Anesthetic Treatment Blocks Synaptogenesis But Not Neuronal Regeneration of Cultured Lymnaea Neurons

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INTRODUCTION

Complex surgical procedures necessitate extensive exposure to anesthetics. Therefore it is important to investigate the impact of long-term anesthetic exposure on nervous system function, regeneration, and repair. Anesthetics have been shown to perturb both excitatory (El Beheiry and Pul 1989; MacIver et al. 1989; Wakasugi et al. 1999) and inhibitory synaptic transmission in the nervous system (Hamakawa et al. 1999a; Pocock and Richards 1991), although the precise site(s) of anesthetic action (pre- or postsynaptic) remain elusive. The reasons for this lack of fundamental knowledge regarding the direct effects of general anesthetics on synaptic transmission are perhaps best pointed out by Perouansky and Hemmings (2003), who state that: “the small size of most nerve terminals in the CNS (<1 μm diameter) hampers direct electrophysiological analysis of presynaptic events, and direct measurements of action-potential-evoked transmitter release from single CNS synapses is not possible.”

Because anesthetics affect synaptic transmission in the nervous system, it is reasonable to assume that nerve regeneration and synapse formation, which rely on activity dependent mechanisms (Lohman et al. 2002), may also be influenced by chronic anesthetic exposure. However, few previous studies have addressed this issue directly. Although, for example, Jevtovic-Todorobic and colleagues (2003) recently have shown that an early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and produces persistent learning defects, the underlying mechanisms remain unknown.

In this study, we used in vitro cell culture techniques to define the mechanisms by which propofol affects synaptic transmission between identified pre- and postsynaptic neurons from the mollusc Lymnaea. We also tested the hypothesis that chronic propofol treatment affects nerve regeneration and synapse formation between cultured Lymnaea neurons.

When cultured in vitro, the adult Lymnaea neurons not only regenerate their neuritic processes but also reform their specific patterns of synaptic connections (Syed et al. 1990). We have previously demonstrated that specific synapses between the identified, cholinergic neuron visceral dorsal 4 (VD4), the presynaptic cell, and its postsynaptic partner left pedal dorsal 1 (LPeD1) can be reconstructed in a soma-soma configuration (Hamakawa et al. 1999b; Smit et al. 2001; Woodin et al. 2002). These soma-soma synapses are ultrastructurally and electrophysiologically similar to synapses in their control counterparts.

After drug washout, appropriate synapses did reform between the sprouted neurons failed to develop chemical (VD4 and LPeD1) and the electrically coupled Pedal A cluster neurons (PeA) did not affect nerve regeneration in cell culture as the neurons continued to exhibit extensive neurite outgrowth. However, these sprouted neurons failed to develop chemical (VD4 and LPeD1) and electrical (PeA) synapses as observed in their control counterparts. After drug washout, appropriate synapses did reform between the neurons in a manner similar to that seen in vivo. Long-term anesthetic treatment of the identified neurons visceral dorsal 4 (VD4) and left pedal dorsal 1 (LPeD1) and the electrically coupled Pedal A cluster neurons (PeA) did not affect nerve regeneration in cell culture as the neurons continued to exhibit extensive neurite outgrowth. However, these sprouted neurons did develop chemical (VD4 and LPeD1) and electrical (PeA) synapses as observed in their control counterparts. After drug washout, appropriate synapses did reform between the cells, although this synaptogenesis required several days. Taken together, this study provides the first direct evidence that the clinically used anesthetic propofol does not affect nerve regeneration. However, the formation of both chemical and electrical synapses is severely compromised in the presence of this drug. This study emphasizes the importance of short-term anesthetic treatment, which may be critical for the restoration of synaptic connections between injured neurons.

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physiologically similar to those seen in vivo and are amenable to direct cellular and synaptic analysis (Feng et al. 1997). This model (Hamakawa et al. 1999a), and various other invertebrate preparations, such as Aplysia (Arimura and Ikemoto 1986), Helix (Judge and Norman 1982), and Lymnaea (Franks and Lieb 1988, 1991; Spencer et al. 1995, 1996) have previously been used to identify the cellular mechanisms by which clinically relevant concentrations of inhalation anesthetic halothane, sevoflurane, isoflurane, enfurane, etc., alter synaptic transmission between identified neurons (see Franks and Lieb 1991). Invertebrate models such as Lymnaea, provide an excellent opportunity to define the precise sites of anesthetic action and to assess whether they also affect neuronal regeneration and synapse formation.

Cell culture, electrophysiological, and fluorescent imaging techniques were used to demonstrate that propofol blocks cholinergic, excitatory synaptic transmission between VD4 and LPeD1 in a dose-dependent and reversible manner and that these effects do not involve presynaptic secretory machinery. Moreover, we provide direct evidence that long-term (12-18 h) anesthetic treatment of soma-soma paired cells blocks synapse formation between VD4 and LPeD1. To test whether propofol affects nerve regeneration of VD4, LPeD1, and PeA neurons, these cells were cultured in the presence of propofol for several days. Although chronic propofol treatment did not affect neurite outgrowth in cell culture, the formation of both chemical (VD4/LPeD1) and electrical (PeA) synapses was severely compromised. These synapses, however, re-formed after prolonged anesthetic washout. We demonstrate that propofol-induced suppression of synaptogenesis between cultured neurons does not involve protein synthesis-dependent steps but rather a failure of presynaptic machinery to assemble at the synaptic sites.

METHODS

Animals

Stocks of the fresh water pond snail Lymnaea stagnalis were maintained at room temperature (18-20°C) in an aquarium containing well-aerated, filtered pond water and were fed lettuce. One-to-two-month-old animals (shell length, 10-15 mm) were used for cell isolation, whereas brain-conditioned medium (CM) was produced from 3- to 4-month-old animals (shell length, 15-25 mm).

Cell culture

Single cells were isolated and cultured as described previously (Ridgway et al. 1991; Syed et al. 1999). Briefly, snails were anesthetized with 10% Listerine (ethanol, 21.9%; menthol, 0.042%) solution in normal Lymnaea saline [containing (in mM) 51.3 NaCl, 1.7 KCl, 4.1 CaCl₂, and 1.5 MgCl₂] buffered to pH 7.9 adjusted with HEPES. The central ring ganglia were removed under sterile conditions and washed with antibiotic saline (gentamycin, 50 µg/ml: 2 washes, 10 min each). The ganglia were then treated with 0.2% trypsin (Sigma type II) for 22 min followed by 0.2% soybean trypsin inhibitor (Sigma type I-S) for 10 min, both in defined medium (DM). DM consisted of serum free, 50% L-15 medium with added inorganic salts [(in mM) 40 NaCl, 1.7 KCl, 4.1 CaCl₂, and 1.5 MgCl₂] buffered to pH 7.9 adjusted with HEPES, pH 7.9 adjusted with 1 N NaOH, and 20 µM gentamycin. The enzymatically treated ganglia were pinned to the bottom of a dissection dish that contained 8 ml high osmolarity DM (DM + 37.5 mM glucose).

The identified cells were isolated by applying gentle suction to a fire-polished and Sigmacote (Sigma, St. Louis, MO) treated pipette. The isolated cells were plated onto poly-1-lysine-pretreated coverslips (Ridgway et al. 1991) in the presence of CM. This CM was previously prepared by incubating central ganglia in DM (12 ganglia / 6 ml DM) for 4 or 5 days and frozen until it was used. Isolated somata of identified neurons were juxtaposed in a soma-soma configuration and left undisturbed overnight (Feng et al. 1997).

Neurite outgrowth

To assess neuronal regeneration in the presence of propofol, identified neurons were isolated in cell culture and plated on poly-1-lysine-coated dishes containing CM. The PeA neurons were selected for neurite outgrowth assays because they comprise a homogenous population of 60 cells in brain and are morphologically and electrophysiologically well-defined (Syed and Winlow 1989). To test for the effects of propofol on neurite outgrowth, the anesthetic was added to the dish 1 hr after cell plating, and the neurons were maintained overnight in the dark. Neuronal sprouting was assessed as described previously (Ridgway et al. 1991). Specifically, only those neurons exhibiting outgrowth (multiple branches, active growth cones, etc.) equivalent to five somata diameter were considered as sprouted. Neurons cultured overnight in CM or CM + 0.1% DMSO (the highest concentration used to dissolve propofol) alone served as controls.

Soma-soma synapse

Soma-soma synapses were prepared by juxtaposing the isolated somata of the identified neurons (Feng et al. 1997). The cells were paired in CM or CM + propofol overnight, which was subsequently washed out prior to electrophysiological recordings. To demonstrate the reversibility of propofol-induced changes, the anesthetic was washed away with normal saline and the cells were maintained in CM for an additional 4-7 h prior to intracellular recording. Synapses were re-tested between the paired cells, and the incidence of synaptic transmission was investigated electrophysiologically.

Neurite-neurite synapse

To test whether neurons sprouted in propofol had developed either chemical or electrical synapses, cells were allowed to extend processes in the presence of this drug. After extensive physical overlap between the neurites of VD4 and LPeD1, chemical synapses were tested electrophysiologically. Similarly, to determine whether PeA cluster neurons with overlapping neurites had developed electrical synapses, either hyperpolarizing or depolarizing square pulses were tested for their ability to pass between the cells.

Electrophysiology

Conventional intracellular recording techniques were used (Syed et al. 1999). Specifically, glass microelectrodes (1.5 mm ID, W/FIL, WPI) were pulled on a vertical electrode puller (Kopf, 700C) and filled with a saturated solution of K₂SO₄ (resistance 30-60 MΩ). Isolated cells were viewed under a Zeiss (Telavaly 31) inverted microscope and impaled using Narishige micromanipulators (Model M0-103, Tokyo, Japan). The intracellular signals were amplified via a preamplifier (Neurodata Model, IR-283), displayed on a storage oscilloscope (Tektronix R5103N), and recorded on a chart recorder (Gould). All experiments were performed at room temperature (18-22°C). Propofol (2, 6 disopropylphenol, Aldrich), was dissolved in DMSO (0.1%) and perfused at a constant rate of 2 ml/min.

Soma-soma pairs were exposed to propofol at concentrations of 5, 10, 25, 50, and 100 µM. The cells were perfused with the anesthetic solution for 5 min to allow the bath to reach the desired concentration. Exogenous application of acetylcholine (ACh) was performed (80-ms
complete recovery from 100/1000 ns (H9262) pulses, 8 psi) in some experiments using 1 μM ACh (Sigma) applied at a distance of 1–2 somata diameter via a Pneumatic PicoPump (PV800, WPI).

FM1-43 imaging

Cells were incubated in 20 μM FM1-43 (Molecular Probes) for 10 min prior to the addition of 50–100 μM propofol to the bath. The presynaptic cell (VD4) was stimulated to generate 100 action potentials (10 spikes/burst) by conventional electrophysiological techniques to facilitate the uptake of FM1-43 either in the presence or absence of propofol. The styryl dye and propofol 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 Axiovert 200M inverted microscope. Excitation light was from a 100-W Hg lamp, excitation filters (480/30 nm), dichroic mirror (505 nm), and emission filters (570 LP nm or 610 nm). Phase and fluorescent images were captured with a Photometrics (Tucson, AZ) Sensys 1400 camera (1- to 100-ms exposure), connected to a computer running Axiovision 3.0 for Windows (Carl Zeiss).

Statistical analysis

Parametric data are expressed as means ± SE and were analyzed for significance using one-way ANOVAs with repeated measures (propofol concentration was the between-subjects factor) and a Tukey’s post hoc test. Nonparametric data are expressed as percentes and were analyzed for significance using the χ² test. Significance was assumed if P < 0.05.

RESULTS

**Propofol blocks excitatory synaptic transmission between VD4 and LPeD1**

Propofol concentrations in human serum during induction of anesthesia range from 8 to 50 μM (Cockshott 1985; Shafer et al. 1988), whereas recovery from anesthesia occurs when its serum concentration drops to 6 μM (Kanto 1988). In experimental animals however, 10–100 times higher concentrations (300 μM) of this intravenous anesthetic are required for anesthesia (see Wakasugi et al. 1999).

When paired in a soma-soma configuration in CM, excitatory synapses between VD4 and LPeD1 develop reliably (100%) within 12–18 h. Specifically, presynaptic action potentials in VD4 generated 1:1 excitatory postsynaptic potentials (EPSPs) in LPeD1, held at a resting membrane potential of -80 mV (Fig. 1A). To investigate whether propofol affects cholinergic synaptic transmission between VD4 and LPeD1, recordings were made from the paired cells in the presence of anesthetic. At a concentration of 25 μM (n = 8), propofol had no significant effect on EPSP amplitude, whereas 50 (n = 9) and 100 μM (n = 8) propofol significantly reduced synaptic transmission between the paired cells (Fig. 1, A and B). Specifically, 50 μM propofol reduced EPSP amplitude from 10.6 ± 1.3 to 7.1 ± 0.1 mV. On washout with normal saline, the EPSP amplitude returned to control values (10.0 ± 1.5 mV). Similarly, 100 μM propofol significantly attenuated EPSP amplitude from 9.0 ± 1.7 to 2.3 ± 0.5 mV. Although a complete recovery from 100 μM propofol-induced suppression required longer washout, significant recovery was nevertheless achieved after 30 min of anesthetic removal (Fig. 1, A and B). The vehicle solution (0.1% DMSO in normal saline) had no effect on neuronal excitability or synaptic transmission (data not shown).

**Propofol-induced suppression of synaptic transmission does not involve the presynaptic neuron**

To determine whether propofol-induced effects on synaptic transmission involved neurotransmitter release, VD4 was soma-soma paired with LPeD1 in culture overnight. After 12–18 h, the presynaptic neuron was impaled with sharp recording electrode. Both propofol (50 or 100 μM) and the fluorescent dye FM1-43 (20 μM) were added to the culture dish for 15
min, and VD4 was subsequently depolarized to induce bursts of spikes. Propofol and FM1-43 were thoroughly washed out and fluorescent images were acquired. We hypothesized that if propofol interfered with transmitter release or the exocytotic/endocytic process, then fluorescent labeling would be absent at the synapse. This assumption was, however, refuted by the experimental evidence, as propofol failed to block both endocytosis and exocytosis in VD4 as intense punctuate labeling of the FM1–43 dye was observed in VD4 at its contact/synaptic site with LPeD1 in both control (n = 10) and in the presence of 50 (n = 7) or 100 μM (n = 6; Fig. 2). Thus these data provide the first direct evidence that propofol does not affect presynaptic secretory capabilities nor is its endocytic process perturbed.

Propofol-induced suppression of synaptic transmission involves postsynaptic mechanisms

To test whether propofol affects postsynaptic (LPeD1) ACh receptors (the neurotoxin used by VD4 at this synapse) (see Woodin et al., 2002), either single or paired cells were cultured in CM overnight. After 12–18 h, neurons were impaled with sharp electrodes and 1 μM ACh was pressure applied directly at the postsynaptic somata (LPeD1) either in the presence or absence of propofol. The postsynaptic, cholinergic responses generated in LPeD1 at a holding potential of ~80 mV were suppressed significantly by all concentrations of propofol used. Specifically, 25 μM propofol (n = 7) suppressed the ACh response from 8.5 ± 0.5 to 4.1 ± 1.0 mV, whereas 50 μM propofol (n = 7) suppressed the response from 9.6 ± 0.7 to 5.4 ± 1.1 mV. On the other hand, 100 μM propofol (n = 5) suppressed the response from 10.5 ± 0.8 to 1.3 mV (±0.4 mV). On washout with normal saline, the cholinergic responses significantly increased for all propofol concentrations (25 μM 6.8 ± 0.8 mV; 50 μM 9.9 ± 1.1 mV; and 100 μM 8.7 ± 0.7 mV), indicating that the effects of this drug are reversible (Fig. 3, A and B). Taken together, the preceding data demonstrate that propofol-induced suppression of synaptic transmission between VD4 and LPeD1 may affect postsynaptic responsiveness to ACh.

FIG. 3. Propofol significantly reduces nonsynaptic cholinergic response in LPeD1. VD4 and LPeD1 cells were soma-soma paired in CM and after 12–18 h in culture, ACh (1 μM) was pressure applied to LPeD1 soma (↑) either in the presence or absence of propofol [25 μM (n = 7), 50 μM (n = 7), 100 μM (n = 5)]. All propofol concentrations tested significantly and reversibly blocked nonsynaptic cholinergic responses in LPeD1. A: summary data with mean peak amplitude of postsynaptic cholinergic response (± SE) obtained from soma-soma pairs before, during, and after washout of various propofol concentrations. [25 μM (n = 7); 50 μM (n = 7); 100 μM (n = 5) propofol]. B: * statistical significance (P < 0.05) from control; ** statistical significance (P < 0.05) from propofol treatment after washout of the drug.

Chronic propofol treatment of the soma-soma pairs blocks synapse formation between VD4 and LPeD1

To test whether chronic propofol treatment alters neuronal ability to establish chemical synapses in culture, VD4 and LPeD1 were soma-soma paired in the presence of 5, 10, 25, or 50 μM propofol. After 12–18 h in propofol, the anesthetic was washed out for several hours, and synapses were tested in the presence of normal saline. We found that the 73% of pairs exposed overnight to propofol failed to develop synapses (n = 22 pairs) as compared with 100% pairs that established synapses under control conditions (n = 6; Fig. 4). Specifically, presynaptic action potentials in VD4 failed to generate EPSPs in LPeD1 in most instances when cells were paired in propofol. In 5 (n = 10) and 10 μM (n = 9) propofol, 30 and 55.6% of the paired cells failed to develop synapses respectively, whereas in 25 μM (n = 8) propofol, 75% of the soma-soma pairs failed to establish chemical synapses in cell culture. On the other hand, 50 μM propofol completely blocked synapto-
aptic release machinery, cells were paired overnight in 50 μM propofol solution DMSO had no effect on synapse formation. All concentrations of propofol (5, 10, and 25 μM) significantly reduced the incidence of synapse formation between the paired cells, and 50 μM propofol was sufficient to completely block synapse formation between VD4 and LPeD1. In control experiments, soma-soma pairs in CM or CM + the carrier solution (0.1% DMSO), established normal excitatory synapses. * statistical significance (P < 0.05) from control.

To test whether chronic anesthetic treatment affects presynaptic release machinery, cells were paired overnight in 50 μM propofol, FM1-43 was subsequently added to the dish for 10 min as described earlier, and fluorescent images were acquired after the intracellular stimulation of VD4. However, despite extensive stimulation, cells paired in propofol but recorded under normal saline conditions, either loaded poorly with FM1-43 (n = 3 of 9) or had no staining at all (n = 6 of 9; Fig. 5).

To test neuronal ability to establish synapses after propofol washout, the anesthetic was replaced with normal saline and then CM was substituted into the dishes. The paired cells were allowed to recover for several hours, and synaptic transmission was subsequently tested via direct intracellular recordings. We found that after 5–6 h of recovery period, all the paired cells had established chemical, excitatory synapses, which were indistinguishable from their control (CM + 0.1% DMSO), established normal excitatory synapses. * statistical significance (P < 0.05) from control.

We next hypothesized that synaptic transmission observed after propofol washout may have involved formation of new synapses. Because excitatory synapse formation between VD4 and LPeD1 has previously been shown to involve de novo protein synthesis (Hamakawa et al. 1999b), we asked whether synaptogenesis after drug washout was also involved new protein synthesis. Cells paired overnight in propofol (50 μM), were washed with normal saline and subsequently incubated in CM containing a nonspecific protein synthesis blocker anisomycin (1.25 μg/ml) (Hamakawa et al. 1999b). After 5–6 h, anisomycin was washed with normal saline and synapses were tested electrophysiologically. Contrary to our expectation, anisomycin failed to block synapse formation, and induced action potentials in VD4 successfully generated 1:1 EPSPs in LPeD1 (n = 6; control n = 5; 50 μM n = 6). Taken together with earlier experiments, these data suggest that propofol does not block the synthesis of synapse specific proteins.

Chronic propofol treatment of VD4 and LPeD1 or PeA cluster neurons does not affect neurite outgrowth, but both chemical and electrical synapses fail to develop

To test whether chronic propofol treatment affects neurite outgrowth from VD4 and LPeD1, these cells were individually isolated and plated on poly-L-lysine-coated dishes containing CM + propofol. After 48 h, cells were examined for their ability to extend neurites. Neurons cultured in the presence of propofol extended neurites (n = 14; Fig. 6A) that were indistinguishable from their control (CM + 0.1% DMSO) counterparts (n = 13, not shown). Intracellular recordings from cells with overlapping neurites, however, failed to reveal synapses between the anesthetic-pretreated cells (Fig. 6B). These data, on one hand, support results obtained from the soma-soma model and on the other hand demonstrate that propofol does not affect neurite regeneration from VD4 and LPeD1.

To test whether the chronic exposure of PeA neurons to propofol affects neurite regeneration, and electrical synapse formation, cells were cultured in CM either in the presence or absence of propofol. After 24–48 h in culture, neurite outgrowth patterns of neurons under the two conditions were analyzed. No significant differences in growth patterns (as described in details previously) (see Ridgway et al. 1991), length of the neurites, and number of processes were observed under both conditions. For example, in 50 μM propofol, 20 of 22 cells exhibited extensive neurite outgrowth (Fig. 7C), whereas in control CM, 26 of 26 cells (Fig. 7A) extended neurites. These data demonstrate that chronic propofol treatment does not affect neurite outgrowth from PeA cluster neurons.

Because PeA neurons are electrically connected both in vivo (Syed and Winlow 1989) and in vitro (Spencer et al. 1996), we next asked whether propofol affects electrical synaptic connections in cell culture. Specifically, cells were washed with normal saline after prolonged propofol treatment, and electrical

**FIG. 4.** Chronic propofol treatment blocks excitatory synapse formation between VD4 and LPeD1. VD4 and LPeD1 were soma-soma paired overnight in CM, either in the presence or absence of various propofol concentrations. After 12–18 h, propofol was washed out with normal saline for 30 min to several hours, and synapses were tested electrophysiologically. All concentrations of propofol (5, 10, and 25 μM) significantly reduced the incidence of synapse formation between the paired cells, and 50 μM propofol was sufficient to completely block synapse formation between VD4 and LPeD1. 10. P = 0.05 from control.

**FIG. 5.** Chronic propofol treatment perturbs presynaptic release machinery. Cells were soma-soma paired in CM either in the presence or absence of 50 μM propofol. After 12–18 h, cells paired in CM were incubated in FM1-43 dye (20 μM) for 10 min, and VD4 was stimulated via direct intracellular current injections (100 action potentials). The dye was subsequently washed out with cold saline, and fluorescent images were acquired. FM1-43 labeled, fluorescent puncta was discernible at VD4's contact point with LPeD1 and also localized to those processes that surrounded the postsynaptic somata. A and B, ↔️️. In contrast, cells paired in propofol either exhibited faint staining (n = 3 of 9; C and D, ↔️️) or no labeling with the styryl dye (n = 6 of 9; E and F). 1. LPeD1; 4, VD4; scale bar = 50 μM.
Connectivity was tested by passing hyperpolarizing and depolarizing current pulses between physically connected cells. We found that chronic propofol treatment blocked the development of gap junctions between the PeA cluster neurons. For instance, neither hyperpolarizing nor depolarizing current pulses (0.2–0.5 nA) injected in either cell elicited a corresponding response in its counterpart (Fig. 7D). These data demonstrate that although propofol does not affect neurite outgrowth from PeA cells, it does however, block the formation of electrical synapses between the cells. After washout with DM and subsequent incubation (12–24 h) in CM, electrical coupling between the paired cells was fully restored (Fig. 7E).

**DISCUSSION**

This study is the first to demonstrate that long-term anesthetic treatment does not affect neurite regeneration. However, both chemical and electrical synapse formation between cultured neurons was found to be severely compromised by propofol. The propofol-induced effects on synapse formation (both electrical and chemical) were reversible and appropriate synapses developed after several hours of drug washout. Regarding the mechanism of propofol actions, the present study provides direct evidence that this intravenous, general anesthetic, which is also used as a sedative for critically ill patients, blocks excitatory, cholinergic synaptic transmission between cultured pre- and postsynaptic neurons. The propofol-induced effects do not appear to involve presynaptic transmitter release machinery nor is the vesicle endocytosis perturbed. Because propofol reduced postsynaptic cholinergic responses, our data thus demonstrated that the propofol-induced suppression of synaptic transmission primarily involves postsynaptic mechanisms. These data are in contrast with previous studies on vertebrate neurons, where propofol did not significantly alter the responsiveness of either glutamate (see Perouansky and Antognini 2003 for details) or glycine receptors (Daniels and Roberts 1998).

Propofol has been found to inhibit endogenous glutamate release from endosomes (see Perouansky and Hemmings 2003) suggesting that it may also affect transmitter release though a similar action on intact, functional synapses has not yet been demonstrated. Other studies on rat hippocampal neurons in vitro (Orser et al. 1995) argue that propofol-induced suppression of synaptic transmission may be mediated indirectly through an enhancement of GABA receptor function (Bai et al. 2001; Hirota et al. 1998; Wakasugi et al. 1999; see Pearce 2003). This notion is consistent with a recent study, which demonstrates that propofol potentiates GABAergic synaptic transmission.
transmission in the brain and that these effects accompany neuronal activity-mediated, enhancement of c-fos expression (Nelson et al. 2002). As propofol and other anesthetics were found to affect GABA$_A$ receptors in the sleep pathway (Nelson et al. 2002), the preceding study thus further underscores the importance of such agents in defining normal patterns of neuronal activity in the nervous system. On the other hand, because mutant mice lacking the $\delta$ subunit of the GABA$_A$ receptor still exhibit normal anesthesia in response to propofol, the precise site and the mode of this anesthetic’s actions still remain controversial (see Homanics and Firestone 2003).

Although the propofol concentrations (50–100 $\mu$M) required to suppress the synaptic transmission between VD4 and LPdD1 was higher than clinical doses, they were nevertheless consistent with those used in other studies on mammalian models (see Wakasugi et al. 1999 for details). As pointed out by Wakasugi et al. (1999), a higher concentration dependency of intravenous anesthetics to exert their effects in experimental models may be due to the “lack of artificial cerebral spinal fluid and/or lack of certain inputs and outputs that normally exist in the intact brain.” In the present study, supratherapeutic concentrations (100 $\mu$M) were required to suppress synaptic transmission between the neuron pairs. Because the drug was applied under a fast perfusion system and was delivered in brief pulses, it is possible that the effective propofol concentration at synaptic sites that were concealed between the somata was much lower than used in the pipette solution. Notwithstanding the fact that supratherapeutic concentrations were required to suppress the synaptic transmission between Lymnaea neurons, we do not believe that these effects involved drug toxicity. Chronic treatment of cultured neurons to propofol did not affect neuronal viability (resting membrane potential and other cellular parameters), neurite outgrowth, or postsynaptic responsiveness to ACh. Interestingly, however, clinically used concentrations of propofol (5 $\mu$M) did significantly block 30% neurons from establishing excitatory synapses in culture, lending further support to the idea that chronic anesthetic treatment is indeed detrimental exclusively for neuronal connectivity. This study tested the effects of propofol on synaptic transmission and synapse formation in an in vitro preparation, which lacks glia and other extrinsic factors that may otherwise modulate the actions of anesthetics in the intact brain. Thus the propofol-induced effects observed in this in vitro study must be interpreted cautiously vis-à-vis its role in general anesthesia.

In contrast to the effects of various anesthetics on ion channels and synaptic machinery, virtually nothing is known about their actions (both inhalation and intravenous) on nerve regeneration and synapse formation that follows injury and/or surgical interventions. An exception is a recent study in which chronic exposure to general anesthetics was shown to induce neuronal degeneration and learning defects in developing rats (Zorumski et al. 2003). In this study, we demonstrate that chronic exposure to anesthetic propofol did not affect neurite outgrowth and extension from identified Lymnaea neurons.

Because PeA cells extended extensive neurites in the presence of propofol, our data also demonstrate that this anesthetic does not affect gene transcription or de novo protein synthesis for various cytoskeletal proteins that might be required for nerve regeneration. These data clearly demonstrate that propofol does not interfere with the regenerative machinery of cultured Lymnaea neurons. It is, however, likely that even longer exposure of Lymnaea neurons to propofol may result in neuronal degeneration as was observed in rats (Zorumski et al. 2003). This degeneration may be due to a neuronal inability to communicate via synaptic activity.

In the present study, cells were treated chronically with propofol in CM. It is therefore possible that the synapse specific gene transcription and protein translation may have remained unperturbed by the anesthetic. These proteins, however, may have not been appropriately targeted to specific sites in the presence of propofol. Consistent with this reasoning are our data, which showed that exocytotic machinery was not functional in chronic propofol-treated presynaptic cells. The precise nature of these proteins and the underlying mechanisms however, remain to be investigated.

Electrical activity dependent mechanisms have previously been shown to be critical for synapse formation, synaptic refinement and plasticity (Desai et al. 2002; Penn et al. 1998). For instance, blocking electrical activity or sensory deprivation results in drastic abnormalities of the neuronal connectivity patterns (Drakow et al. 1999; also see Zhong and Poo 2001). It is therefore reasonable to suggest that propofol-induced effects may have also involved activity-dependent mechanisms. Because Na$^+$ channels and neuronal action potentials in Lymnaea and other mollusks (Haydon and Urban 1986) are insensitive to anesthetics, the activity-dependent mechanism is therefore unlikely to have played an important role in propofol-induced suppression of synapse formation between VD4 and LPdD1. Another possibility is that chronic propofol may have rendered synaptic transmission ineffective by desensitizing or disrupting the synaptic machinery. However, exogenously applied ACh to chronically treated LPdD1 cells did not block cholinergic responses, suggesting that the receptors did not desensitize in response to chronic propofol exposure (not shown). Similarly, if cells were originally paired in CM and subsequently treated with propofol for an additional 12–18 h, normal synapses were detected immediately on anesthetized washout (data not shown).

In summary, the data presented in this study clearly demonstrate that chronic propofol treatment blocks synapse formation in cell culture without affecting nerve regeneration.

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DISCLOSURES

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In the present study, supratherapeutic concentrations (50–100 $\mu$M) did significantly block 30% neurons from establishing excitatory synapses in culture, lending further support to the idea that chronic anesthetic treatment is indeed detrimental exclusively for neuronal connectivity. This study tested the effects of propofol on synaptic transmission and synapse formation in an in vitro preparation, which lacks glia and other extrinsic factors that may otherwise modulate the actions of anesthetics in the intact brain. Thus the propofol-induced effects observed in this in vitro study must be interpreted cautiously vis-à-vis its role in general anesthesia.

In contrast to the effects of various anesthetics on ion channels and synaptic machinery, virtually nothing is known about their actions (both inhalation and intravenous) on nerve regeneration and synapse formation that follows injury and/or surgical interventions. An exception is a recent study in which chronic exposure to general anesthetics was shown to induce neuronal degeneration and learning defects in developing rats (Zorumski et al. 2003). In this study, we demonstrate that chronic exposure to anesthetic propofol did not affect neurite outgrowth and extension from identified Lymnaea neurons.

Because PeA cells extended extensive neurites in the presence of propofol, our data also demonstrate that this anesthetic does not affect gene transcription or de novo protein synthesis for various cytoskeletal proteins that might be required for nerve regeneration. These data clearly demonstrate that propofol does not interfere with the regenerative machinery of cultured Lymnaea neurons. It is, however, likely that even longer exposure of Lymnaea neurons to propofol may result in neuronal degeneration as was observed in rats (Zorumski et al. 2003). This degeneration may be due to a neuronal inability to communicate via synaptic activity.

In the present study, cells were treated chronically with propofol in CM. It is therefore possible that the synapse specific gene transcription and protein translation may have remained unperturbed by the anesthetic. These proteins, however, may have not been appropriately targeted to specific sites in the presence of propofol. Consistent with this reasoning are our data, which showed that exocytotic machinery was not functional in chronic propofol-treated presynaptic cells. The precise nature of these proteins and the underlying mechanisms however, remain to be investigated.

Electrical activity dependent mechanisms have previously been shown to be critical for synapse formation, synaptic refinement and plasticity (Desai et al. 2002; Penn et al. 1998). For instance, blocking electrical activity or sensory deprivation results in drastic abnormalities of the neuronal connectivity patterns (Drakow et al. 1999; also see Zhong and Poo 2001). It is therefore reasonable to suggest that propofol-induced effects may have also involved activity-dependent mechanisms. Because Na$^+$ channels and neuronal action potentials in Lymnaea and other mollusks (Haydon and Urban 1986) are insensitive to anesthetics, the activity-dependent mechanism is therefore unlikely to have played an important role in propofol-induced suppression of synapse formation between VD4 and LPdD1. Another possibility is that chronic propofol may have rendered synaptic transmission ineffective by desensitizing or disrupting the synaptic machinery. However, exogenously applied ACh to chronically treated LPdD1 cells did not block cholinergic responses, suggesting that the receptors did not desensitize in response to chronic propofol exposure (not shown). Similarly, if cells were originally paired in CM and subsequently treated with propofol for an additional 12–18 h, normal synapses were detected immediately on anesthetized washout (data not shown).

In summary, the data presented in this study clearly demonstrate that chronic propofol treatment blocks synapse formation in cell culture without affecting nerve regeneration.

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DISCLOSURES

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