Differential effects of isoflurane on presynaptic voltage-gated calcium channel subtypes in hippocampal neurons

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Introduction:

- Despite the essential role of volatile anesthetics (VAs) in modern medicine, the molecular mechanisms underlying their depression of the central nervous system (CNS) are not fully understood.[1]
- Studies have shown that VAs decrease neurotransmitter release, preferentially affecting excitatory transmission, which offers an explanation for CNS depression.[2,3]
- Presynaptic ion channels are likely an important mediator of anesthetic action.[4-7]
- Neuronal voltage-gated calcium channels (VGCCs) have a fundamental role in neurotransmission.[8]
- Studies exploring the effects of VAs on VGCCs have led to discordant results.[9-12] Therefore, investigation of anesthetic interactions with N- and P/Q-type VGCCs, the predominant CNS subtypes,[8] should provide new insights into the mechanisms of anesthesia.

Objective:

- We sought to study the effect of the inhaled anesthetic isoflurane on VGCC subtype-dependent exocytosis in hippocampal neurons using live-cell fluorescence microscopy and pharmacologic isolation of subtype channels.

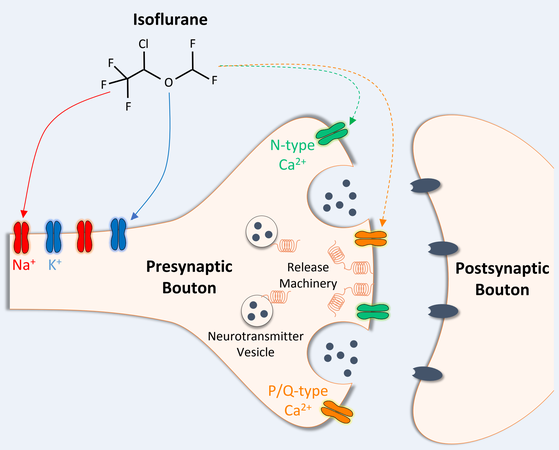

Figure 1| Schematic of the interactions of isoflurane with presynaptic ion channels. Isoflurane inhibits voltage gated Na^+ channels (solid red line) and potentiates K^+ channels (solid blue line), diminishing action potential propagation and decreasing neurotransmission. The signal for neurotransmitter release is the rapid influx of calcium ions, mediated predominantly by N- and P/Q-type VGCCs. The effect of isoflurane on these channels (dotted lines) is less understood. Image adapted from Motifolio Inc.©

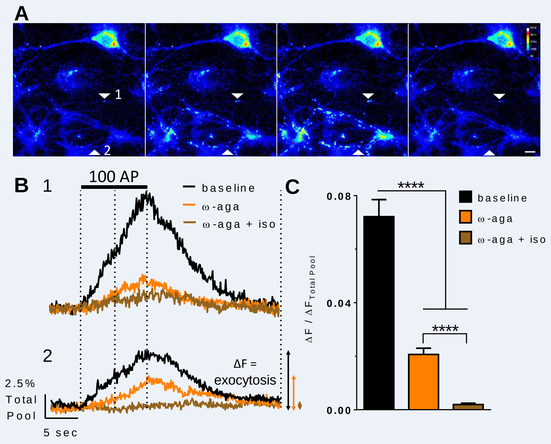

Figure 2| Experimental approach to measure the effect of isoflurane on presynaptic calcium channels. (A) The increase in fluorescence as a hippocampal neuron transfected with vGlut-pH is stimulated by 100 action potentials, false-colored to show changes in intensity. Scale bar, 10 µm. Calibration bar, intensity in arbitrary fluorescence units. (B) Traces of fluorescence intensity over time of the boutons marked in A. The dashed vertical lines correspond to the time points in A. The black trace shows the fluorescence at baseline, and the orange and brown traces show the same boutons after sequential treatment with the P/Q channel inhibitor ω-aga and 2 MAC isoflurane, respectively. (C) Comparison of the average exocytosis of all the boutons in A at baseline, with ω-aga, and 2 MAC isoflurane with ω-aga (n = 127, **** p < 0.0001).

Methods:

- Hippocampal neurons from Sprague-Dawley rats (postnatal days 0-2) were transfected after 7-8 days *in vitro* with vesicular glutamate transporter-1 (vGlut-1) tagged pHluorin (vGpH) to enable quantitative measurements of exocytosis.[13] Experiments were performed 7-17 days after transfection.
- Epifluorescence microscopy was conducted with a Zeiss Axio Observer microscope and a solid state diode-pumped 488nM laser. Excitation and emission were transmitted by a 470/40 nm excitation filter, 495 nm dichroic mirror, and 525/50 nm bandpass filter (Zeiss) using a 40x, 1.3NA objective and a 1.6x Optivar (Zeiss). Images were acquired with an Andor iXon+ CCD.
- Cells on coverslips were loaded onto a custom chamber and perfused with Tyrode's solution (pH 7.4) containing 2 mM $CaCl_2$ and the glutamatergic receptor antagonists CNQX (10 µM) and AP5 (50 µM). Action potentials (APs) were stimulated by ~10V/cm pulses of 1 ms duration delivered by platinum-iridium electrodes.
- VGCC inhibitors were applied for 3 min: 400 nM ω-agatoxin IVA (ω-aga), 500 nM ω-conotoxin GVIA (ω-cono), 1 µM SNX-482, or 10 µM nimodipine.
- Exocytosis was measured with 100 APs in 10s, resting 5 min between stimulation. The fluorescence of the total pool of vesicles was determined by applying 50 mM NH_4Cl to the chamber to alkalinize intracellular vesicles at the conclusion of each experiment.
- Isoflurane was washed into the chamber for 5 min with at 0.72 ± 0.11 mM, corresponding to 2 MAC in rats, as verified by gas chromatography.
- The fluorescence intensity over time for each bouton was measured using an ImageJ plug-in.[14] Exocytosis was calculated as the peak of fluorescence above baseline and normalized to the fluorescence value of the total vesicle pool. Statistical analysis was performed in GraphPad Prism v6, with significance set as p < 0.05.

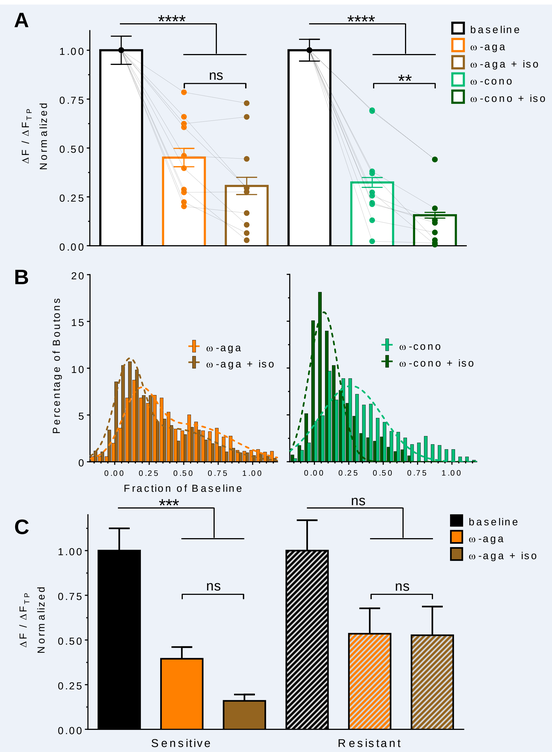

Figure 3| The differential effects of isoflurane on exocytosis after isolation of presynaptic VGCCs. (A) Measurements of exocytosis for individual cells (filled circles) and average across cells (open bars). Exocytosis values for each cell were normalized to the average baseline value. (n = 10, ** p < 0.01, **** p < 0.0001, ns p > 0.05). (B) Boutons for all cells were normalized to their baseline value and binned by the extent of inhibition. The ω-aga treated boutons were fit by the sum of two normal distributions, and the ω-cono treated boutons were fit by a normal distribution. (C) Cells treated with ω-aga were grouped and analyzed by sensitivity (solid bars, n = 6) or resistance (striped bars, n = 4) to isoflurane (cut-off p < 0.05).

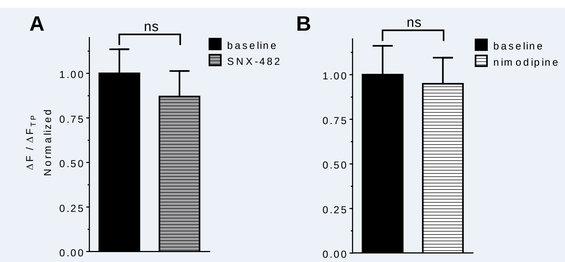

Figure 4| The contribution of presynaptic R-type (A) and L-type (B) VGCCs to exocytosis (n = 4).

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Results:

- Treatment with ω-cono led to greater inhibition than with ω-aga (32% and 45% of baseline, respectively, *n* = 10).
- After application of ω-cono to block N-type channels, treatment with isoflurane led conistently to further inhibition of exocytosis (p < 0.05 in 9/10 cells), with a mean inhibition of 52%.
- In contrast to the results with ω-cono treatment, isoflurane inhibited exocytosis further in only a subset of cells after blocking P/Q-type channels with ω-aga (p < 0.05 in 6/10 cells).
- For ω-aga treated cells classified by their sensitivity or resistance to isoflurane (cut-off p < 0.05), resistant cells demonstrated negligible inhibition by isoflurane (~2%, p > 0.9, *n* = 4) whereas sensitive cells were inhibited by 60%, though the difference was not statistically significant (p = 0.15, *n* = 6).
- Normalizing each bouton to its baseline value, the effect of ω-aga alone or with isoflurane could be fit by the sum of two Gaussian functions (R^2 0.88 and 0.96, respectively).
- Inhibiting L-type channels with nimodipine and presynaptic R-type channels with SNX-482 led to 5% and 13% decrease in exocytosis, respectively (p > 0.2, *n* = 4 for both).

Conclusions:

- N-type VGCCs contribute to exocytosis to a greater degree than P/Q-type channels in this culture preparation, consistent with previous results.[13] Presynaptic R-type and L-type channels contribute minimally.
- Isoflurane inhibits exocytosis in hippocampal neurons to a greater extent and more consistently following block of N-type calcium channels compared to block of P/Q channels.
- Boutons may contain subpopulations of P/Q-type channels with differing sensitivities to ω-aga and isoflurane.

Future Directions:

- Future work will address subtype-specific inhibition of Ca^{2+} influx by isoflurane and the relative contributions of Na^+ and Ca^{2+} channels in mediating inhibition of neurotransmitter release.

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