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ORIGINAL ARTICLE

Lidocaine treatment during synapse reformation periods permanently inhibits NGF-induced excitation in an identified reconstructed synapse of *Lymnaea stagnalis*

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Abstract
Purpose Nerve growth factor (NGF) has been reported to affect synaptic transmission and cause neuropathic pain. In contrast, lidocaine has been used to reduce neuropathic pain; however, the effect of NGF and lidocaine on spontaneous transmitter release and synapse excitation has not been fully defined. Therefore, the effect of NGF and lidocaine on nerve regeneration, synapse reformation, and subsequent spontaneous transmitter release was investigated. We used *Lymnaea stagnalis* soma–soma-identified synaptic reconstruction to demonstrate that a transient increase in both frequency and amplitude of spontaneous events of miniature endplate potentials (MEPPs) occurs following NGF treatment and a short burst of action potentials in the presynaptic cell; in addition, the effect of lidocaine on NGF-induced synapse reformation was investigated.
Methods Using a cell culture and electrophysiological and FM-143 imaging techniques for exocytosis on unequivocally identified presynaptic visceral dorsal 4 (VD4) and postsynaptic somata left pedal (LPeE) neurons from the mollusc *Lymnaea stagnalis*, the effects of NGF and lidocaine on nerve regeneration, synapse reformation, and its electrophysiological spontaneous synaptic transmission between cultured neurons were described.
Results NGF increased axonal growth, frequency, and amplitudes of MEPPs. Lidocaine exposure during synapse reformation periods was drastically and permanently reduced axonal growth and the incidence of synapse excitation by NGF.
Conclusion NGF increased amplitudes and frequencies of MEPPs and induced synaptic excitation by increasing axonal growth and exocytosis. Lidocaine exposure during synapse reformation periods permanently suppressed NGF-induced excitation by suppressing axonal growth and exocytosis of presynaptic neurons in the identified reconstructed synapse of *L. stagnalis*.

Keywords Lidocaine · NGF · Apoptosis · Miniature endplate potentials

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Introduction

Nerve growth factor (NGF) regulates survival, growth, or differentiation of a discrete population of neurons and is involved in neural plasticity. NGF transmits its signals intracellularly via a specific member of the trk family of receptor tyrosine kinases, trkA [1]. NGF not only is critical for the survival of a population of sensory neurons during their development but plays a role in maintaining phenotypes of adult dorsal-root ganglion (DRG) neurons [2]. Therefore, NGF is critically involved in neuropathic pain, such as hyperalgesia [3]. As

neuropathic pain is often unresponsive to conventional analgesics such as opiates and nonsteroidal anti-inflammatory drugs, the choice of treatment has been largely limited. Local anesthetics block the activity of voltage-gated sodium channels, thereby reversibly inhibiting the conduction of nerve impulses along axons and neuron excitation [4]. Based on these mechanisms of action, local anesthetics can be used to reduce neuropathic pain in clinical practice. The amelioration of pain by these drugs sometimes outlasts the duration of the sodium (Na^+)-channel blockade, probably due to their pharmacological properties. Although the underlying mechanisms of this persistent analgesic effect are largely unknown, several hypotheses have been proposed: interrupting nociceptor activity with local anesthetics not only leads to reversible sensitization of spinal-cord neurons but might also induce plastic changes in neurons depending on the timing and period of exposure [5–7]. Identifying the mechanisms of action of local anesthetics to relieve chronic pain might lead to the development of a new strategy to treat neuropathic pain. Therefore, the aim of this study was to clarify the effects of NGF and lidocaine on synaptic excitation during synapse reformation. To clarify these effects, the synaptic regeneration system of *Lymnaea stagnalis* was used.

Materials and methods

Animals and cell culture

All animal experiments were approved by the Animal Care Committee of the University of Miyazaki. Specifically, individual dorsal ganglion neurons from laboratory-raised *L. stagnalis* (freshwater snail) were used at room temperature. The snails were deshelled and transferred to a sterile dissection dish in normal *Lymnaea* saline: [51.3 mM sodium chloride (NaCl), 1.7 mM potassium chloride (KCl), 4.1 mM calcium chloride ($CaCl_2$), 1.5 mM magnesium chloride ($MgCl_2$), and 5.0 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 8, with sodium hydroxide (NaOH)]. Ganglia were treated in a defined medium (DM) [a serum-free 50% Leibovitz L-15 medium (GIBCO-BRL Life Technologies, Burlington, Ontario, Canada) with added inorganic salts, 20 μg/ml of gentamicin, pH 7.9)] for 25 min with 0.2% trypsin type III (Sigma Chemical Co., St. Louis, MO, USA). Neurons were removed by gentle suction with a siliconized fine-polished pipette with a microforge with an outer diameter of 1.5 mm (IB-150 F, WPI, Sarasota, FL, USA). After this, the previsceral dorsal 4 (VD4) and the postsynaptic somata left pedal E (LPeE) were juxtaposed in a soma–soma configuration on poly-L-lysine dishes (Falcon Plastics, Los Angeles, CA, USA) containing 3 ml of the defined medium (DM) or the DM with 10 ng/ml NGF for 24 h before use [8].

Neurite outgrowth

To assess neuronal regeneration in the absence and presence of NGF and lidocaine, identified neurons were isolated the cell culture and plated on poly-L-lysine-coated dishes containing the DM with NGF or DM with NGF and each concentration of lidocaine for 24 h. Presynaptic VD4 and postsynaptic LPeE neurons were selected for neurite outgrowth and synapse formation assays [9]. Neuronal sprouting was assessed as described previously [7]. Specifically, only those neurons exhibiting outgrowth (multiple branches and active growth cones) equivalent to a diameter of five somata were considered as sprouted. To test the hypothesis that NGF promotes neurite regeneration and synapse reformation from isolated identified neurons, cells were cultured in DM either in the absence or presence of NGF and allowed to extend neurites. The extent of outgrowth was calculated as a function of maximum neurite length. Identified presynaptic VD4 neurons were cultured with or without NGF and lidocaine. Specifically, cells were paired in close proximity and allowed to extend neurites. We reasoned that if neurons regenerated their processes, then synapses would develop between the neurites that could then be detected morphologically or electrophysiologically.

Intracellular recording

To assess synaptic reformation in the absence and presence of NGF and lidocaine, the following neuronal activity was monitored using a two-channel intracellular recording [10–12]. Sample neurons were exposed with NGF and lidocaine for 24 h, and after washout in culture solutions, the following experiments were performed: A glass microelectrode with a filament and an outside diameter of 1.5 mm (TW150F-4, WPI) was filled with a KCl pipette solution consisting of 50 mM KCl, 10 mM HEPES, and 2 mM magnesium adenosine triphosphate (Mg-ATP), and the pH was clamped to 7.0 with potassium hydroxide (KOH), yielding a tip resistance of 20–30 MΩ. Electrical signals were amplified with a current–voltage clamp amplifier (Multiclamp-700A, Axon Instruments, CA, USA). For experimental control and data acquisition, an analog–digital and digital–analog converter (Digidata 1322A, Axon Instruments) was used. Data acquisition and analysis were conducted using p-clamp software (p-clamp 9, Axon Instruments). In the somata–somata synapse model between presynaptic VD4 and postsynaptic LPeE neuron, the action potentials in VD4 in the absence of NGF conditions (Fig. 3) generated a 1:1 excitatory postsynaptic potential (EPSP) in

LPeE, and these excitatory responses were mimicked by an exogenous acetylcholine (ACh) puff (at arrow), which was performed (80-ms pulses, 1–2 psi) by applying 1 μM acetylcholine directly to the synaptic site via a Pneumatic PicoPump (PV800; World Precision Instruments) pressure injector under a fast perfusion system. In each synapse model, EPSP and the ACh responses were measured. To determine whether spontaneous transmitter release could be modulated by presynaptic activity, miniature endplate potentials (MEPPs) were recorded before and after a brief burst of action potentials in the presynaptic VD4 (10–15 action potentials at 5–10 Hz) generated by a continuous depolarizing current injection (0.5–1 nA). The holding membrane potential of each VD4 and LPeE neuron was −100 mV.

FM1-43 imaging

Cells were incubated in 20 μM FM1-43 (Molecular Probes, Eugene, USA) for 10 min. The presynaptic cell (VD4) stimulated the generation of 100 action potentials (10 spikes/burst) by conventional electrophysiological techniques to facilitate the uptake of FM1-43 in cells that were paired overnight either in the presence or absence of NGF, as described previously [7]. The probe and cultured medium were then replaced with cold saline to prevent neuronal firing during the washout and to remove background fluorescence. Fluorescent images of the FM1-43-labeled cells were acquired using a TE-300 inverted microscope (Nikon, Tokyo, Japan). Excitation light was from a Xenon lamp, excitation filters (490/30 nm), a dichroic mirror (505 nm), and emission filters (570 nm long-pass filter or 610 nm). Phase and fluorescent images were captured with an EM-CCD camera (Imagem, Hamamatsu Photonics, Tokyo, Japan) connected to a computer running AQAcosmos (Hamamatsu Photonics, Shizuoka, Japan). pixels in the cross-section of the fixed area (200×150 μm^2) were integrated and measured with NIH image software [version 1.62, National Institutes of Health (NIH), Bethesda, MA, USA]. Cells were paired overnight either in the absence or presence of NGF and lidocaine. On day two, the cultured solution was replaced with normal saline containing the dye FM1-43. The dye was then washed away with normal saline, and images were acquired.

Statistical analysis

Parametric data are expressed as mean ± standard error (SE) and were analyzed for significance using one-way analysis of variance (ANOVA) with repeated measures and a Scheffe's post hoc test. Nonparametric data are expressed in percentages and were analyzed for significance using the χ^2 test. Significance was assumed if $P < 0.05$.

Results

Effects of NGF and lidocaine on neurite outgrowth of pre- and postsynaptic neurons and paired pre- and postsynaptic neurons

The addition of NGF to the DM (DM with NGF) significantly enhanced neuron outgrowth, as shown by increases in the length and axon density of cultured presynaptic VD4 and postsynaptic LPeE neurons, which were suppressed by lidocaine in a concentration-dependent manner. In DM with NGF, an increase of outgrowth by NGF was almost completely suppressed by lidocaine at 50 and 100 μM (VD4 neurite length: DM, 59 ± 39; NGF, 162 ± 49; lidocaine 0.01 mM, 136 ± 28; lidocaine 0.1 mM, 38 ± 23; lidocaine 1 mM, 15 ± 9; LPeE neurite length DM, 67 ± 48; DM with NGF (NGF), 195 ± 101; lidocaine 0.01 mM, 154 ± 73; lidocaine 0.1 mM, 28 ± 14; lidocaine 1 mM, 11 ± 5 μm) (Fig. 1a, b). In paired pre- and postsynaptic neurons, the addition of NGF also potentiated neurite regeneration and synapse reformation, as shown by an increase in neurite length and the number of collaterals of the isolated identified neurons. Lidocaine also suppressed the increase of both neurite length and number of collaterals in these paired pre- and postsynaptic neurons, both of which were completely suppressed by 50 and 100 μM lidocaine (neurite length of paired VD4 and LPeE: DM, 64 ± 66; DM with NGF, 354 ± 359; lidocaine 0.01 mM, 259 ± 265; lidocaine 0.1 mM, 79 ± 75; lidocaine 1 mM, 65 ± 62 μm) (Fig. 2a, b).

Electrophysiological change of identified paired pre- and postsynaptic neurons with or without NGF and lidocaine

In DM with NGF, EPSP in LPeE was significantly increased versus DM conditions, and the ACh response also increased significantly more than under the DM conditions ($p < 0.05$). In DM with NGF, lidocaine exposure during the synapse formation period significantly reduced the EPSP in a concentration-dependent manner ($p < 0.05$) ($n = 10$–11) (Fig. 3b). In DM with NGF, the ACh response in LPeE was reduced by lidocaine at a high concentration (100 μM).

A brief burst of action potentials in VD4 (10–15 action potentials at 5–10 Hz) generated by a continuous depolarizing current injection (marked by the projecting bar in the bottom trace in Fig. 4a) significantly increased both MEPP frequency ($p < 0.05$) (Fig. 4a upper trace, Fig. 4b upper graph) and amplitude ($p < 0.05$) (Fig. 4a upper trace, Fig. 4b lower graph) for at least 1 min after cessation of the presynaptic action potential activity in the presence of NGF. In contrast, after the ACh puff in which the main

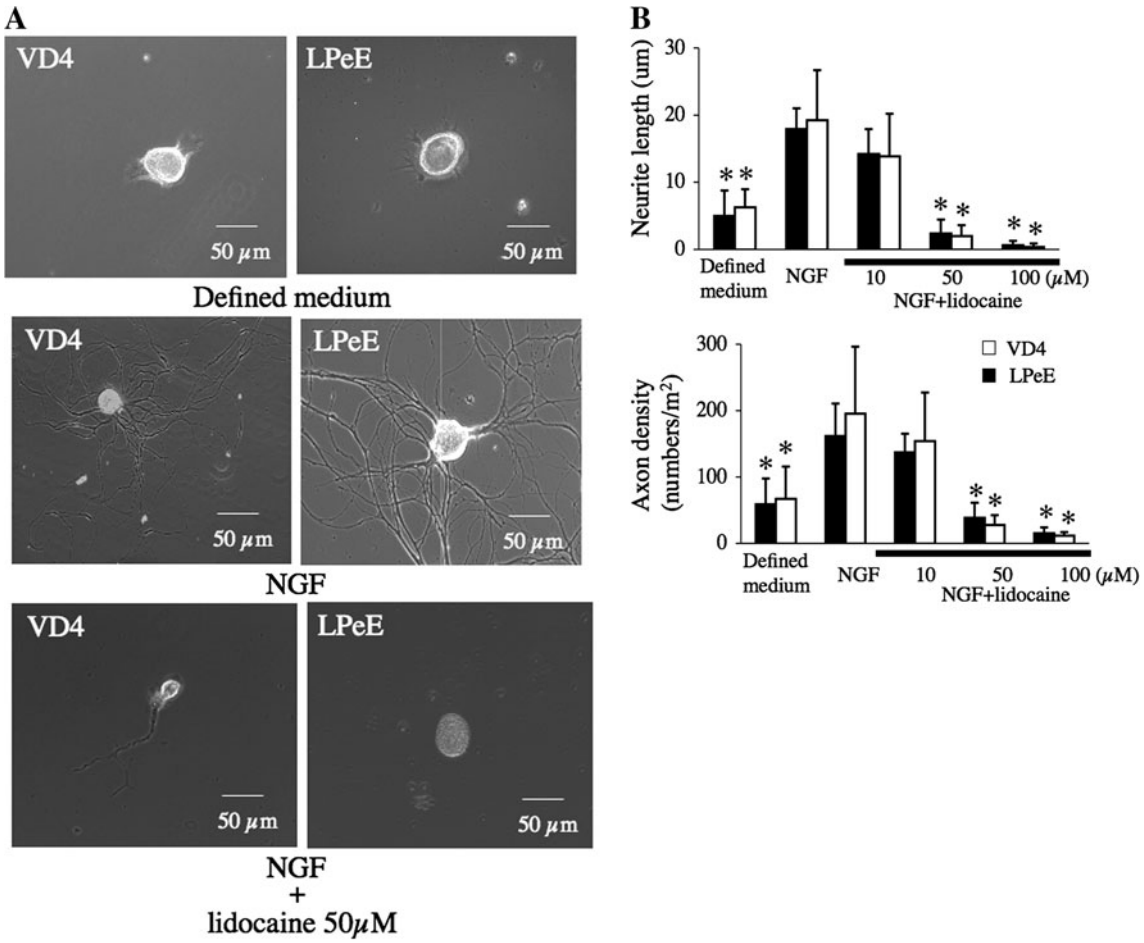

Fig. 1 Effects of nerve growth factor (NGF) and lidocaine on neurite regressions of presynaptic visceral dorsal 4 (VD4) cells. **a** To test the effects of NGF on neurite regeneration, presynaptic VD4 cells and postsynaptic somata left pedal (LPeE) cells were isolated and cultured in a defined culture medium (DM) either in the absence (*upper pictures*) ($n = 11$ in VD4 and $n = 7$ in LPeE) or presence (*middle pictures*) ($n = 13$ in VD4 and $n = 7$ in LPeE) of NGF and then exposed to lidocaine in the presence of NGF for 24 h during synapse reformation periods (*lower pictures*) ($n = 11$ in VD4 and $n = 7$ in LPeE). **b** NGF increased neurite outgrowth extensively. The growth exhibited in the presence of NGF was indistinguishable from that observed in the group without NGF. $*p < 0.05$ compared with the NGF group

transmitter of this synapse was exposed to the postsynaptic LPeE neuron, these MEPPs were not observed (Fig. 4a lower trace, Fig. 4b).

In DM with NGF, both MEPP frequency and amplitude increased significantly more than in the absence of NGF ($p < 0.05$) (Fig. 4c, d), and lidocaine exposure during the synapse reformation period significantly suppressed both MEPP frequency ($p < 0.05$) (Fig. 4c lower trace and Fig. 4d upper graph) and amplitude for at least 1 min after the cessation of presynaptic action potential activity in a concentration-dependent manner ($p < 0.05$) ($n = 11$–13) (Fig. 4c, d).

Exocytosis imaging by FM1-43

In DM with NGF, exclusive labeling of presynaptic cells was discernable at its contact site with LPeE as well as in its processes surrounding the postsynaptic somata (Fig. 5a). In contrast, faint staining of presynaptic cells was observed in VD4 paired in DM with NGF and lidocaine. The pixel values for each category were converted into 3D images by NIH imaging. They are depicted in the corresponding panels, their pixel values are calculated, and summaries are presented in Fig. 5b. In contrast, lidocaine inhibits NGF-promoted nerve regeneration and synapse formation in a concentration-dependent manner, as shown in Fig. 5.

Discussion

Study results demonstrate that NGF promoted neurite outgrowth (Figs. 1, 2) and EPSP amplitudes in response to ACh (Fig. 3) and generated spontaneous MEPPs (Fig. 4).

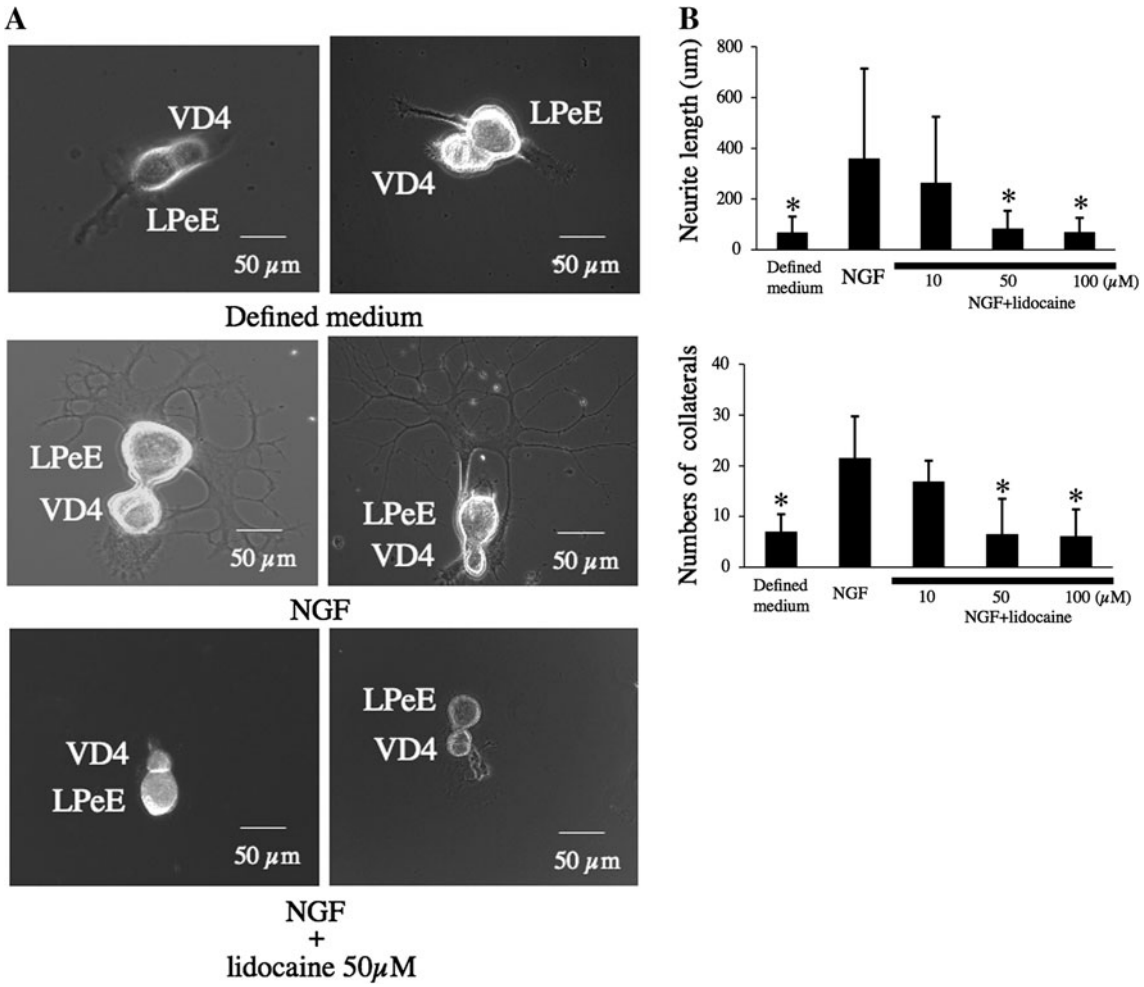

Fig. 2 Effects of nerve growth factor (NGF) and lidocaine on neurite regression in synaptic reconstitution. **a** To test the effects of NGF on neurite regeneration in synapse reconstruction, identified presynaptic visceral dorsal 4 (VD4) and postsynaptic somata left pedal (LPeE) neurons were isolated and cultured with their respective stomata in a defined culture medium (DM) either in the absence (*upper pictures*) ($n = 12$) or presence (*middle pictures*) ($n = 16$) of NGF. Lidocaine exposure for 24 h during synapse reformation periods suppressed NGF-induced neurite outgrowth (*lower pictures*) ($n = 14$). **b** NGF also increased neurite outgrowth extensively in synapse reconstruction; however, the growth exhibited in the presence of NGF was indistinguishable from that of the group without NGF. $*p < 0.05$ compared with NGF group

In contrast, lidocaine suppressed these NGF-promoted neurite outgrowths, EPSP, and MEPPs. Vesicles that include neurotransmitters, such as ACh, for synaptic transmission, as imaged by FM1-43, were stained much more in the presence of NGF than those of NGF with lidocaine, as shown in Fig. 5.

In general, an EPSP is a temporary depolarization of postsynaptic membrane potential caused by the flow of positively charged ions, such as sodium and calcium, into the postsynaptic cell as a result of opening of ligand-sensitive channels, such as the ACh receptor in postsynaptic cells by neurotransmitter stimulation, e.g., ACh, released from presynaptic cells by depolarization of the presynaptic cell. On the other hand, MEPPs are caused by spontaneous leakage of presynaptic neurotransmitter molecules, such as vesicles containing ACh, and MEPP amplitude depends on the amount of neurotransmitter leakage and postsynaptic response to them [13, 14]. Occasionally, ACh is likely to be spontaneously released because there is a basal level of calcium in the presynaptic terminal. Each vesicle, e.g., the vesicular ACh transporter, indeed contains enough transmitters to open >1,000 individual ACh-sensitive channels. Each vesicle refers to those small endplate potentials that occur randomly in the absence of any stimulation. EPSP is due to the summation effects of many vesicles being released at the same time by stimulation, such as action potential. One vesicle produces a potential of about 0.5 mV. The release of 100 of those vesicles at the same time could produce a potential that is 100 times as great (50 mV). Neurotransmitter (ACh) release for EPSP

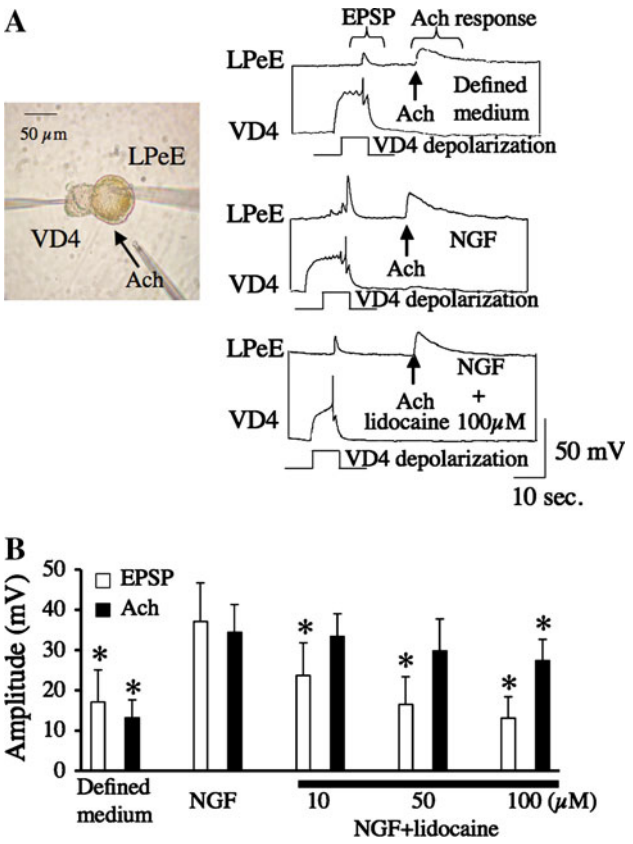

Fig. 3 Effects of nerve growth factor (NGF) and lidocaine on excitatory postsynaptic potential (EPSP) and response to acetylcholine (ACh). **a** Intracellular recordings between visceral dorsal 4 (VD4) and somata left pedal (LPeE) revealed an excitatory synapse in defined culture medium (DM), and the action potentials in the presynaptic cell generated 1:1 EPSP (*upper trace*). These excitatory responses were mimicked by exogenously applied ACh (*arrow*), which generated a compound EPSP. Both synaptic (VD4) and nonsynaptic (ACh) responses were significantly increased by NGF treatment (*middle trace*). VD4 responses were significantly depressed by lidocaine exposure during synapse reformation periods (*lower trace*). In contrast, nonsynaptic (ACh) responses were not suppressed by lidocaine treatment. **b** Results are presented as mean ± standard deviation, $n = 11$. $^{*}p < 0.05$ compared with NGF group

and MEPPs is triggered by intracellular Ca. Therefore, afterburst spikes increase intracellular Ca through voltage-dependent Ca channels, which will increase the amplitude and frequency of MEPPs. Our data also demonstrated that MEPPs were increased in both amplitude and frequency by a burst of presynaptic neurons but were not induced by ACh exposure to postsynaptic (LPeE) neurons, as shown in Fig. 4a, b. Therefore, these MEPP afterburst spikes in this VD4–LPeE synapse model are mainly dependent on the amount of neurotransmitter leakage from presynaptic neurons. Morphologically, the amplitude and frequency of MEPPs increased in the synapses, which have a high density of axons, as shown in Figs. 2 and 5. In FM1-43 imaging, which stains the exocytosis area, under NGF conditions, exclusive labeling of presynaptic VD4 cells was discernable at its contact site with postsynaptic LPeE. In addition, in many cells, the processes and axon collaterals surrounded the postsynaptic somata. It has been reported that NGF increases ACh synthesis and release and vesicular ACh transporter expression [15, 16].

Therefore, it is assumed that many input signals in postsynaptic neurons will be received from presynaptic neurons under NGF conditions and that mechanical inputs and presynaptic activity are important factors for MEPP amplitude and frequency. NGF treatment during synapse reformation periods significantly increased MEPP amplitude and frequency, especially after a burst of presynaptic neurons, as shown in Fig. 4c, d. These results suggest that NGF treatment during synapse reformation periods grows collateral axons that will increase neurotransmitter release from presynaptic neurons, which induces an increase in MEPP amplitude and frequency. In other words, NGF induces an increase in input sites of presynaptic signals, and our results indicate that it is one of the mechanisms of NGF-induced hyperalgesia.

We also demonstrate that long-term exposure to lidocaine during synapse reformation periods permanently suppresses EPSP and the MEPPs in both amplitude and frequency before and after a presynaptic neuron burst. We previously reported that lidocaine suppresses voltage-dependent Ca currents [12], and this is one reason that lidocaine suppresses both EPSP and the MEPPs, because these are triggered by Ca. Lidocaine also suppressed axon growth. Fujii et al. [17] reported that lidocaine suppressed ACh synthesis in 3T3 cells. Our investigation indicates that long-term exposure to lidocaine suppresses both EPSP and MEPP more than it suppresses ACh response in the postsynaptic neuron, and these results indicate that lidocaine suppresses neurotransmitter release from presynaptic neurons. Therefore, continuous exposure to lidocaine during synapse reformation periods will permanently suppress NGF-induced synaptic excitation, such as MEPPs, by reducing ACh synthesis and release and mechanical inputs from presynaptic to postsynaptic neurons.

Lidocaine has been shown in many reports to induce toxicity for axon degeneration [18–20]. Most of them indicate that lidocaine induces morphological changes in axons and neurons. We also reported that long-term exposure to lidocaine induced morphological changes in cone and neurite growth in *Lymnaea* neurons [21]. However, the mechanisms remain obscure. Marques et al. [22] reported that lidocaine changes the distribution of ACh receptors and induces long-term regeneration at the neuromuscular junction. Tsuchiya et al. [23] reported that lidocaine interacts with anionic phospholipid membrane structure dependently to modify fluidity and induces neuronal damage, such as apoptosis and necrosis. On the other hand, lidocaine has also been reported to act as a

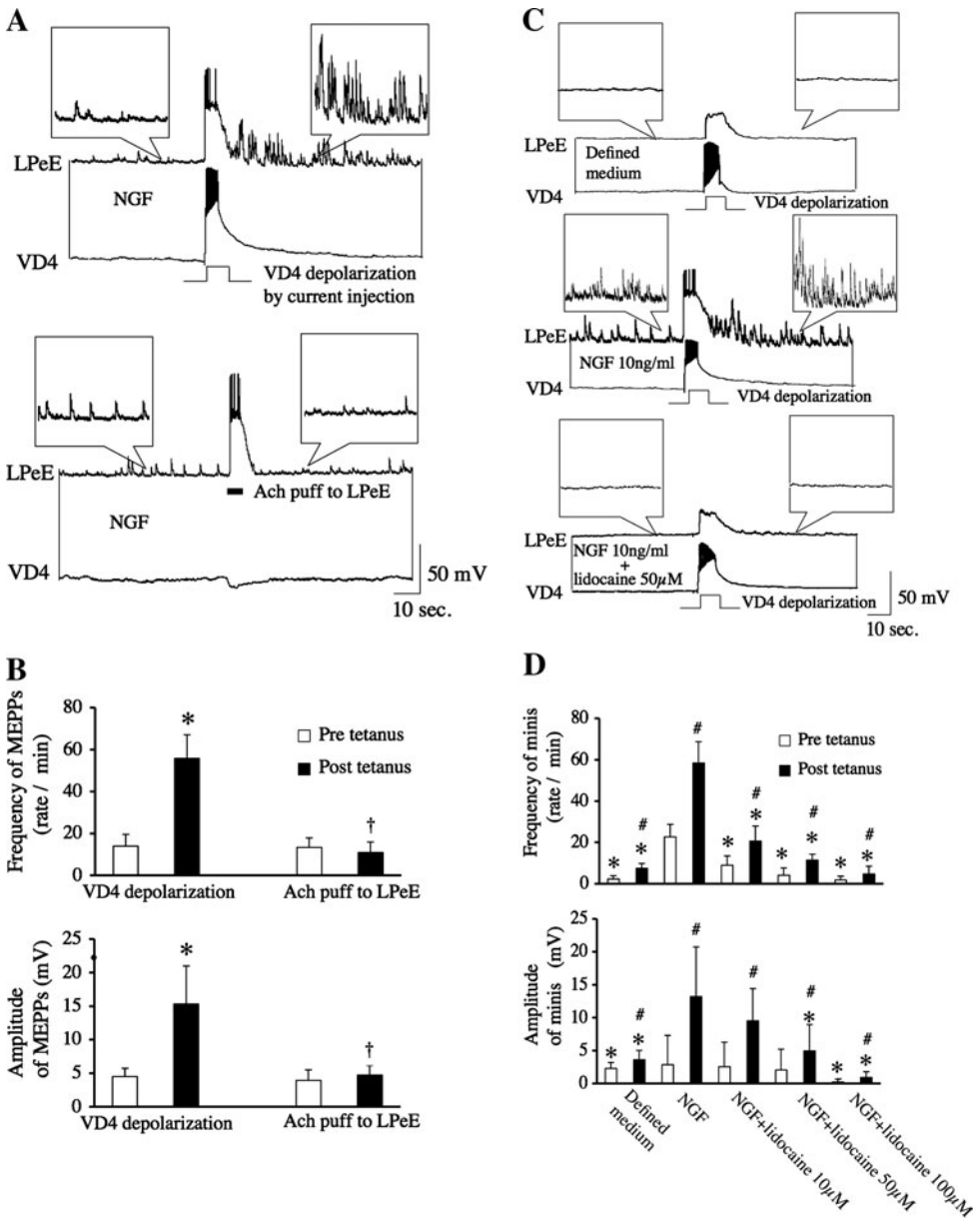

Fig. 4 Effects of nerve growth factor (NGF) and lidocaine on miniature endplate potentials (MEPPs). **a** Intracellular recordings between visceral dorsal 4 (VD4) and somata left pedal (LPeE) revealed an excitatory synapse in defined culture medium (DM) in the presence of NGF, and many MEPPs were observed by a brief burst of action potentials in VD4 (10–15 action potentials) generated by a continuous depolarizing current injection (*upper trace* of **a**). In contrast, following ACh puff to the postsynaptic LPeE neuron, these MEPPs were not obtained (*lower trace* of **a**). **b** *Upper graph* shows the frequency of MEPPs, and *lower trace* shows the amplitudes of MEPPs. Results are presented as the mean ± standard deviation, $n = 8$. *p<0.05 in comparison to baseline of pretetanus. $^{\dagger}p < 0.05$ compared with baseline. **c** Intracellular recordings between VD4 and LPeE revealed an excitatory synapse in DM in the absence of NGF; however, few MEPPs were observed (*upper trace*) ($n = 12$). In contrast, in the presence of NGF, many MEPPs were observed (*middle trace*) ($n = 14$). **d** A brief burst of action potentials in VD4 (10–15 action potentials) generated by a continuous depolarizing current injection significantly increased both MEPP frequency and amplitude for at least 1 min after cessation of presynaptic action potential activity. Lidocaine exposure during synapse reformation periods permanently inhibits these NGF-induced MEPPs (*lower trace*). **d** MEPP data. *Upper graph* shows the frequency of MEPPs, and the *lower trace* shows the amplitudes of MEPPs. Results are presented as mean ± standard deviation. *$p < 0.05$ compared with NGF group. $^{\#}p < 0.05$ compared with pretetanus

multitargeting drug. Lirk et al. [24] reported that lidocaine-induced axonal injury is caused by activation of the p38 mitogen-activated protein kinase. Radwan et al. [25] reported similar results; however, they presented a contrasting view in which the growth cone-collapsing effect of lidocaine on DRG neurons is reversed by several neurotrophic factors, such as NGF. Takatori et al. [26] reported identical results to ours, namely, that lidocaine

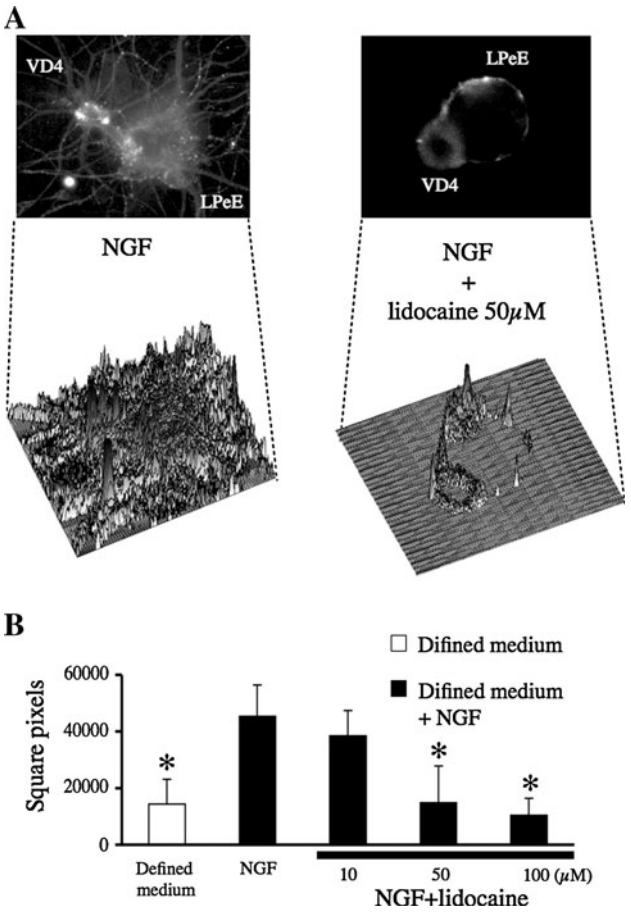

Fig. 5 Effects of nerve growth factor (NGF) and lidocaine on exocytotic profiles. **a** To test whether the transmitter secretory machinery of the presynaptic cell paired overnight in NGF was perturbed, the exocytotic profiles were analyzed through FM1-43 imaging. Visceral dorsal 4 (VD4) cells paired with somata left panel (LPeE) overnight in the presence of NGF (*left-hand pictures*) and NGF with lidocaine (*right-hand pictures*) and recorded under normal saline conditions were loaded with the FM1-43 dye. VD4 was extensively loaded either at its contact site with postsynaptic neurons (LPeE) or in its processes surrounding the postsynaptic somata (*left-hand pictures*). Lidocaine significantly decreased this NGF-induced axonal growth (*right-hand pictures*). *Corresponding panels under each image* represent 3D images of pixel values, **b** and these pixel values are averaged in a *bar graph*. Results are presented as mean ± standard deviation; $n = 8$–10. $^*p < 0.05$ compared with NGF group

suppresses NGF-mediated neurite outgrowth by inhibiting tyrosine kinase A activity. Our results demonstrate that lidocaine exposure during synapse reformation periods is able to suppress NGF-induced MEPPs permanently. Neurotrophic factors, such as NGF, have been reported to act as protein kinase A (PKA) because the inhibitor for PKA was able to inhibit synapse reformation within 24 h. In other words, these messengers of cyclic adenosine monophosphate (cAMP)-PKA pathway may play a novel role in regulating the synaptic efficacy during early synaptogenesis and plasticity induced by neurotrophic factors [27].

NGF is related to several types of neuropathic pain, such as herpetic neuralgia [28, 29], complex regional pain syndrome (CRPS) [30, 31], and cancer pain [32, 33]. In our investigation, we demonstrated that NGF increases MEPPs and induces excitation after synapse reformation, which will cause neuropathic pain, including hyperalgesia or allodynia. Hamakawa et al. [34] reported similar results in which the neurotrophic factor changes from an inhibitory synapse to excitatory synapse in the identified *Lymnaea* reconstructed synapse model. Therefore, lidocaine exposure in the early periods may be beneficial for treating these types of neuropathic pains [35].

In this experiment, human NGF was used. Human NGF is also able to support neurite outgrowth of *Lymnaea* neurons [9, 36]. We took advantage of an ideal model preparation in which synaptic transmission between uniquely identified neurons was investigated at the level of single pre- and postsynaptic neurons. Individually isolated neurons from the mollusc *Lymnaea* not only regenerate their neurites in cell culture but also recapitulate their specific patterns of synapses, which are similar to those observed in vivo.

It is very difficult to observe synaptic reformation morphologically and electrophysiologically in mammals in vivo because there are numerous glial cells and neurons of other cells obstructing the view. In contrast, the somata–somata synapse model of *Lymnaea* is the simplest, as it consists of only two pre- and postsynaptic neurons, which makes it easy to observe synaptic plasticity morphologically and electrophysiologically. Of course, there are species differences between *Lymnaea* and mammals; moreover, this somata–somata synapse model of *Lymnaea* provides a nice opportunity for synaptic plasticity. This in vitro approach using *Lymnaea* neurons has been extensively used in previous studies to decipher both cellular and synaptic mechanisms whereby NGF and various anesthetics affect neuronal function and synaptic transmission [9, 10, 16].

In conclusion, NGF increased axonal growth, EPSP amplitudes, and MEPP amplitudes and frequencies in the identified reconstructed synapse, and lidocaine exposure during synapse reformation periods permanently suppressed them in the identified reconstructed synapse of *L. stagnalis*.

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