# Propofol-induced potentiation of $GABA_A$ receptor-mediated tonic $Cl^-$ currents in the rat insular cortex

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# Index



This thesis is based on the following article and additional results in terms of the effect of bicuculline on spike firing:

Keisuke Kaneko, Yuko Koyanagi, Yoshiyuki Oi, Masayuki Kobayashi (2016)
Propofol-induced spike firing suppression is more pronounced in pyramidal neurons than in fast-spiking neurons in the rat insular cortex. Neuroscience 339:548-560.

## Abstract

Propofol is a major intravenous anesthetic that facilitates $GABA_A$ receptor-mediated inhibitory synaptic currents and modulates hyperpolarization-activated inward current ($I_h$), $K^+$, and voltage-gated $Na^+$ currents. This propofol-induced modulation of ionic currents changes intrinsic membrane properties and repetitive spike firing in cortical pyramidal neurons. However, it has been unknown whether propofol modulates these electrophysiological properties in GABAergic neurons, which express these ion channels at different levels. This study examined whether pyramidal and GABAergic neuronal properties are differentially modulated by propofol in the rat insular cortical slice preparation. Multiple whole-cell patch-clamp recordings were conducted from pyramidal neurons and from GABAergic neurons, which were classified into fast-spiking (FS), low threshold spike, late-spiking, and regular-spiking nonpyramidal neurons. It became clear that 100 μM propofol hyperpolarized the resting membrane potential and decreased input resistance in all types of neurons tested. Propofol also potently suppressed, and in most cases eliminated, repetitive spike firing in all these neurons. However, the potency of the propofol-induced changes in membrane and firing properties is particularly prominent in pyramidal neurons. Using a low concentration of propofol clarified this tendency: 30 μM propofol decreased the firing of pyramidal neurons but had little effect on GABAergic neurons. Pre-application of either $GABA_A$ receptor antagonist, picrotoxin (100 μM) or bicuculline (10 μM), diminished the propofol-induced suppression of neural activities in both pyramidal and FS neurons. These results suggest that GABAergic neurons, especially FS neurons, are less affected by propofol than pyramidal neurons and that propofol-induced modulation of the intrinsic membrane properties and repetitive spike firings are principally induced by $GABA_A$ receptor-mediated tonic currents.

# Introduction

Propofol, a popular intravenous anesthetic, is considered to suppress cortical activities by facilitating $GABA_A$ receptor-mediated inhibitory synaptic currents (Koyanagi et al., 2014). In addition to the potentiation of GABAergic currents, propofol modulates other ionic currents in pyramidal neurons in the cerebral cortex. First, propofol hyperpolarizes the resting membrane potential by suppressing the hyperpolarization-activated inward current ($I_h$) (Higuchi et al., 2003, Chen et al., 2005, Ying et al., 2006), which is involved in depolarization of the resting membrane potential (Pape, 1996). Second, the voltage-dependent $Na^+$ currents are suppressed by propofol, which may result in decreased spike firing frequency in response to injection of depolarizing current pulses (Ratnakumari and Hemmings, 1997, Martella et al., 2005). Third, propofol also suppresses $K^+$ currents (Song et al., 2011, Zhang et al., 2016). This propofol-induced modulation of ionic currents is likely to diminish the electrical activities of cortical pyramidal neurons (Martella et al., 2005).

Pyramidal neurons are a major type of excitatory neurons in the cerebral cortex, and another 10-20% of cortical neurons are GABAergic neurons, which are classified into several types based on their firing and morphological profiles (Kawaguchi and Kubota, 1997, Koyanagi et al., 2010, Kobayashi et al., 2012). Characterizing the effects of propofol on these GABAergic neurons is critical to understanding the mechanisms of the propofol-induced suppression of cortical activity because pyramidal and GABAergic neurons have opposing effects on postsynaptic neurons, i.e., excitation and inhibition, respectively. However, it has remained unknown how propofol modulates membrane properties of GABAergic neurons in the cerebral cortex.

The propofol-mediated modulation of GABAergic neurons in other parts of the brain may be referenced to predict its effect on cortical GABAergic neurons. However, interestingly, propofol shows a wide variety of effects on GABAergic neurons. Propofol treatment of GABAergic neurons in the reticular thalamic nucleus produces increased firing frequency and input resistance due to blocking of SK channels (Ying and Goldstein, 2005). On the other hand, propofol treatment of GABAergic neurons in the ventrolateral preoptic nucleus produces increases in the firing frequency and in the frequency and amplitude of spontaneous EPSCs by blocking NKCC1 (Li et al., 2009). In contrast, in hippocampal CA1 GABAergic neurons, propofol potentiates GABAergic tonic currents, thereby hyperpolarizing the membrane potential and decreasing input resistance, which results in suppression of spike firing (Bieda and MacIver, 2004).

The present study aimed to examine whether pyramidal and GABAergic neurons in the rat insular cortex (IC) are differentially modulated by propofol. Propofol-induced modulation of the passive electrophysiological properties, including the resting membrane potential and input resistance, and repetitive firing properties between pyramidal and GABAergic neurons were compared.

## Materials and Methods

The Institutional Animal Care and Use Committee at Nihon University approved the study protocol, and all experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used as well as their suffering.

***Slice preparations***

The techniques for slice preparation and maintenance were similar to those described previously (Koyanagi et al., 2014, Yamamoto et al., 2015). Briefly, vesicular GABA transporter (VGAT)-Venus line A transgenic rats of either sex (postnatal days 18-35) were deeply anesthetized with isoflurane (5%) and decapitated. Tissue blocks including the IC were rapidly removed and stored for 3 min in ice-cold modified artificial cerebrospinal fluid (ACSF) (in mM: 230 sucrose, 2.5 KCl, 10 $MgSO_4$, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, 2.5 $CaCl_2$, and 10 D-glucose). Coronal slices were cut to a thickness of 350 μm using a microslicer (Linearslicer Pro 7, Dosaka EM, Kyoto, Japan). Slices were incubated at 32°C for 40 min in a submersion-type holding chamber that contained 50% modified ACSF and 50% normal ACSF (in mM: 126 NaCl, 3 KCl, 2 $MgSO_4$, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 D-glucose). Modified and normal ACSF were continuously aerated with a mixture of 95% $O_2$ and 5% $CO_2$. Slices were then placed in normal ACSF at 32°C for 1 hr and thereafter maintained at room temperature until used for recording.

***Cell identification and paired whole-cell patch-clamp recording***

The slices were transferred to a recording chamber that was continuously perfused with normal ACSF with 0.1 % dimethyl sulfoxide (DMSO) at a rate of 2.0 ml/min. Whole-cell patch-clamp recordings were obtained from Venus-positive fluorescent neurons and Venus-negative pyramidal neurons identified in layer V of the IC using a fluorescence microscope equipped with Nomarski optics (x 40, ECLIPSE FN1, Nikon, Tokyo, Japan) and an infrared-sensitive video camera (IR-1000, DAGE-MTI, Michigan City, IN, USA). Electrical signals were recorded by amplifiers (Multiclamp 700B, Molecular Devices, Sunnyvale, CA, USA), and then digitized (Digidata 1440A, Molecular Devices), observed online, and stored on a computer hard disk using Clampex (pClamp 10, Molecular Devices).

The composition of the pipette solution for recordings unless otherwise specified was (in mM) as follows: 135 potassium gluconate, 5 KCl, 5 *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), 2 $MgCl_2$, 2 magnesium ATP, 0.3 sodium GTP, and 5 EGTA. The pipette solution had a pH of 7.3 and an osmolarity of 300 mOsm. As an internal solution including high concentration of $Cl^-$, the following pipette solution was used: 70 potassium gluconate, 70 KCl, 10 HEPES, 15 biocytin, 0.5 EGTA, 2 $MgCl_2$, 2 magnesium ATP), and 0.3 sodium GTP. The liquid junction potentials of the former and latter pipette solutions were -13 mV and -9 mV, respectively. The voltage

was not corrected in the present study. Thin-wall borosilicate patch electrodes (2-5 MΩ) were pulled on a Flaming-Brown micropipette puller (P-97, Sutter Instruments, Novato, CA, USA).

Recordings were obtained at 30 ± 1 °C. The seal resistance was > 5 GΩ, and only data obtained from electrodes with access resistance of 6-20 MΩ and < 20% change during recordings were included in this study. The voltage responses of cells were recorded by the application of long hyperpolarizing and depolarizing current pulse (300 ms) injections to examine basic electrophysiological properties, including input resistance, spike threshold, and repetitive firing patterns. Hyper- to depolarizing ramp current pulse (1 s) was injected to measure the latency of spike firing (Fig. 1Ca). Propofol (2,6-Diisopropylphenol; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO at a concentration of 100 mM and diluted to 100 μM or 30 μM in the perfusate. Picrotoxin (100 μM, picrotoxin; Sigma-Aldrich, St. Louis, MO, USA) or bicuculline (10 μM, (-)-bicuculline methiodide; Tocris Bioscience, UK) were used to antagonize $GABA_A$ receptors.

***Data analysis***

Clampfit (pClamp 10, Molecular Devices) was used for analyses of electrophysiological data. Input resistance was calculated from the relationship between the voltage responses and the injected current intensity (up to -200 pA). The repetitive spike firing properties were evaluated by measuring the maximum spike number responding to rectangular depolarizing current pulse injection (300 ms, up to 300 pA). Spike failure was defined as a case in which no spikes were induced in response to the injection of current at 300 pA. FS neurons often showed irregular spike firing such as stuttering, which disturbed the linear correlation between the current intensity and the number of spikes, quantification of the slope of the spike number versus the injected current amplitude were not performed. The action potential threshold was assigned as the potential at which the third derivative of the membrane potential changed sign from negative to positive (Takei et al., 2010, Ebihara et al., 2013). The reversal potential of propofol-induced suppression of voltage response was obtained as the potential of the intersection of control and propofol traces (Fig. 1Cd arrow). The cases in which the trace during propofol application showed a parallel shift from that of control was excluded.

***Statistics***

The data are presented as the mean ± standard error of the means. Comparisons between the control and propofol application or between a $GABA_A$ blocker application and propofol with a $GABA_A$ blocker in each neuronal subtype were made using paired *t*-tests, in which $P < 0.05$ was considered significant. The degree of the propofol-induced effects on the electrophysiological properties was compared between pyramidal neurons and each GABAergic neural subtype (4 pairs) using Student's *t*-test with the Bonferroni correction. The rate of failure for spike induction was compared between pyramidal neurons and each GABAergic neural subtype (4 pairs) using the multiple $\chi^2$ test. In these analyses, $P < 0.0125$

was considered significant.

# Results

In the present study, the recorded neurons were divided into GABAergic neurons, which express the fluorescent protein Venus, and excitatory pyramidal neurons, whose somata are pyramidal and Venus-negative. The GABAergic neurons were further classified into fast-spiking (FS), low threshold spike (LTS), late-spiking (LS), and regular-spiking nonpyramidal (RSNP) neurons. FS neurons are characterized by a large afterhyperpolarization amplitude with a quick recovery and an extremely high repetitive firing frequency without spike adaptation (Kawaguchi and Kubota, 1997, Koyanagi et al., 2010, Kobayashi et al., 2012). Another characteristic of FS neurons is frequent synaptic inputs. LTS cells were characterized by low threshold spikes and rebound APs after hyperpolarizing current pulse injections (300 ms). Subthreshold responses to hyperpolarizing current pulse injection often showed sag (Kawaguchi and Kubota, 1997, Xiang et al., 2002). LS cells were characterized by a slowly developing ramp depolarization to spike threshold (Kawaguchi and Kubota, 1997).

To examine the effects of propofol on cells' electrophysiological properties, three types of currents were applied: (1) rectangular hyperpolarizing current pulses, (2) depolarizing current pulses, and (3) a ramp current.

***Propofol suppresses excitability of pyramidal neurons***

Pyramidal neurons are electrophysiologically characterized by sag (Fig. 1Aa, arrow) and rebound potential (Fig. 1Aa, arrowhead) in response to a hyperpolarizing current pulse injection, and their repetitive spike firing with adaptation occurs as shown in Fig. 1Ba. Figure 1 shows a typical example of the effect of propofol (100 μM) on the electrophysiological properties of a pyramidal neuron.

Bath application of propofol significantly hyperpolarized the resting membrane potential by 4.3 ± 0.8 mV (n = 19; $P < 0.001$, paired *t*-test), accompanied by a decrease in input resistance by 28.6 ± 5.3 MΩ (n = 19; $P < 0.001$, paired *t*-test; Fig. 1A,D). Propofol diminished the sag and rebound potential as shown in Fig. 1Ab,d.

Repetitive spike firing in response to long depolarizing pulses was suppressed by propofol (100 μM; Fig. 1B). All pyramidal neurons exhibited repetitive spike firing in response to long depolarizing current pulse injection (300 ms) under control conditions, whereas 57.9% of pyramidal neurons did not show spikes under application of propofol. Propofol significantly decreased the maximum number of spikes fired in response to 300 ms depolarizing current pulse (300 pA) injection by 7.9 ± 1.0 spikes/300 ms (n = 19; $P < 0.001$, paired *t*-test). Propofol treatment depolarized the action potential threshold by 2.3 ± 1.0 mV (n = 21; $P < 0.05$, paired *t*-test). These data suggest that propofol suppresses the excitability of pyramidal neurons in the IC.

Repetitive spike firing in response to a hyper- to depolarizing ramp pulse (Fig. 1C) was also suppressed by propofol (100 μM; Fig. 1C). The latency from the onset of the

hyperpolarizing to depolarizing ramp current injection (Fig. 1C, double-headed arrow) was delayed by 292.6 ± 39.4 ms (n = 25; $P < 0.001$, paired *t*-test). The reversal potential of propofol-induced suppression of voltage response was -74.7 ± 2.8 mV (n = 15). The other 10 neurons did not show an apparent cross point of traces in control and propofol application, and therefore, these neurons were excluded from the reversal potential measurement. The propofol-induced suppression of repetitive spike firing was partially recovered by washing out propofol.

***Propofol-induced suppressive effects on FS neurons***

Similar to pyramidal neurons, FS neurons also showed propofol-induced hyperpolarization of the resting membrane potential (1.5 ± 0.4 mV, n = 69; $P < 0.001$, paired *t*-test) with a decrease in input resistance (16.9 ± 3.2 MΩ, n = 69; $P < 0.001$, paired *t*-test; Fig. 2A). Under control conditions, all FS neurons exhibited repetitive spike firing (n = 59), but 13.8% of FS neurons did not show spikes under application of propofol. Propofol (100 μM) tended to decrease repetitive spike firing of FS neurons (Fig. 2B): the maximum number of spikes fired was decreased by 15.9 ± 1.4 spikes/300 ms (n = 63; $P < 0.001$, paired *t*-test). The action potential threshold was depolarized by propofol by 2.7 ± 0.8 mV (n = 42; $P < 0.01$, paired *t*-test). These data suggest that propofol suppresses FS neuron excitability in the IC.

Repetitive spike firing of FS neurons in response to a hyper- to depolarizing ramp pulse was also suppressed by propofol (100 μM; Fig. 2C). The latency from the onset of the hyper- to depolarizing ramp current injection was delayed by 121.7 ± 18.5 ms (n = 69; $P < 0.001$, paired *t*-test). The reversal potential of propofol-induced suppression of voltage response was -70.9 ± 1.3 mV (n = 33). The propofol-induced suppression of repetitive spike firing was partially recovered by washing out propofol.

These results suggest that 100 μM propofol suppresses FS neuron excitability in a manner almost identical to the results obtained for pyramidal neurons (Fig. 1). However, the potency of the propofol-induced suppression in FS neurons appears to be milder than that of pyramidal neurons. These differences are quantitatively examined in a later section.

***Propofol induced suppression of non-FS neuron excitability***

In addition to FS neurons, the effects of 100 μM propofol on other types of GABAergic neurons: RSNP, LTS, and LS neurons were examined (Fig. 3).

Bath application of 100 μM propofol hyperpolarized the resting membrane potential in all these GABAergic neurons: LTS, 4.6 ± 0.9 mV (n = 21; $P < 0.001$, paired *t*-test); LS, 2.1 ± 0.8 mV (n = 20; $P < 0.01$, paired *t*-test); RSNP, 3.4 ± 0.7 mV (n = 41; $P < 0.001$, paired *t*-test). The hyperpolarization of the resting membrane potential was accompanied by a decrease in input resistance: LTS, 43.3 ± 9.9 MΩ (n = 21; $P < 0.05$, paired *t*-test); LS, 21.8 ± 6.8 MΩ (n = 20; $P < 0.001$, paired *t*-test); RSNP, 41.4 ± 7.2 MΩ (n = 41; $P < 0.001$, paired *t*-test).

All LTS (n = 22), LS (n = 17), and RSNP neurons (n = 48) exhibited repetitive spike firing under control conditions, but 18.2% of LTS neurons, 47.1% of LS neurons, and 21.3% of

RSNP neurons did not show spikes under application of propofol. Propofol treatment decreased the maximum numbers of spikes fired by these neurons as follows: LTS, 10.5 ± 1.8 spikes/300 ms (n = 22; $P < 0.001$, paired *t*-test); LS, 12.3 ± 1.9 spike/300 ms (n = 17; $P < 0.001$, paired *t*-test); RSNP, 14.3 ± 1.4 spikes/300 ms (n = 48; $P < 0.001$, paired *t*-test). Spike threshold was depolarized by 100 μM propofol in LTS (2.7 ± 1.3 mV, n = 18; $P < 0.05$, paired *t*-test), LS (3.5 ± 1.3 mV, n = 12; $P < 0.05$, paired *t*-test) and RSNP (6.1 ± 1.2 mV, n = 34; $P < 0.01$, paired *t*-test) neurons. Spike latency was prolonged by propofol in LTS (146.2 ± 40.8 ms, n = 21; $P < 0.01$, paired *t*-test); LS (135.0 ± 32.9 ms, n = 20; $P < 0.05$, paired *t*-test); and RSNP cells (212.7 ± 31.0 ms, n = 41; $P < 0.001$, paired *t*-test). The reversal potential of propofol-induced suppression of voltage response was -79.6 ± 5.2 mV (n = 9) in LTS, -75.7 ± 2.0 mV (n = 7) in LS, and -81.3 ± 3.6 mV (n = 25) in RSNP cells.

These results suggest that 100 μM propofol suppresses GABAergic neuron excitability. In the next section, the suppressive effects of propofol on GABAergic neuronal excitability are statistically compared with those on pyramidal neurons.

***Comparison of propofol-induced effects between pyramidal and GABAergic neurons***

Propofol (100 μM) modulated electrophysiological properties, including the resting membrane potential, input resistance, spike threshold, and repetitive spike firing frequency, in all types of cortical neurons. However, the degree of the propofol-induced changes is dependent on neuronal subtype (Fig. 4).

The most prominent differences are observed between pyramidal and FS neurons. Except for the spike threshold, pyramidal neurons tended to show larger effects of propofol than FS neurons. In particular, the hyperpolarization of the resting membrane potential and the spike latency were significantly milder in FS cells than in pyramidal neurons ($P < 0.001$-$0.05$, Student's *t*-test with the Bonferroni correction). These observations suggest that pyramidal neurons are the most sensitive to propofol-induced neural suppression.

For comparison of the rate of spike failure induced by propofol in pyramidal neurons (57.9%, n = 19) with those in FS (13.8%, n = 58), LTS (18.2%, n = 22), LS neurons (47.1%, n = 17), and RSNP (21.3%, n = 47) neurons, the multiple $\chi^2$ test was performed (Fig. 4F). It became clear that the rates of spike failure in FS, LTS and RSNP neurons were significantly smaller than that in pyramidal neurons ($P < 0.01$).

The reversal potential upon propofol-induced suppression of the voltage response in pyramidal neurons (-74.7 ± 2.8 mV, n = 15) is almost comparable ($P > 0.1$, Student's *t*-test with the Bonferroni correction) to those in FS (-70.9 ± 1.3 mV, n = 33), LS (-75.7 ± 2.0 mV, n = 7), and RSNP neurons (-81.3 ± 3.6 mV, n = 25). However, the reversal potential of LTS cells (-79.6 ± 5.2 mV, n = 9) was significantly hyperpolarized compared to that of pyramidal neurons ($P < 0.01$, Student's *t*-test with the Bonferroni correction).

Based on these comparisons, it is likely that pyramidal neurons are the most sensitive to propofol in terms of their intrinsic membrane properties.

***Lower concentrations of propofol do not change basic properties of FS neurons***

To examine whether pyramidal neurons are more sensitive to propofol than FS cells and other types of GABAergic neurons, it is reasonable to test the effects of propofol at lower concentrations because propofol at 100 μM, a relatively high concentration, may induce saturated effects. Therefore, I examined the effects of 30 μM propofol on the electrophysiological properties of these cell types.

Fig. 5 shows a typical example of simultaneous recordings from pyramidal, FS, and LTS neurons. In terms of pyramidal neurons, application of 30 μM propofol hyperpolarized the resting membrane potential, induced the suppression of input resistance (Fig. 5C) and the frequency of the repetitive firing (Fig. 5D), and delayed spike initiation in response to the hyper- to depolarizing ramp current injection (Fig. 5E). In contrast, both LTS and FS neurons showed less effects of 30 μM propofol on these electrophysiological properties (Fig. 5B-E). These results suggest that a lower concentration of propofol changes the properties of pyramidal neurons but not of FS and LTS neurons when recorded under the same conditions.

In summary, 30 μM propofol significantly hyperpolarized the resting membrane potential (2.6 ± 0.9 mV, n = 10, $P < 0.05$, paired *t*-test), reduced input resistance (28.1 ± 5.2 MΩ, n = 10, $P < 0.001$, paired *t*-test) and the maximum number of spikes fired (2.6 ± 0.8 spikes/300 ms, n = 10, $P < 0.05$, paired *t*-test), and extended the latency of spike induction in response to the ramp current injection in pyramidal neurons (76.0 ± 21.1 ms, n = 9, $P < 0.01$, paired *t*-test). On the other hand, FS neurons showed a significant decrease only in input resistance (7.2 ± 1.8 MΩ, n = 7, $P < 0.01$, paired *t*-test) upon treatment with 30 μM propofol, and other properties (the resting membrane potential, maximum number of spikes fired, and latency of spike induction) were not affected. However, non-FS neurons, including LTS, LS, and RSNP neurons, showed significantly hyperpolarized resting membrane potentials (3.0 ± 0.7 mV, n = 9, $P < 0.01$, paired *t*-test) upon treatment with 30 μM propofol but did not show changes in input resistance, the frequency of repetitive spike firing, and the latency of spike induction. These results suggest that the basic membrane properties of pyramidal neurons are more sensitive to propofol than are those of FS and non-FS GABAergic neurons.

***The roles of $GABA_A$ receptors in the properties of pyramidal and FS neurons***

Propofol potentiates $GABA_A$ receptor-mediated inhibitory synaptic current by prolonging the duration of IPSCs (Kitamura et al., 2003, 2004, Koyanagi et al., 2014). In cortical pyramidal neurons, $GABA_A$ receptors induce tonic $Cl^-$ currents (Salin and Prince, 1996), increasing the possibility that the propofol-induced changes in the electrophysiological properties described above may be caused by an increase in tonic $Cl^-$ currents. To test this hypothesis, I examined the effects of propofol (100 μM) along with the application of either $GABA_A$ receptor antagonist, 100 μM picrotoxin (Fig. 6) or 10 μM bicuculline (Fig. 7).

In pyramidal neurons, propofol under application of picrotoxin had little effect on the resting membrane potential, input resistance, spike threshold, and spike latency (n = 19; Fig.

6A,B). The maximum spike frequency was also unaffected by propofol in combination with picrotoxin (n = 19; Fig. 6A,B).

Similar to pyramidal neurons, FS neurons showed little effect of propofol with picrotoxin on the resting membrane potential, input resistance, spike threshold, and spike latency (n = 22; Fig. 6C,D). In addition, propofol with picrotoxin had little effect on the maximum spike frequency (n = 20; Fig. 6C,D).

The effects of propofol under application of bicuculline on electrophysiological membrane properties were examined. In pyramidal neurons, propofol with bicuculline had little effect on the resting membrane potential, spike threshold, and spike latency, though the input resistance and maximum spike frequency were slightly but significantly affected by propofol with bicuculline (n = 14; $P < 0.05$, paired *t*-test; Fig. 7A,B). On the other hand, FS neurons showed little effect of propofol with bicuculline on the resting membrane potential, input resistance, spike threshold, and spike latency (n = 14; Fig. 7C,D). In addition, propofol with bicuculline had little effect on the maximum spike frequency (n = 14; Fig. 7C,D).

To examine the possibility that potentiation of $GABA_A$ receptor-mediated tonic current is involved in the hyperpolarization of the resting membrane potential by propofol, I examined the shift of the resting membrane potential by propofol using the internal solution including high $Cl^-$ (74 mM), whose equilibrium potential is estimated to be -15 mV. Application of propofol (100 μM) depolarized the resting membrane potential from -67.5 ± 1.2 mV to -58.0 ± 2.2 mV (n = 14; $P < 0.01$, paired *t*-test) in pyramidal and from -64.7 ± 1.4 mV to -59.8 ± 1.6 mV (n = 17; $P < 0.001$, paired *t*-test) in FS neurons.

These results suggest that $GABA_A$ receptors play a major role in the propofol-induced modulation of the passive membrane and firing properties.

# Discussion

The present study aimed to examine whether propofol uniformly suppresses the spike firing of pyramidal and GABAergic neurons by changing the membrane properties or whether propofol differentially modulates their firing properties. It became clear that a high dose of propofol (100 μM) hyperpolarized the resting membrane potential, decreased input resistance, and suppressed repetitive spike firing in pyramidal, FS, and non-FS neurons. However, the propofol-induced changes in the membrane and firing properties were greatest in pyramidal neurons. Indeed, a lower concentration of propofol only affected neural firing in pyramidal neurons. The propofol-induced modulation of the membrane properties is likely to be mediated by potentiation of $GABA_A$ receptor-mediated tonic currents because preapplication of picrotoxin or bicuculline effectively suppressed the effects of propofol.

***Roles of $GABA_A$ receptors in propofol-induced changes in membrane properties***

Propofol is well known to prolong $GABA_A$ currents (Kitamura et al., 2003, 2004, Koyanagi et al., 2014), making it reasonable to postulate that propofol hyperpolarizes the resting membrane potential via $GABA_A$ receptors. Indeed, propofol has been shown to induce potentiation of GABAergic tonic currents, resulting in membrane hyperpolarization with a decrease in input resistance, in neurons of the hippocampus (Bieda and MacIver, 2004), magnocellular neurosecretory cells in the hypothalamic supraoptic nucleus (Jeong et al., 2011), solitary tract nucleus neurons (McDougall et al., 2008), and spinal neurons (Eckle et al., 2015). A previous study (Salin and Prince, 1996) has reported that bicuculline decreases the resting conductance and causes a shift in baseline current in rat cortical pyramidal neurons, indicating that $GABA_A$ receptor-mediated tonic current regulates the resting membrane potential. However, little information is so far available regarding the effects of propofol on the GABAergic tonic currents in the cerebral cortex.

The present findings that either picrotoxin or bicuculline decreased the effects of propofol on the resting membrane potential and input resistance, suggest the involvement of $GABA_A$ receptor potentiation in the propofol-induced modulation of intrinsic membrane properties. This idea is supported by the evidence that propofol depolarized the resting membrane potential with a decrease in input resistance that were recorded using the patch solution containing high $Cl^-$.

The reversal potential of propofol-induced suppression of voltage response ranged from -74.7 mV in pyramidal neurons to -81.3 mV in RSNP neurons. This potential is between the equilibrium potentials of $K^+$ and $Cl^-$ obtained using the Nernst equation, -98.5 mV and -65.2 mV, respectively. Therefore, propofol-induced hyperpolarization of the resting membrane potential is likely to be caused not by modulation of a single current but by effects on the multiple currents mentioned below.

***Possible currents contributing to propofol-induced changes in membrane properties***

In addition to the enhancement of $GABA_A$ receptor-mediated currents, propofol may suppress $I_h$ (Higuchi et al., 2003, Chen et al., 2005, Ying et al., 2006). $I_h$ contributes to depolarization of the resting membrane potential because of its persistent activation at the resting membrane potential (Pape, 1996). This possibility is supported by the present finding of the attenuation of the depolarizing sag potential (Fig. 1Aa, arrow) and the rebound potential (Fig. 1Aa, arrowhead) by propofol. However, the sag potential was little affected by propofol under application of picrotoxin (Fig. 6A) or bicuculline (Fig. 7A). This discrepancy of the sag sensitivity to propofol might be due to a dependency of $I_h$ on intracellular $Cl^-$ concentration. Lenz et al. (1997) have reported that high concentration of $[Cl^-]_i$ attenuates $I_h$ in hippocampal CA1 pyramidal neurons. Therefore, it is reasonable to postulate that an increase in $[Cl^-]_i$ by potentiating tonic $GABA_A$ currents by propofol depresses $I_h$, whereas propofol has little effect on $I_h$ in $GABA_A$ receptor-blocked condition.

A potentiation of leak $K^+$ ($K_{leak}$) currents, which are likely to be mediated by TWIK-related acid-sensitive $K^+$ (TASK) channels and are considered the major determinants of the resting membrane potential and input resistance (Millar et al., 2000, Sirois et al., 2000, Talley et al., 2000, Meuth et al., 2003), is possibly occurred by propofol. A previous study (Putzke et al., 2007) demonstrates that human TASK-1 or TASK-3 channels expressed in oocytes are not sensitive to 50-200 μM propofol. The finding of only a minor effect of propofol on TASK-3 channels is supported by behavioral pharmacological studies that show little difference in the latency and duration of loss of the righting reflex induced by propofol between wild-type and TASK-3 KO mice (Linden et al., 2007). In contrast, TASK-1 KO mice show a longer propofol-induced loss of righting reflex than their littermate controls, suggesting an interaction between propofol and TASK-1 channels (Linden et al., 2008). Thus, the role of TASK-1 in propofol-induced hyperpolarization of cortical neurons remains an open issue.

***Depolarization of the spike threshold and reduction of repetitive spike firing by propofol***

Voltage-gated $Na^+$ channels play a central role in action potential generation. It has been reported that propofol attenuates $Na^+$ influx via voltage-gated $Na^+$ channels in rat cerebrocortical synaptosomes (Ratnakumari and Hemmings, 1997), hippocampal CA1 (Jones et al., 2007), CA3 (Wakita et al., 2013), the neurohypophysis (Ouyang et al., 2003), and a Chinese hamster ovary cells (Rehberg and Duch, 1999). The findings in this study are consistent with these results and extended them by demonstrating that neural firing of both glutamatergic and GABAergic cortical neurons is suppressed by propofol. In addition, pyramidal neurons are more sensitive than FS neurons, which send abundant inhibitory projections to pyramidal neurons. I consider that the higher propofol sensitivity of pyramidal neurons than FS neurons is a reasonable explanation for the clinical action of propofol, which attenuates cortical excitability *in toto*.

However, spike threshold was not affected by propofol under application of $GABA_A$ receptor antagonists, suggesting that voltage-gated $Na^+$ channels are not the sole determinant

of spike threshold in either pyramidal or FS neurons. This issue should be further explored in the future.

***Preferential suppression of spike firing in pyramidal neurons***

The strength of cortical output is determined by the balance between excitation and inhibition. The main source of the output from the cortex is pyramidal neurons; therefore, the simplest circuits in which to consider the effects of propofol on the cerebral cortex consist of glutamatergic excitatory and GABAergic inhibitory inputs to pyramidal neurons. Both the potent and the weak suppression of spike firing in pyramidal neurons and GABAergic neurons, respectively, support the idea that postsynaptic pyramidal neurons receive fewer excitatory input during propofol application. As a result, excitatory outputs from the cerebral cortex are suppressed by propofol, which may contribute to decreasing the level of consciousness.

It is worth noting that the concentration of propofol required to suppress spike firing is rather higher than that necessary to facilitate $GABA_A$ receptor currents. The previous study in pyramidal neurons of the IC demonstrates that $GABA_A$ receptors show effects of propofol at 1 μM (Koyanagi et al., 2014), whereas the present study indicates that spike suppression needs at least ~30 μM of propofol.

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# Figures

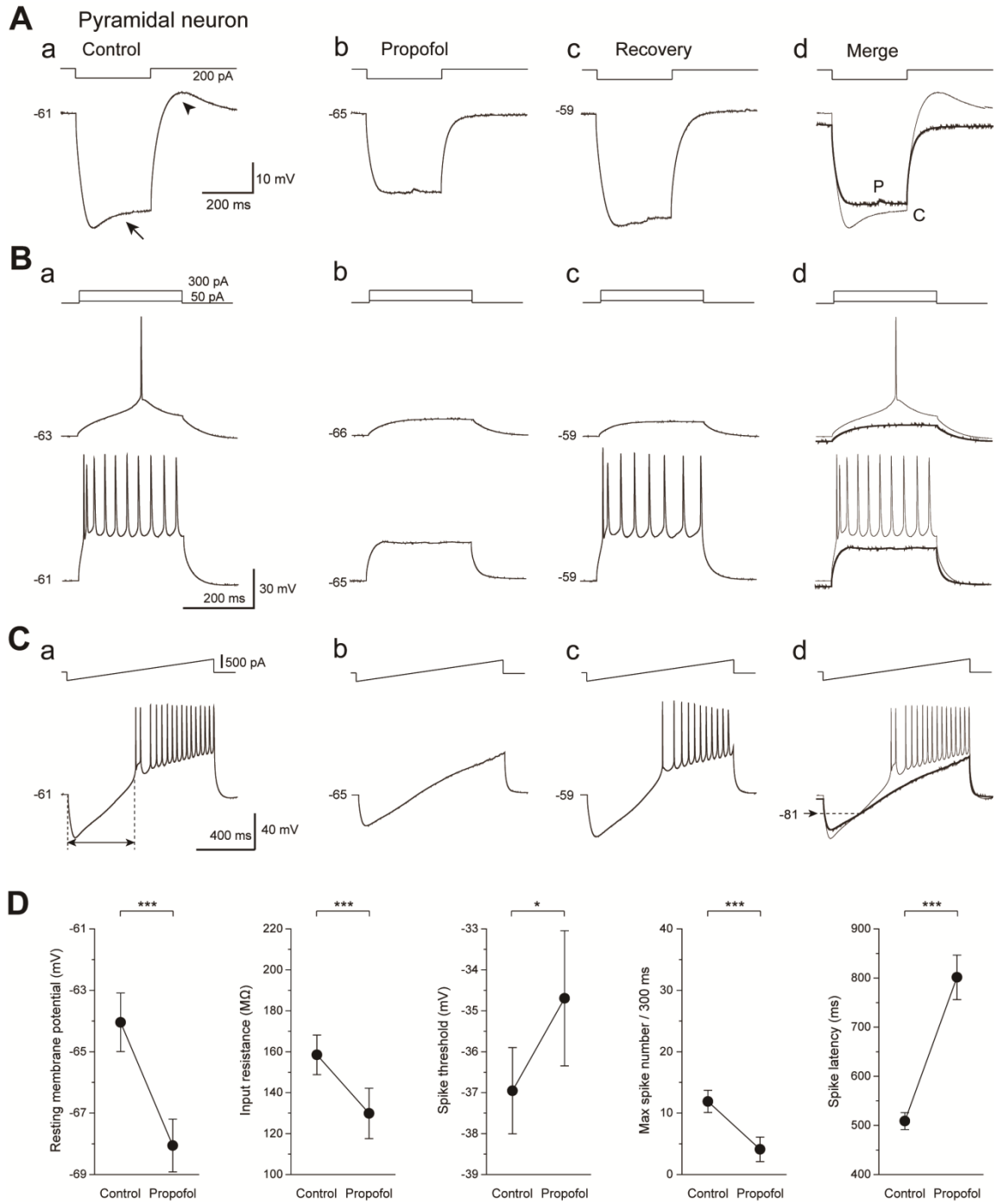


***Figure 1.*** Effects of 100 μM propofol on the membrane properties of pyramidal neurons in the insular cortex (IC). ***A***. Bath application of propofol hyperpolarized the resting membrane potential and reduced the response to a hyperpolarizing current pulse injection (200 pA, 300 ms). The sag (arrow) and rebound potential (arrowhead) were also diminished by propofol. ***B***. Propofol suppressed repetitive spike firing in response to depolarizing current pulse injection (300 ms) in the same pyramidal neuron shown in ***A***. ***C***. Propofol increased the latency of spike firing (double-headed arrow) in response to a hyperpolarizing to depolarizing ramp current injection in the same pyramidal neuron shown in ***A***. The traces in control conditions, under application of propofol, and after propofol washout are shown in columns ***a***, ***b***, and ***c***, respectively. The traces in the control (thin line) and during propofol application (thick line) are merged in column ***d***. The potential of the intersection of the voltage responses in control and under propofol application (arrow) was defined as the reversal potential of propofol-induced suppression of voltage response. The resting membrane potential is shown on the left of each voltage trace. ***D***. Summed changes in the resting membrane potential, input resistance, spike threshold, repetitive firing frequency, and the latency of spike firing obtained from 19-25 pyramidal neurons. *, $P < 0.05$; ***, $P < 0.001$, paired *t*-test.

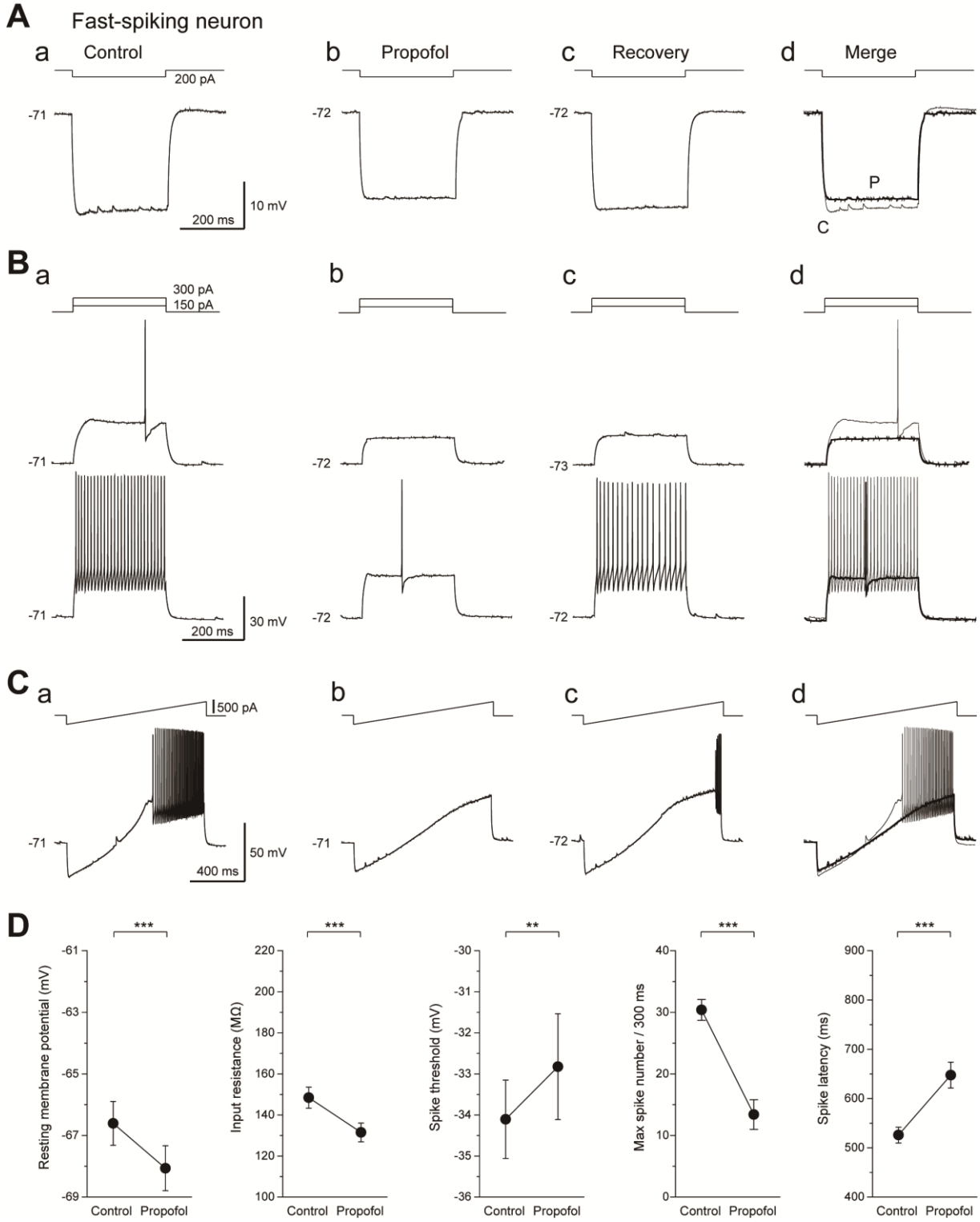


***Figure 2.*** Effects of 100 μM propofol on the membrane properties of fast-spiking (FS) neurons in the IC. ***A***. Propofol hyperpolarized the resting membrane potential and reduced the response to a hyperpolarizing current pulse injection. ***B***. Propofol suppressed repetitive spike firing responding to depolarizing current pulse injection. ***C***. Propofol increased the latency of spike firing in response to a hyperpolarizing to depolarizing ramp current injection. All traces shown in ***A***, ***B***, and ***C*** are obtained from the same FS neuron. The traces in control conditions, under application of propofol, and after propofol washout are shown in columns ***a***, ***b***, and ***c***, respectively, and traces in the control and during propofol application are merged in column ***d***. The resting membrane potential is shown on the left of each voltage trace. ***D***. Summed changes in the resting membrane potential, input resistance, spike threshold, repetitive firing frequency, and the latency of spike firing obtained from 42-69 FS neurons. **, $P < 0.01$; ***, $P < 0.001$, paired *t*-test.

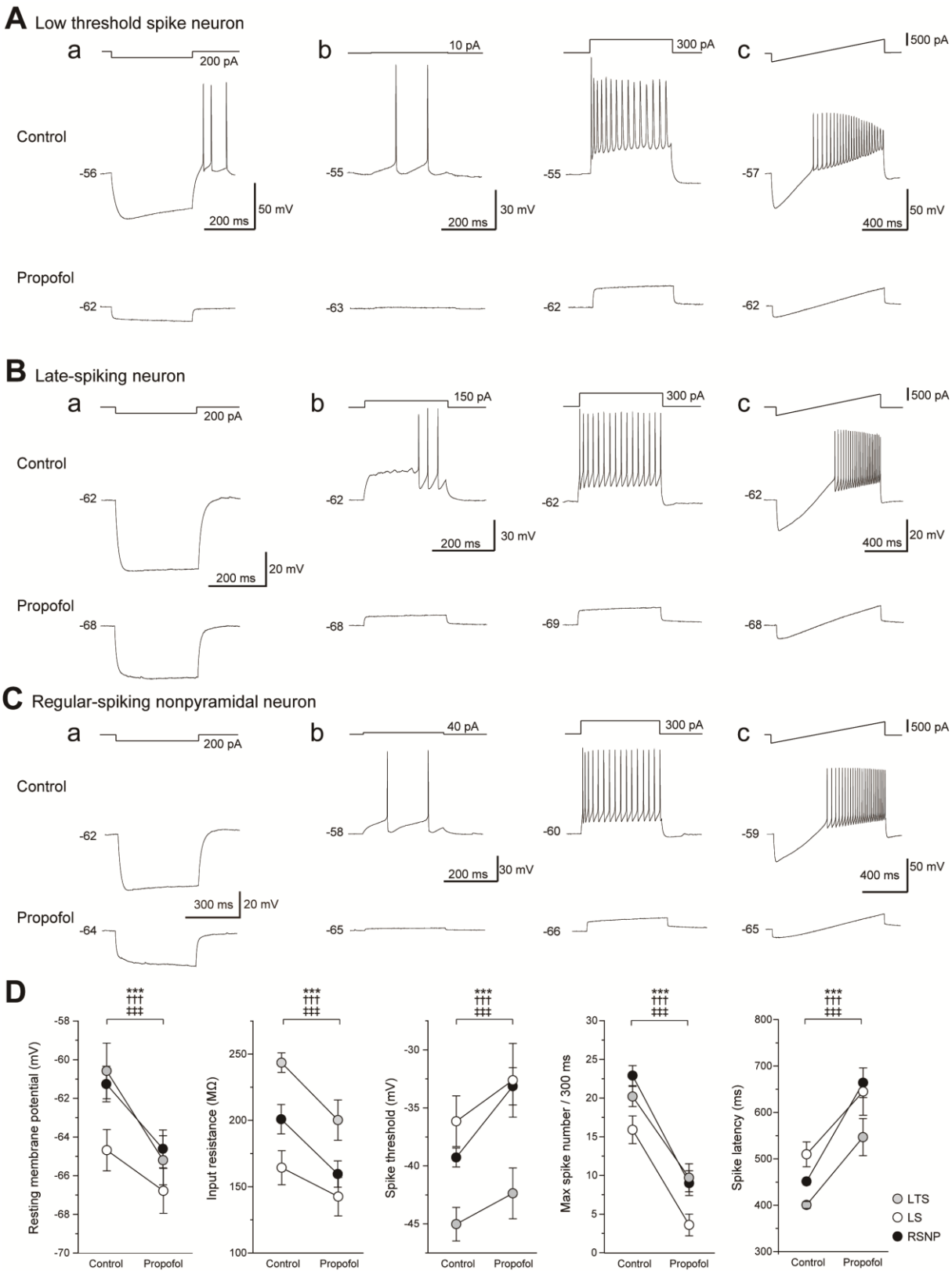


***Figure 3.*** Effects of 100 μM propofol on the membrane properties of low threshold spike (LTS), late-spiking (LS), and regular-spiking nonpyramidal (RSNP) GABAergic neurons in the IC. ***A, B, C***. Effects of propofol on LTS (***A***), LS (***B***), and RSNP neurons (***C***). Propofol hyperpolarized the resting membrane potential, reduced the response to a hyperpolarizing current pulse injection (***a***), suppressed repetitive spike firing (***b***), and increased the latency of spike firing (***c***). All traces in (***A***), (***B***), and (***C***) are obtained from the same LTS, LS, and RSNP neurons, respectively. ***D***. Summed changes in the resting membrane potential, input resistance, spike threshold, repetitive firing frequency, and latency of spike firing obtained from ** LTS, LS, and RSNP neurons. ***, †††, ###: $P < 0.001$, paired *t*-test, in LTS, LS, and RSNP neurons, respectively.

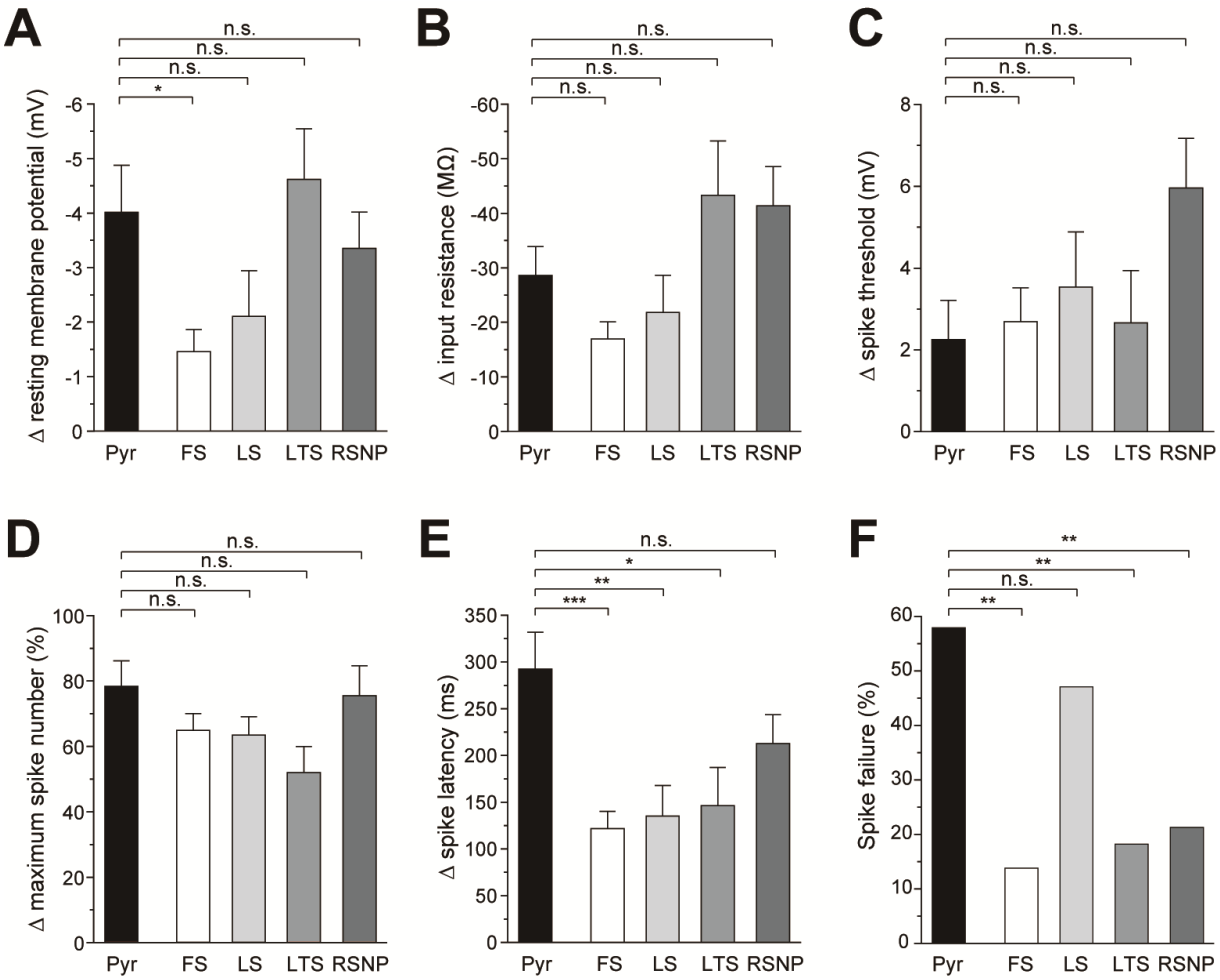


***Figure 4.*** Summary of propofol-induced changes in the resting membrane potential (***A***), input resistance (***B***), spike threshold (***C***), maximum spike number (***D***), latency of spike firing (***E***), and rate of spike failure (***F***) in pyramidal, FS, LTS, LS, and RSNP neurons. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, n.s.: $P > 0.05$, Student's *t*-test (***A-E***) and multiple $\chi^2$ test (***F***) with the Bonferroni correction in comparison to pyramidal neurons.

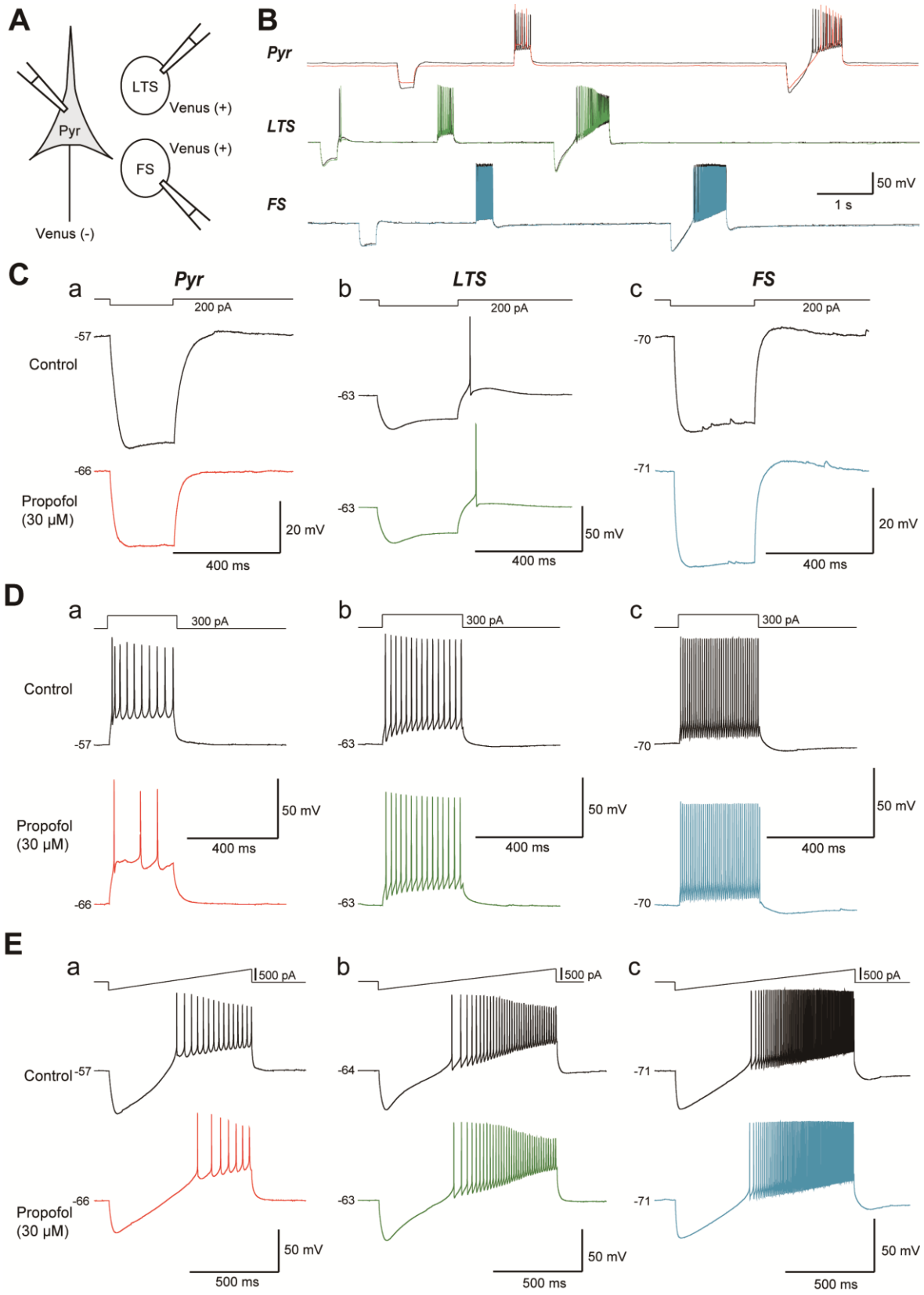


***Figure 5.*** Effects of 30 μM propofol on the membrane properties of pyramidal, LTS, and FS neurons in the IC. ***A***. Triple whole-cell patch-clamp recording from pyramidal, LTS, and FS neurons. ***B***. All test pulses were applied at different times to avoid the possibility of their synaptic inputs to other neurons. ***C***. Propofol (30 μM) hyperpolarized the resting membrane potential and reduced the hyperpolarization response in a pyramidal neuron (***a***), whereas LTS (***b***) and FS neurons (***c***) showed little effect of propofol. ***D***. Propofol suppressed repetitive spike firing in the pyramidal neuron (***a***). However, propofol had little effect on repetitive spike firing in the LTS (***b***) and FS neurons (***c***). ***E***. Propofol increased the latency of spike firing in the pyramidal neuron (***a***) but not in the LTS (***b***) and FS neurons (***c***).

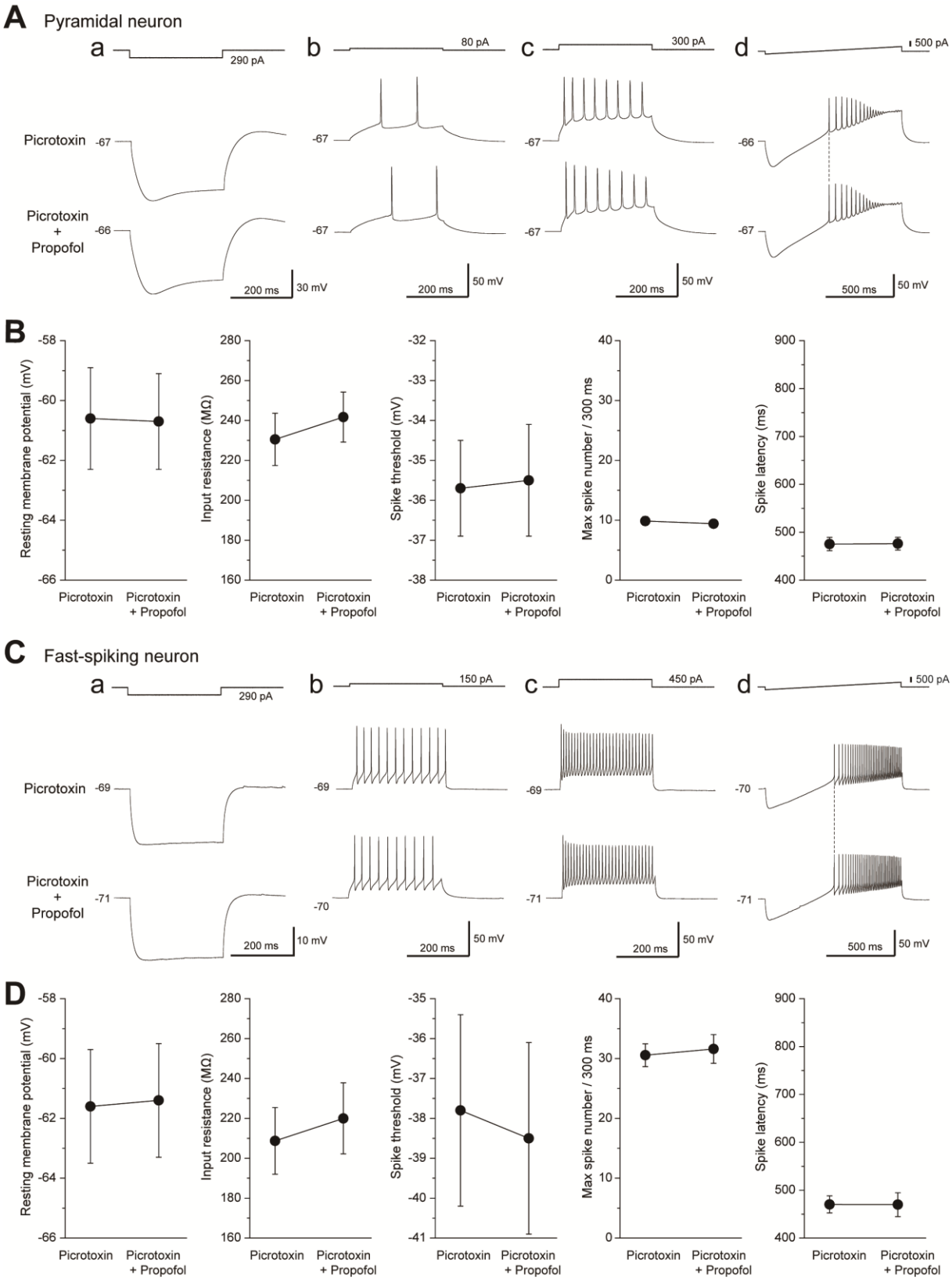


***Figure 6.*** Effects of 100 μM propofol in the presence of 100 μM picrotoxin on the membrane properties of pyramidal (***A,B***) and FS neurons (***C,D***). ***A***. Propofol had little effect on the resting membrane potential (***a-d***), input resistance (***a***), maximum spike number (***c***), and latency of spike firing (***d***) of pyramidal neurons under application of picrotoxin. ***B***. Quantitative analysis of the effect of propofol combined with picrotoxin on the membrane properties of pyramidal neurons. ***C***. In the presence of picrotoxin, propofol had little effect on the resting membrane potential (***a-d***), input resistance (***a***), maximum spike number (***c***), and latency of spike firing (***d***) of FS neurons. ***D***. Quantitative analysis of the effect of propofol combined with picrotoxin on the membrane properties of FS neurons.

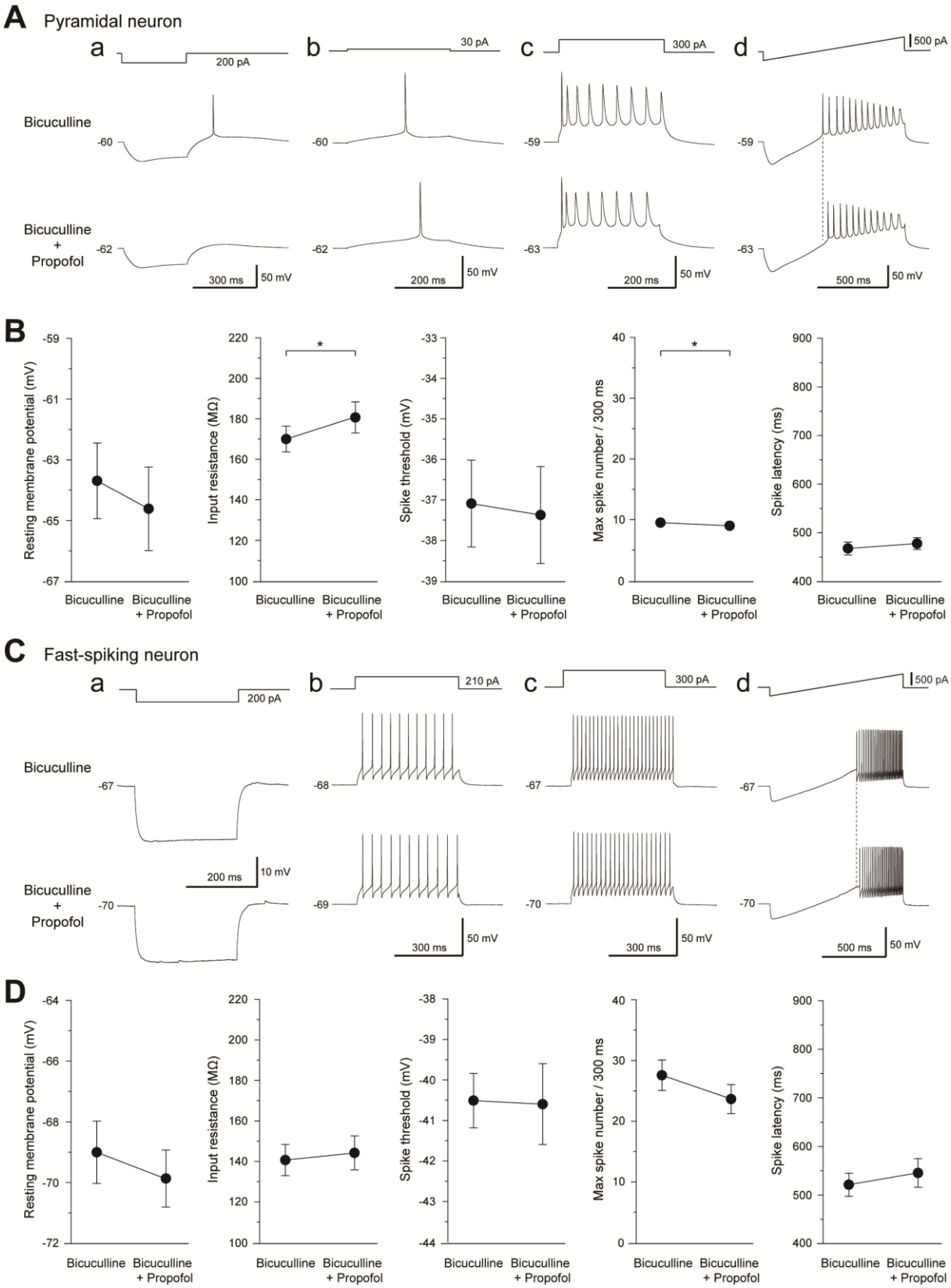


***Figure 7.*** Effects of 100 μM propofol in the presence of 10 μM bicuculline on the membrane properties of pyramidal (***A,B***) and FS neurons (***C,D***). ***A***. Propofol had little effect on the resting membrane potential (***a-d***), and latency of spike firing (***d***) of pyramidal neurons under application of bicuculline. ***B***. Quantitative analysis of the effect of propofol combined with bicuculline on the membrane properties of pyramidal neurons. ***C***. In the presence of bicuculline, propofol had little effect on the resting membrane potential (***a-d***), input resistance (***a***), maximum spike number (***c***), and latency of spike firing (***d***) of FS neurons. ***D***. Quantitative analysis of the effect of propofol combined with bicuculline on the membrane properties of FS neurons.