ANESTHESIOLOGY

L-bupivacaine Inhibition of Nociceptive Transmission in Rat Peripheral and Dorsal Horn Neurons

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Both the therapeutic and toxic effects of local anesthetics are influenced by the stereochemistry of these drugs
- L-bupivacaine has a favorable toxicity profile compared with D-bupivacaine, but possible differential effects on action potential conduction are poorly understood

What This Article Tells Us That Is New

- The concentration of L-bupivacaine required to block C and Aδ neurons was lower than the required concentration of D-bupivacaine
- In the spinal cord dorsal horn, L-bupivacaine appeared to be more selective for blocking nociceptive signal transmission than did D-bupivacaine

ABSTRACT

Background: Although the widely used single L-enantiomers of local anesthetics have less toxic effects on the cardiovascular and central nervous systems, the mechanisms mediating their antinociceptive actions are not well understood. The authors hypothesized that significant differences in the ion channel blocking abilities of the enantiomers of bupivacaine would be identified.

Methods: The authors performed electrophysiologic analysis on rat dorsal root ganglion neurons *in vitro* and on spinal transmissions *in vivo*.

Results: In the dorsal root ganglion, these anesthetics decreased the amplitudes of action potentials. The half-maximum inhibitory concentrations of D-enantiomer D-bupivacaine were almost equal for Aβ (29.5 μM), Aδ (29.7μM), and C (29.8 μM) neurons. However, the half-maximum inhibitory concentrations of L-bupivacaine was lower for Aδ (19.35 μM) and C (19.5 μM) neurons than for A β (79.4 μM) neurons. Moreover, D-bupivacaine almost equally inhibited tetrodotoxin-resistant (mean ± SD: 15.8 ± 10.9% of the control, n = 14, $P < 0.001$) and tetrodotoxin-sensitive (15.4 ± 15.6% of the control, n = 11, $P = 0.004$) sodium currents. In contrast, L-bupivacaine suppressed tetrodotoxin-resistant sodium currents (26.1 ± 19.5% of the control, n = 18, $P < 0.001$) but not tetrodotoxin-sensitive sodium currents (74.5 ± 18.2% of the control, n = 11, $P = 0.477$). In the spinal dorsal horn, L-bupivacaine decreased the area of pinch-evoked excitatory postsynaptic currents (39.4 ± 11.3% of the control, n = 7, $P < 0.001$) but not touch-evoked responses (84.2 ± 14.5% of the control, n = 6, $P = 0.826$). In contrast, D-bupivacaine equally decreased pinch- and touch-evoked responses (38.8 ± 9.5% of the control, n = 6, $P = 0.001$, 42.9 ± 11.8% of the control, n = 6, $P = 0.013$, respectively).

Conclusions: These results suggest that the L-enantiomer of bupivacaine (L-bupivacaine) effectively inhibits noxious transmission to the spinal dorsal horn by blocking action potential conduction through C and Aδ afferent fibers.

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The essential role of the voltage-gated sodium channel involved in acute and chronic pain is emphasized by the clinical use of several drugs in analgesic therapy that have sodium channel-blocking properties, including local anesthetics, antidepressants, and anticonvulsants.[1–4] Recently, the newly developed single stereoisomers have attracted attention as new therapeutics. Most voltage-gated sodium channel blockers used in anesthesiology, such as bupivacaine, have an asymmetric carbon and are still used primarily as racemic mixtures (1:1 mixture of D and L enantiomers).[5–8] It was reported that clinically used voltage-gated sodium channel blockers such as bupivacaine have cardio- and neurotoxic effects, and these effects are primarily dependent on the D-enantiomer.[5,9] However, among voltage-gated sodium channel blockers, the L-enantiomers often have favorable biologic properties. The pharmacodynamic and pharmacokinetic profiles of L-enantiomers are similar to those of D-enantiomers, but *in vitro* and *in vivo* studies have shown that L-enantiomers such as L-bupivacaine and ropivacaine are less toxic to the cardiac and central nervous system.[5,8,10,11] Clinical data suggest that the margin of safety is higher for L-enantiomers than for D-enantiomers. A large margin of safety is necessary for further expanding the application of regional anesthesia. Nevertheless, it is yet to be explored how L-enantiomers affect noxious and innocuous inputs to the spinal dorsal horn.

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We hypothesized that significant differences in the ion channel blocking abilities of the enantiomers of bupivacaine would be identified. Thus, the aim of this study was to investigate how L-bupivacaine and D-bupivacaine could effect the primary sensory nerves on rat dorsal root ganglion neurons and synaptic transmissions in spinal dorsal horn neurons. In the present study, we performed intracellular as well as whole cell patch-clamp recordings from dorsal root ganglion neurons *in vitro*. We investigated whether L-bupivacaine acts on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channel to selective inhibition induced by dorsal root stimuli in dorsal root ganglion neurons. Moreover, we used *in vivo* whole cell patch-clamp recordings from superficial and deep neurons in spinal dorsal horn and examined whether bath application of L-bupivacaine and D-bupivacaine acts on the excitatory synaptic transmissions induced by peripheral nociceptive and innoxious stimuli in adult male and female rats. This new information extends our understanding of the selective inhibition of L-bupivacaine at the spinal cord level in peripheral nociceptive and innoxious stimuli.

Materials and Methods

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Kyushu University (Fukuoka, Japan), the National Institutes of Natural Sciences (Okazaki, Japan), and Toyama University (Toyama, Japan). Experiments involving animals were performed in accordance with the institutional guidelines for animal experiments and in accordance with the ethical guidelines of the International Association for the Study of Pain. All experiments were performed in the afternoon between 9:00 AM and 7:00 PM. All male and female rats were randomly assigned to the electrophysiologic experiment and were tested in sequential order. All efforts were made to minimize animal suffering and the number of animals used for the studies.

Intracellular Recordings from Isolated Rat Dorsal Root Ganglion Neurons

We used 31 rats in this experiment. Male Sprague-Dawley rats (6 to 10 weeks old, Kyudo, Japan), weighting 200 to 330 g, were used for the intracellular recordings. The methods used for obtaining the dorsal root ganglion were similar to those described previously.[12–14] Briefly, rats were anesthetized with urethane (1.2 to 1.5 g/kg intraperitoneally) and decapitated immediately, followed by lumbar laminectomy. L4–L6 dorsal root ganglia were isolated from the rats together with a proximal dorsal root with a length of 9–16 mm. The isolated dorsal root ganglion was then submerged in Krebs solution (containing [in mM] 117 sodium chloride, 3.6 potassium chloride, 2.5 calcium chloride, 1.2 magnesium chloride, 1.2 sodium dihydrogen phosphate, 11 glucose, and 25 sodium bicarbonate) equilibrated with 95% O_2–5% CO_2, maintained at 36 ± 1°C and set in a recording chamber. Antidromic stimulation (0.1 ms duration) of the central end of the dorsal root was performed with a suction electrode, as described in a previous study,[12] where the threshold stimulus intensity for eliciting an action potential was monitored on an isolator (ss-202J; Nihon Kohden, Japan) equipped with a digitized output. Intracellular recordings of the action potentials from dorsal root ganglion neurons were made with glass microelectrodes filled with 4 M potassium acetate and having a direct current tip resistance of 50–100 MΩ. The action potentials were evoked by stimulating the dorsal root at a frequency of 0.2 Hz. The stimulus strength used in the subsequent experiments was 1.2 times the threshold for action potential generation. Signals were acquired with a high-input impedance bridge amplifier (Axoclamp 2B; Molecular Devices, USA) and were monitored on a digital oscilloscope (VC-11; Nihon Kohden, Japan). Artifacts were minimized with low-pass (1,000 Hz) or notch (250 Hz) filters using the pCLAMP program (Molecular Devices). Data from neurons with resting membrane potentials more negative than −55 mV and with action potential amplitudes larger than 60 mV were included in the present study.[12–14] The antidromic action potentials were analyzed with respect to their conduction velocity, action potential duration, and threshold stimulus intensity. The action potential duration was determined as the duration at half of the peak amplitude of the action potential. In some experiments, antidromic action potentials were elicited at frequencies higher than 0.2 Hz to determine whether the action potential was produced one-to-one in response to the stimulation of the dorsal root.

Whole Cell Patch-clamp Recordings from Rat Dorsal Root Ganglion Neurons

We used 11 rats in this experiment. Dorsal root ganglion neurons were isolated as described previously.[15] Briefly, male young and adult Sprague-Dawley rats (3–9 weeks old, 70–300 g, Japan SLC, Japan) were anesthetized with urethane (1.2 to 1.5 g/kg intraperitoneally) and decapitated immediately, followed by lumbar laminectomy. L4–L6 dorsal root ganglia were isolated from the rats. The dorsal root ganglia were incubated at 37°C for 45–60 min in Tyrode solution (for composition, see below) containing 2 mg/ml collagenase (Type 1; Sigma, USA) and 5 mg/ml trypsin.[15] After washing three times with fresh, enzyme-free Tyrode solution, single neuronal cells were obtained by gentle agitation in the Tyrode solution through a small-bore Pasteur pipette on a 35-mm culture dish (Primaria 3801; Becton Dickinson, USA) at room temperature (20 ± 2°C).

Whole cell current clamp recordings were made at room temperature (20 ± 2°C). Patch pipettes with a resistance of 0.5–2.0 MΩ were pulled from glass capillaries using a micropipette puller (P-97; Sutter Instrument, USA). Membrane currents were amplified using patch-clamp amplifier (EPC-9; HEKA Electronic, Germany). Because

the resting membrane potential of dorsal root ganglion neurons has been reported to range from −60 to −80 mV,[16,17] the effects of stereoisomers on both types of sodium currents were further investigated in cells held at −80 mV. Therefore, whole cell membrane sodium currents of DRG neurons were evoked from −80 mV by stepping to 0 mV for 50 ms.[15] Series resistances were monitored online, and the uncompensated series resistance was typically less than 10 MΩ. A series resistance compensation of 70 to 80% was used during recordings from the dorsal root ganglion neurons. Currents were digitized at 100 kHz after low-pass filtering at 10 kHz. Data were acquired using the PULSE program (HEKA, version 8.54). Pulse Fit (HEKA, version 8.54) was used to analyze the obtained data.

The Tyrode solution contained (in mM) 140 sodium chloride, 4.0 potassium chloride, 2.0 magnesium chloride, 10 glucose, and 10 HEPES. It was adjusted to a pH of 7.4 with sodium hydroxide. The pipette solution contained (in mM) 110 cesium sulfate, 5 tetraethylammonium chloride, 0.5 calcium chloride, 2 magnesium chloride, 5 EGTA, 5 HEPES, and 5 magnesium-ATP; it was adjusted to a pH of 7.2. The external solution contained (in mM) 25 sodium chloride, 75 tetramethylammonium chloride, 20 tetraethylammonium chloride, 5.0 cesium chloride, 1.8 calcium chloride, 1.0 magnesium chloride, 25 glucose, and 5 HEPES, and was adjusted to a pH of 7.4 with tetraethylammonium hydroxide solution. Cadmium (cadmium chloride, 100 μM) was added to the external solution to give a final concentration of 100 μM, sufficient to block calcium channel currents.

In Vivo Whole Cell Patch-clamp Recordings from Rat Spinal Dorsal Horn Neurons

We used 18 rats (12 male, 6 female) in this experiment. The methods used for the *in vivo* whole cell patch-clamp recording were similar to those described previously.[18,19] Briefly, male and female Sprague-Dawley rats (6 to 10 weeks old, 180 to 330 g, Japan SLC) were anesthetized with urethane (1.2 to 1.5 g/kg intraperitoneally). Urethane produces a long-lasting steady level of anesthesia which does not require administration of supplemental doses except in a few cases. Oxygen was supplied through a nose cone. To prevent pneumothorax, artificial ventilation was not performed, as reported previously.[18,19] An additional dose of urethane was given during surgery and the data collection period every time a withdrawal reflex to the noxious stimuli was noted. The rectal temperature was kept at 37°–38°C using a heating pad. A thoracolumbar laminectomy was performed to expose the L1–L6 lumbar vertebrae. The animal was subsequently placed in a stereotaxic apparatus. After removing the dura and cutting the arachnoid membrane to make a window large enough to insert a patch electrode into the dorsal horn, the surface of the spinal cord was irrigated with 95% O_2-5% CO_2-equilibrated Krebs solution (10 to 15 ml/min), using glass pipettes at 37 ± 1°C.

A whole cell voltage-clamp technique was applied to substantia gelatinosa neurons. The electrode had a tip resistance of 10 to 15 MΩ and was filled with a potassium gluconate-based solution (in mM: 136 potassium gluconate, 5 potassium chloride, 0.5 calcium chloride, 2 magnesium chloride, 5 EGTA, 5 HEPES, and 5 magnesium-ATP; pH 7.2) to examine excitatory postsynaptic currents at a holding potential of −70 mV. A giga-ohm seal was then formed with neurons at a regular depth of 20 to 350 μm. These cells were within the superficial dorsal horn (20 to 150 μm, lamina I–II) and deep dorsal horn (150 to 350 μm, lamina III–IV). They were accessed using their depth from the surface of the spinal cord in slices obtained from the same spinal level in same-age rats. In some instances, the location and morphological features of the substantia gelatinosa and deep dorsal horn neurons were also confirmed by the injection of neurobiotin. The mechanical stimuli were induced by pinching the skin folds with a toothed forceps and brushing the surface of the skin or the hairs in the ipsilateral hind limb.[18,20,21] The limb point most sensitive to stimulation was different for each cell tested. All the neurons examined had membrane potentials more negative than −55 mV. Series resistance was assessed according to the response to a 5-mV hyperpolarizing step. This value was monitored during the recording session, and data were rejected if values changed by more than 15%. Signals were acquired with a patch-clamp amplifier (Axopatch 700B; Molecular Devices). The data were digitized with an analog-to-digital converter (Digidata 1400A; Molecular Devices), stored on a personal computer with a data acquisition program (Clampex version 10.0; Molecular Devices), and analyzed with a special software package (Clampfit version 10.0; Molecular Devices). The peak amplitudes were not determined, because multiple summations resulting from the high frequency bursting of excitatory postsynaptic currents made it difficult to obtain an accurate estimation. Therefore, we analyzed the change in area surrounded by the baseline and border of the excitatory postsynaptic currents.

Application of Drugs

Drugs were dissolved in Krebs solution, and the solution was applied through perfusion *via* a three-way stop-cork. The external solution, which contained drugs of known concentrations, was perfused without an alteration in the perfusion rate and temperature. The drugs used in this study were L-bupivacaine hydrochloride, ropivacaine hydrochloride, D-bupivacaine hydrochloride, tetrodotoxin (Wako Pure Chemical Industries, Japan), and 6-cyano-7-nitroqui-noxaline-2,3-dione (Tocris Cookson, USA).

Statistical Analysis

All data values were expressed as mean ± SD. No statistical power calculation was conducted before the study, but the sample sizes were based on our previous experience with

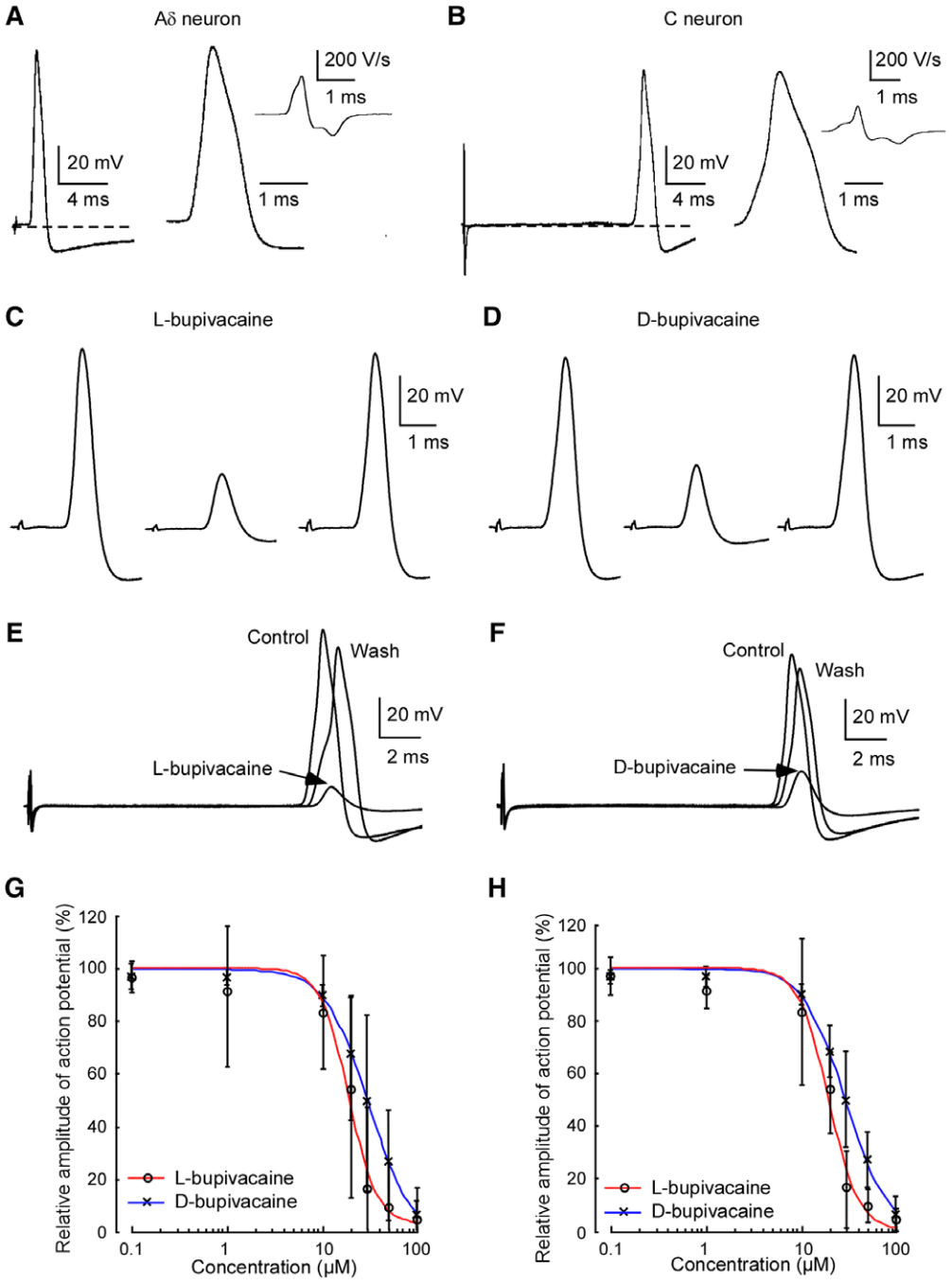

Fig. 1. Effects of L-bupivacaine (50 μM) and D-bupivacaine (50 μM) on action potentials of Aδ and C neurons in the dorsal root ganglion. (*A*) A typical trace of action potentials generated by Aδ-type neurons at a frequency of 0.2 Hz. (*Inset, right*) The Aδ-type action potential shows this maximum rate of depolarization. (*B*) A typical trace of action potentials generated by C-type neurons at a frequency of 0.2 Hz. (*Inset, right*) The C-type action potential shows this maximum rate of depolarization. (*C*) L-bupivacaine decreased the amplitude of action potentials generated by Aδ-type neurons. (*D*) D-bupivacaine decreased the amplitude of action potentials generated by Aδ-type neurons. (*E*) L-bupivacaine decreased the amplitude of action potentials generated by C-type neurons. (*F*) D-bupivacaine decreased the amplitude of action potentials generated by C-type neurons. (*G*) The amplitude of action potentials generated by Aδ fibers as a function of the concentrations of L-bupivacaine (○, *red line*) and D-bupivacaine (×, *blue line*). A 50% reduction in the relative amplitudes of action potentials were observed after L-bupivacaine and D-bupivacaine administration (19.3 μM and 29.7 μM, respectively). Data are presented as mean ± SD. (*H*) The amplitude of action potentials generated by C fibers as a function of the concentrations of L-bupivacaine (○, *red line*) and D-bupivacaine (×, *blue line*). A 50% reduction in the relative amplitudes of action potentials were observed after L-bupivacaine and D-bupivacaine administration (19.5 μM and 29.8 μM, respectively). Data are presented as mean ± SD.

similar studies and were similar to those generally used in the field and were selected based on the available data. No outliers were excluded, but data were not collected when cell series resistance changed by more than 15%. All data were verified for normality of variance using Kolmogorov–Smirnov tests before analysis. Statistical significance in these studies were determined as $P < 0.05$ using paired or independent unpaired two-tailed Student's *t* test. Kolomogorov-Smirnov test was also used for comparing two cumulative distributions of synaptic responses. Randomization and blinding methods were not used to assign subjects. In all cases, n refers to the number of neurons studied.

Results

L-bupivacaine Selectively Suppresses the Action Potentials in C and Aδ Fibers but Not Aβ Fibers

After the classification of the dorsal root ganglion neurons (table 1),[22–25] we examined the effects of L-bupivacaine and D-bupivacaine on the action potentials from three subtypes (Aβ, Aδ, and C fibers) of dorsal root ganglion neurons (fig. 1, A and B and fig. 2A). In Aδ and C neurons, L-bupivacaine (50 μM) significantly decreased the amplitude of the action potentials without affecting resting membrane potentials (Aδ neuron; 17.9 ± 15.7% of that of the control, n = 20, $P < 0.001$, fig. 1C; C neuron; 9.3 ± 7.3% of that of the control, n = 12, $P = 0.001$, fig. 1E). Similarly, D-bupivacaine (50 μM) significantly reduced the amplitude of the action potentials (Aδ neuron; 32.9 ± 47.8% of that of the control, n = 17, $P < 0.001$, fig. 1D; C neuron; 27.2 ± 11.6% of that of the control, n = 11, $P = 0.022$, fig. 1F). L-bupivacaine and R-bupivacaine reduced the amplitude of the action potentials in a dose-dependent manner (ranging from 10 to 100 μM; fig. 1, G and H, and fig. 2D). In both Aδ and C neurons, the dose–response curves suggest that the effect of L-bupivacaine was more selective and potent compared with D-bupivacaine (fig. 1, G and H). In Aδ neurons, a 50% reduction in the relative amplitudes of action potentials was observed after L-bupivacaine and D-bupivacaine administration (19.3 μM and 29.8 μM, respectively). In C neurons, L-bupivacaine and D-bupivacaine were concentration-dependent with half-maximum inhibitory concentrations (IC_{50}) of 19.5 μM and 29.8 μM, respectively.

In Aβ neurons, L-bupivacaine (50 μM) slightly decreased the amplitude of action potentials (82.6 ± 19.2% of that of the control, n = 23, $P = 0.373$, half-maximum inhibitory concentrations = 79.4 μM, fig. 2B). On the other hand, D-bupivacaine caused a reduction in the amplitudes of action potentials without affecting resting membrane potentials in Aβ neurons (27.2 ± 15.3% of the control, n = 19, $P < 0.001$, half-maximum inhibitory concentrations = 29.5 μM, fig. 2C). Taken together, these results suggest that L-bupivacaine selectively inhibits Aδ and C and requires a high dose to suppress Aβ fiber.

The Inhibitory Effect of a L-enantiomer, Ropivacaine, on Action Potentials in Dorsal Root Ganglion Neurons

The effect of another L-enantiomer, ropivacaine (50 μM) significantly decreased the amplitude of the action potentials generated by Aδ and C neurons (Aδ neuron; 29.1 ± 18.7% of that of the control, n = 18, $P < 0.001$, fig. 3A; C neuron; 22.1 ± 20.8% of that of the control, n = 12, $P = 0.0004$, fig. 3B). However, ropivacaine also has less of an effect on Aβ fibers compared with D-bupivacaine (64.5 ± 27.2% of that of the control, n = 18, $P = 0.029$, fig. 3C). Differences in inhibition effect of L-bupivacaine between Aδ and Aβ neurons and between C and Aβ neurons were observed. However, no differences were observed between Aδ and Aβ neurons when other stereoisomers were administered (fig. 3D).

Sodium Currents in Dorsal Root Ganglion Neurons

The short-lasting (activated and subsequently inactivated within 5 ms) sodium currents were almost completely blocked by tetrodotoxin (0.2 μM), indicating that it was generated as a result of the activation of tetrodotoxin-sensitive sodium channels (fig 4A). Because the long-lasting (persisted for more than 20 ms) sodium currents were resistant to or only partly blocked by tetrodotoxin, the long-lasting sodim current was considered to be generated by either tetrodotoxin-resistant sodium channels alone or a mixture

Table 1. Comparison of Electrophysiologic Properties of Dorsal Root Ganglion Neurons

	Resting Membrane Potential (mV)	Conduction Velocity (m/s)	Duration of Action Potential (ms)	Threshold Stimulus Intensity (mA)	Maximum Rate of Depolarization (V/s)
Aβ neurons (n = 58)	–64 ± 6.9 (–72 to –55)	15.0 ± 3.8 (12.1–22.7)	0.35 ± 0.08 (0.21–0.47)	0.11 ± 0.08 (0.02–0.4)	312 ± 84 (251–441)
Aδ neurons (n = 61)	–66 ± 7.0 (–80 to –57)	7.5 ± 3.1 (2.3–11.1)	0.64 ± 0.16 (0.46–0.91)	0.55 ± 1.41 (0.08–4.8)	251 ± 70 (155–385)
C neurons (n = 76)	–70 ± 7.0 (–80 to –64)	0.6 ± 0.9 (0.4–1.0)	1.91 ± 1.13 (0.89–4.55)	2.34 ± 2.53 (0.6–5.7)	181 ± 61 (111–298)

Values are shown as means ± SD. The range of values for each parameter is given in parentheses shown below the mean. Action potentials were evoked at a frequency of 0.2 Hz. Threshold stimulus intensity was determined with a stimulus of 0.1 ms. Duration of action potential measured at its half-maximal amplitude.

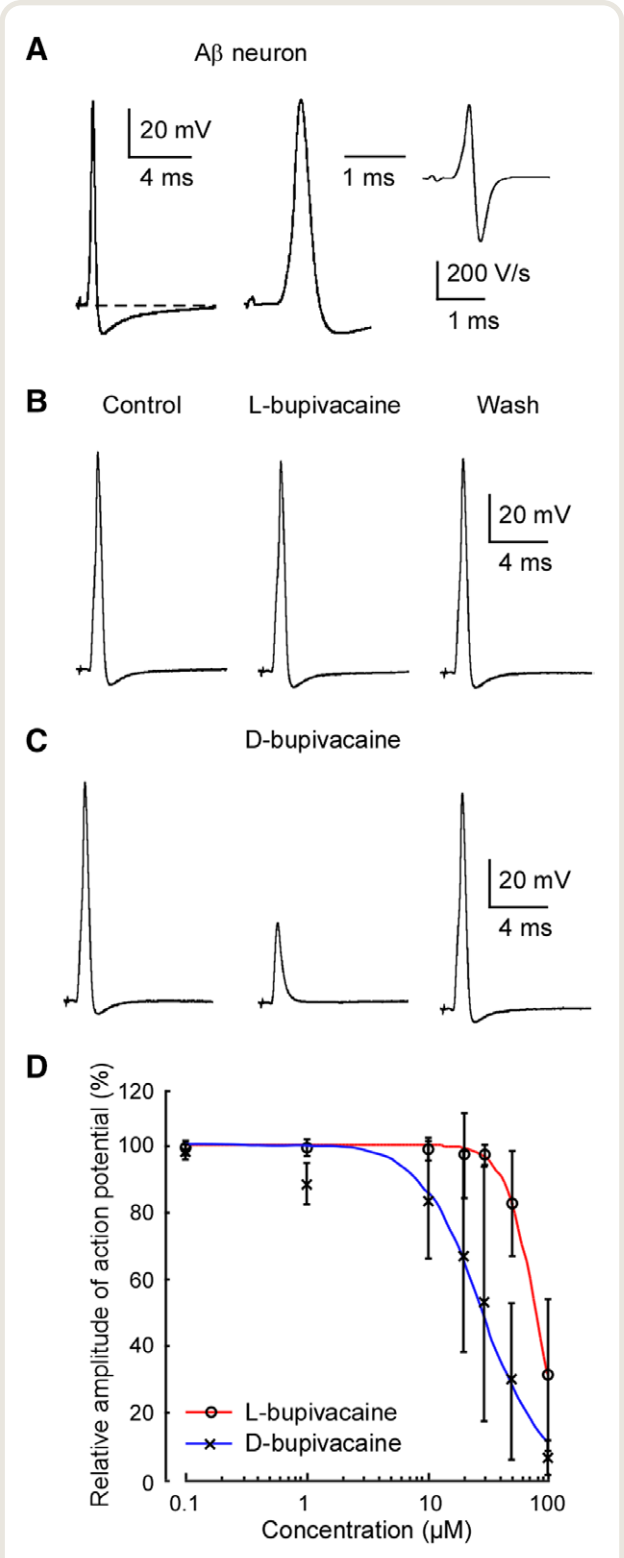

Fig. 2. Effects of L-bupivacaine (50 μM) and D-bupivacaine (50 μM) on action potentials of Aβ neurons in the dorsal root ganglion. (*A*) A typical trace of action potentials generated by Aβ-type neurons at a frequency of 0.2 Hz. (*Inset, right*) The Aβ-type action potential shows this maximum rate of depolarization. (*B*) L-bupivacaine did not decrease the amplitude of action potentials in Aβ neurons. (*C*) D-bupivacaine decreased the amplitude of action potentials in Aβ neurons. (*D*) The amplitude of action potentials generated by Aβ fibers as a function of the concentrations of L-bupivacaine (○, *red line*) and D-bupivacaine (×, *blue line*). Half-maximum inhibitory concentrations for L-bupivacaine (79.4 μM) in Aβ fibers was higher than that for D-bupivacaine (29.5 μM). Data are presented as mean ± SD.

of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels (fig. 4B). The diameters of dorsal root ganglion neurons exhibited either the short-lasting or the long-lasting sodium current (fig. 4C). Short-lasting currents were recorded from 47.7% of neurons with diameters of more than 30 μm, whereas long-lasting currents were recorded from 52.6% of neurons with diameters of less than 30 μm. Among the neurons which generated the long-lasting currents, 60.5% were unaffected by tetrodotoxin.

The Inhibitory Effect of Stereoisomers on Sodium Currents

The effects of L-bupivacaine and D-bupivacaine on typical tetrodotoxin-sensitive and tetrodotoxin-resistant sodium currents in dorsal root ganglion neurons were examined (fig. 4, D and E). L-bupivacaine (50 μM) did not decrease the amplitude of tetrodotoxin-sensitive sodium currents (74.5 ± 18.2% of that of the control, n = 11, P = 0.477; fig. 4, D and F). Notably, L-bupivacaine (50 μM) significantly decreased the amplitude of tetrodotoxin-resistant sodium currents (26.1 ± 19.5% of that of the control, n = 18, P = 0.001; fig. 4, E and F). On the other hand, D-bupivacaine (50 μM) decreased the amplitudes of both tetrodotoxin-sensitive and tetrodotoxin-resistant sodium currents (15.4 ± 15.6%, n = 11, P = 0.004 and 15.8 ± 10.9%, n = 14, P < 0.001 of that of the control, respectively; fig. 4, D–F). These results suggest that L-bupivacaine selectively abolishes the tetrodotoxin-resistant sodium channels without affecting the tetrodotoxin-sensitive sodium channels compared to D-bupivacaine (P = 0.001).

Effects of L-bupivacaine and D-bupivacaine on Responses to Pinch and Touch Stimuli *In Vivo*

Finally, we performed *in vivo* whole cell patch-clamp recordings (fig. 5) from 11 substantia gelatinosa and 9 deep dorsal horn neurons (resting membrane potential in substantia gelatinosa neurons: −63.9 ± 3.6 mV; in deep dorsal horn neurons: −63.4 ± 4.5 mV).[19] Under voltage-clamp conditions holding membrane potential at −70 mV, substantia gelatinosa and deep dorsal horn neurons exhibited spontaneous excitatory postsynaptic currents with average amplitudes of 19.4 ± 12.3 pA in substantia gelatinosa neurons and 19.1 ± 5.4 pA in deep dorsal horn neurons, respectively, and frequencies of 15.8 ± 7.3 Hz in substantia gelatinosa neurons and 17.5 ± 4.8 Hz in deep dorsal horn neurons, respectively.

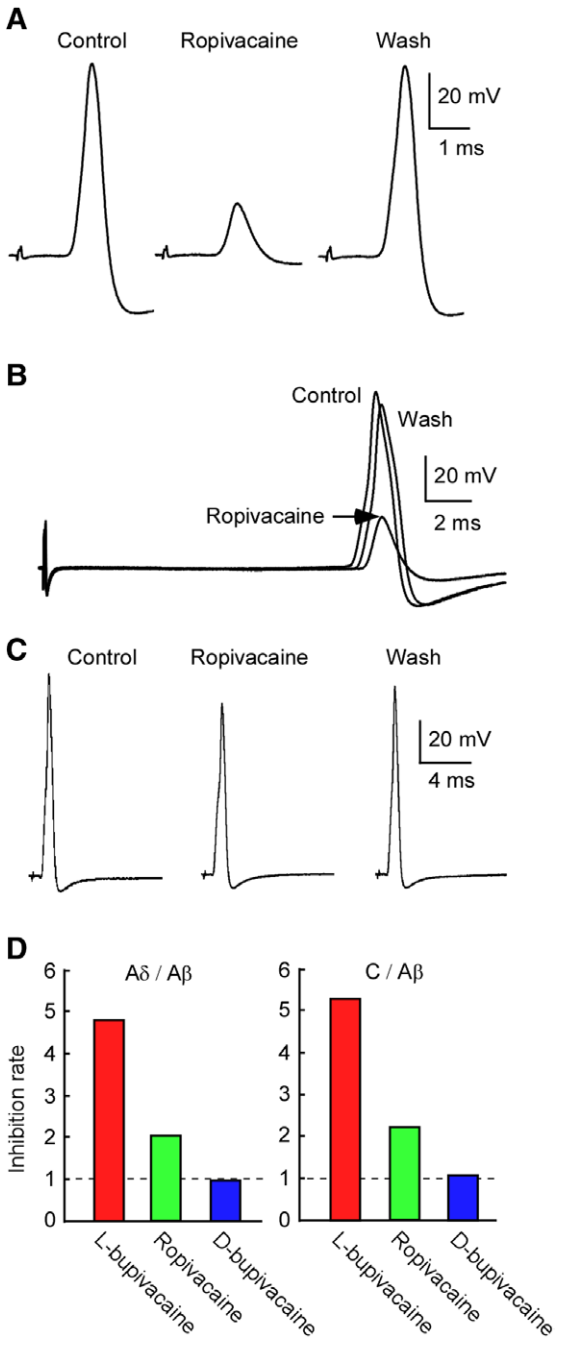

Fig. 3. Effect of the L-enantiomer ropivacaine (50 μM) on action potentials. (*A*) Ropivacaine decreased the amplitude of action potentials in Aδ neurons. (*B*) Ropivacaine decreased the amplitude of action potentials in C neurons. (*C*) Ropivacaine did not decrease the amplitude of action potentials in Aβ neurons. (*D*) Ratios of the amplitude of action potentials of Aδ/Aβ (*left*) and C/Aβ (*right*) at a concentration of 50 μM for the different local anesthetics.

The pinch and touch stimuli elicited a barrage of excitatory postsynaptic currents in the two types of neurons (fig. 5). The pinch-evoked excitatory postsynaptic currents were completely suppressed by either bath application of tetrodotoxin (0.5 μM) or 6-cyano-7-nitroqui-noxaline-2,3-dione (10 μM; fig. 5, C and D). Touch-evoked excitatory postsynaptic currents were similarly suppressed by either tetrodotoxin or 6-cyano-7-nitroqui-noxaline-2,3-dione as well.[18] Stimulating the contralateral hind limb did not elicit any synaptic changes.[18]

When L-bupivacaine (50 μM) or D-bupivacaine (50 μM) were applied to the surface of the spinal cord, the pinch-evoked excitatory postsynaptic currents were decreased in substantia gelatinosa neurons. The area decreased to 39.4 ± 11.3% of that of the control when L-bupivacaine (n = 7, $P = 0.001$) was administered and to 38.8 ± 9.5% of that of the control when D-bupivacaine (n = 6, $P = 0.001$) was administered (fig. 6, A, B, and E–G). However, the bath application of L-bupivacaine (50 μM) did not significantly decrease the area of the excitatory postsynaptic currents evoked by innocuous (brush) mechanical stimuli for deep dorsal horn neurons (the area of the excitatory postsynaptic currents decreased to 84.2 ± 14.5% of that of the control [n = 6, $P = 0.826$; fig. 6, C, E, and G]). On the other hand, the bath application of D-bupivacaine (50 μM) decreased the brush-evoked excitatory postsynaptic currents (42.9 ± 11.8% of that of the control, n = 6, $P = 0.013$; fig. 6, D, F, and H) for all deep dorsal horn neurons. Importantly, L-bupivacaine hardly suppressed the brush-evoked excitatory postsynaptic currents compared with D-bupivacaine ($P < 0.001$; fig. 6H).

We next investigated whether the actions of L-bupivacaine or D-bupivacaine were presynaptic or postsynaptic by analyzing miniature excitatory postsynaptic currents. In the presence of tetrodotoxin (0.5 μM), L-bupivacaine (50 μM) and D-bupivacaine (50 μM) had no significant effect on the frequency and amplitude of the miniature excitatory postsynaptic currents. The administration of L-bupivacaine resulted in a decrease in the frequency to 96.3 ± 6.4% of that of the control (n = 6, $P = 0.766$) and to 96.1 ± 7.3% of that of the control when D-bupivacaine (n = 6, $P = 0.612$) was administered (fig. 6I). The amplitude of the miniature excitatory postsynaptic currents also decreased to 96.8 ± 8.3% of that of the control when L-bupivacaine was administered (n = 6, $P = 0.763$) and to 95.3 ± 9.3% of that of the control when D-bupivacaine was applied (n = 6, $P = 0.727$; fig. 6I). To study the sex difference of the local anesthetics, we also performed the *in vivo* recording from adult female rats (fig. 7). When L-bupivacaine (50 μM) or D-bupivacaine (50 μM) were applied to the surface of the spinal cord, the pinch evoked excitatory postsynaptic currents were decreased in substantia gelatinosa neurons (L-bupivacaine; decreased to 38.0 ± 11.6% of the control, n = 6, $P = 0.001$, fig. 7, A and E; D-bupivacaine; decreased to 37.4 ± 11.4% of that of the control, n = 6, $P = 0.002$, fig. 7, B and E). Similar to the results in male rats, the application of L-bupivacaine (50 μM) did not decrease the area of innocuous (brush) mechanical stimuli

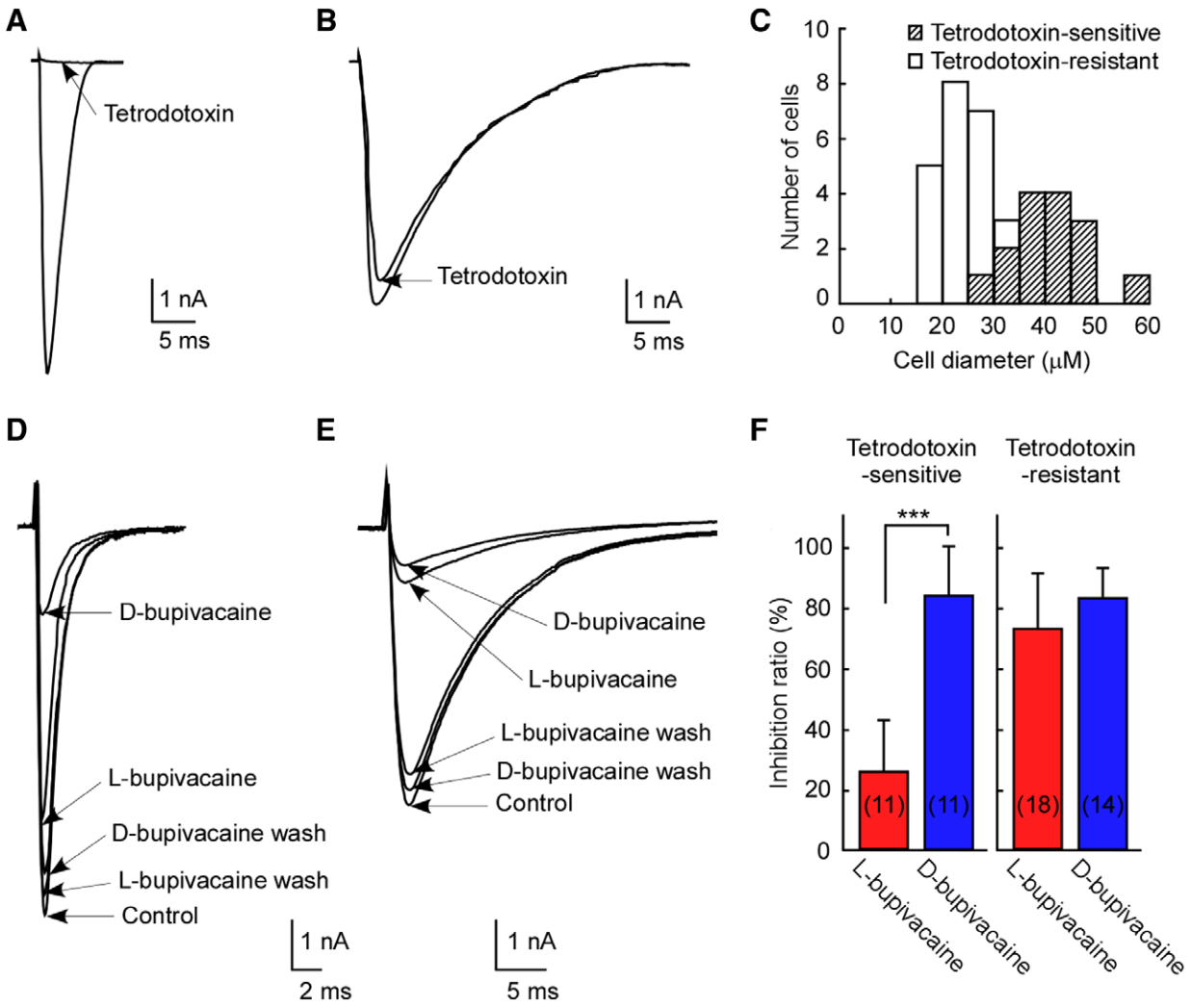

Fig. 4. Effect of L-bupivacaine (50 μM) and D-bupivacaine (50 μM) on sodium currents in dorsal root ganglion neurons. (*A*) A typical trace of tetrodotoxin-sensitive sodium current. Short-lasting currents were completely blocked by 0.2 μM tetrodotoxin, which indicates that these currents are generated as a result of the activation of tetrodotoxin-sensitive sodium channels. (*B*) A typical trace of tetrodotoxin-resistant sodium current. Long-lasting currents were only partly blocked by 0.2 μM tetrodotoxin, which indicates that these currents are generated as a result of activation of a mixture of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. (*C*) Histogram of the distribution of the diameters of dorsal root ganglion neurons that generated tetrodotoxin-sensitive (shaded columns) and tetrodotoxin-resistant (open columns) sodium currents. (*D*) L-bupivacaine slightly decreased the amplitude of tetrodotoxin-sensitive sodium currents. However, D-bupivacaine dramatically decreased the amplitude of tetrodotoxin-sensitive sodium currents. (*E*) L-bupivacaine and D-bupivacaine mostly decreased the amplitude of tetrodotoxin-resistant sodium currents. (*F*) Summary of the blocking effects of L-bupivacaine and D-bupivacaine on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium currents. L-bupivacaine selectively abolishes the tetrodotoxin-resistant sodium channels without affecting the tetrodotoxin-sensitive sodium channels compared to D-bupivacaine (***$P < 0.001$). Data presented as mean ± SD.

evoked excitatory postsynaptic currents in deep dorsal horn neurons. The area of the excitatory postsynaptic currents decreased to 87.9 ± 16.5% of that of the control (n = 5, $P = 0.235$, fig. 7, C and F). On the other hand, the application of D-bupivacaine (50 μM) decreased the area of the brush-evoked excitatory postsynaptic currents to 43.1 ± 8.6% of that of the control (n = 5, $P < 0.001$, fig. 7, D and F) in all deep dorsal horn neurons. L-bupivacaine (50 μM) and D-bupivacaine (50 μM) had no significant effect on the frequency (L-bupivacaine; 99.6 ± 95.3% of the control, $P = 0.974$, n = 6, D-bupivacaine; 98.9 ± 5.1% of the control, n = 6, $P = 0.973$, fig. 7, G and H) and amplitude (L-bupivacaine; 103.6 ± 5.1% of the control, n = 6, $P = 0.803$, D-bupivacaine; 99.3 ± 5.5% of the control, n = 6, $P = 0.955$; fig. 7, G and H) of the miniature excitatory postsynaptic currents.

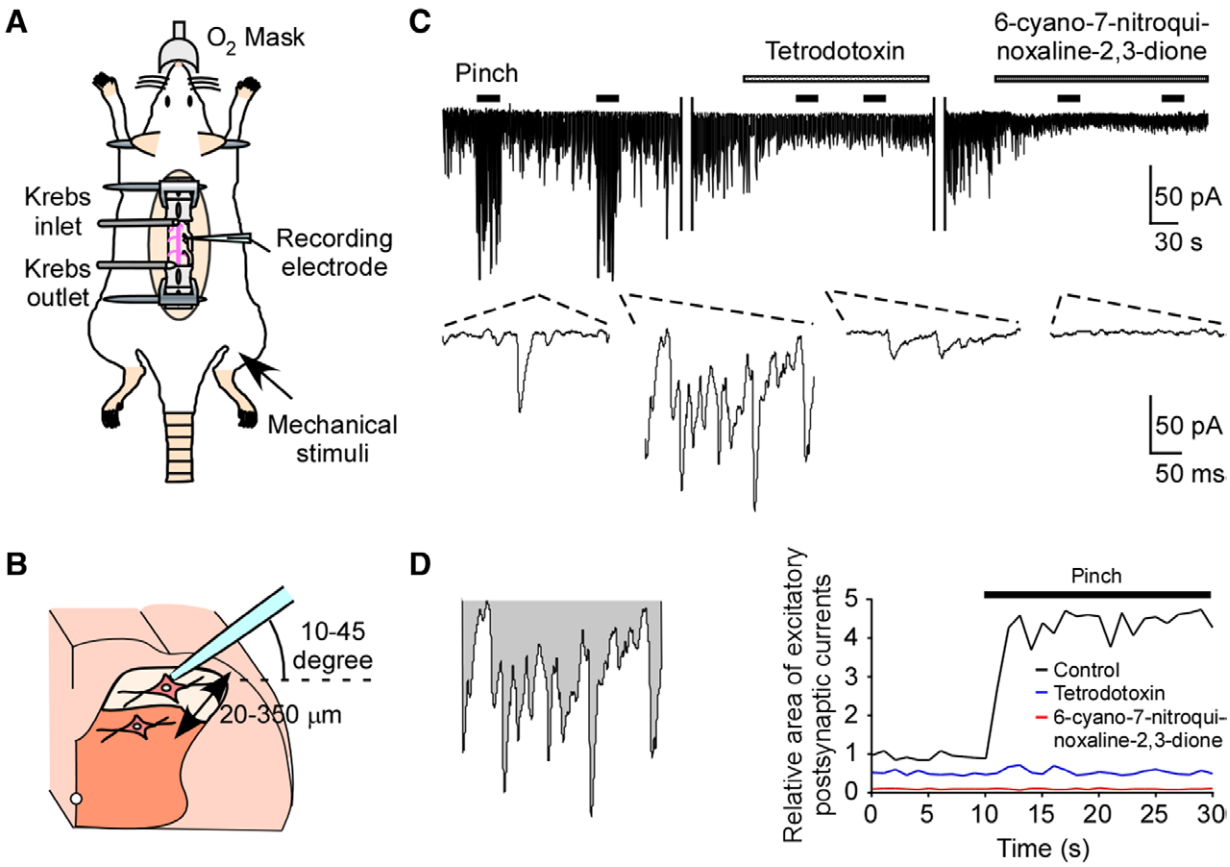

Fig. 5. Schematic diagram of rat preparation for *in vivo* experiment and analysis of responses to the noxious stimuli. (*A*) The lumbar vertebrae were exposed by laminectomy, and the surface of the spinal cord was superfused continuously with Krebs solution. (*B*) Schematic diagrams of a transverse section through a spinal cord and a recording electrode. Recordings were made from substantia gelatinosa and deep dorsal horn neurons at depths ranging from 20 to 350 μm (shown by *arrows*) from the surface of the spinal cord. (*C*) The amplitude and frequency of excitatory postsynaptic currents during the pinch stimuli were completely decreased in the presence of tetrodotoxin (0.5 μM) and 6-cyano-7-nitroqui-noxaline-2,3-dione (10 μM). (*D*) Schematic diagram showing the area of an excitatory postsynaptic currents (*left*). Analysis of area surrounded by the baseline and border of an excitatory postsynaptic current. The area of pinch-evoked excitatory postsynaptic currents was completely decreased in the presence of tetrodotoxin and 6-cyano-7-nitroqui-noxaline-2,3-dione (*right*). Note that the remaining excitatory postsynaptic currents with small amplitudes in the presence of tetrodotoxin are miniature excitatory postsynaptic currents.

Discussion

In the present study, we evaluated the effects of the voltage-gated sodium channel blockers D-bupivacaine and L-bupivacaine on action potentials generated by rat lumbar dorsal root ganglion neurons in response to stimulation of the dorsal roots. We demonstrated that L-bupivacaine more effectively blocked synaptic inputs to dorsal horn neurons from Aδ and C fibers than did D-bupivacaine. Importantly, there was no difference in the effect of L-bupivacaine and D-bupivacaine on male *versus* female rat spinal dorsal horn neurons.

Desirable Drugs

Bupivacaine has long been used as a common local anesthetic for regional and obstetric anesthesia.[6] However, the disadvantage of using bupivacaine is that it can cause cardio- and neurotoxicity.[5,9] The benefits of using bupivacaine in comparison with using other contemporary local anesthetics were highlighted by researchers in the effort to understand the fundamental mechanisms underlying its toxicity, which was shown to be attributable to the stereochemistry of its compound. Bupivacaine is actually a racemic mixture of L- and D-enantiomers. Subsequent studies revealed that the D-enantiomer of bupivacaine is the culprit behind its toxic effects. This led to the development of single L-enantiomers, such as L-bupivacaine and ropivacaine.

The Selective Actions of Local Anesthetics in Dorsal Root Ganglion Neurons

Several studies have demonstrated that single L-enantiomers of amid-type local anesthetics are less potent than racemic

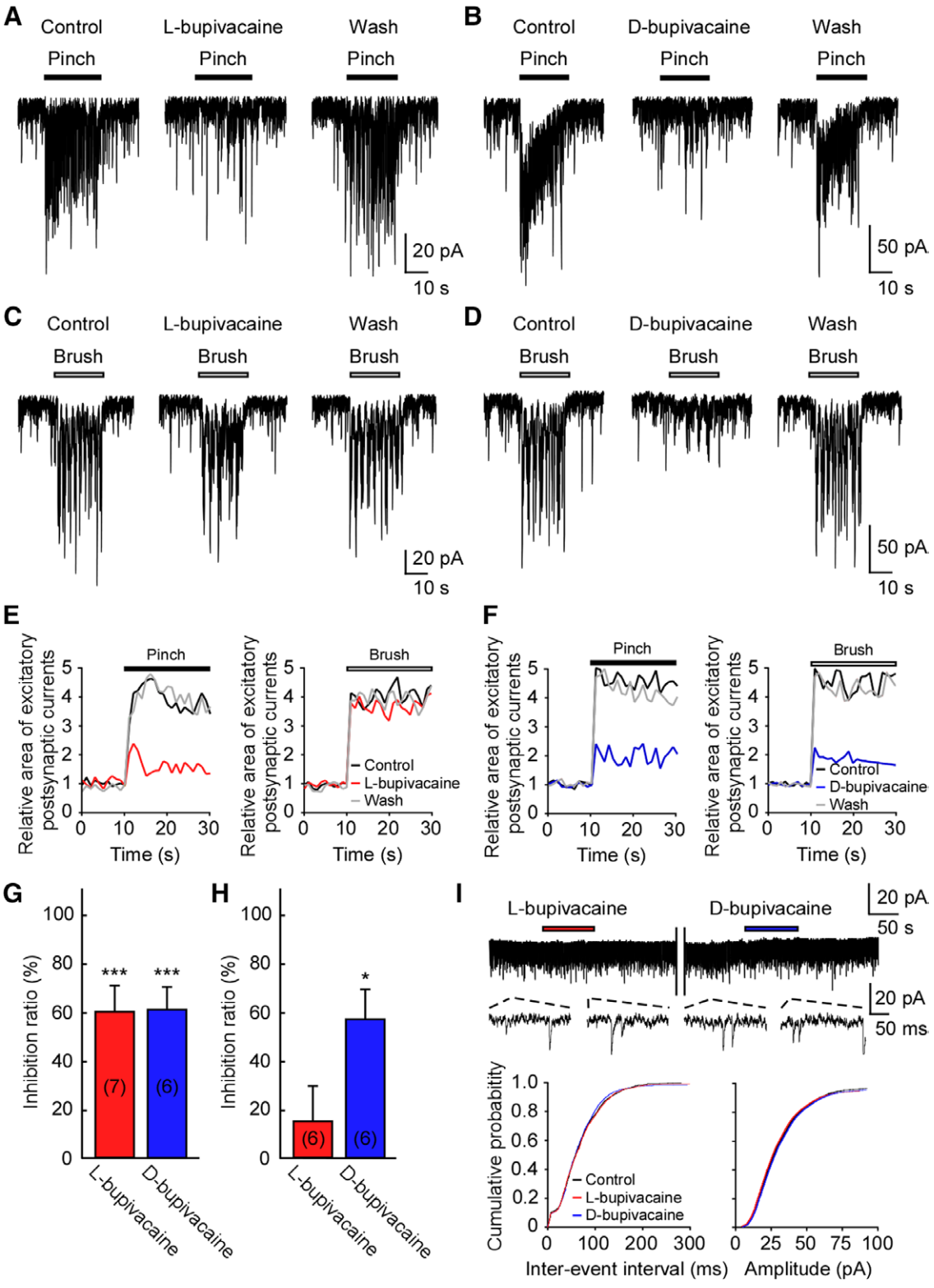

Fig. 6. Effect of L-bupivacaine and D-bupivacaine on excitatory postsynaptic currents evoked by noxious and innocuous mechanical stimuli. (*A*) Representative effects of L-bupivacaine (50 μM) on pinch-evoked excitatory postsynaptic currents recorded from substantia gelatinosa neurons. The pinch-evoked excitatory postsynaptic currents were reduced in the presence of L-bupivacaine. (*B*) Representative effects of D-bupivacaine (50 μM) on pinch-evoked excitatory postsynaptic currents recorded from substantia gelatinosa neurons. The pinch-evoked excitatory postsynaptic currents were reduced in the presence of D-bupivacaine. (*C*) Representative effects of L-bupivacaine (50 μM)

bupivacaine.[5,11] However, our results suggest that L-bupivacaine as well as ropivacaine might be more potent in blocking noxious sensory information than bupivacaine. The unmyelinated C and thinly myelinated Aδ fibers carry noxious information from the periphery to the central level, whereas Aβ fibers mainly transmit tactile information.[26] In our present study, although action potentials generated by all three types of fibers were blocked by all three local anesthetics tested, the half-maximum inhibitory concentration value of L-bupivacaine was low for Aδ and C fiber-evoked action potentials, suggesting a preferential inhibition of impulses in nociceptive neurons by the latter local anesthetic. The differential blockade of distinct types of sodium channels by the local anesthetics might underlie such differences. Although the mechanisms underlying regional anesthesia using local anesthetics is generally believed to be associated with the blockade of tetrodotoxin-sensitive sodium channels, recent studies have demonstrated that some local anesthetics can block tetrodotoxin-resistant sodium channels.[27] The distribution of tetrodotoxin-resistant and tetrodotoxin-sensitive sodium channels in the peripheral nerves differs considerably. It is reported that tetrodotoxin-resistant sodium channels are preferentially expressed in small-diameter dorsal root ganglion neurons and contribute to the generation of action potentials within the cell body and axons of these neurons, which are mainly nociceptive in nature.[2,28–34] Additionally, in the present study, tetrodotoxin-resistant sodium currents were observed in small diameter dorsal root ganglion neurons and were strongly blocked by these local anesthetics. Therefore, the preferential blockade of evoked action potentials by C and Aδ fibers in dorsal root ganglion neurons by the single L-enantiomers L-bupivacaine and ropivacaine, which we observed in our study, might be attributable to their preferential blockade of tetrodotoxin-resistant sodium channels. On the other hand, lidocaine and bupivacaine have been reported to block tetrodotoxin-sensitive sodium channels more potently than tetrodotoxin-resistant sodium channels in rat dorsal root ganglion neurons.[35] Our results showed that D-bupivacaine, but not the L-enantiomer L-bupivacaine, strongly suppressed tetrodotoxin-sensitive sodium currents. Indeed, the preferential blockade of tetrodotoxin-resistant sodium currents in small diameter dorsal root ganglion neurons compared with tetrodotoxin-sensitive sodium currents in large diameter dorsal root ganglion neurons in rats by the L-enantiomer ropivacaine have been reported.[15] Therefore, our results suggest that the selective preferential blockade of action potentials in Aδ and C neurons by the L-enantiomers L-bupivacaine and ropivacaine could be attributable to the stereo-selective effects of local anesthetics on sodium channels expressed in these fibers. The lipophilicity of local anesthetics has been reported to be another determining factor for the differential blocking properties of local anesthetics.[36,37] However, this factor mainly affects the pharmacokinetics of the drug molecules instead of the types of sodium channels to be blocked. Thus, the blocking potencies of local anesthetics on tetrodotoxin-resistant sodium channels in dorsal root ganglion neurons was reported to be independent of their lipophilicity.[38] Moreover, the selective effect of L-bupivacaine on action potentials in nociceptive Aδ and C neurons is important because local anesthetics are increasingly being used intraoperatively for the prevention of postoperative pain.[39] The sensitization of spinal dorsal horn neurons by noxious stimuli during surgical processes is known to cause pain. Therefore, because of the preferential blockade of action potentials in nociceptive Aδ and C neurons by L-bupivacaine, its use as an anesthetic would be beneficial for postoperative pain management. Another finding of the present study is that L-bupivacaine is more effective than ropivacaine in blocking action potentials in Aδ and C fibers. Clinical evidence also supports this finding.[40] In patients undergoing axillary brachial plexus block for anesthesia of the upper extremity, L-bupivacaine was reported to provide more postoperative analgesia in comparison with ropivacaine.[41,42] In clinical settings, L-bupivacaine was reported to produce a longer sensory analgesia than ropivacaine.[40]

Fig. 6. (*Continued*). on brush-evoked excitatory postsynaptic currents recorded from deep dorsal horn neurons. The brush-evoked excitatory postsynaptic currents were hardly changed in the presence of L-bupivacaine. (*D*) Representative effects of D-bupivacaine (50 μM) on brush-evoked excitatory postsynaptic currents recorded from deep dorsal horn neurons. The brush-evoked excitatory postsynaptic currents were reduced in the presence of D-bupivacaine. (*E*) Effects of L-bupivacaine (50 μM) on the relative area of pinch- (*A*) and brush- (*C*) evoked excitatory postsynaptic currents. (*F*) Effects of D-bupivacaine (50 μM) on the relative area of pinch- (*B*) and brush- (*D*) evoked excitatory postsynaptic currents. (*G*) Summary of the inhibition ratio of L-bupivacaine (n = 7, ***$P < 0.001$) and D-bupivacaine (n = 6, ***$P < 0.001$) on the area of pinch-evoked excitatory postsynaptic currents. Data presented as mean ± SD. (*H*) Summary of the inhibition ratio of L-bupivacaine (n = 6, $P = 0.826$) and D-bupivacaine (n = 6, $P = 0.013$) on the area of brush-evoked excitatory postsynaptic currents. Data presented as mean ± SD. (*I*) Effect of L-bupivacaine and D-bupivacaine on miniature excitatory postsynaptic currents. In the presence of tetrodotoxin (0.5 μM), L-bupivacaine and D-bupivacaine did not affect the frequency (L-bupivacaine: n = 6, $P = 0.766$; D-bupivacaine: n = 6, $P = 0.612$) or amplitude (L-bupivacaine: n = 6, $P = 0.763$; D-bupivacaine: n = 6, $P = 0.727$) of miniature excitatory postsynaptic currents. Cumulative distributions of the interevent interval and amplitude of miniature excitatory postsynaptic currents were shown. Both L-bupivacaine (*red line*) and D-bupivacaine (*blue line*) did not change the interevent interval ($P = 0.798$ and $P = 0.685$, respectively, Kolomogorov–Smirnov test) and amplitude ($P = 0.977$ and $P = 0.961$, respectively, Kolomogorov–Smirnov test) of miniature excitatory postsynaptic currents.

Inhibition of Synaptic Inputs to Spinal Dorsal Horn Neurons by L-bupivacaine and D-bupivacaine

The present results indicate that L-bupivacaine, D-bupivacaine, and ropivacaine inhibit pinch-evoked excitatory synaptic inputs to substantia gelatinosa neurons from Aδ and C fibers.

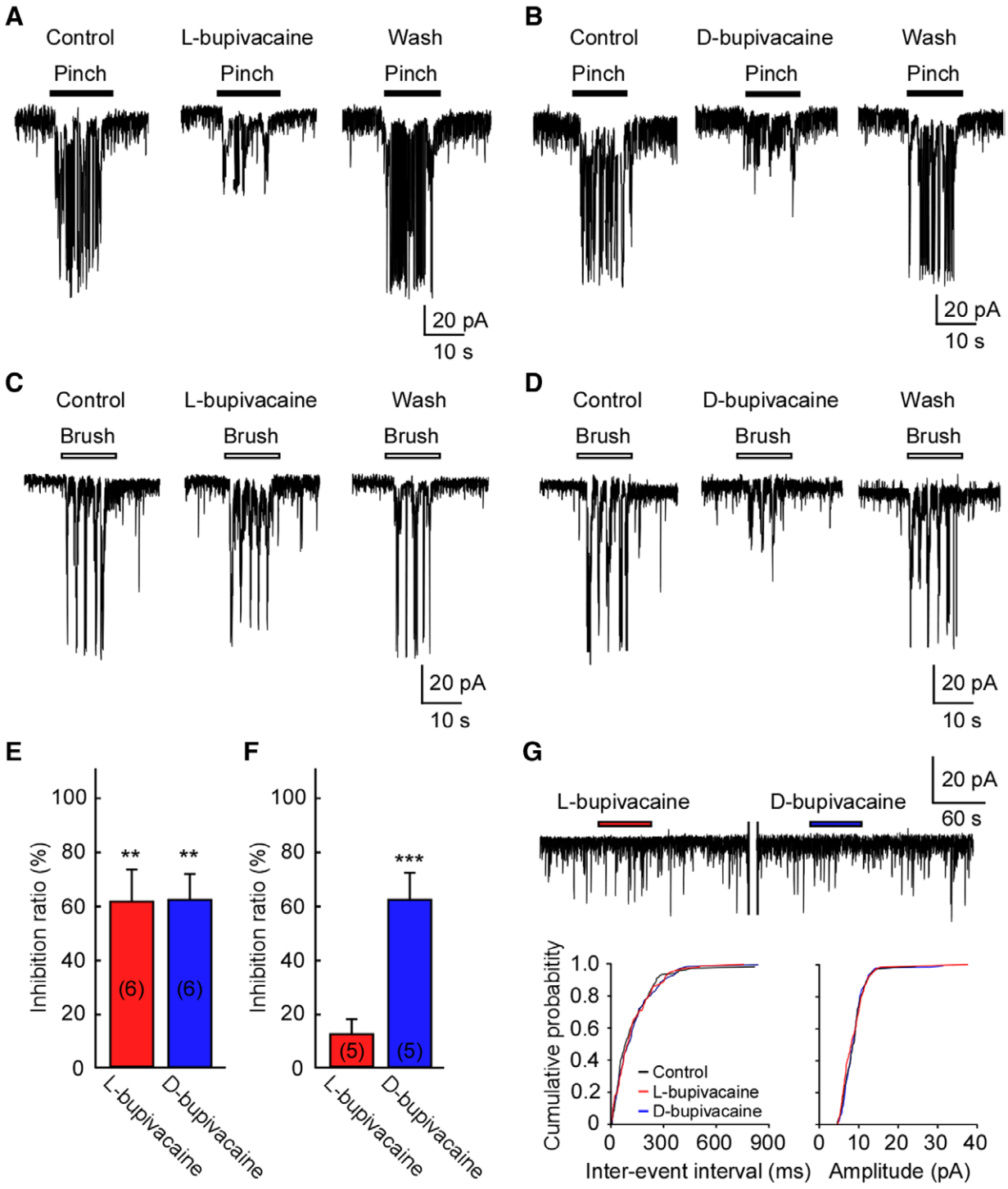

Fig. 7. Effect of L-bupivacaine and D-bupivacaine on excitatory postsynaptic currents evoked by noxious and innocuous mechanical stimuli in female rats. (*A*) Representative effects of L-bupivacaine (50 μM) on pinch-evoked excitatory postsynaptic currents recorded from substantia gelatinosa neurons. The pinch-evoked excitatory postsynaptic currents were reduced in the presence of L-bupivacaine. (*B*) Representative effects of D-bupivacaine (50 μM) on pinch-evoked excitatory postsynaptic currents recorded from substantia gelatinosa neurons. The pinch-evoked excitatory postsynaptic currents were reduced in the presence of D-bupivacaine. (*C*) Representative effects of L-bupivacaine (50 μM) on brush-evoked excitatory postsynaptic currents recorded from deep dorsal horn neurons. The brush-evoked excitatory postsynaptic currents were hardly changed in the presence of L-bupivacaine. (*D*) Representative effects of D-bupivacaine (50 μM) on brush-evoked excitatory postsynaptic currents recorded from deep dorsal horn neurons. The brush-evoked excitatory postsynaptic currents were reduced in the presence

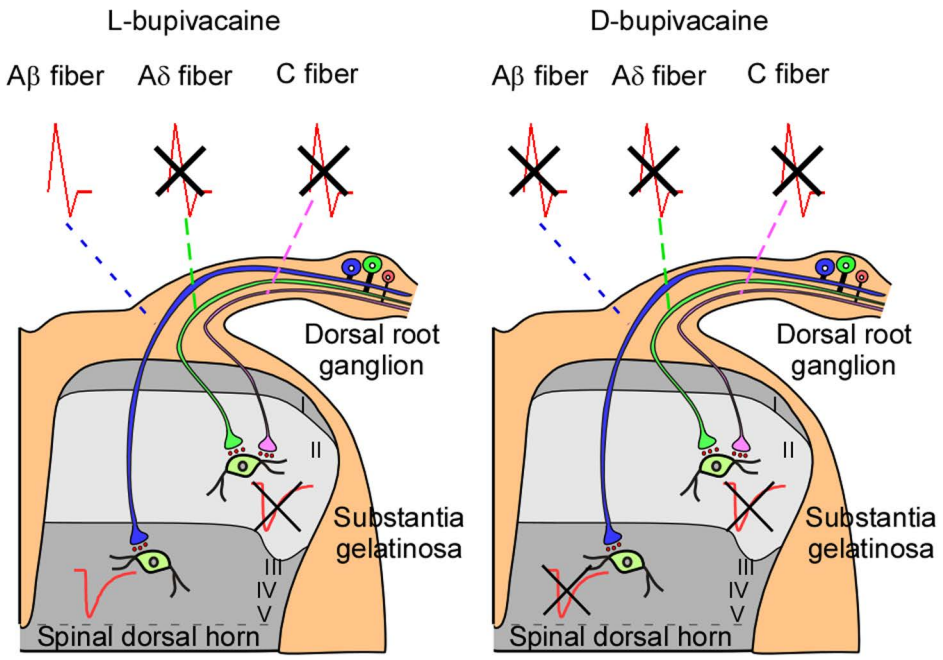

Fig. 8. Schematic diagram of the mechanism of inhibition of nociceptive transmission in the spinal dorsal horn. The half-maximum inhibitory concentration of L-bupivacaine was lower for Aδ and C dorsal root ganglion neurons than that for Aβ neurons. Therefore, L-bupivacaine is capable of effectively selectively inhibiting noxious responses in the spinal dorsal horn mediated by Aδ and C afferent fibers (*left*). However, the half-maximum inhibitory concentration of D-bupivacaine for Aβ, Aδ, and C dorsal root ganglion neurons were almost equal. Thus, D-bupivacaine is not effectively selectively inhibiting noxious responses in the spinal dorsal horn (*right*).

These synaptic inputs propagate through the primary afferent fibers, resulting in the release of glutamate from the terminals of the primary afferent fibers, after the depolarization of the presynaptic membrane, onto spinal dorsal horn neurons.[18] In the present study, pinch-evoked excitatory postsynaptic currents and antidromic action potentials generated by Aβ, Aδ, and C fibers were almost completely suppressed by all three local anesthetics at the same concentration. Previous studies reported a similar inhibition to action potential firing and voltage-gated sodium channels in small dorsal root ganglion neurons by local anesthetics.[15] On the other hand, local anesthetics did not change the frequency and amplitude of miniature excitatory postsynaptic currents in dorsal horn neurons, which received monosynaptic inputs from primary afferents in the present study (fig. 6I). Consistent with the well-known effect of local anesthetics, these results suggest that local anesthetics suppressed pinch-evoked excitatory synaptic inputs to substantia gelatinosa neurons by blocking action potential conduction through Aδ and C fibers without changing the glutamate release probability at the presynaptic terminals and the activity of postsynaptic glutamate receptors. In deep dorsal horn neurons, D-bupivacaine decreased the amplitudes of excitatory postsynaptic currents evoked by touch stimuli and antidromic action potentials in Aβ neurons. However, L-bupivacaine hardly decreased touch-evoked excitatory postsynaptic currents and antidromic action potentials in Aβ neurons. In the present study, our findings are based solely on the results from male and female naïve rats. Further study is needed to validate the selective effectiveness of L-bupivacaine on nociceptive information in animal models of chronic pain.

Fig. 7. (*Continued*). of D-bupivacaine. (*E*) Summary of the inhibition ratio of L-bupivacaine (n = 6, $^{**}P$ = 0.001) and D-bupivacaine (n = 6, $^{**}P$ = 0.002) on the area of pinch-evoked excitatory postsynaptic currents. Data presented as mean ± SD. (*F*) Summary of the inhibition ratio of L-bupivacaine (n = 5, P = 0.235) and D-bupivacaine (n = 5, $^{***}P$ < 0.001) on the area of brush-evoked excitatory postsynaptic currents. Data presented as mean ± SD. (*G*) Effect of L-bupivacaine and D-bupivacaine on miniature excitatory postsynaptic currents. In the presence of tetrodotoxin (0.5 μM), L-bupivacaine and D-bupivacaine did not affect the frequency (L-bupivacaine: n = 6, P = 0.974; D-bupivacaine: n = 6, P = 0.973) or amplitude (L-bupivacaine: n = 6, P = 0.803; D-bupivacaine: n = 6, P = 0.955) of miniature excitatory postsynaptic currents. Cumulative distributions of the interevent interval and amplitude of miniature excitatory postsynaptic currents were shown. Both L-bupivacaine (*red line*) and D-bupivacaine (*blue line*) did not change the interevent interval (P = 0.977 and P = 0.961, respectively, Kolomogorov–Smirnov test) and amplitude (P = 0.803 and P = 0.955, respectively, Kolomogorov–Smirnov test) of miniature excitatory postsynaptic currents.

In conclusion, L-bupivacaine preferentially blocks action potentials in small diameter Aδ and C neurons, which are primarily nociceptive in nature (fig. 8). This nociceptive fiber-specific effect of L-bupivacaine, in addition to its low cardio- and neurotoxicity, makes it a desirable compound for regional anesthesia. Moreover, the present findings further support the use of L-bupivacaine intraoperatively for postoperative pain management because of its selective effects on nociceptive Aδ and C neurons.

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Competing Interests

The authors declare no competing interests.

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