Bupivacaine Indirectly Potentiates Glutamate-induced Intracellular Calcium Signaling in Rat Hippocampal Neurons by Impairing Mitochondrial Function in Cocultured Astrocytes

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ABSTRACT

Background: Bupivacaine induces central neurotoxicity at lower blood concentrations than cardiovascular toxicity. However, central sensitivity to bupivacaine is poorly understood. The toxicity mechanism might be related to glutamate-induced excitotoxicity in hippocampal cells.

Methods: The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), mitochondrial membrane potential, and reactive oxygen species generation were measured by fluorescence and two-photon laser scanning microscopy in fetal rat hippocampal neurons and astrocytes.

Results: In astrocyte/neuron cocultures, 300 μM bupivacaine inhibited glutamate-induced increases in $[Ca^{2+}]_i$ in astrocytes by 40% ($P < 0.0001$; n = 20) but significantly potentiated glutamate-induced increases in $[Ca^{2+}]_i$ in neurons by 102% ($P = 0.0007$; n = 10). Ropivacaine produced concentration-dependent effects similar to bupivacaine (0.3 to 300 μM). Tetrodotoxin did not mimic bupivacaine's effects. In pure cell cultures, bupivacaine did not affect glutamate-induced increases in $[Ca^{2+}]_i$ in neurons but did inhibit increased $[Ca^{2+}]_i$ in astrocytes. Moreover, bupivacaine produced a 61% decrease in the mitochondrial membrane potential (n = 20) and a 130% increase in reactive oxygen species generation (n = 15) in astrocytes. Cyclosporin A treatment suppressed bupivacaine's effects on $[Ca^{2+}]_i$, mitochondrial membrane potential, and reactive oxygen species generation. When astrocyte/neuron cocultures were incubated with 500 μM dihydrokainic acid (a specific glutamate transporter–1 inhibitor), bupivacaine did not potentiate glutamate-induced increases in $[Ca^{2+}]_i$ in neurons but still inhibited glutamate-induced increases in $[Ca^{2+}]_i$ in astrocytes.

Conclusions: In primary rat hippocampal astrocyte and neuron cocultures, clinically relevant concentrations of bupivacaine selectively impair astrocytic mitochondrial function, thereby suppressing glutamate uptake, which indirectly potentiates glutamate-induced increases in $[Ca^{2+}]_i$ in neurons. **(Anesthesiology 2018; 128:539-54)**

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BUPIVACAINE is a local anesthetic used for caudal, epidural, and spinal anesthesia and is widely used in clinics to manage acute and chronic pain. However, bupivacaine is markedly more cytotoxic than other local anesthetics.[1,2] Systemic exposure to excess levels of bupivacaine mainly results in central nervous system (CNS)[3] and cardiovascular[4,5] effects. The effects of bupivacaine on the CNS, including numbness of the tongue, lightheadedness, visual disturbances, muscular twitching, convulsions, and coma, usually occur at lower blood plasma concentrations than cardiovascular effects.[6] The detailed mechanisms of neurotoxicity induced by bupivacaine and other local anesthetics have not yet been clarified. The mechanism underlying the anesthetic action of the majority of local anesthetic agents involves the blockade of voltage-gated sodium channels in nerve membranes, thereby preventing depolarization.[7] Nonetheless, the neurotoxicity of local anesthetics does not result from blockade of voltage-gated sodium channels *in vivo* and *in vitro*.[3,8,9] In Schwann cell lines, human SH-SY5Y neuroblastoma cells, and the ND7 cell line derived from rat dorsal root ganglion, only high concentrations of bupivacaine (0.5 to 10 mM) increased the intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$),[10] mitochondrial injury,[11] and reactive oxygen species (ROS) production,[12] which ultimately triggered the apoptosis pathway.[10–12]

What We Already Know about This Topic

- Bupivacaine neurotoxicity is characterized by neuronal network dysfunction and the development of seizures. The mechanism by which bupivacaine injures neurons is not clear.

What This Article Tells Us That Is New

- At clinically relevant concentrations, bupivacaine impaired mitochondrial function and reduced mitochondrial membrane potential in astrocytes but not in neurons.
- Astrocyte glutamate uptake was decreased by bupivacaine.
- The results suggest that, by reducing astrocyte glutamate uptake, bupivacaine increases neuronal exposure to glutamate, thereby causing excitotoxic injury.

Local anesthetics have been reported to inhibit hippocampal long-term potentiation, the form of synaptic plasticity underlying learning and memory, in rat hippocampal slices.[13,14] Although the hippocampus is a crucial structure

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<zdoi;10.1097/ALN.0000000000002003>

involved in learning and long-term memory functions,[15] both the hippocampus and amygdala were previously reported to be highly sensitive to the convulsive activity of local anesthetics.[16]

In the CNS, neurons are responsible for transmitting electrical signals and processing information, whereas astrocytes perform many important supporting functions for neurons.[17] Recently, astrocytes have been recognized as an integral modulatory component of the synapse.[18–21] However, little published information is available concerning the effects of local anesthetics on primary cultures of hippocampal cells. In preliminary experiments, bupivacaine produced opposite effects on the glutamate-induced increase in $[Ca^{2+}]_i$ in rat hippocampal astrocytes (down-regulation) and neurons (up-regulation) in an astrocyte/neuron coculture system. Glutamate is the most important synaptic transmitter responsible for higher brain functions in humans.[22] Our new observation increases the possibility that the opposite regulatory effects of bupivacaine on neuron–astrocyte cocultures might not result from a direct action on glutamate receptors but are probably due to its actions on one cell type, which then affects the other cell type. In subsequent studies, digitized video fluorescence microscopy and two-photon excitation laser scanning microscopy were used to investigate bupivacaine-induced changes in the $[Ca^{2+}]_i$, mitochondrial membrane potential ($mt\Delta\Psi$), and ROS generation in primary hippocampal neurons and astrocytes from rat fetuses to clarify the underlying mechanisms. Local anesthetics exhibit time- and dose-dependent toxic effects on a variety of tissues, including the CNS,[3,23,24] and reach 0.1 to 1.0 mM concentrations in the spinal cord after a direct spinal injection.[25–28] The maximum plasma concentration of bupivacaine is approximately 4.13 μM in patients undergoing epidural anesthesia for elective cesarean section,[29] and unintentional intravascular injection of bupivacaine (150 mg) might result in a peak blood concentration of approximately 100 μM.[30] Therefore, comparatively low concentrations (0.3 to 300 μM) were chosen to investigate the neurotoxic mechanisms of bupivacaine in the current study.

Materials and Methods

Animals

Male and female Sprague-Dawley rats weighing 300 to 350 g were provided by the Hebei Laboratory Animal Center (Shijiazhuang, China) and housed at a controlled temperature (23 ± 1°C) and humidity (50 ± 5%) on a constant 12-h light/dark cycle (lights on from 08:00 to 20:00) with free access to standard lab chow and tap water. All animals were allowed to habituate to the animal maintenance facilities for a period of at least 3 days before breeding. The current study was approved by the Hebei Medical University Ethics Committee for Animals (Shijiazhuang, China). Animal care and experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Main Chemicals

Neurobasal medium, Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), fura-2-acetoxymethyl ester, sodium pyruvate, glucose, B-27, L-glutamine, HEPES, and Hibernate-E were all obtained from Invitrogen (USA). Vitamins A and E, glutathione, ropivacaine, cyclopiazonic acid (CPA), tetrodotoxin, intracellular fluorescence ROS kit, poly-D-lysine, L-glutamic acid monosodium salt, 5-fluoro-1,3-dimethyluracil, NaCl, KCl, $MgCl_2$, $CaCl_2$, and penicillin/streptomycin were purchased from Sigma (USA). Cyclosporin A (CsA), JC-1, 1,2-Bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) xestospongin C, and dihydrokainic acid (Dih) were obtained from Abcam (USA). Bupivacaine, 2',7'-dichlorofluorescin-diacetate (DCFH-DA), EGTA, dantrolene, and adenosine triphosphate (ATP) were purchased from TCI (Japan), Beyotime (China), TaKaRa (Japan), U.S. Pharmacopeia (USA), and SERVA (Germany), respectively. Bupivacaine was dissolved in sterile water at a concentration of 5.8 mM and diluted with HEPES buffer (see Medium and Buffer Preparation) to final concentrations of 0.3 to 300 μM.

Medium and Buffer Preparation

The chemically defined medium (CDM) contained neurobasal medium, glucose-HEPES, 2% B27, 0.2 μM vitamin A, 3 μM vitamin E, penicillin (50 U/ml)/streptomycin (50 μg/ml), 4 μM glutathione, 5 mM L-glutamine, and 1 mM sodium pyruvate. HEPES buffer contained (in mM) 145 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH). The Ca^{2+}-free solution plus 5 mM EGTA (Ca^{2+}-free solution) contained (in mM) 145 NaCl, 3 KCl, 4 $MgCl_2$, 10 glucose, 10 HEPES, and 5 EGTA (adjusted to pH 7.4 with NaOH).

Cell Culture

Mixed cultures of primary hippocampal neurons and astrocytes were prepared from Sprague-Dawley rat fetuses at gestational days 18 to 20. The hippocampal cell suspension was prepared using previously described methods[31] and plated in 24-well plates coated with poly-D-lysine at a density of 2.5×10^4 cells/cm^2. Cultures were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. Four hours after the initial plating, the medium was replaced with fresh CDM and was refreshed every 3 days by replacing half of the volume of the medium with an equal volume of fresh and prewarmed CDM.

In pure cultures of primary hippocampal neurons, the medium was replaced with fresh CDM supplemented with 10 μM 5-fluoro-1,3-dimethyluracil to inhibit the replication of nonneuronal cells 3 days after the initial plating. Afterward, the medium was replaced with fresh CDM and

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refreshed every 3 days by replacing half of the volume of the medium with an equal volume of fresh and prewarmed CDM. In pure cultures of primary hippocampal astrocytes, cells were cultured in DMEM supplemented with 10% FBS for the first 3 days after the initial plating, and then the medium was replaced with pure DMEM.

Calcium Imaging

After 7 to 10 days in culture, rat hippocampal cells were loaded with fura-2-acetoxymethyl (2 μM) in the dark for 20 min at 37°C. After loading, hippocampal cell cultures were washed twice with HEPES buffer to remove the extracellular dye and placed in a recording chamber that was continuously perfused with HEPES buffer at a flow rate of 2 ml/min at room temperature (23 ± 1°C). Ratiometric calcium imaging was performed at room temperature using previously described methods.[31] Ca^{2+} signals were excited at 340 and 380 nm. The ratio of values at 340/380 nm, the fluorescence intensity at 340 nm divided by that at 380 nm, was calculated using the equation $[Ca^{2+}]_i$ (340/380 nm) = {[P(340/380 nm) − B(340/380 nm)] / B(340/380 nm)} × 100%, where P(340/380 nm) is the maximum ratio after the intervention and B(340/380 nm) is the baseline ratio before the intervention. We applied the positive markers KCl and ATP to hippocampal cells at the end of each experiment to distinguish neurons (KCl) and astrocytes (ATP).

Measurement of the Mitochondrial Membrane Potential

Hippocampal neurons and astrocytes in culture were loaded with JC-1 dye (10 μg/ml, 20 min, 37°C), a mitochondrial-specific lipophilic cationic fluorescence probe, to monitor the mtΔΨ. After loading, cells were washed with HEPES buffer and perfused using the same method as calcium imaging method. Images of the mtΔΨ were generated and captured with a Leica DMi8 two-photon confocal laser scanning microscope (Leica Microsystems Inc., Germany) at 3-s intervals. The mtΔΨ signals were excited at 488 and 519 nm, and mtΔΨ (519/488 nm) represented the ratio of red/green JC-1 fluorescence that was used as a marker to show the changes in mtΔΨ. A decrease in mtΔΨ (519/488 nm) indicated a collapse of mtΔΨ and depolarization of the mitochondrial membrane.

Measurement of ROS Generation

Intracellular ROS generation induced by the drug intervention was assessed by measuring fluorescence intensity using DCFH-DA or an intracellular fluorescence ROS kit. Hippocampal neurons and astrocytes in culture were loaded with DCFH-DA or reagents from the ROS kit in the dark at 37°C for 20 min or 60 min. After loading with DCFH-DA, hippocampal cell cultures were washed and perfused with HEPES buffer using the same method as the calcium imaging method. In contrast, cells loaded with the fluorescent dye from the ROS kit were directly placed under the microscope without washing. ROS signals were excited at 488 nm using a Leica DMI3000B microscope (Leica Microsystems Inc.) equipped with a ratiometric imaging system and recorded at 1-s intervals using a cooled electron multiplying charge coupled device camera (Andor, Germany). ROS generation, as indicated by the fluorescence intensity at 488 nm, in cells loaded with DCFH-DA or the fluorescent dye from the ROS kit was calculated using the equation ROS generation (488 nm) = [(P488 nm − B488 nm) / B488 nm] × 100%, where P488 nm is the peak fluorescence intensity after the intervention and B488 nm is the baseline fluorescence intensity before the intervention.

Drug Administration

All agents were dissolved in HEPES buffer and applied locally to the hippocampal cells through a micropipette (with a tip diameter of 100 μm) connected to an 8-channel pressure-controlled drug application system (ALA Scientific, USA). In the primary hippocampal mixed astrocyte/neuron cell cultures or pure cultures of a particular cell type, two exposures to glutamate at the concentration evoking the half-maximal response (EC_{50}; 1 mM) for 10 s induced reproducible increases in $[Ca^{2+}]_i$ in the solvent control group, and the increased $[Ca^{2+}]_i$ was restored to the baseline level after a washout of the agent. Before the second exposure to glutamate in the same cell, a pretreatment with bupivacaine, ropivacaine, tetrodotoxin, CPA, or Dih was administered through a 5-min perfusion, according to the research plan.

Statistical Analysis

Values are presented as means ± SD. A one-way ANOVA followed by Dunnett's test was used to analyze concentration-response curves of bupivacaine or ropivacaine, and a two-way ANOVA followed by Bonferroni *post hoc* test was used to evaluate any differences between the two sets of concentration-response curves. Differences between pre- and posttreatment values in the same cell were determined using a paired *t* test, and differences between the two groups were determined using an unpaired *t* test. The EC_{50} values (molar concentration of agonist that produced 50% of the maximal response) and E_{max} values (the maximal response) for bupivacaine or glutamate were calculated with a nonlinear regression analysis using GraphPad Prism 5.00 software (GraphPad Software Inc., USA) and compared using an unpaired *t* test. A *P* value less than 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism software and SPSS software (v. 20.0; SPSS, USA). No *a priori* statistical power calculations were performed to guide sample size considerations. The sample sizes were based on previous international research experience and published papers. No randomization methods were used in this study. Experimenters were not blinded to the conditions under study. Our experiments did not involve any missing data, lost data, or excluded data.

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Results

Effects of Bupivacaine on Glutamate-induced Increases in $[Ca^{2+}]_i$ in Neurons and Astrocytes

A 10-s incubation with 1 mM glutamate produced an increase in $[Ca^{2+}]_i$ in neurons and astrocytes in either astrocyte/neuron cocultures or pure cultures of a particular cell type. The $[Ca^{2+}]_i$ in hippocampal cells increased by 169 ± 20% (cocultured neurons, n = 14), 233 ± 42% (cocultured astrocytes, n = 14), 229 ± 56% (pure neurons, n = 16), and 238 ± 44% (pure astrocytes, n = 20) from baseline to peak values. The increased $[Ca^{2+}]_i$ in hippocampal cells returned to baseline levels after washout. After 5 min, a second exposure to 1 mM glutamate in the same cells produced a similar increase in $[Ca^{2+}]_i$ of 172 ± 26% (cocultured neurons), 233 ± 42% (cocultured astrocytes), 237 ± 59% (pure neurons), and 227 ± 43% (pure astrocytes). A significant difference in the peak values was not observed between the two glutamate exposures in the same cell type (P = 0.533 for cocultured neurons, P = 0.836 for cocultured astrocytes, P = 0.266 for pure neurons, and P = 0.918 for pure astrocytes). We used a confocal fluorescence microscope to observe hippocampal cultures (appendix 1A). The administration of 10 μM ATP significantly increased $[Ca^{2+}]_i$ specifically in astrocytes, and 30 mM KCl increased $[Ca^{2+}]_i$ specifically in neurons (appendix 1B).

The glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons was significantly attenuated by a 5-min treatment with 300 μM bupivacaine (fig. 1A). The peak value decreased from 229 ± 55% (first glutamate exposure) to 178 ± 41% (second exposure) with a decrease of 40% (P < 0.0001), and the area under the curve (AUC) and Tau values were also significantly changed (P < 0.0001; fig. 1B). In contrast, the glutamate-induced $[Ca^{2+}]_i$ response in neurons cocultured with astrocytes was significantly potentiated by the same treatment with bupivacaine (fig. 1C), and its peak value increased significantly from 168 ± 21% (first glutamate exposure) to 231 ± 37% (second exposure) with an increase of 102% (P = 0.0007). The AUC and Tau values were also significantly increased (P < 0.0001 for AUC and P = 0.0001 for Tau values; fig. 1D).

The peak value of $[Ca^{2+}]_i$ increase induced by the first exposure of glutamate at 1 mM in the pure culture of astrocytes (fig. 1, E and F) was 239 ± 40%, which was not significantly different from that in astrocytes cocultured with neurons (233 ± 42%; P = 0.810). However, the peak value of $[Ca^{2+}]_i$ increase (240 ± 81%) induced by the first exposure of glutamate at 1 mM in the pure culture of neurons (fig. 1, G and H) was significantly greater than that in neurons cocultured with astrocytes (169 ± 20%; P < 0.0001).

The $[Ca^{2+}]_i$ response to the second glutamate exposure in pure astrocyte cultures was significantly reduced by an incubation with 300 μM bupivacaine (fig. 1E). The peak value decreased from 239 ± 40% (first glutamate exposure) to 189 ± 34% (second glutamate exposure; P < 0.0001), and its AUC and Tau values were also significantly altered (fig. 1F). These three values changed in a similar manner to astrocytes cocultured with neurons. However, the $[Ca^{2+}]_i$ response to the second glutamate exposure in pure neuronal cultures was not significantly changed by the incubation with bupivacaine compared with the first exposure (P = 0.788; fig. 1H).

In the astrocyte/neuron coculture system, bupivacaine regulated the glutamate-induced increases in $[Ca^{2+}]_i$ in the two cell types in an opposite, concentration-dependent manner. Bupivacaine initiated its effect on the glutamate-induced increase in $[Ca^{2+}]_i$ at 0.3 μM and peaked at 300 μM (fig. 2). The EC_{50} values calculated from the peak increase in $[Ca^{2+}]_i$ were 1.64 μM in astrocytes cocultured with neurons and 1.76 μM in neurons cocultured with astrocytes. Ropivacaine, another local anesthetic, had the same effects as bupivacaine, but its EC_{50} values were 15.51 μM and 16.18 μM in the two types of cells, respectively (fig. 2).

Effects of Tetrodotoxin and CPA on Glutamate-induced Increases in $[Ca^{2+}]_i$ in the Astrocyte/Neuron Coculture System

A 5-min incubation with a sodium channel blocker (tetrodotoxin at 1 μM) did not change the glutamate-induced increase in $[Ca^{2+}]_i$ in either neurons or astrocytes in the mixed culture system (fig. 3, A–D). When 10 μM CPA, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, was added to the mixed cultures to deplete the intracellular Ca^{2+} stores, both types of hippocampal cells showed an elevation in baseline $[Ca^{2+}]_i$. The CPA incubation significantly decreased the 1 mM glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons, and its peak value dropped from 225 ± 46% to 130 ± 11% (P < 0.0001). CPA decreased the AUC value and increased the Tau value (fig. 3, E and F). However, the CPA incubation did not significantly affect the peak and AUC values of the glutamate-induced increase in $[Ca^{2+}]_i$ in neurons cocultured with astrocytes (P = 0.068 for peak and P = 0.624 for AUC; fig. 3, G and H).

At 1 mM, glutamate did not produce an obvious increase in $[Ca^{2+}]_i$ in pure cultures of neurons perfused with a Ca^{2+}-free solution plus 5 mM EGTA (Ca^{2+}-free solution) compared to neurons perfused with a normal solution, and a pretreatment with 300 μM bupivacaine did not change the action of 1 mM glutamate in pure cultures of neurons perfused with the Ca^{2+}-free solution (appendix 2). A 10-μM dantrolene treatment did not change the increase in $[Ca^{2+}]_i$ in response to 1 mM glutamate in pure neuronal cultures compared to neurons that were not treated with dantrolene (P = 0.106), and 300 μM bupivacaine did not exert any effect on the 1-mM glutamate-induced increase in $[Ca^{2+}]_i$ in pure neuronal cultures that had been pretreated with 10 μM dantrolene (P = 0.148; appendix 3).

Effects of Bupivacaine on the Mitochondrial Membrane Potential of Astrocytes

The JC-1 fluorescent probe and two-photon microscopy were used to measure the change in mtΔΨ in hippocampal cells

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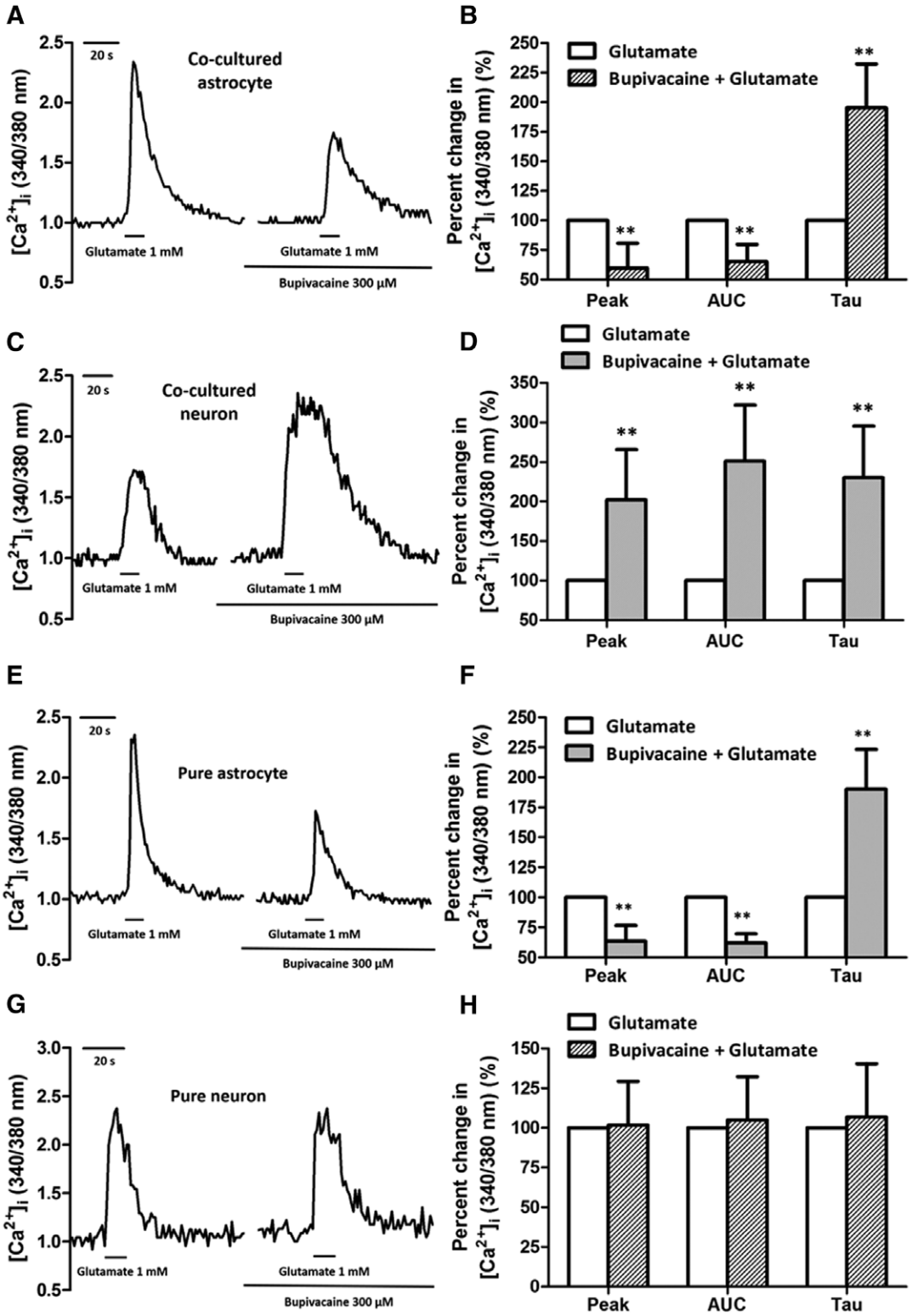

Fig. 1. Inhibitory effect of a 5-min incubation with 300 μM bupivacaine on the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (*A*, a typical trace; *B*, changes in peak, area under the curve (AUC), and Tau values, n = 20), a potentiating effect of the same treatment with bupivacaine on neurons cocultured with astrocytes (*C*, a typical trace; *D*, changes in peak, AUC, and Tau values, n = 10), and effects of the same treatment with bupivacaine on the glutamate-induced increase in $[Ca^{2+}]_i$ in pure cultures of astrocytes (*E*, a typical trace; *F*, changes in peak, AUC, and Tau values, n = 13) and neurons (*G*, a typical trace; *H*, changes in peak, AUC, and Tau values, n = 18). Cells were exposed to 1 mM glutamate for 10 s, and the data are expressed as the means ± SD. $^{**}P < 0.01$ compared with the glutamate-induced responses before the bupivacaine treatment using a paired *t* test.

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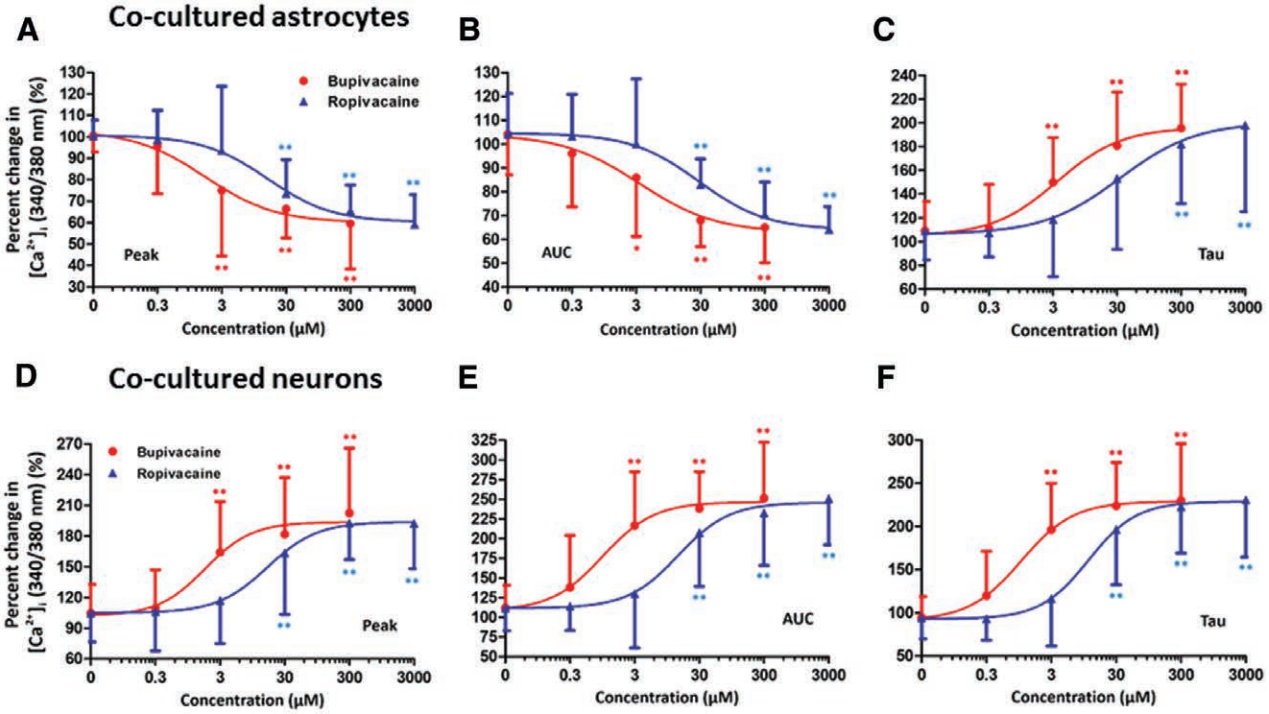

Fig. 2. Concentration-response curves for the inhibitory effects of 5-min treatments with bupivacaine (*red*; n = 12, 14, 16, 14, and 20 for 0, 0.3, 3, 30, and 300 μM) or ropivacaine (*blue*; n = 12, 12, 15, 15, 13, and 17 for 0, 0.3, 3, 30, 300, and 3,000 μM, respectively) on the glutamate-induced increases in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (*A–C*), and the potentiating effects of the same treatments with bupivacaine (*red*; n = 14, 14, 14, 12, and 10 for 0, 0.3, 3, 30, and 300 μM, respectively) or ropivacaine (*blue*; n = 14, 14, 11, 10, 10, and 13 for 0, 0.3, 3, 30, 300, and 3,000 μM, respectively) on the glutamate-induced increases in $[Ca^{2+}]_i$ in neurons cocultured with astrocytes (*D–F*). Cells were exposed to 1 mM glutamate for 10 s, and the data are expressed as the means ± SD. **P* < 0.05 and ***P* < 0.01 compared with the glutamate-induced responses before treatment with bupivacaine or ropivacaine using a one-way ANOVA followed by Dunnett's test. AUC = area under the curve.

(fig. 4, A–J). The administration of 300 μM bupivacaine significantly decreased the mtΔΨ in astrocytes cocultured with neurons by 60% (from 1 to 0.40 ± 0.05), but it did not affect the mtΔΨ in neurons cocultured with astrocytes (fig. 4, A–C).

At 300 μM, bupivacaine also significantly decreased the mtΔΨ by 61% (from 1 to 0.39 ± 0.20; fig. 4, G and H) in pure astrocyte cultures but not pure neuronal cultures (fig. 4, D–F). Bupivacaine (0.3 to 300 μM) dose-dependently decreased the mtΔΨ of pure astrocyte cultures (fig. 4J), with a maximum inhibition of 61% and a half maximal inhibitory concentration of 1.64 μM. Pretreatment with 1 μM CsA, an inhibitor of the mitochondrial permeability transition pore (mPTP), significantly inhibited the bupivacaine-induced decrease in the mtΔΨ in pure astrocyte cultures (fig. 4, G–I). At 300 μM, bupivacaine significantly decreased the mtΔΨ in pure astrocyte cultures pretreated with a Ca^{2+}-free solution, 5 μM BAPTA-AM, 10 μM xestospongin C, or 10 μM dantrolene, and the decrease in mtΔΨ was not significantly different from the decrease induced by 300 μM bupivacaine in astrocytes lacking the treatments mentioned above (*P* = 0.486 for Ca^{2+}-free solution, *P* = 0.396 for BAPTA-AM, *P* = 0.790 for xestospongin C, and *P* = 0.616 for dantrolene; appendix 4, A and B).

Effect of Bupivacaine on ROS Generation in Pure Astrocyte Cultures

Pure cultures of neurons or astrocytes were used to measure the change in ROS generation (fig. 5, A–D). Bupivacaine (300 μM) did not affect ROS generation in pure neuronal cultures loaded with the DCFH-DA dye (fig. 5A) but significantly and dose-dependently increased ROS generation by 130% in pure astrocyte cultures (fig. 5, A and C). A pretreatment with 1 μM CsA for 5 min significantly inhibited bupivacaine-induced ROS generation by 81% in pure astrocyte cultures (fig. 5, A and B). An intracellular ROS kit was utilized to reconfirm the ROS generation in pure astrocyte cultures, and bupivacaine (0.3 to 300 μM)–induced ROS production was also significantly suppressed by a 5-min incubation with 1 μM CsA (fig. 5D). At 300 μM, bupivacaine induced significant ROS generation in pure astrocyte cultures pretreated with a Ca^{2+}-free solution, 5 μM BAPTA-AM, 10 μM xestospongin C, or 10 μM dantrolene, and ROS production was not significantly different from the levels induced by 300 μM bupivacaine in astrocytes lacking the treatments mentioned above (*P* = 0.336 for Ca^{2+}-free solution, *P* = 1.000 for BAPTA-AM, *P* = 0.721 for xestospongin C, and *P* = 0.924 for dantrolene; appendix 4, C and D).

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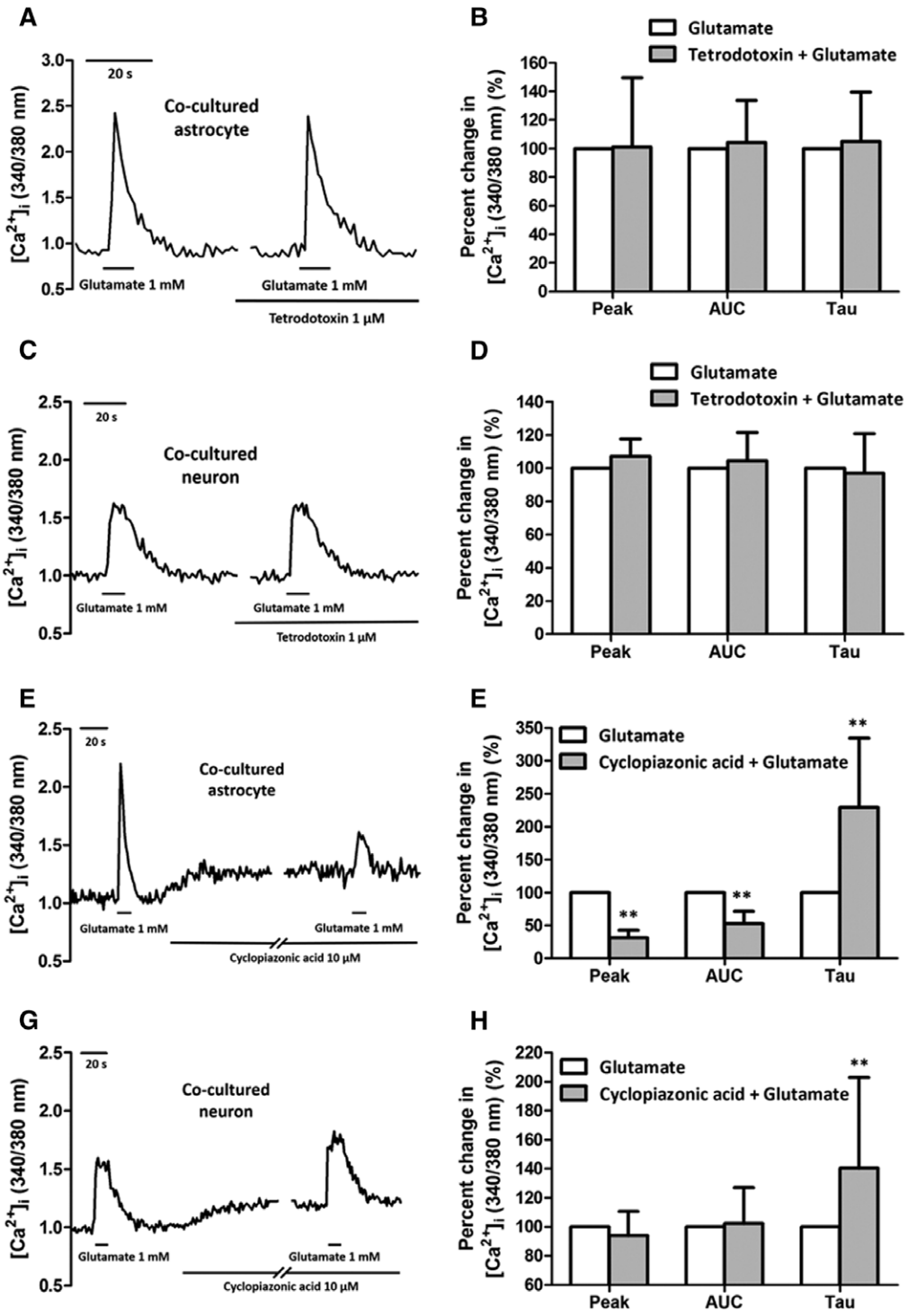

Fig. 3. Effects of a 5-min incubation with 1 μM tetrodotoxin on the glutamate-induced increases in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (*A*, a typical trace; *B*, changes in peak, area under the curve (AUC), and Tau values, n = 10) and in neurons cocultured with astrocytes (*C*, a typical trace; *D*, changes in peak, AUC, and Tau values, n = 16), as well as the effects of 5-min incubation with 10 μM cyclopiazonic acid on the glutamate-induced increases in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (*E*, a typical trace; *F*, changes in peak, AUC, and Tau values, n = 31) and in neurons cocultured with astrocytes (*G*, a typical trace; *H*, changes in peak, AUC, and Tau values, n = 29). Cells were exposed to 1 mM glutamate for 10 s, and the data are expressed as the means ± SD. $P > 0.05$ compared with the glutamate-induced responses before the tetrodotoxin treatment using a paired *t* test. $^{**}P < 0.01$ compared with the glutamate-induced responses before cyclopiazonic acid treatment using a paired *t* test.

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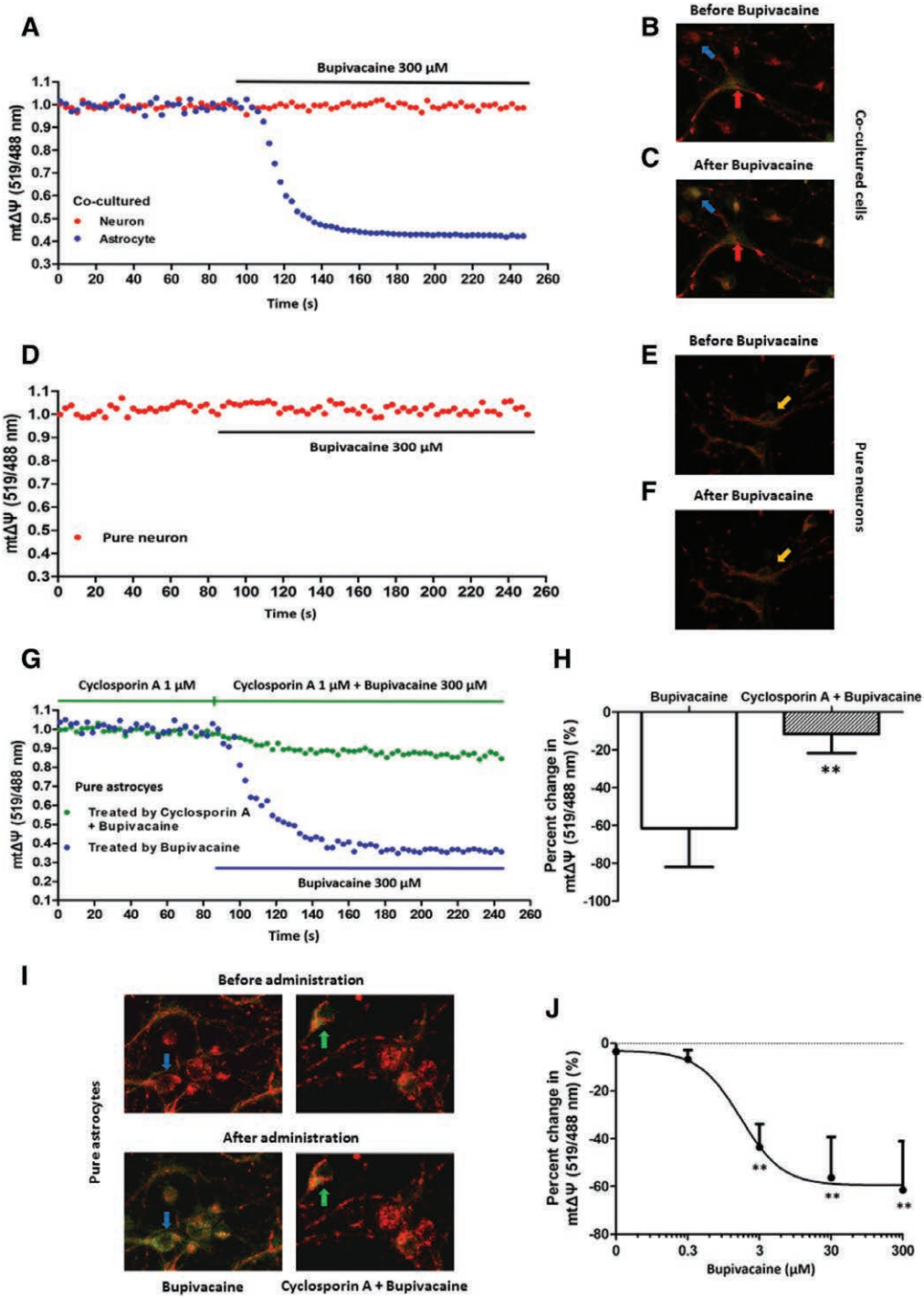

Fig. 4. Representative traces showing the decrease in the mtΔΨ induced by 300 μM bupivacaine, as measured by ratio of the intensity of red/green JC-1 fluorescence in astrocytes (*A*, *blue circles*; n = 24) cocultured with neurons. A decrease in the mtΔΨ (converting the normally red fluorescence to green) was observed only in astrocytes before (*B*) and after (*C*) bupivacaine treatment in the neuron (*red arrows*)/astrocyte (*blue arrows*) coculture system. The mtΔΨ of the pure culture of neurons (*D*, n = 11) or the mixed culture of neurons (*A*, *red circles*; n = 20) was not affected by 300 μM bupivacaine. Fluorescence color remained the same before (*E*) and after (*F*) bupivacaine treatment in pure cultures of neurons (*yellow arrows*). A decrease in mtΔΨ was induced by treatment with 300 μM bupivacaine in pure cultures of astrocytes (*G*, *blue circles*; n = 20), and the effect was reversed by 1 μM cyclosporin A (*G, green circles*; n = 20); the data were analyzed in (*H*). Analysis of JC-1 fluorescence before and after treatment with 300 μM bupivacaine (*blue arrows*) or with 1 μM cyclosporin A plus 300 μM bupivacaine (*green arrows*) in pure cultures of astrocytes (*I*). The mtΔΨ of the pure culture of astrocytes was decreased by 0.3 to 300 μM bupivacaine (*J*, n = 20, 17, 16, 17, 20 for 0, 0.3, 3, 30, 300 μM, respectively). Data are expressed as the means ± SD. $^{**}P < 0.01$ compared with the bupivacaine group using an unpaired *t* test (*H*). $^{**}P < 0.01$ compared with the value before treatment with bupivacaine using a one-way ANOVA followed by Dunnett's test (*J*). mtΔΨ = mitochondrial membrane potential.

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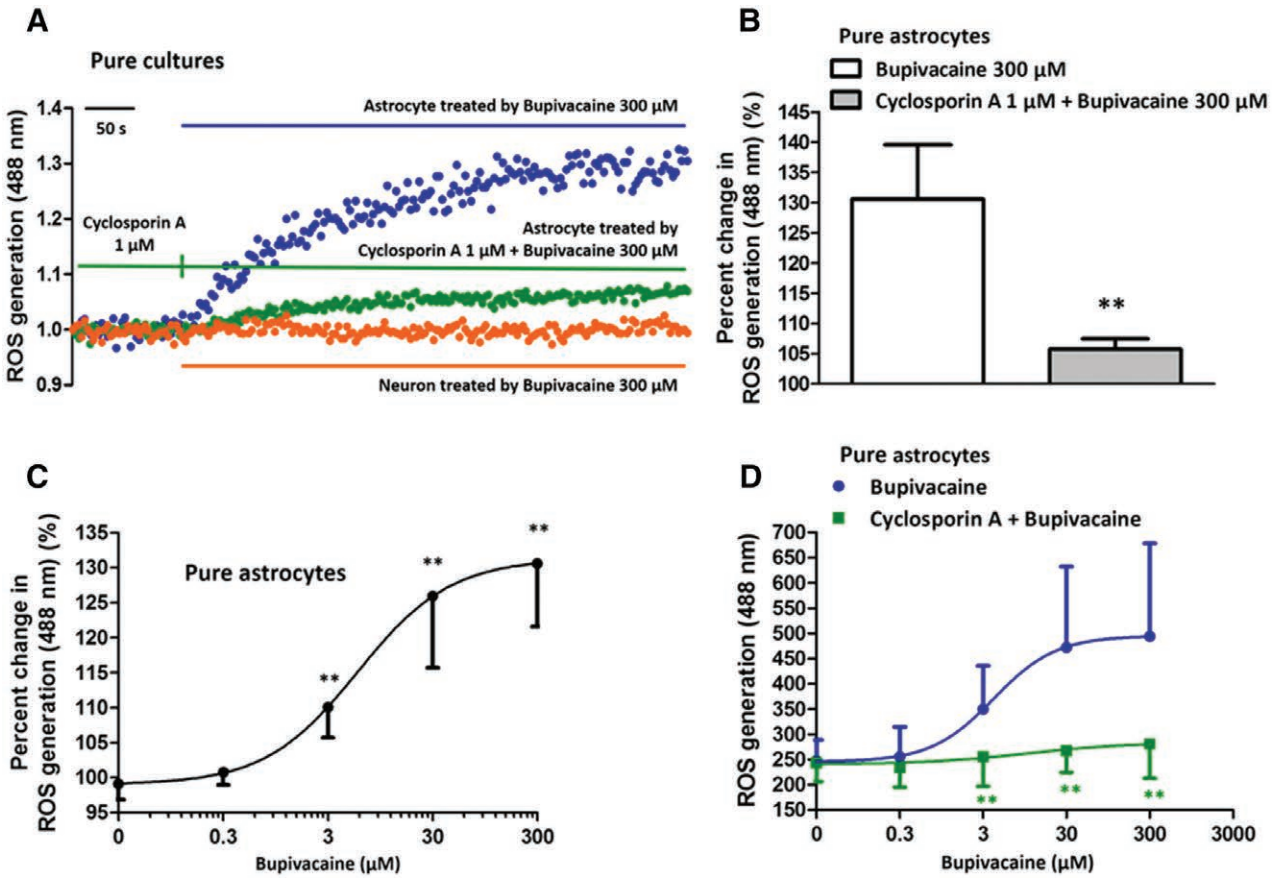

Fig. 5. Intracellular ROS generation induced by 300 μM bupivacaine was measured by labeling with the DCFH-DA dye in pure cultures of astrocytes (*A*, *blue circles*; n = 15), and a 5-min incubation with 1 μM cyclosporin A reversed this effect (*A*, *green circles*; n = 22); datasets are shown in (*B*). The effects of 0.3-300 μM bupivacaine on pure cultures of astrocytes were measured by labeling with the DCFH-DA dye (*C*). The effect of cyclosporin A on reversing bupivacaine-induced ROS generation was re-confirmed using an intracellular ROS kit (*D*). Data are expressed as the means ± SD. $^{**}P < 0.01$ compared with the bupivacaine group using an unpaired *t* test (*B*), $^{**}P < 0.01$ compared with the value before treatment with bupivacaine using one-way ANOVA followed by Dunnett's test (*C*, n = 23, 21, 18, 22, and 15 for 0, 0.3, 3, 30, and 300 μM bupivacaine, respectively), and $^{**}P < 0.01$ compared with the bupivacaine group using a two-way ANOVA followed by Bonferroni *post hoc* test (*D*, n = 46, 52, 52, 52, and 51 for 0, 0.3, 3, 30, and 300 μM bupivacaine; n = 44, 21, 52, 23, and 24 for 0, 0.3, 3, 30, and 300 μM bupivacaine plus cyclosporin A, respectively). ROS = reactive oxygen species.

Effect of CsA on the Bupivacaine-induced Inhibition of Glutamate-induced Increases in $[Ca^{2+}]_i$ in Astrocytes

The inhibitory effect of bupivacaine on the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (fig. 6, A and B) and the bupivacaine-mediated potentiation of the glutamate-induced increase in $[Ca^{2+}]_i$ in neurons cocultured with astrocytes (fig. 6, C and D) almost completely disappeared when mixed cultures were pretreated with the combination of 1 μM CsA and 300 μM bupivacaine for 5 min. Bupivacaine (0.3 to 300 μM) dose-dependently inhibited the glutamate-induced increase in $[Ca^{2+}]_i$ in pure astrocyte cultures (appendix 5). When pure astrocyte cultures were treated with a combination of 1 μM CsA and bupivacaine (0.3 to 300 μM), the peak and AUC values of the glutamate-induced increase in $[Ca^{2+}]_i$ were not significantly different from the values observed before treatment (appendix 5, A and B), and the Tau values of the glutamate-induced increase in $[Ca^{2+}]_i$ changed slightly at 30 μM (appendix 5C).

Effects of a Glutamate Transporter Inhibitor on the Opposite Regulatory Effects of Bupivacaine on Hippocampal Cells

In the astrocyte/neuron coculture system, glutamate (0.01 to 100 mM) increased astrocytic and neuronal $[Ca^{2+}]_i$ in a concentration-dependent manner (fig. 7, A and B) with an E_{max} of 334% and an EC_{50} of 0.86 mM calculated from the peak value of the $[Ca^{2+}]_i$ increase in astrocytes as well as an E_{max} of 312% and an EC_{50} of 3.18 mM in neurons. After pretreatment with 300 μM bupivacaine, the E_{max} of glutamate in astrocytes cocultured with neurons decreased to 232% ($P < 0.0001$), and the EC_{50} was 1.27 mM ($P = 0.561$; fig. 7A); meanwhile, the E_{max} of glutamate in neurons cocultured with astrocytes remained at 311% ($P = 0.898$), but the EC_{50} decreased to 0.48 mM ($P < 0.0001$; fig. 7B). The concentration-response curve of the glutamate-induced increase in $[Ca^{2+}]_i$ in pure neuronal cultures almost completely overlapped the curve for

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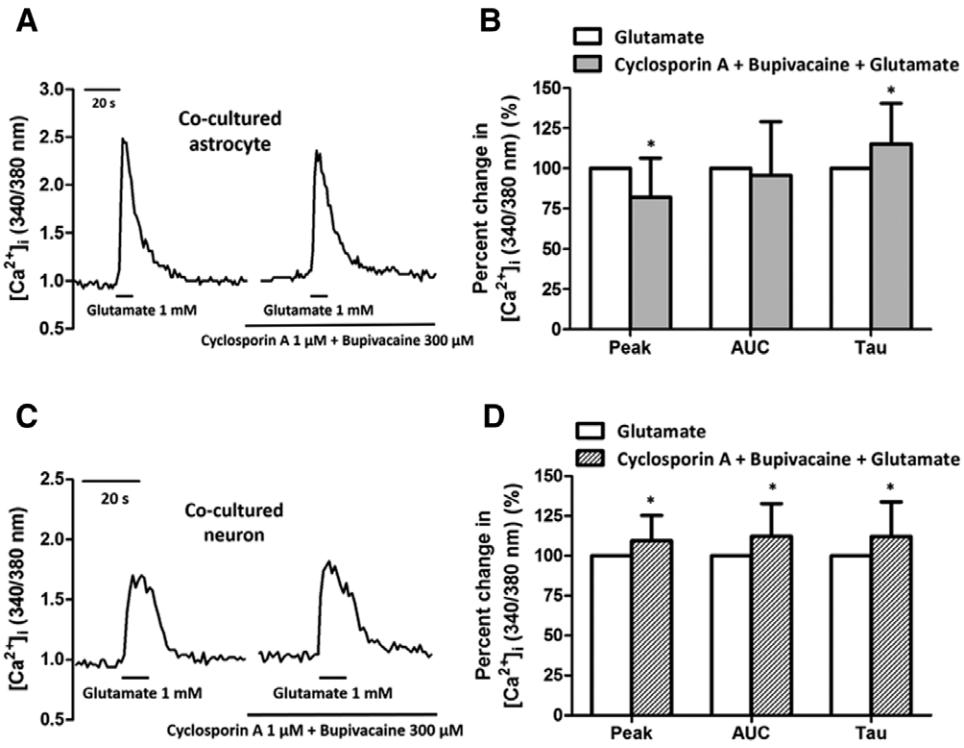

Fig. 6. Effects of a 5-min incubation with the combination of 1 μM cyclosporin A plus 300 μM bupivacaine on the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (*A*, a typical trace; *B*, changes in peak, area under the curve (AUC), and Tau values, n = 14) and in neurons cocultured with astrocytes (*C*, a typical trace; *D*, changes in peak, AUC, and Tau values, n = 20). Cells were exposed to 1 mM glutamate for 10 s, and the data are expressed as the means ± SD. *$P < 0.05$ compared with the glutamate-induced responses before bupivacaine treatment using a paired *t* test.

neurons cocultured with astrocytes that had been pretreated with bupivacaine (fig. 7B).

When 500 μM Dih, a specific inhibitor of glutamate transporter–1, was added to the astrocyte/neuron coculture system (fig. 7, C–F), the peak values of the glutamate-induced increase in $[Ca^{2+}]_i$ (first exposure) were 237 ± 156% in neurons (fig. 7E) and 284 ± 53% in astrocytes (fig. 7C). In the presence of Dih, 300 μM bupivacaine did not affect the 1-mM glutamate-induced increase in $[Ca^{2+}]_i$ in neurons cocultured with astrocytes (fig. 7F) but strongly inhibited the peak value of the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (fig. 7D).

Discussion

In the current study, KCl and ATP were used as neuron-specific[32] and astrocyte-specific[33] positive markers, respectively. At concentrations of 0.01 to 100 mM, glutamate increased $[Ca^{2+}]_i$ in a concentration-dependent manner in astrocytes and neurons, whereas 0.3 to 300 μM bupivacaine did not affect the baseline $[Ca^{2+}]_i$ in the two types of cells in mixed cultures. However, bupivacaine significantly potentiated the glutamate-induced increase in $[Ca^{2+}]_i$ in neurons cocultured with astrocytes, and inhibited the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons. At 3 to 3,000 μM, ropivacaine produced effects similar to bupivacaine. However, the highly selective neuronal sodium channel blocker tetrodotoxin did not mimic any of the above effects of bupivacaine or ropivacaine, indicating that the opposite regulatory effects of bupivacaine on the glutamate-induced increase in $[Ca^{2+}]_i$ in the two types of cells are not the result of sodium channel blockade.

We observed its effects on pure cultures of hippocampal astrocytes and of neurons to confirm whether bupivacaine exerts indirect effects on the glutamate-induced increase in $[Ca^{2+}]_i$ in the two types of cells. Interestingly, bupivacaine (0.3 to 300 μM) did not affect the glutamate-induced response in pure neuronal cultures, but it still inhibited the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes in a concentration-dependent manner. Therefore, bupivacaine likely acted directly on astrocytes, thereby indirectly affecting the glutamate-induced increase in $[Ca^{2+}]_i$ in cocultured neurons.

Therefore, we investigated the mechanisms underlying the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes and neurons in the fetal rat hippocampus. Treatment with CPA, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase that depletes intracellular Ca^{2+} stores, significantly inhibited the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes but not in neurons, suggesting that the increase in $[Ca^{2+}]_i$ observed in glutamate-stimulated astrocytes was mainly due to release from intracellular stores. Further evidence for an indirect mechanism was that glutamate

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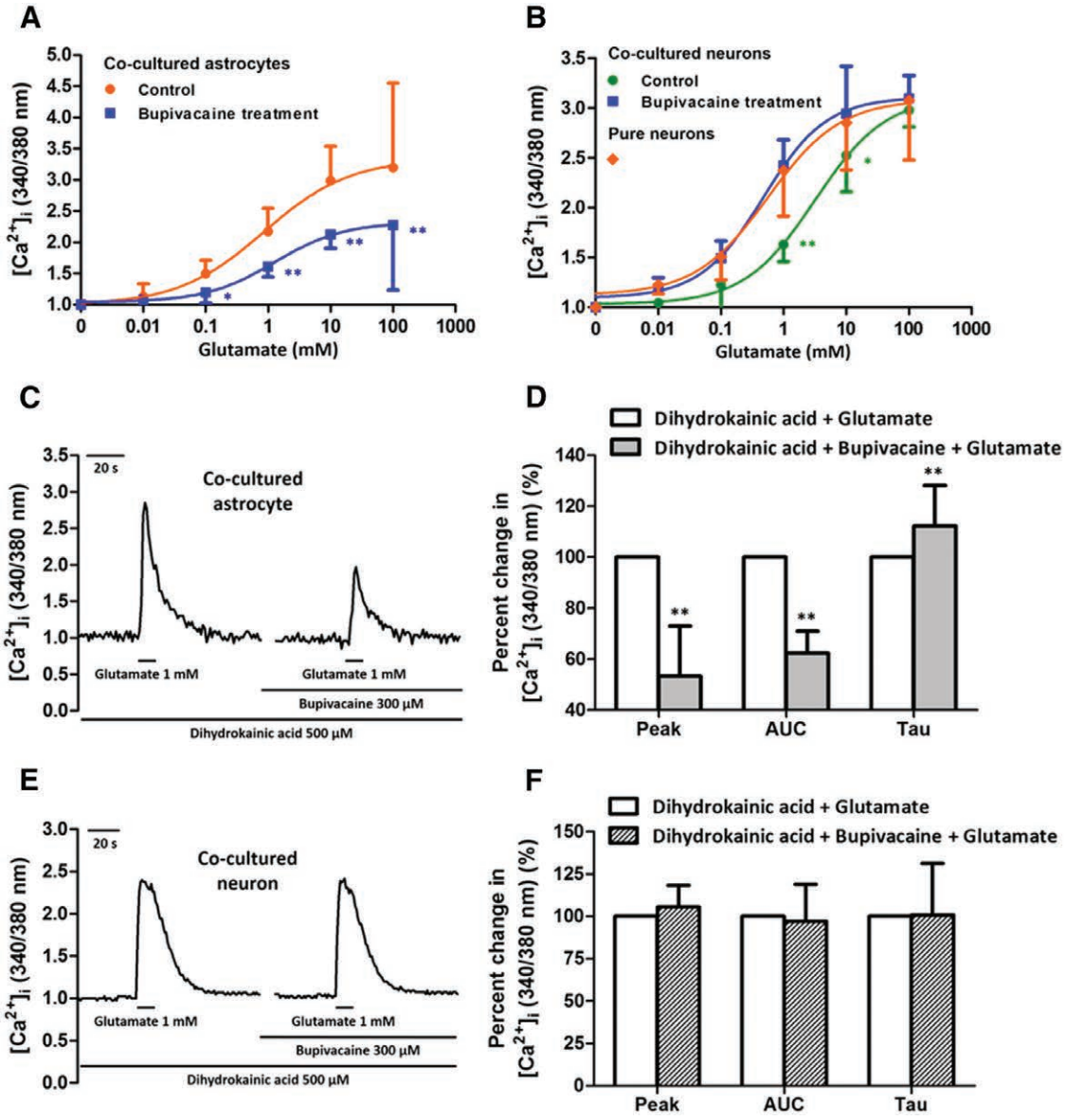

Fig. 7. Concentration-response curve of the glutamate-induced increase in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (*A*, *orange*; n = 21, 49, 22, 22, and 20 for 0.01, 0.1, 1, 10, and 100 mM, respectively), and in the same cells pretreated with bupivacaine (300 μM, *A, left*, *blue*; n = 21, 49, 22, 22, and 20 for 0.01, 0.1, 1, 10, and 100 mM, respectively). *Right*, the concentration-response curve for the glutamate-induced increase in $[Ca^{2+}]_i$ in neurons cocultured with astrocytes (*B*, *green*; n = 26, 10, 25, 10, and 25 for 0.01, 0.1, 1, 10, and 100 mM, respectively), the curve for the same cells pretreated with bupivacaine (300 μM, *B*, *blue*; n = 26, 10, 25, 10, and 25 for 0.01, 0.1, 1, 10, and 100 mM, respectively), and the curve for the pure culture of neurons without any pretreatment (*B*, *orange*; n = 36, 23, 15, 38, and 26 for 0.01, 0.1, 1, 10, and 100 mM, respectively). Effects of a pretreatment with 500 μM dihydrokainic acid on the regulatory effects of bupivacaine on the glutamate-induced increases in $[Ca^{2+}]_i$ in astrocytes cocultured with neurons (*C*, a typical trace; *D*, changes in peak, area under the curve (AUC), and Tau values, n = 31) and in neurons cocultured with astrocytes (*E*, a typical trace; *F*, changes in peak, AUC, and Tau values, n = 20). Cells were exposed to 1 mM glutamate for 10 s, and the data are expressed as the means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group (without bupivacaine treatment) of cocultured cells using a two-way ANOVA followed by Bonferroni *post hoc* test (*A* and *B*) and $^{**}P < 0.01$ compared with the glutamate-induced responses before bupivacaine treatment using a paired *t* test (*D* and *F*).

did not increase $[Ca^{2+}]_i$ in the pure culture of neurons perfused with a Ca^{2+}-free solution plus EGTA. At concentrations of 0.5 mM or more, bupivacaine noncompetitively inhibited recombinant *N*-methyl-D-aspartate receptors expressed in *Xenopus laevis* oocytes, with an a half maximal inhibitory concentration of approximately 1 mM.[34] Nishizawa *et al.*[35] did not observe an inhibitory effect of clinically relevant concentrations of bupivacaine on *N*-methyl-D-aspartate–induced currents in CA1 mouse pyramidal neurons using the whole cell patch clamp technique, with the exception of 1 mM bupivacaine. As a result, the effects of 0.3 to 300 μM bupivacaine on the

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glutamate-induced increase in $[Ca^{2+}]_i$ were not likely due to a direct action on the glutamate receptors because bupivacaine was not able to produce an effect on pure cultures of hippocampal neurons.

Bupivacaine has been the subject of extensive investigation to elucidate its documented neuro- and myotoxicity, and a number of studies have identified the mitochondria as a target of bupivacaine due to a severe impairment in oxidative phosphorylation.[36] Furthermore, in the study by Cela *et al.*,[6] the bupivacaine-induced depression of cellular respiration was closely related to the specific inhibition of respiratory chain complexes I and III accompanied by ROS production in a human hepatoma cell line. Unexpectedly, 0.3 to 300 μM bupivacaine only significantly decreased the mtΔΨ and increased ROS generation in rat hippocampal astrocytes in a concentration-dependent manner in our study. Treatment with the mPTP inhibitor CsA suppressed the decrease in the mtΔΨ and the increase in ROS generation induced by bupivacaine. The observation that the inhibitory effect of bupivacaine on the glutamate-induced increase in $[Ca^{2+}]_i$ was also suppressed by CsA in astrocytes cocultured with neurons is particularly interesting. Based on these results, clinically relevant concentrations of bupivacaine selectively target the astrocytic mitochondria to dissipate the mtΔΨ and produce excess ROS, ultimately leading to mitochondrial dysfunction and intracellular Ca^{2+} dysregulation.

Although glutamate is the primary excitatory neurotransmitter in the brain, overstimulation of glutamate receptors is highly toxic to neurons.[37] Hippocampal astrocytes express glutamate transporters responsible for glutamate reuptake and clearance from the synaptic cleft to prevent excitotoxic increase in glutamate concentrations, thus providing a form of neuroprotection.[38–40] Consequently, the simplest and most reasonable explanation for the potentiating effect of bupivacaine on neurons cocultured with astrocytes is that the glutamate uptake function of the cocultured astrocytes was impaired by bupivacaine, which in turn resulted in an accumulation of extracellular glutamate in the coculture system. This hypothesis is supported by the following facts. First, the concentration-response curve of intracellular Ca^{2+} signaling induced by 0.01 to 100 mM glutamate in pure neuronal cultures shifted to the left in a parallel manner without changing the maximal response compared with the curve for neurons cocultured with astrocytes (fig. 7B). This response occurred because extracellular glutamate was rapidly taken up by the cocultured astrocytes, thereby reducing the glutamate concentration around neurons. Second, the concentration-response curve of intracellular Ca^{2+} signaling induced by glutamate in neurons cocultured with astrocytes that had been pretreated with 300 μM bupivacaine was almost the same as in the curve for pure neuronal cultures without the pretreatment. Bupivacaine likely impaired the astrocytic mitochondria, subsequently inhibiting glutamate uptake. Moreover, glutamate transport into astrocytes is an energy-consuming process.[41,42] The extracellular glutamate concentration around neurons in the coculture system was increased by the bupivacaine treatment, reaching an extracellular glutamate concentration similar to pure cultures of neurons. Third, a pretreatment with 0.5 mM Dih, a selective inhibitor of glutamate transporter–1, suppressed the bupivacaine-induced potentiation of the glutamate-induced increase in $[Ca^{2+}]_i$ in neurons cocultured with astrocytes. Moreover, 0.5 mM Dih enhanced Ca^{2+} signaling in response to glutamate in neurons cocultured with astrocytes, mimicking the potentiating effect of bupivacaine (fig. 7E).

In the current study, a 50% reduction in glutamate uptake was observed in rat astrocytes cultured with 1.64 μM bupivacaine. According to a previous study by Lin *et al.*,[43] 1 μM bupivacaine inhibited glutamate release from rat cerebral cortex synaptosomes by only 15%. Therefore, we speculate that at lower toxic concentrations, bupivacaine might inhibit glutamate uptake by astrocytes before its inhibition of glutamate release from neurons, causing the extracellular accumulation of glutamate.

In the astrocyte/neuron coculture system, bupivacaine significantly increased the Tau values of both astrocytes and neurons, indicating that the time for glutamate-induced peak $[Ca^{2+}]_i$ levels to recover to baseline levels was prolonged by bupivacaine, which might be related to its central neurotoxicity. Because of the limitation of the experimental conditions, we were not able to determine why bupivacaine selectively damaged the astrocytic mitochondria, even though both astrocytes and neurons contained equivalent numbers of mitochondria.[41] Details of the precise relationship among the bupivacaine-induced mtΔΨ collapse, mPTP opening, and ROS generation in the astrocytic mitochondria require further clarification using mitochondria isolated from astrocytes and neurons and a high-resolution oximetry technique.

In conclusion, in primary cocultures of rat hippocampal astrocytes and neurons, clinically relevant concentrations of bupivacaine selectively impair astrocytic mitochondria, thereby suppressing glutamate uptake and indirectly potentiating the glutamate-induced increase in $[Ca^{2+}]_i$ in neurons.

Research Support

Support was provided by the National Science Foundation of China (No. 81573416; Beijing, China), the Ministry of Education (the Young Thousand Talent Program to Dr. W. Zhang; Beijing, China), the Hebei Natural Science Foundation (No. H2016206030; Shijiazhuang, China), and the High Talent Science Research Project of Education Bureau Hebei Province (No. GCC2014015; Shijiazhuang, China).

Competing Interests

The authors declare no competing interests.

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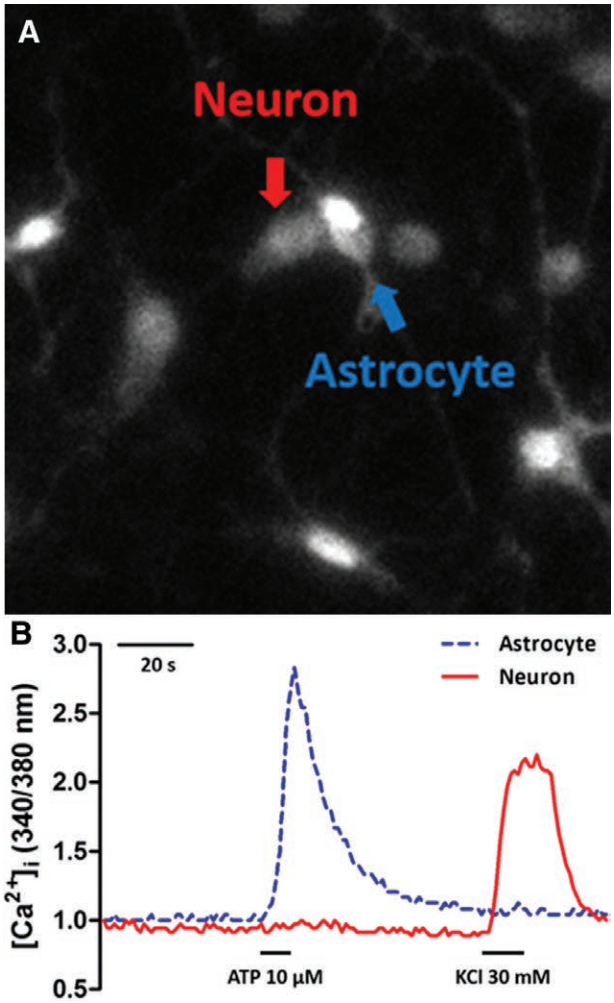
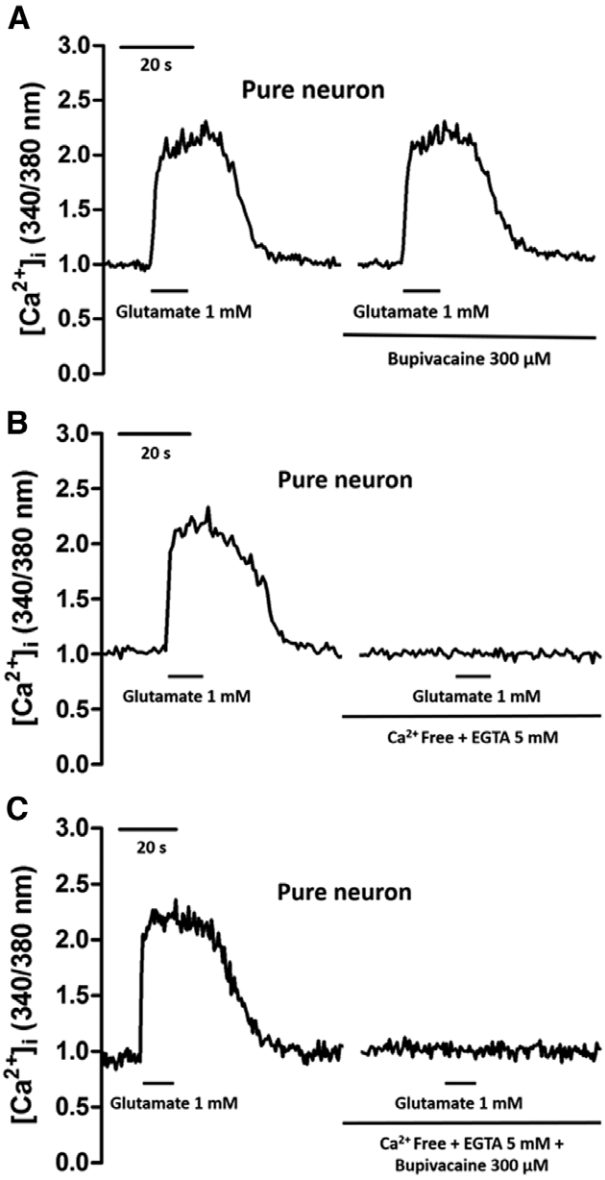
Appendix 1

Hippocampal neurons (*red arrow*) and astrocytes (*blue arrow*) in the astrocyte/neuron coculture system (*A*) and increases in $[Ca^{2+}]_i$ induced by 10 μM ATP in astrocytes (*B*, *red solid line*) cocultured with neurons or by 30 mM KCl in neurons (*B*, *blue dotted line*) cocultured with astrocytes were observed under a fluorescence microscope.



Appendix 2

Effects of a 5-min treatment with 300 μM bupivacaine (*A*, n = 16), a perfusion with a Ca^{2+}-free solution plus 5 mM EGTA (Ca^{2+}-free + EGTA; *B*, n = 37), and a combination treatment of bupivacaine with Ca^{2+}-free + EGTA (*C*, n = 34) on glutamate-induced increases in $[Ca^{2+}]_i$ in pure neuronal cultures. Cells were exposed to 1 mM glutamate for 10 s.



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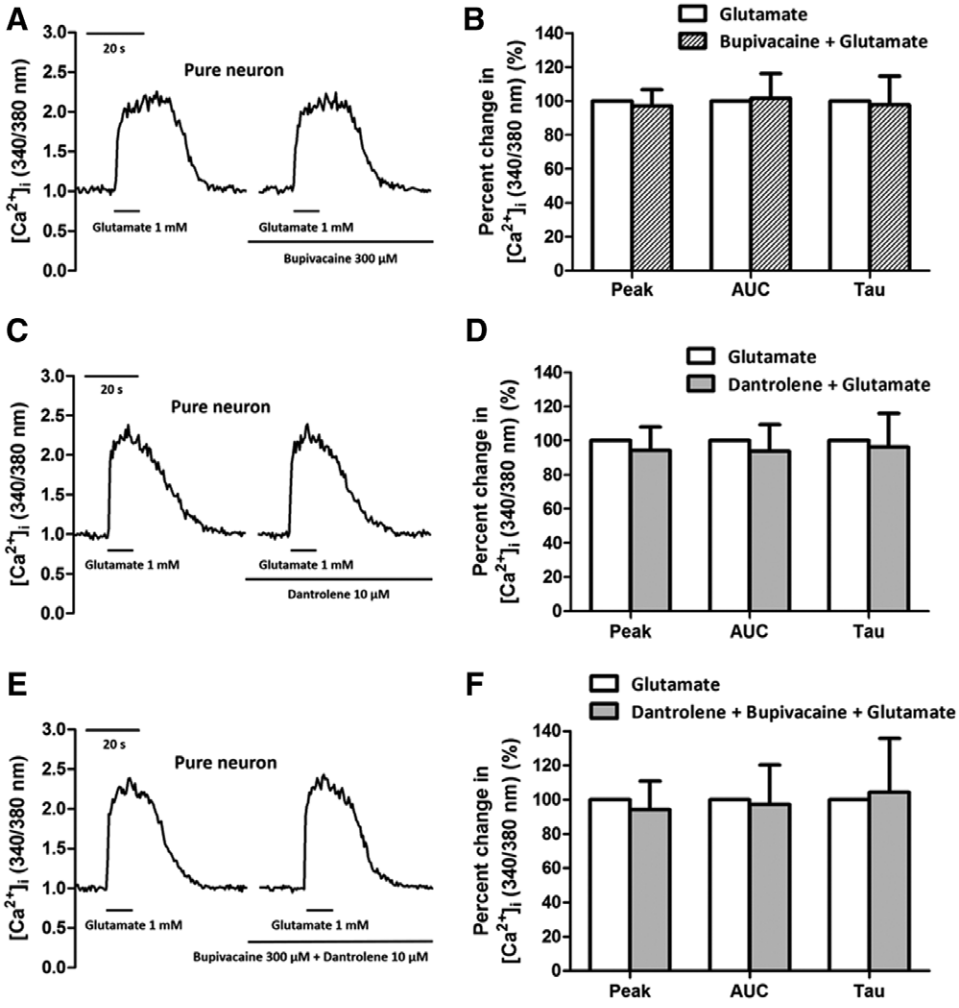
Appendix 3

Effects of a 5-min incubation with 300 μM bupivacaine (*A*, a typical trace; *B*, changes in peak, area under the curve (AUC), and Tau values, n = 13), 10 μM dantrolene (*C*, a typical trace; *D*, changes in peak, AUC, and Tau values, n = 17), or 10 μM dantrolene plus 300 μM bupivacaine (*E*, a typical trace; *F*, changes in peak, AUC, and Tau values, n = 18) on the glutamate-induced increases in $[Ca^{2+}]_i$ in pure neuronal cultures. Cells were exposed to 1 mM glutamate for 10 s, and the data are expressed as the means ± SD. $P > 0.05$ compared with the glutamate-induced responses before treatment using a paired *t* test.



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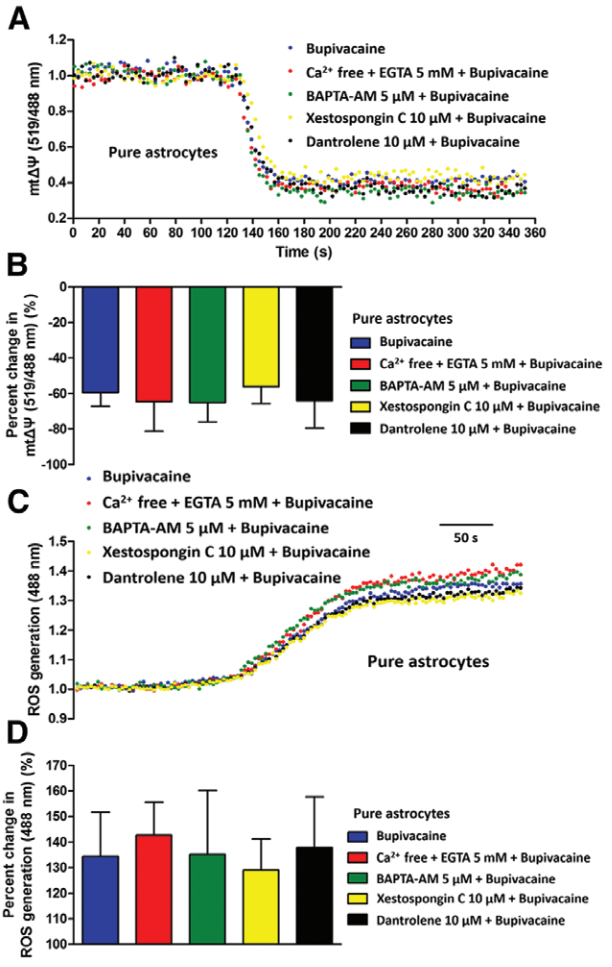
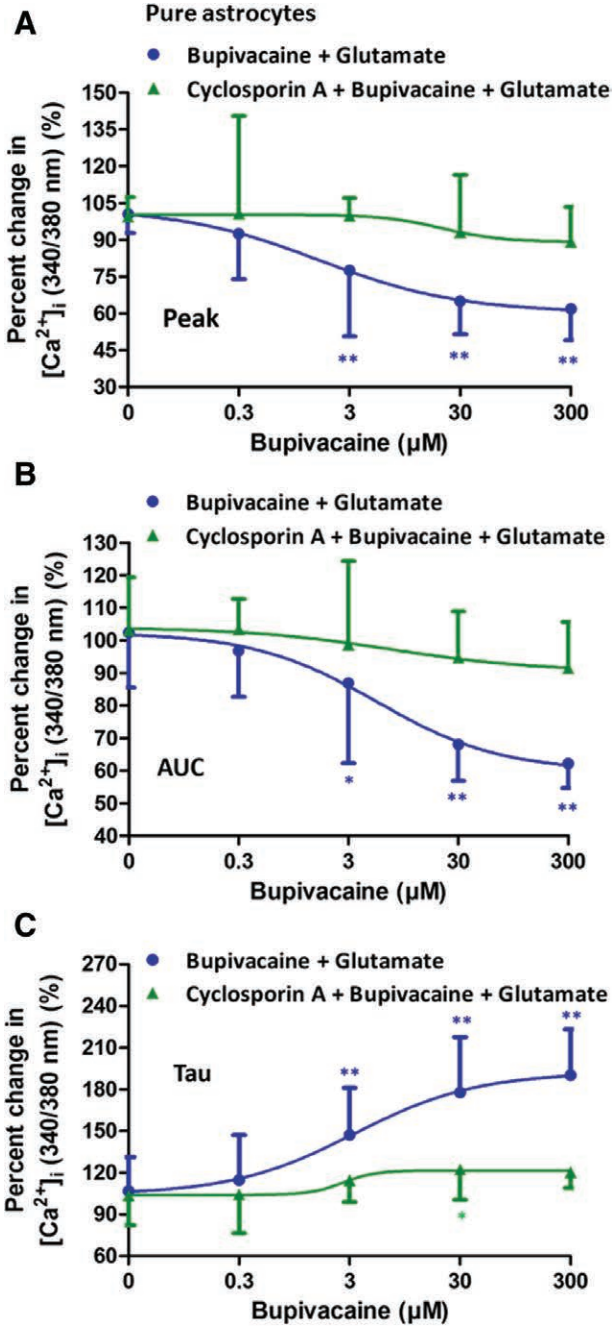
Appendix 4

Representative traces showing the decrease in mtΔΨ induced by 300 μM bupivacaine (*blue*), a combination of 300 μM bupivacaine and Ca^{2+}-free solution plus 5 mM EGTA (Ca^{2+}-free + EGTA, *red*), a combination of 300 μM bupivacaine and 5 μM 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM, *green*), a combination of 300 μM bupivacaine and 10 μM xestospongin C (*yellow*), and a combination of 300 μM bupivacaine and 10 μM dantrolene (*black*) in pure cultures of astrocytes (*A*). The mtΔΨ was measured by determining the ratio of red/green JC-1 fluorescence intensity. No significant differences were observed among the five groups (*B*; n = 20, 23, 22, 23, and 21 for blue, red, green, yellow and black, respectively). Representative traces showing reactive oxygen species (ROS) generation induced by 300 μM bupivacaine (*blue*), a combination of 300 μM bupivacaine and Ca^{2+}-free + EGTA (*red*), a combination of 300 μM bupivacaine and 5 μM BAPTA-AM (*green*), a combination of 300 μM bupivacaine and 10 μM xestospongin C (*yellow*), and a combination of 300 μM bupivacaine and 10 μM dantrolene (*black*) in pure cultures of astrocytes (*C*). ROS generation was measured using 2',7'-dichlorofluorescin-diacetate dye. No significant differences were observed among the five groups (*D*; n = 18, 29, 29, 25, 19 for blue, red, green, yellow and black, respectively). Data are expressed as the means ± SD. $P > 0.05$ compared with the bupivacaine group using a one-way ANOVA followed by Dunnett's test. mtΔΨ = mitochondrial membrane potential.



Appendix 5

Concentration-dependent inhibitory effects of a 5-min bupivacaine incubation on the glutamate-induced increase in $[Ca^{2+}]_i$ in pure cultures of astrocytes (*A–C*, *blue circles*, changes in peak, area under the curve (AUC), and Tau values, n = 17, 14, 16, 14, and 13 for 0, 0.3, 3, 30, and 300 μM bupivacaine, respectively) and the effects of a 5-min incubation with 1 μM cyclosporin A plus bupivacaine on the same parameter in pure cultures of astrocytes (*A–C*, *green circles*, changes in peak, AUC, and Tau values, n = 18, 19, 14, 14, and 20 for 0, 0.3, 3, 30, and 300 μM bupivacaine plus cyclosporin A, respectively). Cells were exposed to 1 mM glutamate for 10 s, and the data are expressed as the means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the glutamate-induced response before treatment with bupivacaine or bupivacaine plus cyclosporin A using a one-way ANOVA followed by Dunnett's test.



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