Long-term Exposure to Local but Not Inhalation Anesthetics Affects Neurite Regeneration and Synapse Formation between Identified Lymnaea Neurons

Shin Onizuka, M.D.,* Mayumi Takasaki, M.D., Ph.D.,† Naweed I. Syed, Ph.D.‡

Background: General and local anesthetics are used in various combinations during surgical procedures to repair damaged tissues and organs, which in almost all instances involve nervous system functions. Because synaptic transmission recovers rapidly from various inhalation anesthetics, it is generally assumed that their effects on nerve regeneration and synapse formation that precede injury or surgery may not be as detrimental as that of their local counterparts. However, a direct comparison of most commonly used inhalation (sevoflurane, isoflurane) and local anesthetics (lidocaine, bupivacaine), vis-à-vis their effects on synapse transmission, neurite regeneration, and synapse formation has not yet been performed.

Methods: In this study, using cell culture, electrophysiologic and imaging techniques on unequivocally identified presynaptic and postsynaptic neurons from the mollusc *Lymnaea*, the authors provided a comparative account of the effects of both general and local anesthetics on synaptic transmission, nerve regeneration, and synapse formation between cultured neurons.

Results: The data show that clinically used concentrations of both inhalation and local anesthetics affect synaptic transmission in a concentration-dependent and reversal manner. The authors provided the first direct evidence that long-term overnight treatment of cultured neurons with sevoflurane and isoflurane does not affect neurite regeneration, whereas both lidocaine and bupivacaine suppress neurite outgrowth completely. The soma–soma synapse model was then used to compare the effects of both types of agents on synapse formation. The authors found that local but not inhalation anesthetics drastically reduced the incidence of synapse formation. The local anesthetic–induced prevention of synapse formation most likely involved the failure of presynaptic machinery, which otherwise developed normally in the presence of both sevoflurane and isoflurane.

Conclusion: This study thus provides the first comparative, albeit preclinical, account of the effects of both general and local anesthetics on synaptic transmission, nerve regeneration, and synapse formation and demonstrates that clinically used lidocaine and bupivacaine have drastic long-term effects on neurite regeneration and synapse formation as compared with sevoflurane and isoflurane.

MOST surgical procedures require either general or local anesthetic treatments, which last from a few minutes to several hours. Although surgical interventions necessitate the use of various anesthetic agents, their choices should be based on the ones with the least deleterious effects on neuronal function, nerve regeneration, and synaptic repair. This information is important in light of better choices vis-à-vis various agents that are available to date and are used extensively in clinical practices. To render such choices feasible, comparable data for the long-term effects on synaptic transmission, regeneration, and synapse formation are needed; however, with a few notable exceptions†‡ for both general and local anesthetics, no such data are currently available.

Notwithstanding the fact that various inhalation (such as sevoflurane, halothane, and isoflurane), intravenous (propofol, thiopental, and ketamine), and local anesthetic agents (such as lidocaine and bupivacaine) perturb nervous system functions by disrupting either synaptic transmission or nerve conductions, their precise modes of actions vary considerably from preparation to preparation." Inhalation anesthetics, for example, affect synaptic transmission by either blocking presynaptic transmitter release3 or suppressing postsynaptic receptor function5–9 at both excitatory10–12 and inhibitory synapses,13,14 and these responses may invoke a variety of ion channels and second messengers.12,15–18 Similarly, intravenous anesthetics such as propofol bring about synaptic depression by enhancing either the function of γ-aminobutyric acid receptors8,12,19–21 or perhaps by suppressing presynaptic glutamate release.3 Despite our current understanding of the cellular basis of anesthesia, the precise mechanisms by which both general and intravenous agents perturb nervous system function remain largely unknown. This limited understanding of how anesthetics affect neuronal communications in the nervous system stems from anatomical challenges that are often met in most mammalian preparations where direct cell–cell interactions are difficult to study unequivocally. Moreover, as compared with their intravenous and inhalation counterparts, even less is known about the mechanisms by which local agents affect synaptic transmission during pain, surgery, and functional recovery.

In addition to some undesired side effects of all acutely applied anesthetics,22 long-term treatments of neuronal tissue with both inhalation23 and local agents24,25 cause widespread learning defects and degeneration. Whether such long-term (hours to days) exposure of the neuronal
tissue to these anesthetics also affects nerve regeneration and synapse formation has not yet been determined.

In this study, we took advantage of an ideal model preparation in which synaptic transmission between uniquely identified neurons can be investigated at the level of single presynaptic and postsynaptic neurons. Individually isolated neurons from the mollusc Lymnaea not only regenerate their neurites in cell culture but also recapitulate their specific patterns of synapses, which are similar to those seen in vivo. This in vitro approach using Lymnaea neurons has been used extensively to decipher both cellular and synaptic mechanisms by which various anesthetics affect neuronal function and synaptic transmission.35,26–34

Here, we sought to determine and compare how clinically relevant concentrations of inhalation (sevoflurane and isoflurane) and local anesthetics (lidocaine and bupivacaine) affect synaptic transmission and whether these actions involve presynaptic mechanisms, postsynaptic mechanisms, or both. Moreover, we provide the first direct and comparative account of how inhalation and local anesthetics affect neurite regeneration and synapse formation. Specifically, our data show that both sevoflurane/isoflurane and lidocaine/bupivacaine block cholinergic synaptic transmission between the paired presynaptic and postsynaptic neurons. Long-term (overnight) sevoflurane/isoflurane treatment of the cultured neurons did not affect neurite outgrowth, whereas the cultured cells did not exhibit regeneration in the presence of both lidocaine and bupivacaine. Because cells did not extend neurites in the presence of local anesthetics, we thus adopted the soma–soma model (synapses develop between the cell bodies in the absence of neurites) to ask the question whether lidocaine and bupivacaine also affect synapse formation between the paired cells. Although both anesthetics reduced the incidence of synapse formation between the paired cells, this synaptogenesis was severely compromised by lidocaine and bupivacaine. Taken together, our data provide the first direct evidence that long-term treatment of neurons with local but not inhalation anesthetics is severely detrimental for neurite outgrowth and synapse formation.

Materials and Methods

Animals

Laboratory-raised stocks of the fresh water pond snail Lymnaea stagnalis were maintained at room temperature (18–20°C) in an aquarium containing well-aerated and dechlorinated tap water and were fed lettuce. Animals aged 1–2 months (shell length, 10–15 mm) were used for cell isolation while the brain-conditioned medium (CM) was prepared from 3- to 4-month-old animals (shell length, 15–25 mm). (Animal Care Certification is not required for invertebrate species such as L. stagnalis at the University of Calgary Animal Resource Centre, Calgary, Alberta, Canada.)

Cell Culture

Cells were isolated individually and cultured as described previously.34 Briefly, snails were anesthetized with 10% Listerine (21.9% ethanol, 0.042% menthol; Pfizer Inc., New York, NY) solution in normal Lymnaea saline (containing 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, and 1.5 mM MgCl₂) buffered to pH 7.9 with HEPES. The central ring ganglia were removed under sterile conditions and washed with antibiotic saline (40 µg/ml gentamycin; two washes, 10 min each). The ganglia were then treated with 0.2% trypsin (Sigma type III; Sigma Chemical Company, St. Louis, MO) for 22 min followed by 0.2% soybean trypsin inhibitor (Sigma type 1-S; Sigma Chemical Company) for 10 min, both in defined medium (DM). DM consisted of serum-free 50% L-15 medium with added inorganic salts (40 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, and 10 mM HEPES). The pH was adjusted to 7.9 with 1 N NaOH, and 20 µg/ml gentamycin was added. The enzymatically treated ganglia were pinned to the bottom of a dissection dish that contained 8 ml high-osmolarity DM (DM with 37.5 mM glucose).

The cells were isolated by applying gentle suction to a fire-polished and Sigmacote (Sigma Chemical Company)-treated pipette. The isolated cells were plated on poly-l-lysine pretreated coverslips glued to the bottom of a 35-mm Falcon dish in the presence of CM. The CM was prepared by incubating central ganglia in DM (12 ganglia/6 ml DM) for 4 or 5 days and frozen until used. Isolated somata of identified neurons were either allowed to extend neurites or were juxtaposed in a soma-soma configuration and left undisturbed overnight.

Neurite Outgrowth

To assess neuronal regeneration in either the absence or the presence of each anesthetic, identified neurons were isolated in cell culture and plated on poly-l-lysine-coated dishes containing CM plus the anesthetics. Presynaptic (visceral dorsal 4 [VD4]) and postsynaptic (left pedal dorsal 1 [LPeD1]) neurons were selected for neurite outgrowth and synapse formation assays. To test for the effects of each anesthetic agent on neurite outgrowth and synapse formation, the anesthetic was added to the dish 1 h after cell plating, and the neurons were maintained overnight in the dark. Neuronal sprouting was assessed as described previously.36 Specifically, only those neurons exhibiting outgrowth (multiple branches, active growth cones, and so forth) equivalent to five somata diameter were considered as sprouted. The extent of outgrowth was calculated as a function of maximum neurite length. Neurons cultured overnight in CM alone served as controls.
Neurite–Neurite Synapses
To prepare neurite–neurite synapses, the isolated cells were plated in CM a few soma diameters apart and were allowed to extend neurites. After 24–48 h, the cells exhibited extensive outgrowth with multiple overlapping neurites. The cells were cultured overnight either in CM alone or CM plus the anesthetic, which was subsequently washed out during electrophysiologic recordings. To demonstrate the reversibility of anesthetic-induced effects, the compound was washed away with normal saline for more than an hour before the intracellular recordings.

Electrophysiologic Recording
Conventional intracellular recording techniques were used.34 Specifically, glass microelectrodes (1.5 mm ID, with filament; World Precision Instruments, Sarasota, FL) were pulled on a vertical electrode puller (Kopf, 700C; David Kopf Instruments, Tujunga, CA) and filled with a saturated solution of K2SO4 (resistance, 30–60 MΩ). Isolated cells were viewed under a Zeiss (Telaval 31; Carl Zeiss Canada Ltd., North York, Ontario, Canada) inverted microscope and impaled using Narishigi micromanipulators (model MO-103; Narishigi Instruments, Tokyo, Japan). The intracellular signals were amplified via a preamplifier (Neurodata model IR-283; Cygnus Technology Inc., Delaware, PA), displayed on a storage oscilloscope (Tektronix R5103N; Tektronix, Montreal, Quebec, Canada), and recorded and stored on a computer using Digidata software (Axon Instruments, Union City, CA). All experiments were performed at room temperature (18°–22°C).

Drugs
Sevoflurane (Sevofrane; Maruishi, Osaka, Japan) and isoflurane (Forane; Abbott, Abbott Park, IL) were selected as inhalation anesthetics, whereas lidocaine (Xylocaine; AstraZeneca, London, United Kingdom) and bupivacaine (Anapin; AstraZeneca) were diluted into CM and served as local agents. VD4 and LPeD1 synapse pairs were exposed to sevoflurane and isoflurane (bubbled overnight) at concentrations of 0.2, 2, 10, 20, and 100 μM, and lidocaine and bupivacaine were used at concentrations of 0.001, 0.005, 0.01, 0.1, and 1 μM. Exogenous application of acetylcholine (Sigma Chemical Company), was performed (80-ms pulses, 1–2 psi) in some experiments using 1 μM acetylcholine applied directly to the synaptic site via a Pneumatic PicoPump (PV800; World Precision Instruments) pressure injector.

FM1-43 Imaging
Cells were incubated in 20 μM FM1-43 (Molecular Probes, Eugene, OR) for 10 min before the addition of 0.01 mM lidocaine or 5 mM sevoflurane to the bath. The presynaptic cell (VD4) was stimulated to generate 100 action potentials (10 spikes/burst) by conventional electrophysiologic techniques to facilitate the uptake of FM1-43 in cells that were paired overnight in either the presence or the absence of each anesthetic. The styryl dye and anesthetics were then replaced with cold saline to prevent neuronal firing during the washout and to remove background fluorescence. Fluorescent images of the FM1-43-labeled cells were acquired using a Zeiss (Carl Zeiss Canada Ltd.) Axiovert 200M inverted microscope. Excitation light was from a 100-W Hg lamp, excitation filters (490/30 nm), a dichroic mirror (505 nm), and emission filters (570 low pass nm or 610 nm). Phase and fluorescent images were captured with a Photometrics (Tuscon, AZ) Sensys 1400 camera (1- to 100-ms exposure), connected to a computer running Axiovision 3.0 for Windows (Carl Zeiss Canada Ltd.). The pixels in cross-section of the fixed area (200 × 150 μm2) were integrated and measured with NIH image software (version 1.62; National Institutes of Health, Bethesda, MD).

Results
Specific Synapse Formation between Identified Neurons in Cell Culture
A well-established model of identified *Lymnaea* neurons35 was used to reconstruct synapses in either a neurite-neurite33,37 or a soma–soma configuration.35,37–40 Specifically, identified presynaptic neuron VD4 and its postsynaptic partner LPeD1 were isolated from the adult animals and cultured overnight in the presence of CM. Within 12–24 h, neurons (n = 20) exhibited extensive outgrowth (fig. 1A). Simultaneous intracellular recordings revealed that current injection in VD4 (at arrow, fig. 1B) generated 1:1 excitatory postsynaptic potentials (EPSPs) in a manner similar to those observed in vivo.38 Similarly, when paired in a soma–soma configuration (n = 32; fig. 1C) in CM, synapses (90–100%) similar to those observed in a neurite-neurite mode also developed (80–100%), albeit in the absence of neurite outgrowth.41 Specifically, action potentials in VD4 (at arrow, fig. 1D) resulted in 1:1 EPSPs in LPeD1. This synaptic transmission has previously been shown to be cholinergic.38,39 It is important to note that the efficacy of synaptic transmission (consistency of EPSP amplitude) between VD4 and LPeD1 was more consistent in our soma–soma model as compared with the neurite-neurite configuration. Therefore, we opted to use the soma-
Fig. 1. Specific synapses reform between *Lymanea* neurons in a neurite–neurite and soma–soma configuration. Identified presynaptic (visceral dorsal 4 [VD4]) and postsynaptic (left pedal dorsal 1 [LPeD1]) neurons were isolated from visceral and left pedal ganglia, respectively. (A) Neurons were plated in close proximity and allowed to extend neurites in conditioned medium. Extensive outgrowth occurred within 24–48 h, and neurites overlapped. (B) Simultaneous intracellular recordings were made from both cells. Induced action potentials in VD4 (at arrows) generated 1:1 excitatory postsynaptic potentials (EPSPs) in LPeD1 (n = 20). (C) Neurons were isolated individually and paired in conditioned medium in a soma–soma configuration. Although neurons did not exhibit neurite outgrowth, the induced actions potentials in VD4 (D) generated 1:1 EPSPs in LPeD1 (n = 32), which were similar to those seen between neurite–neurite pairs.

soma preparation to test for the effects of anesthetics on synaptic transmission. Similar results, however, were obtained from the neurite–neurite preparation.

Both Inhalation and Local Anesthetics Block Excitatory Synaptic Transmission between VD4 and LPeD1

To test and compare the effects of inhalation (sevoflurane and isoflurane) and local anesthetics (lidocaine and bupivacaine) on cholinergic synaptic transmission between VD4 and LPeD1, the soma–soma paired cells were recorded in either the absence or the presence of various anesthetics. Synapses were tested electrophysiologically. Action potentials in VD4 under control saline conditions (fig. 2A) generated 1:1 EPSPs in LPeD1, and these excitatory responses were mimicked by exogenous acetylcholine (at arrow), which was pressure-applied directly at the synapse under a fast perfusion system.42 The perfusion solution was then switched to the saline containing 5 mM sevoflurane, and the synaptic transmission was tested again. We found that both the synaptic transmission and the cholinergic responses were reduced significantly (control, 11.9 ± 0.7 mV; 0.3 mM, 6.4 ± 1.1 mV; 5 mM, 2.2 ± 0.3 mV [n = 11 for each case]) in a concentration-dependent and reversible manner (fig. 2B). Similar results were obtained in the presence of isoflurane (control, 13.9 ± 3.6 mV; 0.3 mM, 7.1 ± 2.6 mV; 5 mM, 2.3 ± 0.9 mV [n = 10 for each]) (fig. 2B).

The above data thus show that both sevoflurane and isoflurane depress synaptic transmission between VD4 and LPeD1 and also the postsynaptic cholinergic responsiveness in LPeD1. These effects were concentration-dependent, and the synaptic transmission recovered within minutes of washout with normal saline (fig. 2B).

Next, we sought to determine the effects of local anesthetics lidocaine and bupivacaine on cholinergic synaptic transmission between VD4 and LPeD1. Synapses were reconstructed as described above, and both the synaptic transmission and the cholinergic responses were tested in either the absence or the presence of lidocaine (fig. 3A). Action potentials in VD4 induced 1:1 EPSPs in LPeD1, and these responses were mimicked by exogenous acetylcholine (applied at arrow). Although lidocaine significantly reduced the amplitude of VD4-induced EPSPs in LPeD1 (EPSP amplitude: control, 11.4 ± 2.6 mV [n = 10]; 0.1 mM lidocaine, 5.6 ± 1.5 mV [n = 8]; 1 mM lidocaine, 1.9 ± 0.8 mV [n = 9]) (fig. 3A), the cholinergic response remained unperturbed as compared with sevoflurane (acetylcholine amplitude: con-
Local but Not Inhalation Anesthetics Suppress Neurite Outgrowth from Cultured Neurons

To test the hypothesis that both inhalation and local anesthetics affect neurite regeneration and synapse formation from isolated *Lymnaea* neurons, cells were cultured in CM in either the absence or the presence of any given anesthetic agent and were allowed to extend neurites. Specifically, cells were paired in close proximity and were allowed to extend neurites. We reasoned that if neurons regenerated their processes, the synapses would develop between the neurites, which could then be detected electrophysiologically. Under normal culture conditions, both cells extended neurites (fig. 4), and when tested electrophysiologically, synapses were detected as described previously (fig. 1; not shown here). Similarly, cells cultured in 5 mM sevoflurane (bubbled and superfused overnight) exhibited extensive outgrowth, which was indistinguishable from control (fig. 4). To demonstrate that the inhalation anesthetics had not evaporated or catalyzed overnight, the synaptic transmission was first tested electrophysiologically in the presence of the incubating medium (CM plus sevoflurane) before washout with normal saline. Under these experimental conditions, action potentials in VD4 did not generate any response in the postsynaptic cell (n = 11; data not shown). However, within a few minutes after washout with normal saline, synapses similar to those observed under control conditions were detected (data not shown). In contrast to the general anesthetics (control, 1.4 ± 0.4 mm [n = 12]; 2 mM sevoflurane, 1.4 ± 0.5 mm [n = 10]; 5 mM sevoflurane, 1.2 ± 0.4 mm [n = 11]), the cultured neurons did not extend neurites in the presence of lidocaine or bupivacaine (0.01 mM...
lidocaine, 0.4 ± 0.3 mm [n = 10]; 0.1 mM lidocaine, 0.2 ± 0.3 mm [n = 10]; 0.01 mM bupivacaine, 0.3 ± 0.3 mm [n = 10]; 0.1 mM bupivacaine, 0.1 ± 0.2 mm [n = 11]) (fig. 5).

These data thus demonstrate that both lidocaine and bupivacaine significantly retard neurite outgrowth from cultured neurons, whereas in the presence of their inhalation counterparts, both neurite processes and synapses develop normally (fig. 5). Because cells paired in local anesthetics did not extend neurites (and thus the synapse formation), their effects on synapse formation could not be tested and compared with sevoflurane and isoflurane. To test their effects on synaptogenesis directly, we therefore resorted to our soma–soma model where synapses could develop in the absence of neurite outgrowth.

Local but Not Inhalation Anesthetics Retard Synapse Formation between Soma–Soma Pairs

To test and compare the effects of both general and local anesthetics on synapse formation, cells were soma-soma paired in CM overnight in either the absence or the presence of various agents. The incidence of synapse formation between the paired cells was tested electrophysiologically. Under normal CM conditions, the incidence of synapse formation ranged between 90 and 100% (fig. 6). Similarly, neurons paired within the clinical concentration range of sevoflurane/isonflurane also formed normal synapses, although the percent of cells forming excitatory synapses decreased with increasing anesthetic concentrations (10 mM sevoflurane, 70% [n = 30; P < 0.05]; 2 mM sevoflurane, 90% [n = 18]; 0.3 mM sevoflurane, 100% [n = 12]; 10 mM isoflurane, 65% [n = 20]; 2 mM isoflurane, 80% [n = 20; P < 0.05]; 0.3 mM isoflurane, 95% [n = 15]). Cells paired overnight in the presence of both local anesthetics, however, did not develop synapses within the clinical range, even though morphologically they seemed healthy and exhibited normal intrinsic membrane properties (resting membrane action potential parameters and others) (1 mM lidocaine, 0% [n = 10]; 0.1 mM lidocaine, 0% [n = 10]; 0.01 mM lidocaine, 6% [n = 11]; 0.005 mM lidocaine, 15% [n = 11]; 0.001 mM lidocaine, 0% [n = 15]; 1 mM bupivacaine, 0% [n = 10]; 0.1 mM bupivacaine, 3% [n = 9]; 0.01 mM bupivacaine, 11% [n = 9]; 0.005 mM bupivacaine, 21% [n = 9]; 0.001 mM bupivacaine, 50% [n = 12]) (fig. 6). It is interesting to note that in instances in which cells did form synapses in lower concentrations of both local agents, the synaptic transmission between these pairs failed rapidly and after 5–10 min of stimulation; no synaptic transmission was detectable. After several minutes of rest, however, the synaptic transmission resumed, although the amplitude of postsynaptic potential was significantly reduced (data not shown).

Figure 7 shows examples of representative traces from cells cultured under control conditions and in the presence of sevoflurane, isoflurane, lidocaine, and bupiva-
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Fig. 7. Local anesthetics block synapse formation between visceral dorsal 4 (VD4) and left pedal dorsal 1 (LPeD1). Cells were paired overnight in either the absence or the presence of various anesthetics. Normal synapses were detected in conditioned medium (control), and the postsynaptic cell exhibited a depolarizing response to exogenously applied acetylcholine (ACh) (at arrow). Similarly, both synaptic and cholinergic responses recorded from cells paired in sevoflurane and isoflurane were identical to those of control. Cells paired in lidocaine and bupivacaine (0.01 mM) exhibited significantly reduced incidence of electrophysiologically detectable synapses, whereas their cholinergic responses were normal.

caine. Synapses similar to those observed under control conditions developed in sevoflurane and isoflurane. Moreover, the cells treated with general anesthetic exhibited responses similar to those of cells with exogenously applied acetylcholine, as was seen under control conditions (fig. 7). Conversely, cells paired in both lidocaine (0.01 mM) and bupivacaine (0.01 mM) did not develop synapses, whereas their responses to exogenously applied acetylcholine were similar to those of control and both sevoflurane and isoflurane. These data are summarized in figure 8 (EPSP amplitude: control, 10.3 ± 3.4 mV [n = 38]; 5 mM sevoflurane, 8.8 ± 1.7 mV [n = 18]; 5 mM isoflurane, 8.2 ± 1.7 mV [n = 20]). Similarly, the amplitude of acetylcholine-induced depolarizing responses in LPeD1 observed in sevoflurane and isoflurane was similar to that observed under control conditions (acetylcholine amplitude: control, 19.8 ± 3.4 mV [n = 38]; 5 mM sevoflurane, 14.7 ± 5.1 mV [n = 18]; 5 mM isoflurane, 13.7 ± 4.9 mV [n = 20]) (fig. 8). Interestingly, although the incidence of synapse formation between cells paired in local anesthetics was significantly reduced (P < 0.05) (EPSP amplitude: 0.01 mM lidocaine, 0.5 ± 0.4 mV [n = 11]; 0.01 mM bupivacaine, 0.8 ± 0.3 mV [n = 9]) (fig. 8), their cholinergic responses in LPeD1 were similar to those of control and the inhalation anesthetics (acetylcholine amplitude: 0.01 mM lidocaine, 16.5 ± 4.8 mV [n = 11]; 0.01 mM bupivacaine, 19.6 ± 2 mV [n = 9]) (fig. 8). These data lend further support to the idea that neuronal failure to develop synapses in the presence of local anesthetic is not due to its toxic effects on neuronal excitability or the desensitization of the postsynaptic receptors.

We next sought to determine whether the absence of synapses between VD4 and LPeD1 cultured in local anesthetics could be due to the fact that the presynaptic machinery may not have developed in their presence. Specifically, cells were paired overnight in either the absence or the presence of anesthetics (fig. 9). On day 2, the anesthetic solution was replaced with normal saline containing the dye FM1-43, and the presynaptic cell was stimulated electrically. We reasoned that if cells possessed normal, functional presynaptic machinery (exocytosis and endocytosis), we should expect labeling of the presynaptic cell at its contact with the postsynaptic neuron. To test this possibility, VD4 was stimulated to fire action potentials via current injections (100 action potentials) in the presence of the dye FM1-43. The dye was then washed away with normal saline, and images were acquired. We found that under both normal (figs. 9A–C) and sevoflurane (figs. 9D–F) conditions, exclusive labeling of the presynaptic cells was discernible at its contact site with LPeD1 and also in its processes surrounding the postsynaptic somata (fig. 9B, control, and fig. 9E, sevoflurane). In contrast, faint or no staining of the presynaptic cell was observed in VD4 paired in the presence of lidocaine (figs. 9G and H).

Fig. 8. Summary data depicting the amplitude of excitatory postsynaptic potentials (EPSPs) and cholinergic responses recorded from cells paired in various anesthetics. Visceral dorsal 4 paired with left pedal dorsal 1 in lidocaine and bupivacaine did not generate EPSPs in its postsynaptic partner, whereas cholinergic responses were similar to those of control. Normal synapses developed in sevoflurane and isoflurane, and the amplitude of both synaptic and cholinergic responses were identical to those of control. Cells paired in both lidocaine and bupivacaine (0.01 mM) exhibited significantly reduced incidence of electrophysiologically detectable synapses, whereas their cholinergic responses were normal.
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Fig. 9. Long-term lidocaine treatment perturbs the development of presynaptic machinery. To test whether the transmitter secretory machinery of the presynaptic cell paired overnight in lidocaine was perturbed, the exocytotic and endocytotic profiles were analyzed through FM1-43 imaging. Visceral dorsal 4 (VD4) cells paired overnight under control (A and B), sevoflurane (D and E), or lidocaine (G and H) but recorded under normal saline conditions were loaded with the dye FM1-43. Specifically, VD4 was stimulated (100 action potentials) in the presence of FM1-43, and the dye was washed away with normal saline. Images were acquired under both control (B) and sevoflurane (E) conditions. VD4 was extensively loaded at either its contact site with left pedal dorsal 1 (LPedD1) or in its processes surrounding the postsynaptic somata (at arrow). The corresponding panels under each image (C, F, I) represent the pixel values, and these values are averaged in J. * Data were statistically different from the control value.

Discussion

This study provides the first direct comparative account of the effects of general and local anesthetics on synaptic transmission (short term), nerve regeneration, and synapse formation (long term). We have demonstrated that although both general (sevoflurane and isoflurane) and local anesthetics used at clinically relevant concentrations affect synaptic transmission between identified neurons in a concentration-dependent and reversible manner, long-term lidocaine and bupivacaine but not sevoflurane and isoflurane treatment retards neurite outgrowth and synapse formation. The neuronal failure to exhibit outgrowth and synapse formation in the presence of local agents, however, cannot be attributed to their toxic effects on intrinsic membrane properties or postsynaptic cholinergic receptors; rather, the presynaptic secretory machinery seems to have been perturbed.

The state of anesthesia almost exclusively involves either the suppression of excitatory or an enhancement of inhibitory synaptic transmission between any given pair or sets of functionally related neurons. Therefore, an imbalance between excitatory and inhibitory synaptic transmission is invoked by various agents, used in a variety of combinations, to create desired results for pain management. However, despite their extensive use in surgical procedures and pain management, our understanding is limited vis-à-vis the modes and sites of action of various anesthetic agents. Notwithstanding our recent progress toward elucidating how inhalation and intravenous anesthetics affect neuronal communications in the brain and achieve desired effects, much less is known about local agents (oral, injected, local) such as lidocaine, which are multimodel and serve as antiarrhythmics, antiepileptics, and anticonvulsants agents. Because the core of all nervous system functions resides at the synaptic transmission between any given presynaptic and postsynaptic neuron or a subset of such neurons, it is therefore pivotal to examine how anesthetics affect neuronal communication at the level of individual neurons. Such an approach may not be relevant for our understanding of the state of unconsciousness that is experienced during anesthesia or how pain perception is managed by the nervous system; nevertheless, it will provide basic understanding of how anesthetics function at a fundamental level. With this caveat in mind, we took advantage of a simple model system approach and used in vitro reconstructed synapses between functionally well-defined neurons. VD4 and LPedD1 are members of the cardiorespiratory network that comprises the respiratory central pattern generator. The synaptic connections between various central pattern-generating neurons are well characterized, and the entire network has been reconstituted in cell culture, where it generates the patterned rhythmic activity in a manner similar to that observed in vivo. Therefore, adult Lymnaea neurons not only regenerate but also recapitulate their specific connections in cell culture. Moreover, specific synapses between the central pattern generator neurons can also be reconstructed in a soma–soma configuration. The soma–soma synapses are also target cell contact specific and require new protein synthesis and gene transcription. Therefore, both morphologically and electrophysiologically, the soma–soma synapses are similar to those seen in vivo. The soma–soma prep-
aration has also been used in a number of other species to define the cellular mechanisms underlying synapse formation, synaptic transmission, and plasticity at a resolution not achievable elsewhere (Aplysia, Heli-
soma, leech; Nicholls). The usefulness of identified Lymnaea neurons for anesthetic research has previously been validated by a number of laboratories (halothane, sevoflurane, and propofol). In this study, we have compared the effects of both inhalation and local anesthetics on cholinergic synaptic transmission, which was suppressed equally by these agents. The sevoflurane- and iso- flurane-induced suppression most likely involved their actions on postsynaptic receptors because cholinergic synaptic transmission, which was suppressed equally by these agents, the synaptic depression induced by the local agents may involve presynaptic mechanisms.

In contrast with their inhalation counterparts, which exert myriad effects on a variety of presynaptic and postsynaptic ion channels, the local anesthetics primarily affect Na⁺ channels and block action potential propagation across the nerve. Their direct effects on either cell bodies or the synaptic sites, however, have not been fully investigated. Local anesthetics, such as procaine, suppressed nerve propagation in squid giant axon by blocking action potentials, whereas in this study, we could successfully generate spikes in Lymnaea neurons, even in the presence of higher concentrations of lidocaine and bupivacaine. Interestingly, within a few seconds after anesthetic perfusion, both cells were depolarized (5–10 mV), and this observation is consistent with an earlier study in which lidocaine increased intracellular Na⁺ concentration in Lymnaea neurons. It is also important to note that even though we could trigger single spikes in VD4, compound action potentials often did not occur (not shown) in response to a sustained depolarizing pulse. A similar voltage-induced inactivation of spikes has also been reported for Lymnaea neurons. It is therefore plausible that local anesthetics may not immediately affect Na⁺ channels required for a single action potential at rest (tonic block), but their depolarizing effects may shift the steady state inactivation curve, thus reducing the availability of various Na⁺ channels for spike generation. The neuronal inability to generate repetitive spikes can also be attributed to “use-dependent” or “frequency-depen- dent block” of Na⁺ channel function. Additional experiments are required to test for the direct versus indirect actions of local anesthetics on various intrinsic neuronal properties. In addition, to deduce whether local anesthetics may also affect presynaptic Ca²⁺ channels, the soma–soma model offers a wonderful opportunity for such research. It is also noteworthy that synaptic recovery from lidocaine and bupivacaine took much longer (several tens of minutes) upon drug washout as compared with sevoflurane and isoflurane, and these long-term effects may also be due to a slower removal of these compounds from the target sites and/or the hydrolysis resistance nature of these ester-type compounds.

In addition to their short-term effects on synaptic transmission, long-term exposure of the neuronal cells to a variety of general anesthetics has been shown to cause widespread degeneration and learning defects in developing rats. Similarly, long-term treatment of the rabbit facial nerve with lidocaine and bupivacaine perturbed its degeneration and regeneration whereas in other studies, these agents were reported to induce apoptosis in various cell types. Lidocaine has also been reported previously to cause axonal degeneration in the posterior root of rats. To add to the list of these toxic effects, the current study demonstrated that lidocaine and bupivacaine also completely suppress neurite outgrowth from cultured neurons and blocked synapse formation between the soma–soma paired cells. Regarding the effects of local anesthetic on synapse formation, these data are consistent with our previous study in which long-term propofol treatment of the soma–soma paired cells also blocked synapse formation between VD4 and LPeD1. However, it is interesting to note that the cells paired in propofol did reestablish their specific synapses after several hours of anesthetic washout, whereas in the current study, synapses did not develop even after a day of the anesthetic removal (5 µM propofol, 70% [n = 10]; 10 µM, 60% [n = 9]; 25 µM, 25%, [n = 8] 50 µM, 0% [n = 6]). One may argue that a long-term treatment of the paired cells with local anesthetics may have rendered the synaptic transmission undetectable. To rule out this possibility, we performed the experiments in which cells were first allowed to develop synapses in CM and then the pair was exposed overnight to the local anesthetics. On day 3, the anesthetic was washed, and within an hour of medium removal, normal synaptic transmission was detected (not shown). These results therefore argue in favor of the idea that local anesthetics block synapse formation between VD4 and LPeD1. Another interesting difference between propofol and local anesthetic was that in the presence of the former, the cells that normally form chemical synapses exhibited electrical coupling, which is not observed in vivo between the cells. However, this electrical coupling was not observed in either lidocaine or bupivacaine, further under-
scoring their effects on both chemical and electrical synapses.

When comparing the effects of sevoflurane, isoflurane, and propofol with lidocaine/bupivacaine on neurite outgrowth, both inhalation (sevoflurane/isoflurane, this study) and intravenous (propofol)\textsuperscript{5} anesthetics did not affect neuronal sprouting, and cells extended processes that were indistinguishable from those of cells cultured in CM. This negative result could not be attributed to the volatile nature of this compound because no synaptic transmission was observed between the paired cells before the anesthetic washout with saline. However, neurite outgrowth was completely retarded by both local anesthetics (lidocaine and bupivacaine). Consistent with these data are earlier studies in which lidocaine was found to induce the collapse of growth cones from dorsal root ganglia neurons,\textsuperscript{2} and these effects may involve an intracellular enhancement of growth cone Ca\(^{2+}\) concentration in a manner similar to that observed after tetracaine treatment.\textsuperscript{3} On one hand, these studies demonstrate a Na\(^{+}\) channel-independent action of local anesthetics on neuronal tissues, and together with our data, they provide unequivocal evidence that long-term exposure of neurons to local but not general anesthetics is detrimental to nerve regeneration. However, these effects could not be attributed to the local anesthetic-induced toxic influences on neuronal viability or a perturbation of their intrinsic membrane properties because all neurons seemed healthy and electrophysiologically viable (firing action potential and response to acetylcholine pulses). Therefore, as demonstrated previously, one of the likely mechanisms by which local agents may prevent neurite outgrowth is to increase intracellular concentration in the growth cones from a growth permissive to a growth suppressive range.\textsuperscript{55–57} Whether the local anesthetic-induced suppression observed here also involved intracellular Ca\(^{2+}\) remains to be determined.

We have previously demonstrated that voltage-induced Ca\(^{2+}\) hot spots develop between soma-soma paired cells and that these are both target cell and contact site specific.\textsuperscript{42} Consistent with these data are the studies that showed that the presynaptic secretory machinery is specialized at the contact site between the cells. Specifically, the dye FM1-43 (which labels vesicles during exocytosis and is subsequently internalized during endocytosis) did not label presynaptic neurons that had not developed synapses after long-term propofol treatment.\textsuperscript{55} In the current study, we have demonstrated that cells treated chronically with local anesthetics also did not label with the dye, suggesting that in the presence of these anesthetics, the transmitter secretory machinery responsible for exocytosis and endocytosis of cholinergic vesicles does not assemble at the contact site between the paired cells Whether this failure is due to neuronal inability to target Ca\(^{2+}\) channels at the synaptic site or a disruption of other candidate molecules in the secretory pathway remains to be investigated.

In summary, notwithstanding the need to develop better therapeutic drugs for long-term pain management, analgesia, and anesthesia, the current preclinical study underscores the importance of a rigorous screening of such agents regarding their long-term effects on nerve regeneration and synapse formation. The ageing population and advanced surgical procedures necessitate the use and development of long-lasting local anesthetics for both short- and long-term pain management. However, these strategies should not be at the expense of their deleterious effects on nerve regeneration and synapse formation, which are pivotal for functional recovery after the loss of all nervous system function after surgery. On one hand, the data presented here provide a comparison between various anesthetics for their effects on synaptic transmission, regeneration, and synapse formation, and on the other hand, this model system provides us with an unparalleled opportunity to identify various genes and their products that are differentially regulated in single cells after long-term anesthetic treatment.

The authors thank Wali Zaidi (Technician, Calgary Brain Institute, University of Calgary, Calgary, Alberta, Canada) for excellent technical support.

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