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Inhibition of T-type calcium current in rat thalamocortical neurons by isoflurane

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Abstract

Thalamocortical (TC) neurons provide the major sensory input to the mammalian somatosensory cortex. Decreased activity of these cells may be pivotal in the ability of general anesthetics to induce loss of consciousness and promote sleep (hypnosis). T-type voltage-gated calcium currents (T-currents) have a key function regulating the cellular excitability of TC neurons and previous studies have indicated that volatile general anesthetics may alter the excitability of these neurons.

Using a patch-clamp technique, we investigated the mechanisms whereby isoflurane, a common volatile anesthetic, modulates isolated T-currents and T-current-dependent excitability of native TC neurons in acute brain slices of the rat.

In voltage-clamp experiments, we found that isoflurane strongly inhibited peak amplitude of T-current, yielding an IC₅₀ of 1.1% at physiological membrane potentials. Ensuing biophysical studies demonstrated that inhibition was more prominent at depolarized membrane potentials as evidenced by hyperpolarizing shifts in channel availability curves. In current-clamp experiments we found that isoflurane decreased the rate of depolarization of low-threshold-calcium spikes (LTCSs) and consequently increased the latency of rebound spike firing at the same concentrations that inhibited isolated T-currents. This effect was mimicked by a novel selective T-channel blocker 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2). In contrast, isoflurane and TTA-P2 had minimal effect on resting membrane potential and cell input resistance. We propose that depression of thalamic T-currents may contribute to some clinical properties of isoflurane.

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Keywords

T-type calcium current; rodent thalamocortical neurons; isoflurane; anesthetics; burst firing; low-voltage-activated

1. INTRODUCTION

The mechanisms causing general anesthetics to produce loss of consciousness are not completely understood, but it is likely that they involve specific effects on multiple classes of ion channels that control the excitability of nerve cells (Urban et al. 2006). Many studies suggest that general anesthetics act through specific ion channels to inhibit neuronal excitability (reviewed by (Franks 2008)). Voltage-gated calcium channels are ubiquitous membrane proteins and have a high level of electrophysiological and pharmacological diversity (Khosravani and Zamponi 2006). On the basis of the membrane potential at which they activate, these channels are subdivided into high voltage-activated (HVA) and low voltage-activated (LVA) or transient T-type calcium channels (T-channels). In neurons, these channels can modulate neurotransmitter release and govern action potential formation, respectively. The cloning of pore-forming $\alpha 1$ subunits of T-channels has shown the existence of at least three subtypes: G ($\text{Ca}_v3.1$), (Perez-Reyes et al. 1998), H ($\text{Ca}_v3.2$), (Cribbs et al. 1998) and I ($\text{Ca}_v3.3$), (Lee et al. 1999), which are likely to contribute to the heterogeneity of T-currents in native cells (Herrington and Lingle 1992), (Todorovic and Lingle 1998). The major functions of T-channels in neurons include: promoting calcium-dependent burst firing, regulating low-amplitude intrinsic neuronal oscillations, promoting calcium entry, and boosting synaptic signals. T-channels are new considerations in anesthesia research, but the part they take in pain transmission (reviewed by (Todorovic and Jevtovic-Todorovic 2011) and the neuronal sleep pathway (reviewed by (Orestes and Todorovic 2010) make them important targets for further investigation. Furthermore, studies indicate that some voltage-gated calcium channels, including T-channels, are affected by volatile general anesthetics at concentrations that likely occur in clinical conditions (Takenoshita and Steinbach 1991), (Study 1994), (Herrington et al. 1991), (McDowell et al. 1996), (Todorovic and Lingle 1998), (Joksovic et al. 2005a), (Joksovic et al. 2005b). This is important since multiple subtypes of neuronal voltage-gated calcium channels have a crucial function in the control of cellular excitability and fast synaptic transmitter release.

Ion channels expressed in various thalamic nuclei have recently received significant attention as possible cellular targets for general anesthetics. Sensory stimuli from the periphery reach the cortex via thalamocortical projections (Bruno and Sakmann 2006b). Studies with *in vivo* extracellular recordings have shown that the processing of sensory input in thalamocortical neurons may be pivotal in providing anesthetic features such as hypnosis and analgesia (Detsch et al. 1999). Furthermore, functional imaging studies in humans and other animals have led to the theory that direct and indirect depression of thalamocortical neurons provides a convergent point for neural pathways of anesthetic action leading to a sleep-like state (Alkire et al. 2000). However, cellular mechanisms for such effects of anesthetics have not been thoroughly studied.

Thalamocortical (TC) relay neurons exhibit phasic behaviors such as tonic and burst firing, which represent different functional modes (McCormick and Bal 1997). During tonic firing, which predominates during awake states, there is a faithful transfer of sensory information to cortical neurons with characteristic low-amplitude, high-frequency electroencephalographic (EEG) patterns. In contrast, during slow oscillations, which occur with T-channel-dependent low-threshold-calcium spikes (LTCSs) and the burst firing pattern of these neurons, there is impairment of sensory transfer and gradual transition to

sleep states. This is manifested on EEG recordings by slow-frequency, high-amplitude wave patterns such as sleep spindles and δ -waves. The current theory is that during natural sleep, and early stages of anesthesia, predominant burst firing in the TC loop indicates impairment of sensory transfer (Franks 2008), (Steriade 2001). In agreement with this, $\text{Ca}_v3.1$ T-channel knockout mice not only show slower anesthetic induction with isoflurane (Petrenko et al. 2007), but have an abnormal natural sleep phenotype that likely results from the lack of T-channels and underlying burst firing in TC neurons (Lee et al. 2004), (Anderson et al. 2005). We have shown that, similarly, $\text{Ca}_v3.2$ knockout mice display delayed anesthetic induction and decreased minimum alveolar concentration (MAC) with isoflurane (Orestes et al. 2009). However, since existing data suggests that T-channel -dependent bursting is an important property of thalamic cells during natural sleep and anesthesia, it remains to be addressed how inhibition of T-channels in thalamocortical neurons may play a role in clinical effects of anesthetics. Inhibition of T-current by isoflurane was previously shown in native reticular thalamic neurons (nRT), which exclusively express $\text{Ca}_v3.2$ and $\text{Ca}_v3.3$ isoforms of T-channels (Joksovic et al.2005a), (Joksovic et al.2005b). Thus, we investigated the effect of this commonly used volatile anesthetic on rodent TC neurons, which largely express the $\text{Ca}_v3.1$ isoform of T-channels (Talley et al. 1999). We asked if inhibition of native T-currents in TC neurons occur within clinically relevant concentrations of isoflurane; if so, what is the mechanism of inhibition; and does isoflurane modulate LTCSs in TC cells? Finally, we discuss the possible relevance of T-channel inhibition by isoflurane in TC cells to clinically useful properties of this prototypical volatile anesthetic.

MATERIAL AND METHODS

1.1. In vitro tissue slice preparation

Experiments were done in accordance with institutional and federal guidelines, including the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 8023, revised 2002). Our *in vitro* slice preparation technique has been described previously (Eckle and Todorovic 2010). Briefly, young Sprague-Dawley rats of either sex (postnatal days 8–15) were briefly anesthetized with isoflurane and decapitated. Brain tissue was rapidly removed and placed in ice-cold slicing solution containing, in mM, 260 sucrose, 26 NaHCO_3 , 10 D-glucose, 3 KCl, 1.25 NaH_2PO_4 , 2 MgCl_2 , and 2 CaCl_2 , adjusted to pH 7.4 with carbogen gas (95% O_2 -5% CO_2). Coronal slices (300 μm thick) containing the sensory ventral posteromedial and posterolateral thalamic nuclei (stereotaxic levels -2.85 to -3.70 mm below bregma)(Swanson 1992) were cut on a microslicer (NVSLM1, World Precision Instruments, Sarasota, FL) and incubated for 30 min in 35°C artificial cerebrospinal fluid containing, in mM, 124 NaCl, 4 KCl, 26 NaHCO_3 , 1.25 NaH_2PO_4 , 2 MgCl_2 , 10 D-glucose, and 2 CaCl_2 adjusted to pH 7.4 with carbogen gas. After incubation, slices were held at room temperature at least 45 min before recording procedures were done.

2.2 Recording procedure

Brain slices were placed in a recording chamber (1-ml bath volume, RC-24N, Warner Instruments, Hamden, CT) that was steadily superfused at a rate of 2 ml/min with carbogenated recording solution containing, in mM, 125 NaCl, 1 MgCl_2 , 25 D-glucose, 25 NaHCO_3 , 1.25 NaH_2PO_4 , 2.5 KCl, 2 CaCl_2 , and 0.001 tetrodotoxin. Saturation with carbogen gas led to a pH of 7.4. For current-clamp recordings, tetrodotoxin was not added to the external solution. For isoflurane application, external solution was continuously gassed during recording, using a bubbling stone attached with high-grade tygon or teflon tubing to the agent-specific vaporizer with a constant flow of carbogen gas. All recordings were done at room temperature. TC relay neurons of the ventral posterior thalamic nucleus were visually identified by videomicroscopy (high-performance CCD camera, Cohu, San Diego,

CA) combined with a BX51WI microscope with oblique illumination (Olympus, Hamburg, Germany). Intracellular solution contained, in mM, 130 potassium gluconate, 5 EGTA, 4 NaCl, 10 HEPES, 0.5 CaCl₂, 2 Mg-ATP, 0.3 Tris-GTP. Calculated liquid junction potential was -14.7 mV and presented data was not corrected. To eliminate glutamatergic excitatory currents, some current-clamp recordings were done in the presence of 5 μ M NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) and 50 μ M d-APV (2*R*-amino-5-phosphonovaleric acid; AP5, (2*R*)-amino-5 phosphonopentanoate) in the bath solution. To eliminate inhibitory synaptic currents, some current-clamp recordings were done in the presence of 50 μ M picrotoxin (a noncompetitive GABA_A antagonist) in the bath solution.

2.3 Electrophysiological recordings

Using a whole-cell voltage clamp technique, T-currents were obtained from TC neurons of the ventral posterior thalamic nucleus. Thin-walled glass capillary electrodes (Corning 7056, Warner Instruments, Hamden, CT) had initial resistances of 3–5 M Ω . For quality reasons, whole-cell configurations had to display gigaseal formation (> 2 G Ω). We compensated for series resistance and cell capacitance to the greatest extent possible. After equilibration with intracellular solution, currents were monitored by periodic voltage steps until the current stabilized. Recordings typically began 10 min after whole-cell access. We recorded membrane currents with a Multiclamp 700B patch-clamp amplifier (Molecular Devices, Foster City, CA) equipped with a CV-7B headstage. Voltage commands and the digitalization of membrane currents were done with Clampex 10.0 of the pClamp software package (Molecular Devices, Foster City, CA). Neurons were typically depolarized from holding potential (V_h) to test potential (V_t) of -50 mV every 20 s to evoke inward calcium currents. Records were filtered at 1.4 kHz with a low-pass filter (Bessel 8-pole). In most experiments, we used a P/5 protocol for online leak subtractions.

2.4 Analysis of current

Results are typically presented as mean $\pm$ SEM unless stated otherwise. The amplitude of T-current was defined as the maximal inward current in response to the depolarization test potential. Curve fitting was done with Origin 7.0 software (OriginLab, Northampton, MA). Concentration-response curves were fitted by the following Hill equation:

$y = V_{\max} [x^n / (IC_{50} + x^n)]$, where $V_{\max}$ is the maximal current block, IC_{50} the concentration of isoflurane that produces 50% of maximal inhibition, and n the apparent Hill coefficient for blockade. The voltage dependence of steady-state activation was described with a single Boltzmann distribution:

$G(V) = G_{\max} / (1 + \exp[-(V - V_{50})/k])$, where $G_{\max}$ is the maximal estimated conductance, V_{50} is the half-maximal voltage, and k (units of mV) represents the voltage dependence of the distribution.

The voltage-dependence of steady-state inactivation was described with a single Boltzmann distribution: $I(V) = I_{\max} / (1 + \exp[(V - V_{50})/k])$, where $I_{\max}$ is the maximal current, V_{50} is the half-maximal voltage, and k (units of mV) represents the voltage dependence of the distribution.

The time course of the macroscopic current inactivation was fitted using a single-exponential equation: $y = A_1 * e^{(-x/t_1)} + y_0$, where A_1 is the amplitude, t_1 the decay constant, and y_0 the offset.

Statistical analysis was performed with a Student's *t*-test. Significant differences were determined by a level of $p < 0.05$.

2.5 Drugs and chemicals

Isoflurane was obtained from Baxter (Deerfield, IL). Tetrodotoxin was obtained from Tocris Bioscience (Ellisville, MO). All other salts and chemicals were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

2.6 Methodological considerations relevant to intact brain slices

Recordings from intact brain slices offer great advantages for studying neurons in an intact setting *in vitro*. However, the presence of extensive cell processes compromises voltage control in whole-cell recordings from slices. Accordingly, we paid close attention to the signs of good voltage control. Specifically, there was no extensive delay in the onset of current; also, the onset and offset kinetics depended on voltage, not on the amplitude of current. In whole-cell experiments, because intact TC neurons have long processes, rapid components of recorded current, such as tail current, are unlikely to reflect the true amplitude and time course of calcium current behavior. All our measurements of amplitudes from holding, peak, and steady-state currents were made at time points sufficient to ensure reasonably well-clamped current conditions.

Similarly, delivery of anesthetic solutions *in vitro* could be compromised in slices due to decline in drug concentration along the length of the bath and diffusion through the sliced tissue (Chesney et al. 2003). To alleviate this problem, we recorded mostly from cells in the superficial 50 μm of slices to facilitate equilibration of IFL and tissue slice. We applied IFL until an apparent steady-state effect on peak T-current was achieved (e.g., Fig. 1B). Apparent saturation of respective IFL solutions was reached after 10 min gassing in a tight glass syringe before application. It is worth mentioning that we performed our experiments at room temperatures that can slightly increase (about 20%) the solubility of volatile anesthetics (Franks and Lieb, 1994).

3 RESULTS

3.1 Isoflurane inhibits T-current in TC neurons in concentration-dependent manner

We first used whole-cell patch-clamp recordings in voltage-clamp mode to determine the effects of escalating concentrations (0.4, 1, 2, 3, and 4%) of isoflurane (IFL) on well-isolated T-currents in TC cells. Figure 1A shows traces in a representative TC cells before, during, and after application of 4% IFL, which reversibly inhibited about 90% of the current. Since T-currents need to be reprimed at negative membrane potentials, our voltage protocol included a hyperpolarized holding membrane potential (V_h) of -90 mV and a depolarizing test potential (V_t) of -50 mV. Panel B shows the temporal recording from the same cell as that shown in panel A of Fig. 1, with the gray bar indicating the time of application of IFL. Note stable recordings of T-current baseline amplitudes, relatively fast onset and almost complete inhibition of the current by IFL, and essentially full recovery of current amplitude after IFL was washed out.

To gain information about the sensitivity of T-currents in TC cells to IFL at more physiological membrane potentials, we did similar experiments evoking T-currents in multiple TC cells from V_h of -70 mV to V_t of -50 mV, then compared the concentration-response curves to IFL in two conditions (Fig. 1, panel C). Insets with representative traces and the graph with average data on Fig. 3C show that IFL was more potent in inhibiting T-currents in TC cells at V_h -70 mV and IC_{50} of 1.1% (black symbols) than it was at V_h -90 mV with an IC_{50} of 1.8% (open symbols). The straight gray arrow at the left on graph denotes the value of IFL needed for *in vivo* effects in rodents. These effects included loss of the righting reflex (LORR), which usually indicates that an anesthetic has hypnotic properties. The straight gray arrow on the right indicates the value of IFL needed in rodents

for *in vivo* effects such as minimum alveolar concentration (MAC), which is defined as the inhaled concentration of an anesthetic needed to prevent movement of 50% of subjects in response to a painful stimulus such as a surgical incision. Importantly, IFL inhibited T-current amplitude in TC cells at physiological membrane potentials (V_h -70 mV) from about 35% (LORR) to about 55% (MAC). At more hyperpolarized membrane potentials (V_h -90 mV), the effects are somewhat less prominent, with about 15% and 38% inhibition of T-current amplitudes for LORR and MAC, respectively. Thus, our data indicate that at likely clinically relevant concentrations, IFL inhibited T-currents in TC cells only partially.

3.2 IFL accelerated T-current inactivation kinetics while voltage-dependence of steady-state activation of T-current is not significantly affected by IFL in TC neurons

The biophysical mechanisms of IFL inhibition of T-current in TC neurons can drastically affect the ability of IFL to influence the cellular excitability of these neurons. Panel A of Fig. 2 shows the effect of 2% IFL on macroscopic T-current inactivation kinetics. In this figure, single exponential fits of decaying portions of original current waveforms before and after the application of IFL from a representative TC cell (V_h -70 mV, V_t -50 mV) indicate that IFL increased an apparent current inactivation rate from estimated inactivation τ of about 66 msec to about 45 msec. On average, we found that IFL accelerated macroscopic inactivation of T-current in TC cells by the $25 \pm 5\%$ decrease in the inactivation τ ($n = 8$, $p = 0.001$, data not shown). Figure 2B illustrates the average current-voltage (I - V) curves from 9 cells in which amplitudes of currents were measured (V_h -90 mV) after a series of escalating depolarizing steps from -80 mV to -20 mV before (black symbols) and after 10 min of applications of 2% IFL (open symbols). It is evident that IFL depressed peak current over the range of all examined test potentials (V_t). From these experiments, we calculated the effects of IFL on the conductance of T-channels in TC neurons (Fig. 2C shows actual data; Fig. 2D presents normalized data). Even though IFL greatly depressed T-channel conductance in the range of membrane potentials more positive to -65 mV (Fig. 2C), the effect of IFL on the midpoint of activation (V_{50}) in these cells was only mildly shifted in depolarized potentials by about 2 mV (control -54 , IFL -52 mV; $p > 0.05$). Thus, we conclude that the voltage-dependence of steady-state activation of T-currents was not significantly altered by 2 vol% application of IFL in TC cells.

3.3 The effect of IFL on steady-state inactivation of T-current in TC cells

Binding to inactivated states is an important property of many drugs that modulate ion channels because this binding can provide selectivity to their action. Transitions from closed to inactivated states can be measured using long conditioning prepulses at varying potentials, producing what are commonly referred to as steady-state inactivation curves. We assessed steady-state inactivation curves using our standard double-pulse protocol with 3.5-s long prepulses to variable voltages (from -110 to -50 mV in 5-mV increments) and V_t to -50 mV (Eckle and Todorovic 2010). The two panels of Figure 3A show representative traces of the currents in the same TC cell before the application of IFL (control, left) and after 10 min of application of 2 vol% of IFL (right). The results of these experiments in TC cells ($n = 8$) before (filled symbols) and after the application of IFL (open symbols) are summarized in panels B and C of Figure 3. We found that IFL caused a significant hyperpolarizing shift of the steady-state inactivation curve of about 7 mV in TC cells (control -69 mV; IFL -76 mV, $p < 0.001$). The pronounced hyperpolarizing shift in V_{50} could be essential for the resulting inhibitory effect of IFL on T-current in TC neurons. For example, at prepulse potentials of -90 mV, IFL inhibited only about 40% of peak T-current, while in the same cells at more depolarized prepulse potentials (e.g., -65 mV) the same concentration of IFL inhibited about 80% of peak T-current.

T-channels activate and inactivate over similar membrane potentials. This property allows for a range of membrane potentials at which T-channels are available for activation, but have not yet been completely inactivated. This phenomenon, represented by the overlapping regions of the Boltzmann curves for steady-state activation and inactivation, is referred to as window current. Using electrophysiological and computational modeling approaches, evidence of window currents has been obtained in TC neurons, where they are important in signal amplification and controlling transitions between different functional states of these cells (Williams et al. 1997), (Hughes et al. 1999), (Dreyfus et al. 2010). Thus, we assessed whether the effects of IFL on steady-state activation and steady-state inactivation decrease window current in TC cells. Figure 4 shows overlapping curves obtained with steady-state activation and steady-state inactivation in control (solid line) and during application of 2% IFL (dashed lines) over the range of membrane potentials from -70 mV to -50 mV. Clearly, IFL greatly diminished the area under the curves representing window current.

3.4 The different effects of IFL on passive membrane properties and spike firing of TC cells

It is generally accepted that T-currents greatly contribute to burst firing mode of TC cells (Perez-Reyes, 2003). Thus, based on our biophysical studies, we hypothesized that IFL at clinically relevant concentrations may modulate T-channel-dependent LTCSs and associated burst firing of TC neurons. We examined the effects of IFL on spike firing and passive membrane properties of TC cells in current-clamp experiments. For current-clamp recordings, tetrodotoxin was not added to the external solution. To study the burst firing mode of these cells, we injected a hyperpolarizing current of 300 ms duration to assess membrane input resistance (R_{in}) and to evoke rebound burst firing. Figure 5A (top trace) shows typical membrane responses to hyperpolarizing current injections in control conditions. Lower panel Figure 5A shows representative trace in the same cell after application of 1% IFL for 5 minutes. In this cell, IFL decreased the number of APs in LTCS from 5 in control conditions to 2. Furthermore, same traces are overlapped on slower time scale on Figure 5B. It is evident that IFL (gray trace) delayed the onset of AP spike when compared to the control (black trace). In contrast, resting membrane potential (RMP) and the amplitude of the membrane response to the hyperpolarizing pulse (R_{in}) changed very little upon applications of IFL. Average data from multiple experiments are shown in Figure 5C. IFL had no statistically significant effect on the passive membrane properties of TC cells such as RMP, ($n = 5$, $p > 0.05$), and R_{in} ($n=5$, $p>0.05$), as shown in panel 5C. In contrast, IFL significantly affected active membrane properties in these cells as evident by increased latency to the first AP ($24\pm7\%$, $p<0.05$, $n=5$) and decreased number of APs in burst ($63\pm0\%$ of control, $p<0.01$, $n=5$). In agreement with the lack of effects of IFL on passive membrane properties of TC cells, we found in voltage-clamp experiments that baseline holding current (-108 ± 25 pA) was not significantly affected by application of IFL (-123 ± 28 pA, $n=8$, $p>0.05$).

We reasoned that, if the effects of IFL on active membrane properties of TC cells are mediated by suppression of T-currents, it should be mimicked by the application of a selective T-channel blocker. For the ensuing experiments, we used TTA-P2, a potent and selective T-channel blocker that inhibits TC cell T-currents in brain slices (Dreyfus et al. 2010). Figure 6A shows traces from a representative TC cells in control condition (top panel, black trace) and after 3 minutes of application of $1\ \mu\text{M}$ TTA-P2 (lower panel, gray trace). Traces from the same experiment are overlapped on Figure 6B and presented on slower time scale. It is obvious that TTA-P2 (gray trace) had very little effect on resting membrane potential and R_{in} , as well as amplitude of LTCS, but it delayed the onset of the first AP as compared to the predrug control in the same cell from 50 to 75 ms (measured from the point of termination of hyperpolarizing pre-pulse). As expected, longer application (> 5 minutes) of TTA-P2 in these cells resulted in complete inhibition of LTCS and

associated burst firing (data not shown). In similar experiments on TC cells, we measured active membrane properties of cells exposed to 1% IFL in the presence of pharmacological blockers of inhibitory and excitatory synaptic transmission (see Methods). Comparison of these data with IFL and TTA-P2 are depicted on bar graphs of Figure 6C. It is evident that both TTA-P2 and IFL significantly increased latency of the first AP spike in bursts ($37 \pm 7\%$, $p < 0.01$, $n = 4$; and $44 \pm 7\%$, $p < 0.01$, $n = 4$; respectively) while the number of APs in bursts was not significantly affected when compared to pre-drug controls. Furthermore, neither TTA-P2 nor IFL had significant effect on RMP and R_{in} in these cells (data not shown). Thus, it appears that, due to its inhibitory effect on T-current, IFL may modulate neuronal excitability of TC cells by affecting the timing of spike firing. However, since effects of IFL are different in the presence of synaptic blockers, the effects of IFL on other voltage-gated and ligand-gated currents may have also contributed to its effect on frequency of APs in burst firing mode of these cells.

4 DISCUSSION

Previous studies have demonstrated that both native and recombinant variants of T-channels are inhibited by various general anesthetics, particularly volatile general anesthetics at clinically relevant concentrations (Todorovic and Lingle 1998), (Takenoshita and Steinbach 1991), (Todorovic et al. 2000), (Herrington et al. 1991), (Study 1994), (McDowell et al. 1996), (Joksovic et al. 2005a), (Joksovic et al. 2005b), (Orestes et al. 2009). The conclusion that a drug at clinically relevant concentrations blocks any relevant ion channel *in vitro* is usually based on comparisons of the apparent IC_{50} for the effect *in vitro* with the plasma concentrations reached *in vivo*. For mammals, IC_{50} clinically relevant concentrations of IFL are thought to be in the range of 0.5–1.5 minimum alveolar concentration (MAC) which corresponds to 0.7–2.1 vol% application (Lukatch et al. 2005). In this range, neocortical burst suppression could be shown, indicating impairment of sensory information transfer (Lukatch et al. 2005). In our experiments, concentrations that produce about 50% current inhibition (IC_{50} for T-current block) in TC cells yielded 1.8 vol% IFL at $V_h -90$ mV and 1.1 vol% of IFL at $V_h -70$ mV, both of which are likely clinically relevant concentrations. Thus, our study establishes that T-currents in TC relay cells could be an important cellular target for clinically useful effects of IFL.

In contrast to our study, Ries and Puil found that hyperpolarization of resting membrane potential and decreased input resistance were predominantly caused by IFL application in TC neurons, presumably by activation of background potassium conductance (Ries and Puil 1999a). This could be caused by ionic current shunting indirectly inhibiting T-current and voltage-gated sodium currents and consequently inhibiting spike firing of these neurons (Ries and Puil 1999b). However, direct effects of IFL on T-currents and other voltage-gated conductances could not be ruled out in that study. Surprisingly, we found that IFL did not affect resting membrane potential and input resistance. These findings are in line with the previously reported inhibitory effect of another anesthetic, aliphatic alcohol octanol, on T-current, along with unchanged passive membrane properties in TC neurons (Eckle and Todorovic 2010). A possible explanation of these divergent findings between our and Puil's group is not immediately obvious, but some possibilities include: internal biological variability within the rat phenotypes; different environmental, nutritional, and housing conditions; and a possible difference in the recording conditions. Using in-situ hybridization, it has been shown that in TC neurons, among various background potassium channels, TASK-3 is highly expressed, while TASK-1, TRAAK, and TWIK-1 are present at moderate levels (Talley et al. 2001). Furthermore, the TREK-1 potassium channel, which is considered to be important for the effect of anesthetics (Franks and Honoré 2004) is not expressed in TC neurons (Talley et al. 2001). Thus, the molecular basis for the effects of IFL on TC neurons reported by Ries and Puil remains unclear. Among different variants of T-

channels, it is likely that the Cav3.1 ($\alpha 1G$) isoform of T-channels is the main target for IFL action since it is highly expressed in native TC cells (Talley et al. 1999). Furthermore, we found corresponding sensitivity and similar voltage-dependent inhibition of recombinant Cav3.1 currents to IFL (Todorovic et al. 2000), (Orestes et al. 2009).

We also found that IFL affected some parameters of active membrane properties of TC cells in brain slices. For example, in the absence of synaptic current blockers, 1% IFL decreased the number of AP spikes in a burst, and prolonged the latency for bursting of TC cells in response to hyperpolarizing stimuli (Figure 5). In contrast, when we repeated the same experiments in the presence of synaptic current blockers like APV, NBQX and picrotoxin, we found that IFL caused only a significant prolongation of latency to the first AP, without altering number of APs per burst. The fact that prolongation of latency of APs by IFL was completely mimicked by selective T-channel blocker TTA-P2, strongly indicates that antagonism of T-currents is critical for this effect (Figure 6). The similar effect of other structurally unrelated T-channel blockers on latency of AP in burst in TC cells was earlier reported (Porcello et al. 2003). A delay in burst onset may, in effect, render a shorter duration threshold current stimulus or excitatory postsynaptic potential (EPSP) insufficient to evoke action potentials in the presence of IFL. We propose that this effect of IFL could affect fidelity of neurotransmission in these cells.

In addition to the T-channels, our study strongly suggests that the effects of IFL on other ion channels, particularly ligand-gated conductances, could have contributed to the observed inhibition of frequency of burst firing in these cells (Figure 5). We did not observe this effect in the presence of synaptic current blockers (Figure 6c). It is well established that at these concentrations IFL strongly potentiates GABA_A-gated ionic currents (Franks 2008), (Urban 2006). Thus, one possible explanation for this inhibitory effect of IFL on burst firing frequency could be shunting effects mediated by GABA_A-gated currents.

Recently, it was shown in TC neurons that complete and selective T-channel blockade with TTA-P2 induced inhibition of burst firing of TC cells while tonic firing mode was not affected (Dreyfus et al. 2010). Hence, in our experiments we focused on the effects of IFL on burst firing mode of TC cells. Here, we report that IFL inhibits T-current in voltage-clamp experiments in a concentration-dependent manner and delays the onset of burst firing patterns in these cells. Furthermore, we found that this effect was fully mimicked by concentrations of TTA-P2 shown to be selective for T-currents (Dreyfus et al., 2010). It has been well established that T-channels in TC cells are necessary for generation of burst firing mode of these cells which in turn supports δ waves (frequency 1–4 HZ) in EEG recordings during anesthesia and sleep-like states (Franks 2008; Steriade 2001; Contreras, 2006). In addition, T-channels in TC cells are crucial for the generation of intrinsic membrane oscillations manifested by slow (< 1Hz waves) corticothalamic sleep rhythms in humans and other animals (Steriade 2001). Importantly, recent evidence strongly supports the idea that this slow sleep rhythm is critically dependent on T-type window current in TC and nRT cells (Crunelli and Hughes 2010). Since we found that IFL inhibited the window component of T-current in TC cells (Figure 4) it would be logical to infer that IFL should diminish slow sleep rhythms as well. Indeed, patch-clamp *in vivo* recordings from anesthetized rats have shown that IFL markedly decreases the frequency of the slow oscillations (Doi et al. 2007). On the other hand, it may be seemingly paradoxical that IFL inhibits T-currents in TC cells, yet it increases *in vivo* δ -oscillations in thalamocortical circuitry (Franks 2008). However, a recent paper by Dreyfus and colleagues (2010) supports the idea that IFL inhibition of T-currents in TC cells may modulate slow waves and δ -waves of thalamocortical networks. The authors found that partial inhibition of T-channels with TTA-P2 gradually decreases duration of slow waves while increases δ oscillations (Dreyfus et al. 2010, Fig. 7). Furthermore, preliminary data using simulations with a biophysical model of slow and δ

oscillations of TC neurons have shown that 20–40% reduction of T-current promoted δ oscillations by suppressing the occurrence of slow oscillations (David et al. 2010). Furthermore, the same study showed that *in vivo* administration of TTA-P2 at lower doses leads to enhancement of δ -waves in EEG recordings. Importantly, we found that at clinically relevant concentrations IFL inhibits about 20–60% of T-current amplitude in TC cells. Hence, partial inhibition of T-current in TC cells by IFL may initiate a switch in thalamic oscillations leading to anesthetized state. In agreement with this point of view, mice lacking Cav3.1 isoform of T-channels display a significant delay in anesthetic induction (Petrenko et al. 2007). Towards this end, recent studies clearly demonstrate sedative effects as a consequence of selective antagonism of T-channels in CNS *in vivo* with TTA-related compounds (Kraus et al. 2010), (Uebele et al. 2009).

Experiments with selective blockers of T-currents like TTA-P2 have demonstrated that complete inhibition of T-currents must be achieved (>80%) in order to abolish the amplitude of LTCS in TC cells (Dreyfus et al. 2010). This could potentially be achieved with IFL only under the deepest levels of anesthesia, which would be associated with more complete direct inhibition of T-currents and profound hyperpolarization of neuronal membrane due to activation of postsynaptic GABA_A receptors. Both of these would lead to eventual cessation of the thalamo-cortical rhythmicity, transition to burst-suppression pattern and, finally, to isoelectric line in EEG recordings (Franks 2008). Extensive biophysical, molecular, and modeling studies are needed to resolve the individual contributions of T-type and other ion channels to the effects of IFL on the excitability of TC cells and their relationship to clinical effects.

In conclusion, our study establishes that T-currents in TC cells are inhibited by clinically relevant concentrations of the commonly used volatile anesthetic IFL. Given the importance of TC cells in sensory information transfer and states of arousal and sleep, the inhibition of T-currents in these cells may lead to the alterations in thalamocortical oscillations and may contribute to the important clinical properties of IFL.

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HIGHLIGHTS

- Thalamocortical neurons provide the major sensory input to the cortex.
- T-currents are key regulators of cellular excitability of these neurons.
- Isoflurane strongly inhibited T-current in at clinically relevant concentrations.
- Isoflurane delayed burst firing of these cells at the same concentrations.
- Isoflurane had no effect on resting membrane potential and cell input resistance.

Figure 1. Concentration-dependent inhibition of T-currents in TC cells by IFL

A: Representative T-current traces showing the reversible inhibitory effect of IFL on peak current. Bars indicate calibration. V_h -90 mV, V_t -50 mV. **B:** Time course from the same experiment as that shown in panel A demonstrating relatively fast onset, about 90% inhibition of the peak T-current, and almost complete washout of the effect of IFL on T-current in a representative TC cell. Horizontal gray bar represents the time of application of IFL; solid points are collected during brief membrane depolarizations every 20 seconds. **C:** This panel summarizes the average percent inhibition of T-currents in TC cells by escalating concentrations of IFL at V_h -90 mV (open circles) and V_h -70 mV (solid circles). Solid

lines are best fits of data points using Hill's equation yielding IC_{50} and slope (n) values as follows for $V_h -90$ mV ($IC_{50} 1.8 \pm 0.1$ vol%, $n = 1.9 \pm 0.3$) and for $V_h -70$ mV ($IC_{50} 1.1 \pm 0.1$ vol%, $n = 1.5 \pm 0.2$). All fits are constrained to 100% maximal inhibition. All data points are averages from at least 4 different cells. Short vertical black bars indicate SEM. Long vertical gray arrows indicate anesthetic end-points of IFL, such as LORR (left) and MAC (right). The data are taken from our previous publication (Orestes et al. 2009). Insets show T-current traces before (black lines) and after application of 1 vol % IFL (gray lines) in the same cell at $V_h -90$ mV (top panel) and $V_h -70$ mV (bottom panel). Cell files: 10118019 (A) and 10112034(35) (C).

Figure 2. The effects of IFL on T-current inactivation and steady-state activation kinetics

A: This graph illustrates IFL-speeded current inactivation kinetics of T-current in a representative TC cell. Inactivation time constants (τ) from decaying portions of current waveforms are calculated using single exponential fits in control conditions (solid black line) and after the application of 2 vol% IFL (gray solid line). In the presence of IFL, inactivation τ decreased from about 80 to 50 msec. Cell file 10105065. **B:** The data points indicate average I-V relationships before (●) and after 5 min (○) of application of 2 vol% IFL, with vertical bars indicating standard errors of the mean (SEM). Currents were elicited by progressing from -80 mV to 20 mV in 10-mV increments from a holding potential of -90 mV ($n = 9$ cells). Note the depression of the current amplitude at most potentials in the presence of IFL. **C, D:** Average conductance (**C**) and normalized conductance (**D**) in whole-cell recordings from I-V experiments shown in panel B are used to construct steady-state

activation curves. Steady-state activation curves were obtained from shown IV-curves and plotted against test potential using the equation $G = I/V_{\text{test}} - V_{\text{rev}}$, where I is the elicited peak current, V_{test} is the voltage command, and V_{rev} is the extrapolated reversal potential, which was taken to be +55 mV. The average half-conductance (V_{50}) was in control -54.0 ± 0.3 mV ($k = 2.9 \pm 0.2$ mV) and in the presence of IFL was -52 ± 0.3 mV ($k = 3.4 \pm 0.2$ mV). Fits were done using the Boltzmann equation ($p > 0.05$).

Figure 3. The effects of IFL on steady-state inactivation curves of T-currents in TC cells

A: Original traces from our double-pulse protocol were used to generate steady-state inactivation curves showing control conditions and after 5 min of IFL 2 vol% application measured using 3.5 s inactivating pulses (-95 mV to -65 mV), followed by a short step to test potential at -50 mV. **B:** Averaged data from 9 cells of control before (●) and after 5 min of the application of 2 vol% and isoflurane (○) are fitted using Boltzmann equations. Peak currents are normalized to control conditions. **C:** To demonstrate shift in the inactivation of T-current, the same data from panel B are normalized to the peak current of each condition. Isoflurane induced a shift of about 7 mV in steady-state inactivation to more negative potentials (control $V_{50} -68.8 \pm 0.1$ mV versus IFL 2 vol% -75.6 ± 0.4 mV, $p < 0.001$). There was a smaller change in the slope of the curve from control 4.0 ± 0.1 mV to

4.8 ± 0.3 mV in the presence of IFL. Cell files: 10119033 (panel A, left) and 10119034 (panel A, right).

Figure 4. The effects of IFL on T-type window current in TC cells

Graph depicts overlay area of steady-state activation (from Fig. 2C) and steady-state inactivation (from Fig. 3B) and demonstrates the reduction of T-type window current under application of 2 vol% IFL (dotted lines) when compared to pre-drug controls in the same cells (solid lines).

Figure 5. The effects of IFL on burst firing of TC cells

A: Current-clamp traces from a representative TC cell depicting burst spike firing activity in response to a hyperpolarizing current injection (-300 pA). Top trace indicates our current injection protocol. Middle trace shows hyperpolarizing pulse and resulting rebound LTCS crowned with 5 AP spikes in control conditions. Bottom (gray) trace depicts resulting cell membrane response in the presence of 1 % IFL. Note that in the presence of IFL cell fired only 2 APs in the response to identical current injection as in control conditions. The initial membrane potential (dotted line) measured just before injections of hyperpolarizing current changed very little during applications of 1% IFL.

B: Control (black) trace and trace after application of IFL (gray trace) from panel A are superimposed on slower time scale. Note that IFL prolonged latency for first AP spike in the burst. Bars indicate calibration.

C: Averaged data from similar experiments as depicted on panels A and B of this figure are plotted with bar graphs with different symbols and with vertical lines indicating SEM from multiple cells ($n=5$). Note that IFL application had very little effect on RMP and R_{in} , while latency to first AP was increased by about 25% and number of APs in burst was decreased by about 40%. *, indicates $p<0.05$.

Cell files: 10115003 (top panel A and black trace on panel B) and 10115004 (bottom panel A and gray trace on panel B).

Figure 6. TTA-P2 mimics the effects of IFL on burst firing in TC neurons

A: Current-clamp traces from a representative TC cell depicting burst spike firing activity in response to a hyperpolarizing current injection (-300 pA). Top trace indicates our current injection protocol. Middle trace shows response of cell's membrane to hyperpolarizing pulse and resulting rebound LTCS crowned with 4 AP spikes in control conditions. Bottom (gray) trace depicts the same cell in the presence of $1 \mu\text{M}$ TTA-P2 in the response to identical current injection as in control conditions. The baseline membrane potential (dotted line) changed very little during applications of TTA-P2.

B: Control (black) trace and trace after application of TTA-P2 (gray trace) from panel A are superimposed on slower time scale. Note that TTA-P2 prolonged latency for first AP spike in the burst. Bars indicate calibration.

C: Averaged data from experiments comparing effects of 1 μ M TTA-P2 and 1% IFL are plotted with different symbols of bar graphs and with vertical lines indicating SEM from multiple cells (n=4 per condition). IFL was applied in the presence of synaptic blockers in bath solution. Note that both IFL and TTA-P2 applications had very little effect on number of APs in burst while latency to first AP was increased by about 44% and 37%, respectively. *, indicates $p < 0.01$.

Cell files: 11o19001 (top panel A and black trace on panel B) and 11o19002 (bottom panel A and gray trace on panel B).