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Isoflurane, But Not the Nonimmobilizers F6 and F8, Inhibits Rat Spinal Cord Motor Neuron Ca_v1 Calcium Currents

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Abstract

Background—Volatile anesthetics decrease Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Ca^{2+} influences neurotransmitter release and neuronal excitability. Because volatile anesthetics act specifically on the spinal cord to produce immobility, we examined the effect of isoflurane and the nonimmobilizers F6 (1, 2- dichlorohexafluorocyclobutane) and F8 (2, 3- dichlorooctafluorobutane) on Ca_v1 and Ca_v2 Ca^{2+} channels in spinal cord motor neurons and dorsal root ganglion neurons.

Methods—Using patch clamping, we compared the effects of isoflurane with those of F6 and F8 on Ca_v1 and Ca_v2 channels in isolated, cultured adult rat spinal cord motor neurons and on Ca_v1 and Ca_v2 channels in adult rat dorsal root ganglion sensory neurons.

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The authors declare no conflicts of interest.

Disclosures

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Results—In spinal cord motor neurons, isoflurane, but not F6 or F8, inhibited currents through Ca_v1 channels. Isoflurane and at least one of the nonimmobilizers inhibited currents through Ca_v1 and Ca_v2 channels in dorsal root ganglion neurons and Ca_v2 in spinal cord motor neurons

Conclusion—The findings that isoflurane, but not nonimmobilizers, inhibited Ca_v1 Ca^{2+} channels in spinal cord motor neurons are consistent with the notion that spinal cord motor neurons might mediate isoflurane-induced immobility. Additional studies are required to examine whether inhibition of Ca_v1 calcium currents in spinal cord motor neurons are sufficient, or whether actions on other channels/proteins also contribute to isoflurane-induced immobility.

Introduction

Neurons transform electrical activity into chemical information via calcium (Ca^{2+}) signaling.¹ Volatile anesthetics (VA) have been shown to alter Ca^{2+} entry through voltage-dependent calcium channels²⁻⁶ and to decrease neurotransmitter release.⁷⁻¹⁰ Calcium ions are important for both excitatory and inhibitory neurotransmission. Since neurotransmitter release depends on the 3rd or 4th power of cytoplasmic Ca^{2+} concentration, a small effect on Ca^{2+} entry via the voltage dependent calcium channels could result in a large effect on neurotransmitter release.^{11,12} Therefore, volatile anesthetic-induced effects, such as anesthesia, analgesia, and immobility, may involve volatile anesthetic actions on neuronal Ca^{2+} regulation.

The spinal cord is the major site at which volatile anesthetics act to induce immobility as measured by MAC.¹³⁻¹⁶ While isoflurane has little effect in the peri-MAC range on spinal cord dorsal horn neurons, it appears that locomotor networks in the ventral horn of the spinal cord are the predominant site of volatile anesthetic-induced immobility.^{15,17-19}

Dorsal root ganglion neurons (DRGN) have their terminals within the spinal cord and some synapse with spinal cord motor neurons (SCMN).^{14,16,17} Since it has been established that anesthetic-induced immobility occurs primarily through an action on the spinal cord^{14,16,20} we examined the effect of isoflurane and the non-immobilizers, F6 (1,2-dichlorohexafluorocyclobutane) and F8 (2,3-dichlorooctafluorobutane), on adult rat DRGN and on SCMN. F6 and F8 are compounds that are predicted by the Meyer-Overton rule, on the basis of their oil solubility, to be anesthetics²¹ but, unlike isoflurane, they do not inhibit movement in response to a noxious stimulus. We focused our attention on two of the high-voltage activated sub-types of the voltage-dependent calcium channels, Ca_v1 (L-type), and Ca_v2 (N-type). These channels are involved in regulating neuronal excitability and neurotransmitter release.²² We postulated that if F6 and/or F8 inhibited Ca_v1 and/or Ca_v2 calcium channels in SCMN and/or DRGN, such channels would not be relevant mediators for isoflurane - induced immobility.

Methods

Cell Culture and Solutions

Primary cultures of adult DRGN (Dorsal root ganglia neurons) and adult SCMN (spinal cord motor neurons) were prepared using Adult Sprague–Dawley rats (70–100 days) following the guidelines approved by the New York University Langone Medical Center Institutional

Animal Care and Use Committee. Primary cultures of adult rat DRGN and SCMN were prepared and maintained as previously described.^{23,24} DRGN were studied within 48-72 hrs; SCMN were studied after 2-6 weeks. SCMN were identified by their morphology. One rat was used for each cell culture preparation. The data shown in this study came from 18 cell culture preparations: 10 DRGN cell preparations, 6 to examine Ca_v1 channels, and 4 to examine Ca_v2 channels; 8 SCMN cell preparations, 3 to examine Ca_v1 channels, and 5 to examine Ca_v2 channels. Sufficient cell preparations with response to electrical stimulation, low noise upon recording and demonstrating reversibility to drug effect were made such that n, the number of cells studied from independent preparations, was greater than or equal to 3.

Ca_v2 and Ca_v1 Calcium Channel Recordings

Total whole-cell Ca_v2 calcium currents were recorded in adult rat SCMN and adult rat DRGN. The pipette solution contained (in mM) 85 CsCl, 10 TEA-Cl (potassium channel blocker), 10 EGTA, 2 MgCl₂, 10 Hepes, 4 CaCl₂, 2 Na₂ATP at pH 7.4 with CsOH and the bath solution contained (in mM) 110 NaCl, 10 BaCl₂, 2 MgCl₂, 10 glucose, 10 Hepes, pH 7.4, and (in μM) 100 picrotoxin (GABA receptor antagonist), 50 bicuculline (GABA receptor antagonist), 10 nitrendipine (Ca_v1 channel blocker) and 1 tetrodotoxin (TTX, voltage-dependent sodium channel blocker) (all from Sigma-Aldrich). Recordings were done initially with bath solution to assure that the level of Ca_v2 currents was stable. Cells were perfused continuously while recording, first with bath solution followed by drug, either isoflurane or F6 or F8, and then with bath solution alone. For SCMN, current-voltage relationships were obtained using a series of depolarizing pulses (95 ms duration) from -50 mV to +30 mV in steps of 10 mV from a holding potential of -80 mV (20ms), with 2 s intervals when the potential was held at -60mV. For DRGN, the duration of the pulse was 40 ms, and the pulses were from -50mV to +50mV, the holding potential was -90 mV (15 ms), and the interval was 4 s when the potential was held at -70mV. Currents were filtered at 2 kHz and digitally sampled at every 5-12 μsec. The membrane capacitance and series resistance were compensated by about 80%. Linear leakage currents and residual capacitance were digitally subtracted on-line with P/4 routines. The current-voltage curves were constructed by measuring the peak current level at each membrane potential. The time-averaged currents at +10mV were measured.

Since Ca_v1 currents display strong rundown under the whole cell configuration, single Ca_v1 channel currents were measured using the cell-attached configuration. The pipette solution contained (in mM): 15 BaCl₂, 110 Sucrose, 10 TEA-Cl, 2 EGTA, 2 Hepes, and (in μM): 100 picrotoxin, 50 bicuculline, 0.9 ω-Conotoxin GVIA (Ca_v2 blocker, Tocris Bioscience, Bristol, UK), 0.1 ω-agatoxin IVA (P-type Calcium channel blocker, Tocris Bioscience), 1 TTX, and 2 (S)-(-)-BayK8644 at pH 7.4. The bath solution contained (in mM) 120 K-Gluconate, 25 KCl, 2 MgCl₂, 0.5 CaCl₂, 2 EGTA, 2 Hepes, and (in μM): 100 picrotoxin, 50 bicuculline, and 1 M TTX, and 2 (S)-(-)-BayK8644 at pH 7.4. Current-voltage relationships were done to confirm the nature of the currents. Consecutive pulses of 10 mV increments from -80 to +40 mV were applied from a holding potential of -90 mV (20 ms). For SCMN the pulse duration was 250 ms and the interval was 2 sec. For DRGN, the pulse duration was 150 ms and the interval was 4 sec. In both cases during the interval, the potential was held at -60 mV. For SCMN, 60-80 traces and for DRGN 36 traces at 0mV were used to measure

the time-averaged current before, during and after drug exposure. Currents were filtered at 1-2 kHz and digitally sampled at every 5-12 μ sec. The background conductance and capacitance transients were subtracted using a fit to a trace without single channel current events.

A two-electrode voltage clamp was used (Dagan 3900; Dagan Corporation, Minneapolis, MN or Axopatch 200B, Axon Instruments, Molecular Devices, LLC). Output from the voltage clamp amplifier was sent to a microcomputer using a data acquisition interface (Lab master; Axon Instruments, Foster City, CA). Pipette resistances were 1.3-3 Mohm. Experiments were conducted at room temperature.

Preparation of Isoflurane and Nonimmobilizers

Saturated isoflurane (Forane ®, Baxter, IL) solutions (14.6 mM) were prepared in the respective bath solutions. The solutions were prepared in advance in a Teflon-sealed glass container and diluted to the final concentrations used in each experiment immediately before use as previously described.²⁵ Saturated solutions of the nonimmobilizers 1, 2-Dichlorohexafluorocyclobutane (F6) (Interchim, Montuçon;) and 2,3-Dichlorooctafluorobutane (F8) (Interchim, Montuçon; SynQuest Laboratories, Inc, Alachua, FL) were prepared according to the method of Cardoso (1999).²⁶ The saturated solutions of F6 and F8 were prepared the day before the experiments. Saturated F6 (225 μ M) and F8 (35 μ M) solutions were prepared by adding 200 μ L of the nonimmobilizers in 20 ml buffer or 85 μ L of the nonimmobilizers in 8.5ml buffer in Teflon-sealed glass container. The solutions were mixed overnight at room temperature and protected from light. Prior to removing the appropriate volume of saturated solution, the solutions were left to rest for at least 3 hours. Stock solutions were used only once. Final concentrations were obtained by adding the appropriate volume (collected with a small gas tight syringe, avoiding the bottom of the bottle) of the saturated solution into a large gas-tight syringe containing bath solution.

The large gas-tight syringe was open only when the drugs (isoflurane, F6 or F8) were being perfused. These drugs were directly applied on the cells through a puffer located directly on top of the cells (perfusion manifold MMF, Scientifica, East Sussex, United Kingdom). Teflon tubing was used between all connections. Finally, measurements were made during continuous perfusion (puffer rate = 0.25ml/min) with either bath solution alone or bath solution containing isoflurane, F6, or F8

Data Analysis

Electrophysiology recordings—Data were acquired and analyzed by using Pclamp8-10 (Axon Instruments, Foster City, CA); figures were done using Clampfit (Axon Instruments, Foster City, CA) and Prism 5.04 (Graph Pad Software Inc. San Diego, CA) software.

Statistical Analysis

Statistical analysis was performed by GraphPad Prism 6 (GraphPad, San Diego, CA). Comparison between groups was performed with one-way ANOVA followed by Bonferroni's Multiple Comparison Test. The denominator for each multiple comparison is indicated in the figure legend.

Results

In SCMN Nonimmobilizers Inhibit Ca_v2 (N-type) but not Ca_v1 (L-type) Currents.

The effect of isoflurane at 0.6 mM (~ 2 MAC) and of F8 and F6 at a concentration equivalent to ~ 2 MAC_{eq} (equivalent MAC) (17.6 μM of F8, 38 μM of F6) on Ca_v2 currents in SCMN were compared. Current traces at +10 mV and their corresponding current-voltage relationships for cells before, during drug exposure (isoflurane, F8 or F6) and after drug removal (Figure 1A-C) are shown. The current-voltage relationships confirm that the currents were Ca_v2 currents, since they start activating at ~-30 mV, and their reversal potential was above +40 mV. F6 had the strongest inhibitory effect, followed by F8 and isoflurane (Figure 1A-C). Figure 2 shows the mean of the normalized time-averaged Ca_v2 current values at +10 mV for control and cells exposed to isoflurane and the nonimmobilizers. The magnitude of the time-averaged current for cells treated with drug was normalized for each cell to that measured during the initial perfusion with control solution and then averaged. Isoflurane at 0.6 mM did not significantly inhibit Ca_v2 currents, $P=0.4963$ (6 comparisons), while F8 and F6 did, $P<0.0001$, and their inhibition was reversible (Figure 2).

The effects of isoflurane at 0.3 mM (~ 1 MAC) with those of F8 (8.8 μM ~1 MAC_{eq}) and F6 (35.6 μM ~ 2 MAC_{eq}) on Ca_v1 single-channel currents in SCMN were compared. Figure 3 shows single-channel current traces at various holding potentials and the corresponding single channel current-voltage relationship that gave a single-channel conductance of ~21pS, a property consistent with Ca_v1 single-channel currents. Figure 3B demonstrates single-channel currents prior to, during, and after exposure to 0.3mM, ~1 MAC, isoflurane. Ca_v1 currents were statistically significantly inhibited by 0.3 mM (~1 MAC) isoflurane, $P=0.0002$, (Figure 4), while F6, $P=0.9240$, and F8, $P=0.3958$, at 1 and 2 MAC_{eq} respectively, had no significant effects (Figure 4).

In DRGN Nonimmobilizers Inhibit Ca_v2 (N-type) and Ca_v1 (L-type) Calcium Currents.

Ca_v2 current traces in DRGN were measured at +10 mV, and their corresponding current-voltage relationships for a control cell (Figure 5A) and for cells before, during drug exposure (isoflurane or F6) and after drug removal (Figure 5B-C) are shown. F6, $P<0.0001$, significantly inhibited Ca_v2 currents in DRG neurons (Figure 6) while the effect of isoflurane was not consistent, $P=0.037$.

In DRGN, we found that isoflurane at ~2 MAC (0.6 mM), $P=0.0081$, and F6 at ~2 MAC_{eq} (35.6 μM), $P=0.0117$, but not F8 (17.6 μM), $P=0.3326$, significantly inhibited Ca_v1 currents (Figure 7). Although the sample size was small, Lilliefors' and Shapiro Wilk's tests of normality and Levene's test for equal variance of the residuals of the ANOVA for drug administration, and drug administration plus the wash period, respectively, demonstrated normal distributions and equal variances as indicated in the legend of Figure 7. Since F6 had an inhibitory effect on Ca_v2 and Ca_v1 currents in DRGN, these channels in DRGN are unlikely to be involved in anesthetic-induced immobility.

Discussion

SCMN and DRGN were exposed to the nonimmobilizers, F6 and F8, to determine whether Ca_v1 and/or Ca_v2 channels in these neurons might be involved in isoflurane-induced immobility. Ca_v1 channels in SCMN were strongly inhibited at a clinically relevant isoflurane concentration (80% inhibition at 0.3mM isoflurane ~ 1MAC), but were not inhibited by F6 or F8 at their 1 or 2 MAC_{eq}, suggesting that SCMN Ca_v1 channels might contribute to isoflurane-induced immobility. On the other hand, SCMN Ca_v2 channels were strongly inhibited by F6 and F8 but not by isoflurane (at 2 MAC), suggesting that Ca_v2 channels do not contribute to isoflurane-induced immobility. Furthermore, Ca_v1 and Ca_v2 Ca²⁺ channels in rat DRGN were inhibited by F6, suggesting that Ca_v1 and Ca_v2 channels in DRGN do not contribute to isoflurane-induced immobility.

It is well known that Ca_v2 and Ca_v1 calcium channel functions are highly regulated by post-translational modifications (e.g. phosphorylation) as well as by interactions with other cellular proteins, e.g. G-proteins, hence the different responses of a given voltage dependent calcium channel in SCMN and DRGN could reflect differences not only in their structure but also in modulation by other cellular processes.^{5,27} Regardless of the possible underlying structural or regulatory mechanisms that lead to the different responses to isoflurane and nonimmobilizers for a given channel type in different neuronal cells, our results suggest that calcium signaling, and specifically Ca_v1 calcium channels in SCMN, may be involved in the mechanism of isoflurane-induced immobility.

AMPA, NMDA, glycine and GABA_A receptors are reportedly involved in the overall anesthetic effect,^{28,29} but other receptors and channels may also be relevant. It has been shown that enflurane at 1 MAC depressed synaptic currents in SCMN.³⁰ A paradigm involving multiple effects of volatile anesthetics on several ligands and channels has been described that could explain how different ligand and receptors, including voltage-dependent calcium channels, may participate in isoflurane-induced immobility.³¹ Our data support Ca_v1 channels in SCMN as one of several targets involved in volatile anesthetic-induced immobility. It is not yet clear how Ca_v1 channels are modulated by volatile anesthetics, which can act directly on the Ca_v1 channel or indirectly through secondary effectors. It is known that isoflurane can act on the channel by increasing current inactivation and prolonging recovery time after inactivation; for example, halothane inhibits Ca_v1 calcium channels in SH-SY5Y cells through stabilization of non-conducting or inactivated states.⁴ We have also shown that activation of G proteins reduces isoflurane inhibition of Ca_v2 calcium currents in SH-SY5Y cells⁵ and in DRGN (unpublished data).

Inhibition of Ca_v1 calcium channels by volatile anesthetics has been demonstrated. Recombinant cardiac Ca_v1 calcium channels are inhibited by halothane.² Ca_v1 calcium channels are involved in neurotransmission, gene expression and regulating neuronal excitability.^{32,33} Ca_v1 calcium currents mediate rhythmic activity induced by a cholinergic antagonist in motor neurons, suggesting that entry of calcium through Ca_v1 calcium channels is a significant pathway involved in agonist-modulated locomotor activity.³⁴ Ca_v1 calcium channels also contribute to NMDA-induced intrinsic oscillations in mature turtle spinal cord motor neurons.³⁵ There are also studies showing the effects of volatile

anesthetics on calcium regulation, and specifically on voltage-dependent calcium channels.³⁶ Our results direct attention to Ca_v1 channels in SCMN as a relevant target of volatile anesthetic-induced immobility. The action of the nonimmobilizer F6 on rat DRGN, but not rat SCMN Ca_v1 calcium channels, suggests that these channels in DRGN are not a prominent site of isoflurane-induced immobility.^{19,15} Furthermore, the inhibition by isoflurane of Ca_v1 channels in rat SCMN, but not the nonimmobilizers, supports the work of others describing anesthetic-induced immobility as an effect mediated through the ventral spinal cord.¹⁷⁻¹⁹

Using the lamprey spinal cord preparation, Jinks et al. concluded that ventral SCMN involved in central pattern locomotor activity were an important site of isoflurane-induced immobility.³⁷ They subsequently used electrical microstimulation of the mesencephalic locomotor region in decerebrated rats and determined that volatile anesthetics produce “immobility mainly by action on ventral spinal cord locomotor networks”.¹⁵ However, motor neurons themselves have been shown to express persistent oscillatory activity sensitive to Ca_v1 and Ca_v2 Ca²⁺ channels and are potentially related to locomotor networks.^{34,35,38-41} SCMN express Ca_v1.2,3(L-), Ca_v2.1(N-), Ca_v2.2(P/Q), Ca_v2.3(R), and Ca_v3(T-)type channels, but mainly Ca_v1.2,3(L-) and Ca_v2.1(N-)type.⁴² Both Ca_v1.2 and Ca_v1.3 L-type channels are found in the CNS,, and Ca_v1.3 (α_{1D}) channels are involved in persistent inward currents which give SCMN the property of bistability and sustained activation.^{39,41,43}

Based on electromyographic studies in the rat, King and Rampil suggested that isoflurane can depress SCMN.¹⁷ They stimulated the tibial nerve of rats and measured the amplitude of the orthodromic (M-wave) and antidromic (F-wave) electromyogram and showed that only the F-wave was inhibited by isoflurane.^{17,18} The F-wave results from the antidromic transmission back to the motor neuron followed by orthodromic transmission from the motor neuron. The Ca_v1 calcium channel is found predominantly in the soma but also in the proximal dendrites of rat SCMN.^{44,45} The action of isoflurane to inhibit calcium movement through the Ca_v1 channel could result in a higher threshold for activating an action potential resulting in inhibition of neurotransmission. The Ca_v1 channel in the motor neuron has approximately a 75% homogeneity with the Ca_v1 channel in skeletal muscle, yet, as shown in the studies of Rampil, although the F-wave is markedly depressed by isoflurane, the M-wave, representing direct stimulation of the motor endplate, was unaffected by isoflurane. That result suggests that the Ca_v1 channel of the motor neuron and skeletal muscle are sufficiently different that, at the clinically relevant concentrations studied, isoflurane had little effect on the skeletal muscle Ca_v1 calcium channel, despite inhibiting calcium movement through Ca_v1 channels in the SCMN.

Although we have presented evidence that Ca_v1 channels in SCMN are a likely target for isoflurane-induced immobility several limitations must be considered. Our studies were performed *in vitro* on cultured DRGN and SCMN. Even though the identified channels demonstrated characteristics of Ca_v1 and Ca_v2 channels, we cannot rule out the possibility that the isolation process might have altered the channels' response to isoflurane and nonimmobilizers. A further limitation is that the concentrations of isoflurane, and F6 and F8 in the solutions bathing the SCMN and DRGN were not measured. However, recordings

were performed while the chamber was continuously perfused; moreover, the observed inhibitory effects of F6 and F8, on Ca_v2 channels in SCMN, and Ca_v1 and Ca_v2 channels in DRGN support our methodology, and suggest that F6 and F8 were present in the chamber solutions used to examine Ca_v1 channels, despite there being no effect on these channels. Finally, we studied only one volatile anesthetic, isoflurane. Is it unique in its target or do other volatile anesthetics also induce immobility by targeting the SCMN Ca_v1 channels? There is supporting evidence in other cell types, including primary cultures and expression systems, that some of these volatile anesthetics can alter Ca_v1 function; however, none of them include Ca_v1 channels from SCMN. Whether Ca_v1 in SCMN are also targeted by other volatile anesthetics, such as halothane, enflurane, desflurane and sevoflurane, remains to be determined.

The physiologic implication of our findings is related to the fact that calcium contributes to the regulation of neuronal excitability through its actions on many sites within a cell.^{46,47} Activation of Ca_v1 calcium channels leads to increased excitability of neurons while inhibition of Ca_v1 channels can lead to decreased excitability. Volatile anesthetics, including isoflurane, have multiple effects on Ca²⁺ homeostasis in neuronal cells. The proposed isoflurane-induced decreased excitability of motor neurons can be related not only to Ca_v1 channel inhibition but might also involve modification of Na⁺ channel conductance and other ion channel conductances involved in intracellular Ca²⁺ regulation. In summary, we found that Ca_v1 calcium channels in SCMN could contribute to isoflurane-mediated immobility. Additional studies are required to examine whether inhibition of Ca_v1 calcium channels is sufficient, or whether isoflurane actions on other channels in SCMN also contribute to isoflurane-induced immobility.

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FIGURE 1. Current Traces of Ca_v2 Ca²⁺ Channels in Rat SCMNs

Current traces at +10 mV (top) and their corresponding current-voltage relationships (bottom) during sequential perfusions of bath solution with no additions (W), with the addition of A. isoflurane, 0.6 mM (~ 2 MAC, red), B. F8, 17.6 μM (~2MAC_{eq}, green) or C. F6, 35.6 μM (~2 MAC_{eq}, blue), and subsequent perfusions with bath solution (W₁, W₂, W₃).

FIGURE 2. Summary Data of F6 and F8 inhibition of $\text{Ca}_v2 \text{Ca}^{2+}$ Currents in Rat SCMN
 Normalized time-averaged currents at +10mV, before (W), during drug exposure (D) and after washing out (W1, W2, and W3) the drug. One-way analysis of variance ($P < 0.0001$). Bonferroni's Multiple Comparison Test (denominator = 6): $\wedge P < 0.0001$ between each group and the corresponding control group (n = the number of cells); control $n=13$, except W3 $n=9$; isoflurane (0.6mM, $\sim 2 \text{ MAC}$) $n=6$; F8 (17.6 μM , $\sim 2 \text{ MAC}_{\text{eq}}$) $n=6$, except W3 $n=3$; F6 (35.6 μM , $\sim 2 \text{ MAC}_{\text{eq}}$) $n=3$, except W2 & W3 $n=1$.

FIGURE 3. Isoflurane inhibits Cav1 currents in Rat SCMNs

A. Single channel current traces at various membrane potentials. Channel openings are downward, and the corresponding single channel current-voltage relationship has a slope of 21.3 pS. B. Current traces at 0 mV before (Control), during exposure to 0.3mM (~1 MAC) isoflurane (ISO), and after removal of isoflurane (Wash).

FIGURE 4. Summary Data of Isoflurane, but not F6 and F8, inhibition of Ca_v1 Currents in Rat SCMN

Normalized-time averaged current traces at 0mV, control $n=11$, isoflurane $n=4$ (red, 0.3 mM, ~ 1 MAC), F6 $n=4$ (green, 35.6 μM , ~2 MAC), F8 $n=3$ (blue, 8.8 μM , ~1 MAC), before, during and after washing out the drug. One-way analysis of variance ($P=0.0019$). Bonferroni's Multiple Comparison Test with a denominator of 6: Isoflurane vs. control * $P=0.0002$; F8 vs. control $P=0.3968$; and F6 vs. control $P=0.9240$.

FIGURE 5. Isoflurane and the Nonimmobilizer F6 reduced the Cav2 calcium currents in DRGN Current traces at +10 mV for a control cell (A) a cell exposed to isoflurane (B, red, 0.3 mM, ~1MAC) and a cell exposed to F6 (C, blue, 17.6 μ M, ~2 MAC_{eq}), before (black traces), during drug (color traces) and after washout (gray traces) and their corresponding current-voltage relationships (bottom).

FIGURE 6. Summary data of Isoflurane and F6 inhibition of Ca_v2 calcium currents in DRGN Normalized time-averaged current traces at +10 mV, before, during drug exposure and after washing out the drug. Control (black, $n=5$), Isoflurane $n=3$ (red, 0.3 mM, ~1 MAC) and F6 $n=4$ (blue, 17.6 μM , ~2 MAC_{eq}) One-way analysis of variance ($P<0.0001$). Bonferroni's Multiple Comparison Test, denominator = 5: # $P=0.0371$, and ^ $P=0.0002$ vs control. Both isoflurane W_1 ($P=0.0085$) and F6 W_1 ($P=0.0002$) are different from control.

FIGURE 7. Summary data of F6 inhibition of Ca_V1 currents in DRGN

Normalized time-averaged current traces at 0mV, before (W), during drug exposure (D), and after washing out the drug (W₁). Control n=4 (black), F8 n=3 (green, 17.6 μM , ~2 MACeq), and F6 n=7 (blue, 35.6 μM , ~2 MAC). One-way analysis of variance ($P=0.0003$) considering exposure to control and F6, and F8. Bonferroni's Multiple Comparison Test, denominator = 3: # $P=0.0117$ between F6 and control. The Levene's Test yields $P=0.4500$ for drug administration, and $P=0.1049$ when the wash periods are included in the analysis, indicating equivalence of variance amongst groups. The Lilliefors test for normality yields $P>0.1000$ for drug administration and $P>0.1000$ when wash periods are included. The Shapiro-Wilk test for normality yields $P=0.9765$ for drug administration and $P=0.7215$ when

wash periods are included. Both tests demonstrate normal distributions of the residuals of the ANOVA.