Effects of clonidine on lidocaine-induced inhibition of axonal transport in cultured mouse dorsal root ganglion neurones

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Background. The α2-adrenoceptor agonist clonidine is used in combination with lidocaine for anaesthesia. Lidocaine inhibits axonal transport and neurite growth, whereas α2-adrenoceptor agonists have neurotrophic effects. Here we have investigated whether clonidine reduces lidocaine-induced inhibition of axonal transport in cultured mouse dorsal root ganglion neurones.

Methods. Axonal transport of organelles and neurite growth were assessed by video microscopy in cells treated with clonidine and lidocaine for 1 h. Stable responses were achieved within this period.

Results. Clonidine (10 and 100 μM) increased and lidocaine (10, 100 μM, and 1 mM) decreased axonal transport. The inhibitory effects of lidocaine were reduced by simultaneous treatment with clonidine. The actions of clonidine were antagonized by the α2-adrenoceptor antagonist yohimbine. Since clonidine was reported to block N-type channels, we further investigated the role of ion channels in the antagonistic action of clonidine on the lidocaine response. The action of lidocaine on axonal transport was not mimicked by the Na+ channel blocker tetrodotoxin and not blocked by the Na+ channel activator veratridine. The action of lidocaine was not blocked by the L-type Ca2+ channel blocker nifedipine, but was blocked by the N-type channel blocker α-conotoxin MVIIA. These effects on axonal transport correlated with the effects on neurite growth.

Conclusions. Inhibition of axonal transport induced by lidocaine, which may be mediated by N-type channel activation, can be blocked by clonidine. Clonidine may alleviate the effects of lidocaine on neuronal dysfunction.

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Lidocaine exhibits an analgesic action and is widely used as a local anaesthetic. This agent acts on axons of sensory neurones to block conduction of action potentials by closing voltage-dependent Na+ channels, which is thought to be closely related to its analgesic effect. Lidocaine also has an attenuating effect on transport of materials in axons.1–3 We previously demonstrated that lidocaine inhibits axonal transport even at low concentrations (10–100 μM) in cultured mouse dorsal root ganglion (DRG) neurones and that this effect is associated with inhibition of neurite growth.3 Several other studies also demonstrated that lidocaine inhibits neurite growth in cultured neurones.4–7 Clonidine, an α2-adrenoceptor agonist, is increasingly used in combination with local anaesthetics including lidocaine for spinal, epidural, and peripheral nerve blocks because of a prolonged and enhanced analgesic effect.8–11 In contrast to lidocaine, α2-adrenoceptor agonists seem to contribute to neurotrophic actions. Activation of α2-adrenoceptors induces neurite growth in transfected PC12 cells.12 Norepinephrine is neurotrophic via activation of α2A-receptors during fetal cortical development.13 The α2A-adrenoceptor agonists promote dendrite growth in cultured cortical neurones.14 Thus, it seems likely that lidocaine and clonidine have opposite effects on axogenesis. The present study investigated whether clonidine reduces inhibition of axonal transport induced by lidocaine in cultured mouse DRG neurones. We also examined neurite growth which is strongly correlated with axonal transport.3 15–18
Methods

Cell culture
The Animal Experimentation and Ethics Committee of Kitasato University School of Medicine approved all animal use in this study. Adult male C57BL/6 mice (8 weeks old) were killed with ether and the dorsal root ganglia removed. The ganglia were incubated for 90 min at 37°C with 2 mg ml⁻¹ collagenase (Worthington Biochemical, Freehold, NJ, USA) in Ham’s F-12 medium (Gibco, Grand Island, NY, USA), and then incubated for 15 min at 37°C in Ca²⁺- and Mg²⁺-free Hank’s balanced salt solution (Gibco) containing 2.5 mg ml⁻¹ trypsin (Sigma, St Louis, MO, USA). The trypsin activity was inhibited with 0.125 mg ml⁻¹ trypsin inhibitor (Sigma). After a rinse with enzyme-free Ham’s F-12 medium, the ganglia were triturated using a fire-polished pipette (inner diameter: 0.5 mm) to dissociate cells. The cells were plated onto the coverslips coated with poly-L-lysine (10 µg ml⁻¹) and cultured for 48 h at 37°C under 5% CO₂ in Ham’s F-12 medium containing 10% fetal bovine serum and penicillin (100 units ml⁻¹) – streptomycin (100 µg ml⁻¹).

Experimental preparation
Figure 1A illustrates the chamber used for video microscopic observation of neurites. The coverslip on which neurones were grown was attached with a waterproof tape to the underside of a 0.5 mm thick stainless steel chamber (50 × 80 mm) having a hexagonal hole (25 × 35 mm). The topside of the chamber was covered with another coverslip, leaving a small opening on both sides for injection of solution. The culture medium was then replaced with physiological salt solution (PSS, pH 7.4, 37°C), and the chamber mounted onto the stage of an inverted Zeiss Axiomat microscope equipped with Plan Apo ×25 and ×100 objectives (Carl Zeiss, Ober-Kochen, Germany). The stage was maintained at 37°C. Drug-containing solution was injected into one opening using a Pasteur pipette, and the solution spilling from the other opening was removed by a suction pump.

Solutions and pharmacological agents
The composition of PSS was (in millimoles): 135 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 5.5 glucose (all from Wako, Osaka, Japan), with the pH adjusted to 7.4 with NaOH. Lidocaine hydrochloride monohydrate (Sigma), clonidine (Sigma), ω-conotoxin MVIIA (Sigma), yohimbine (Sigma), and tetrodotoxin (TTX, Wako) were directly dissolved in PSS. Nifedipine (Sigma) and veratridine (Calbiochem, Madison, WI, USA) were first dissolved in dimethyl sulphoxide (DMSO) and then diluted in PSS. The final DMSO concentration was 0.5% and this concentration of DMSO had no effect on axonal transport and neurite growth. Yohimbine, ω-conotoxin MVIIA, and nifedipine were used at the minimum concentrations necessary for maximum blocking effects in our experimental system. The pH value of all drug solutions was 7.4.

Video microscopy
Figure 1B shows the schematic diagram of the video microscopy system. Nomarski images obtained using an inverted microscope were transformed into video images with enhanced contrast using a video camera (Harpicon, Hamamatsu Photonics, Hamamatsu, Japan) and a video image processor (DVS-20, Hamamatsu Photonics). Video images were displayed on a video monitor (C1864, Hamamatsu Photonics) and stored on a tape (Sony Betacam SP, Sony, Tokyo, Japan) using a video recorder (PVW-2800, Sony).

Observation of axonal transport
Neurite axonal transport in cultured DRG neurones was observed under high-resolution video microscopy using a ×100 objective, and analysed during videotape playback. The movement of membrane-bound organelles, such as mitochondria and lysosomes (Fig. 1), and clearly visible, mobile organelles (organelle diameter ≥ 50 nm) was counted. On the monitor, the number of organelles crossing the line drawn perpendicular to the long axis of the neurites was counted for 1 min at 3 min intervals before and during pharmacological manipulation. In the control extracellular medium (PSS, pH 7.4, 37°C), the mean numbers of organelles (per minute) transported in anterograde (towards the neurite terminal) and retrograde (towards...
the cell body) transport were 43.4 (n=95) and 48.7 (n=95), respectively.

**Measurement of neurite growth rate**

Lengths of neurites arising from the cell body were measured by video microscopy using a ×25 objective. Neurite growth rate was determined from any increase in neurite length during the 1 h treatment period.

**Statistical analysis**

Data from experiments on axonal transport are expressed as mean (SD) of five experiments and reported as percentages of the baseline value (before the addition of pharmacological agents). Analysis of variance (ANOVA) was used to evaluate the statistical significance of fluctuations over time. Differences between values before and during treatment were evaluated for statistical significance using Student’s paired t-test. In addition, differences between mean values (SEM) obtained at the end of treatment were evaluated by ANOVA followed by Fisher’s protected least significant difference (PLSD) test. Rate of neurite growth is expressed as mean (SEM). The statistical significance of difference between treatment groups was determined by ANOVA followed by Fisher’s PLSD test. Differences in neurite behaviour (extension, quiescence, and retraction) between drug treatment and control were analysed by χ² test. Statistical significance was determined as P<0.05.

**Results**

**Effects on axonal transport**

Neurones incubated in PSS showed no change in the number of organelles transported in anterograde and retrograde directions during the 1 h treatment period (Fig. 2A). As shown in Figure 2B, treatment with clonidine at a concentration of 10 μM induced a significant increase in axonal transport in both anterograde and retrograde directions. Responses were rapid (within 5 min) and reached a plateau (140–150% of baseline) after 25 min from the start of treatment. Figure 3A shows the post-treatment values obtained from various concentrations of clonidine (1, 10, and 100 μM). Increases in axonal transport were concentration-dependent, with anterograde and retrograde transport after 10 and 100 μM treatment significantly higher than the control (PSS treatment) (Fig. 3A). Figure 2C shows that treatment with lidocaine at 100 μM resulted in a rapid and significant decrease in anterograde and retrograde transport. The decrease reached 50–60% of baseline after 25 min. The effects of lidocaine were concentration-dependent over the range of 10 μM to 1 mM (Fig. 3B). These values were all significantly lower than control (Fig. 3B). Simultaneous addition of clonidine reduced the inhibitory effect of lidocaine in a concentration-dependent manner (Figs 2D and 3C). Clonidine at concentrations of ≥10 μM completely abolished the effect of 100 μM lidocaine, and similarly clonidine at 100 μM completely

![Fig 2](image_url)

**Fig 2** Changes in axonal transport of organelles before and during treatment with PSS (A), 10 μM clonidine (B), 100 μM lidocaine (C), a combination of 10 μM clonidine and 100 μM lidocaine (D), and a combination of 10 μM yohimbine, 10 μM clonidine, and 100 μM lidocaine (E). The number of organelles moving in anterograde and retrograde directions was counted for 1 min at 3 min intervals by video microscopy. Horizontal axis indicates the time from the start of the treatment period (indicated by horizontal bar). Data are expressed as mean (SD) of five experiments in each treatment. *P<0.05, **P<0.005, ***P<0.0005 vs the value before treatment (ANOVA and paired t-test).
abolished the effect of 1 mM lidocaine (Figs 2a and 3c). The effects of clonidine (10 μM) on the lidocaine (100 μM) response were counteracted by simultaneous treatment with an α2-adrenoceptor antagonist yohimbine (10 μM) (Fig. 2b), confirming that the effects of clonidine were mediated by α2-adrenoceptors. These results indicate that clonidine stimulates and lidocaine inhibits axonal transport in cultured DRG neurons and that clonidine can reduce lidocaine-induced inhibition of axonal transport.

We next investigated the mechanisms by which clonidine inhibits the axonal transport block induced by lidocaine. Our previous study showed that inhibition of axonal transport by lidocaine at a low concentration (30 μM) was blocked in Ca2+-free extracellular medium, suggesting a role for Ca2+ influx. Several studies have shown that clonidine can block Ca2+ influx through N-type Ca2+ channels. We therefore hypothesized that clonidine may reduce Ca2+ channel activity activated by lidocaine. We then examined the involvement of ion channels in treated neurons, to further clarify the inhibitory effects of lidocaine. Since lidocaine is an agent that mainly blocks Na+ channels, we first tested the involvement of Na+ channels. The Na+ channel blocker TTX did not change axonal transport at a concentration of 10 μM (Fig. 4a), which sufficiently blocks action potential generation in neurons. Veratridine, an Na+ channel activator, at 100 μM decreased axonal transport to approximately 70% of baseline (Fig. 4b), and simultaneous treatment with lidocaine (100 μM) augmented the inhibitory effect of veratridine (Fig. 4c). These results suggest that the Na+ channel blocking action of lidocaine is unrelated to the lidocaine-induced inhibition of axonal transport.

We next investigated the involvement of Ca2+ channels. Simultaneous treatment with the N-type voltage-dependent Ca2+ channel blocker ω-conotoxin MVIIA (1 μM) completely blocked the inhibitory effects induced by lidocaine (Fig. 4b), whereas the L-type voltage-dependent Ca2+ channel blocker nifedipine (10 μM) had no significant effects (Fig. 4e). ω-Conotoxin MVIIA alone at 1 μM and nifedipine alone at 10 μM had no significant effect on axonal transport (data not shown). These data are summarized in Figure 5. Since N-type Ca2+ channels seem to be responsible for the block of Ca2+ influx by clonidine, clonidine and lidocaine may have opposite actions on N-type channels and hence axonal transport.

Effects on neurite growth rate

Our previous studies demonstrated that the growth rate of primary neurites arising from the cell body reflects changes in axonal transport. We therefore investigated the growth rate of primary neurites during the 1 h observation period. As shown in Table 1, 67.1% of neurites was extended in control DRG neurones (PSS treatment, total neurites n=76), whereas 27.6% of neurites showed no change in length. Only 5.3% of neurites retracted. The average rate of control neurite growth was 6.1 (1.0) μm h⁻¹ (mean [SEM]) for 1 h (Fig. 6). DRG neurones treated with 10 μM clonidine exhibited a larger number of extended neurites (80.6%, total neurites n=98, P<0.05 vs control; Table 1) and a neurite growth rate of 10.2 (1.1) μm h⁻¹, which was significantly higher than control (Fig. 6). In DRG neurones treated with 100 μM lidocaine, less neurites extended (51.36%, total neurites n=115, P<0.05) and more neurites retracted (27.8%, P<0.005) compared with control neurones (Table 1). As a result, the neurite growth rate in lidocaine-treated DRG neurones was 2.8
 Effects of clonidine and lidocaine on axons

(0.7) μm for 1 h, which was significantly lower than that in control neurones (Fig. 6). In neurones simultaneously treated with clonidine (10 μM) and lidocaine (100 μM), neurite growth rate was not different from control (Fig. 6). This was antagonized by simultaneous addition of 10 μM yohimbine (Fig. 6). We next investigated the involvement of ion channel mechanisms as suggested by axonal transport experiments. The growth rate in DRG neurones treated with 10 μM TTX was not different from control (Fig. 6). Veratridine (100 μM) reduced the growth rate and this reduction was enhanced by simultaneous treatment with lidocaine (100 μM) (Fig. 6). Neurite growth rate in neurones treated with a combination of ω-conotoxin MVIIA (1 μM) and lidocaine (100 μM) was not different from control (Fig. 6). In contrast, nifedipine (10 μM) had no effect on lidocaine-induced inhibition (Fig. 6). Reduced growth rate observed in neurones treated with drugs was associated with less extended neurites and more retracted neurites compared with control (Table 1). ω-Conotoxin MVIIA alone at 1 μM and nifedipine alone at 10 μM had no significant effect on neurite growth (data not shown). Neurite growth (Fig. 6) and axonal transport (Fig. 5) data are similar, suggesting a correlation of the two phenomena. These results suggest that clonidine stimulates and lidocaine inhibits neurite growth and that clonidine reduces the inhibition induced by lidocaine. ω-Conotoxin MVIIA-sensitive N-type Ca^{2+} channels may be involved in these phenomena.

![Fig 4](image LINK)

Changes in axonal transport of organelles before and during treatment with 10 μM TTX (A), 100 μM veratridine (B), a combination of 100 μM veratridine and 100 μM lidocaine (C), a combination of 1 μM ω-conotoxin MVIIA and 100 μM lidocaine (D), and a combination of 10 μM nifedipine and 100 μM lidocaine (E). Data are expressed as mean (sd) of five experiments in each treatment. *P<0.05, **P<0.005, ***P<0.0005 vs the value before treatment (ANOVA and paired t-test).

![Fig 5](image LINK)

Axonal transport after a 1 h treatment with PSS, 10 μM clonidine, 100 μM lidocaine, a combination of 10 μM clonidine and 100 μM lidocaine, a combination of 10 μM yohimbine, 10 μM clonidine, and 100 μM lidocaine, 10 μM TTX, 100 μM veratridine, a combination of 100 μM veratridine and 100 μM lidocaine, a combination of 1 μM ω-conotoxin MVIIA and 100 μM lidocaine, and a combination of 10 μM nifedipine and 100 μM lidocaine. Data are expressed as mean (SEM) of five experiments in each treatment. ***P<0.0005 vs corresponding direction in PSS treatment. *P<0.05, **P<0.005, ***P<0.0005 vs corresponding direction in veratridine alone treatment. Significance of the difference was examined by ANOVA and Fisher’s PLSD test.
Table 1 Neurite behaviour during drug treatment. Neurite length was measured before and after the 1 h drug treatment period. An increase, no change, or decrease in length is indicated as extension, quiescence, and retraction, respectively. n, total number of neurites; PSS, physiological salt solution; TTX, tetrodotoxin. *P<0.05, **P<0.005 vs control by χ² test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Neurite behaviour (%)</th>
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<tbody>
<tr>
<td></td>
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<td>Extension</td>
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<tr>
<td>PSS</td>
<td>76</td>
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<tr>
<td>Clonidine (10 μM)</td>
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<td>Lidocaine (100 μM)</td>
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<td>Clonidine (10 μM)+lidocaine (100 μM)</td>
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<td>Yohimbine (10 μM)+clonidine (10 μM)+lidocaine (100 μM)</td>
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<td>TTX (10 μM)</td>
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<td>Veratridine (100 μM)</td>
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<tr>
<td>Nifedipine (10 μM)+lidocaine (100 μM)</td>
<td>71</td>
<td>49.3*</td>
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Fig 6 Neurite growth rate in cultured DRG neurones. Neurite growth was measured by video microscopy in DRG neurones during a 1 h treatment with PSS, 10 μM clonidine, 100 μM lidocaine, a combination of 10 μM clonidine and 100 μM lidocaine, a combination of 10 μM yohimbine, 10 μM clonidine, and 100 μM lidocaine, 10 μM TTX, 100 μM veratridine, a combination of 100 μM veratridine and 100 μM lidocaine, a combination of 1 μM ω-conotoxin MVIIA and 100 μM lidocaine, and a combination of 10 μM nifedipine and 100 μM lidocaine. Data are expressed as mean (SEM). The numbers in the bars indicate the number of neurites examined. *P<0.05, **P<0.005 vs PSS treatment (ANOVA and Fisher’s LSD test).

Discussion

The present in vitro study indicated that clonidine is able to counteract the action of lidocaine on axonal transport, neurite growth, and neurotrophic effects. We further investigated the cellular mechanisms underlying these effects. The present study suggests that the inhibitory action of lidocaine on axonal transport and neurite growth may be mediated through activation of N-type voltage-dependent Ca²⁺ channels but not through inhibition of Na⁺ channels. These are partially supported by previous work on cultured rat DRG neurones or ND7 cells (cell line derived from the fusion of rat DRG neurones and a neuroblastoma cell line), showing that lidocaine increases intracellular Ca²⁺ concentration. This effect may result from Ca²⁺ influx rather than Ca²⁺ release from intracellular stores. In contrast, α₂-δ-adrenoceptors can block Ca²⁺ influx in neurones, mainly mediated through N-type voltage-dependent Ca²⁺ channels. Thus, the most likely hypothesis is that α₂-δ-adrenoceptors inhibit the lidocaine-induced Ca²⁺ entry through N-type channels and the subsequent increase in intracellular Ca²⁺. However, we did not confirm these mechanisms on axonal transport and neurite growth because of the lack of selective N-type channel activator. It should also be noted that reports of lidocaine-activated Ca²⁺ channels are rare. An electrophysiological study in rat hippocampal neurones demonstrated that lidocaine inhibits Ca²⁺ channels with a half-maximal inhibitory concentration (IC₅₀) of 360 μM. Moreover, an inhibitory effect of lidocaine (≥1 mM) on evoked intracellular Ca²⁺ increase was reported in rat pituitary cell line cells. In addition, radioligand binding assays indicated that lidocaine at relatively higher concentrations (IC₅₀ = 1–10 mM) interacts with L-type voltage-dependent Ca²⁺ channels in rat cortical and cerebrocortical membranes. The inconsistency between these studies and our findings is difficult to explain. However, such inconsistency was observed in the case of Na⁺ channels; although lidocaine is well known to stabilize Na⁺ channels to block conduction of action potentials in peripheral axons, recent evidence suggests that in Lymnaea central respiratory pacemaker neurones, lidocaine (0.01–1 mM) induces depolarization, increases intracellular Na⁺ concentration, and activates action potentials through activating voltage-dependent Na⁺ channels. Thus, although lidocaine directly suppresses some types of voltage-dependent Ca²⁺ channels, it could activate other types of voltage-dependent Ca²⁺ channels directly or via depolarization. Different actions (activation or inhibition) of lidocaine on Ca²⁺ channels may depend on various factors including concentration and cell type. Further studies are required to provide insights into the role of Ca²⁺ channels on lidocaine action.
As indicated in the present and previous studies, local anaesthetics including lidocaine inhibit axonal transport and related activity such as synaptic function \(^{10}\) \(^{30}\) and neurite growth.\(^{3–7}\) These may be related to clinically encountered nerve dysfunction after treatment with local anaesthetics, such as paralysis, sensory loss, and numbness. The addition of clonidine to lidocaine may prevent such undesired events caused by lidocaine. Also, the inhibitory effects of local anaesthetics on axons may possibly be utilized to inhibit aberrant sprouting of primary afferents within the dorsal horn, which may be one of the causes of neuropathic pain.\(^{31}\) In this case, clonidine may interfere with these effects of local anaesthetics on axonal function and morphology.

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