic appearance of the neurite shaft was changed.

Dose–Response Study of Bupivacaine

Three neuronal tissues showed significantly different dose–responses both at 60 min and at 24 h of exposure to bupivacaine (fig. 5; $P < 0.05$ between DRG and sympathetic ganglion tissues at 24 h of exposure; $P < 0.01$ among three neuronal types at 60 min of exposure, and between DRG and retinal tissues, and retinal and sympathetic ganglion tissues at 24 h of exposure), as observed in the case of tetracaine. Statistically significant growth cone collapse was observed when bupivacaine concentration exceeded 0.1 mM in DRG culture, 0.03 mM in retinal culture, and 0.32 mM in sympathetic ganglion culture, both at 60 min and at 24 h after application. The ED_{50} values of bupivacaine at 60 min and at 24 h were significantly higher than the corresponding values of tetracaine in retinal and sympathetic neurons ($P < 0.01$; table 2). At 60 min after the application of bupivacaine, ED_{50} values were significantly different for each of the three neuronal tissue types ($P < 0.01$). At 24 h after exposure, the ED_{50} value for retinal neurons was significantly lower than the value for sympathetic neurons ($P < 0.01$, table 2).

Discussion

Sensitivity of Neurons

In the current study, growth cone collapse was the first observed morphologic change. The growth cone is the most sensitive part of a neurite and functions as the guiding structure that orients the extension of the neurite.[17,18] It senses conditions in the environment with special receptor molecules.[19,20] In neurobiologic devel-

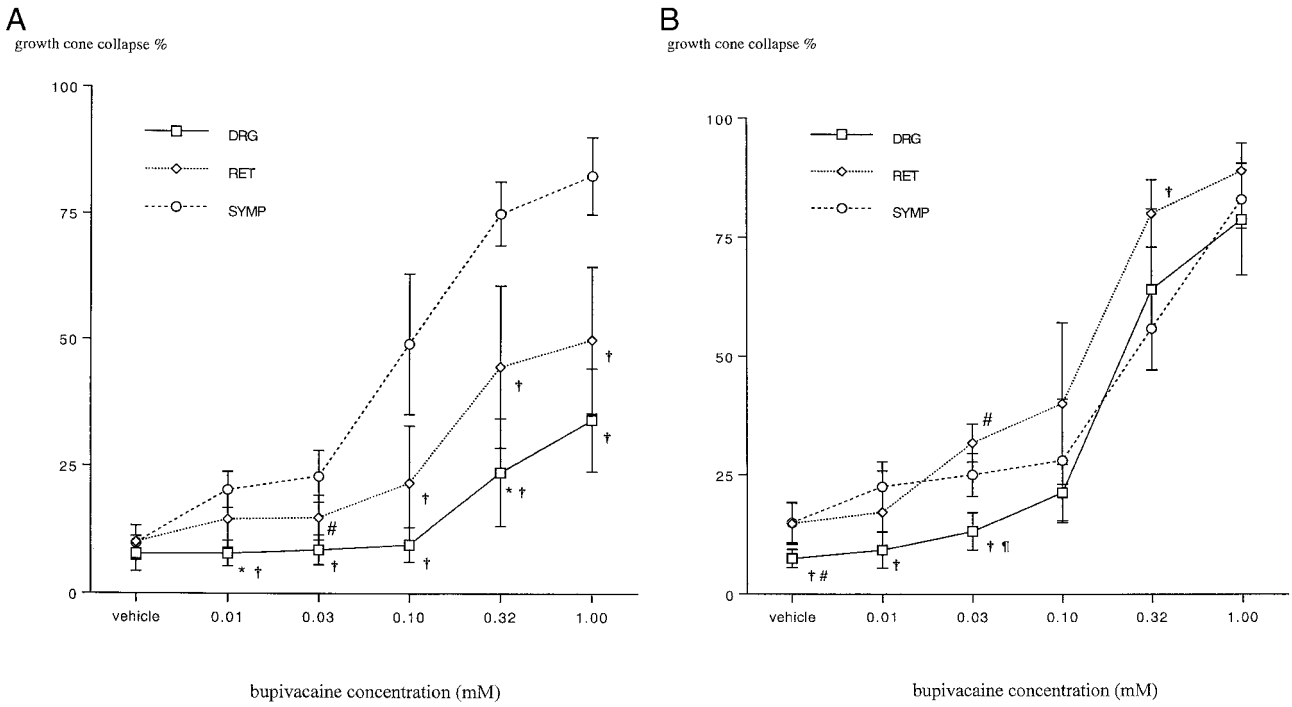

Fig. 5. Dose–response relations of bupivacaine induced growth cone collapse. Growth cone collapse percentage was examined at 60 min after bupivacaine exposure (*A*) and at 24 h after the exposure (*B*). One-way analysis of variance for repeated measurements indicated that three dose–response curves at each time point were significantly different from each other ($P < 0.05$ between dorsal root ganglion [DRG] and sympathetic ganglion [SYMP] neurons at 24 h of exposure; $P < 0.01$ among three neuronal types at 60 min of exposure, between dorsal root ganglion and retinal [RET] neurons, and between retinal and sympathetic ganglion neurons at 24 h of exposure). *¶Significantly different from the corresponding value of retinal neurons (*$P < 0.05$, ¶$P < 0.01$). #†Significantly different from the corresponding value of sympathetic neurons (#$P < 0.05$, †$P < 0.01$). Values are mean ± SD.

opment, growth cone collapse is not necessarily the first step in neuronal destruction. Both *in vivo* and *in vitro*, growth cone collapse and neurite retraction are usually reversible. As observed by Fan *et al.*,[21] when a growth cone encounters a repellent molecule expressed on a guidepost cell, the growth cone collapses. Then, at the tip of the neurite, a new growth cone extending filopodia toward another direction is formed. However, when a growth cone is surrounded by repellent molecules or toxic substances, a new growth cone cannot be formed after growth cone collapse.[22]

In the present study, the ED_{50} value of tetracaine determined by the growth cone collapse assay was lowest in embryonic sympathetic neurons and greatest in DRG neurons. The DRG neurons are mostly sensory neurons, and the retinal ganglion cell layer neurons are categorized as a part of the central nervous system.[15] Thus, this result implies that sympathetic neurons are the most sensitive to the neurotoxicity of tetracaine and that central nervous system neurons have moderate sensitivity. Peripheral sensory neurons are considered to be the most insensitive to the neurotoxicity of tetracaine. However, characteristics of neurons are not exactly the same among species and could change with the processes of maturing and aging.[12] Especially, cytoskeletal elements and neurotrophic factor receptors were developmentally regulated for their expression in neurons.[23,24] Therefore, the results of the current study cannot be directly applied to adult human cases.

Previous histopathologic studies have demonstrated that 1% (33 mM) tetracaine induces neuronal damage in rabbits[25] and 3% (100 mM) in rats, when administered intrathecally.[8] Electrophysiologic investigation by Lambert *et al.*[26] demonstrated that 0.5% (17 mM) tetracaine could induce neuronal damage in bullfrog nerves with sheaths removed. Because the exposure methods were completely different for each of these studies, it is difficult to compare their results. However, the results of the current study imply that growing and regenerating neurons are vulnerable to the toxicity of tetracaine. Because higher concentration of NGF in culture media did not increase the ED_{50} of the neurotoxicologic effect of tetracaine at 1 h and at 24 h after the exposure, it is probable that neurotrophic factor, NGF, had no effect on the neurotoxicity of tetracaine.

Because bupivacaine, which is categorized as an amide type of local anesthetic, had similar cell biologic effects on cultured neurons, the toxicity observed for tetracaine may not be unique to the drug. However, the sensitivities to bupivacaine were not identical to the sensitivities to tetracaine among three neuronal types. Sympathetic neurons were resistant to the bupivacaine toxicity compared with retinal ones. Also, ED_{50} values of bupivacaine were higher than those of tetracaine in retinal and sym-

pathetic ganglion neurons. These results were consistent with the idea that the degree of neurotoxicity is different among local anesthetics.[1]

Because of the results of *in vivo* experiments, external ischemia around neurons[7] and the breakdown of the blood–nerve barrier[27] have been proposed as possible mechanisms of the neurotoxicologic effects of local anesthetics. However, the results of other histologic studies imply that local anesthetics directly damage the structure of axons.[1,3] Kroin *et al.*[28] demonstrated that 4% lidocaine induced functional and morphologic degeneration of nerve fibers when directly infused into a cuff surrounding the sciatic nerve. Hashimoto *et al.*[29] observed that 5% lidocaine preferentially affected nerve roots. Also, Takenami *et al.*[8] observed that 3–20% lidocaine induced axonal degeneration at the sites where the axons were devoid of myelin sheath. Degeneration of the axonal shaft observed in the current study was consistent with these reports. Furthermore, our results demonstrate that, in regenerating neurons, the leading edges of neuronal shafts (growth cones) are the areas most rapidly impaired when exposed to a local anesthetic.

When cell biologic toxicity is assessed in culture experiments, physicochemical conditions of culture media should be maintained within a narrow range. In the current study, osmolality was considered to be maintained within acceptable range. Because, in our previous study, sodium chloride, sodium salicylate, and sodium sulfate did not show any significant growth cone–collapsing activity at concentrations up to 100 mM[11]. Phenol red in culture media showed that pH was not changed by the addition of tetracaine. Thus, it is probable that the neurotoxicity observed in the current study was mostly brought about by tetracaine.

Time Course and Reversibility

In our investigation of time course, we observed that morphologic changes occur slowly in DRG neurons. In contrast, retinal ganglion cell layer neurons and sympathetic neurons reacted rapidly to tetracaine; the first morphologic changes were detected within 10 min after exposure to 1 mM solution, and growth cone collapse exceeded 90% within 120 min. Because of the technical difficulty, we could not quantitatively assess neurite destruction. However, neurite destruction followed growth cone collapse in all neuronal types and always in a similar manner. In addition to growth cone collapse, neurites were quickly destroyed by tetracaine in sympathetic ganglion and retinal neurons. These observations suggest that not only the dose–response but also the time course of reactions was different among neuronal types. Whereas the effects of tetracaine occur slowly in resistant neurons, sensitive neurons can be quickly (within several minutes) damaged by exposure.

Table 2. ED_{50} (mM) Values of Growth Cone Collapsing Effects of Bupivacaine

	DRG Neurons	Retinal Neurons	Sympathetic Neurons
60 min after exposure	2.32 ± 0.50	0.96 ± 0.16*	0.18 ± 0.05*
24 h after exposure	0.34 ± 0.09	0.21 ± 0.06*	0.45 ± 0.10*

At 60 min after the application of bupivacaine, ED_{50} values were significantly different among three neuronal tissue types ($P < 0.01$). At 24 h after exposure, the ED_{50} for retinal neurons was significantly lower than the value for sympathetic neurons ($P < 0.01$). Values are mean ± SD.

* Significantly ($P < 0.01$) higher values compared with the corresponding values of tetracaine (table 1).

DRG = dorsal root ganglia.

Previous histopathologic studies have assessed neuronal damage at several hours or several days after exposure to local anesthetics.[3] Recent electrophysiologic and cell biologic studies showed neuronal damage shortly after exposure. Hodgson *et al.*[1] demonstrated that 15-min exposure to 40 mM lidocaine induced partially irreversible electrophysiologic changes in frog sciatic nerves. Also, Kanai *et al.*[30] showed that 15-min exposure to 80 mM lidocaine induced irreversible alterations in the resting membrane potential and action potential of crayfish giant axon. Using a cell biologic method, Gold *et al.*[9] showed that 4-min exposure to 30 mM lidocaine provoked neuronal cell death in rat DRG neurons. Although early effects of tetracaine have never been examined electrophysiologically or cell biologically in other studies, the time courses of growth cone collapse and neurite retraction observed in the current study were comparable to the alterations observed after exposure to lidocaine in those studies. It is possible that these early cell biologic and electrophysiologic changes were the primary events preceding the histopathologic changes in nerve roots and spinal cords.

Reversibility of growth cone morphologic appearance was not observed in sympathetic ganglion neurons, in which neurites were destroyed immediately after growth cone collapse. However, when retinal neurons were exposed to 1 mM tetracaine for 10 min, partial reversibility was observed. Also, when DRG neurons were exposed to 1 mM tetracaine for 60 min, reversibility was observed. These observations of reversibility were not the result of new growth of other neurites from cell bodies after destruction of neurites. Perhaps, when neurotoxic effects of tetracaine are confined to growth cones, neurons can survive and begin regeneration. Another remarkable fact is that even when tetracaine was removed from the culture media after a brief exposure time, growth cone collapse and neurite destruction proceeded further. It is possible that, in a short time, a process leading to neuronal destruction is initiated by exposure to a local anesthetic of high concentration. Because reversibility was observed only before the destruction of neurites, damage to neurite shafts may be critical for neuronal survival.

In our time-course and washout investigations, we observed that the degree of growth cone collapse and neurite degeneration increased at 72–98 h after exposure to tetracaine, even though DRG and retinal neurons did not show any change immediately after exposure. These late effects of tetracaine could be the results of a mechanism that is independent of the acute toxicity to growth cones and neurites. Previously, Fink and Kish[31] showed that 20 mM (0.54%) lidocaine inhibits axonal transport in neurites. More recently, Fagiolini *et al.*[32] demonstrated that 0.2% lidocaine interrupted axonal transport of neurotrophic factors and induced apoptosis in rat retinal ganglion cells. According to these reports, the late neural damage observed in the current study could be the result of deficiency of neurotrophic factors in cell bodies, a condition that is known to initiate enzymatic activation resulting in nuclear fragmentation. Future studies that focus on the axonal transport of NGF might clarify the mechanism of the delayed neuronal damage brought about by tetracaine.

In conclusion, short-term exposure to tetracaine produced irreversible changes in growing neurons. Growth cones were quickly affected, and neurites degenerated subsequently. Sensitivity varied with neuronal type and was not influenced by the concentration of NGF. Because a similar phenomenon was observed after exposure to bupivacaine, the toxicity to growing neurons may not be unique to tetracaine.

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