HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2024 January 08.

Published in final edited form as:

Neuropharmacology. 2023 December 01; 240: 109705. doi:10.1016/j.neuropharm.2023.109705.

Volatile anesthetics inhibit presynaptic cGMP signaling to depress presynaptic excitability in rat hippocampal neurons

Iris Speigel[1], **Kishan Patel**[1], **Vanessa Osman**[2], **Hugh C Hemmings Jr**[1,2,*]

[1.]Department of Anesthesiology, Weill Cornell Medicine, New York, NY, 10065, USA

[2.]Department of Pharmacology, Weill Cornell Medicine, New York, NY, 10065, USA

Abstract

Volatile anesthetics alter presynaptic function through effects on Ca^{2+} influx and neurotransmitter release. These actions are proposed to play important roles in their pleiotropic neurophysiological effects including immobility, unconsciousness and amnesia. Nitric oxide and cyclic guanosine monophosphate (NO/cGMP) signaling has been implicated in presynaptic mechanisms, and disruption of NO/cGMP signaling has been shown to alter sensitivity to volatile anesthetics *in vivo*. We investigated volatile anesthetic actions NO/cGMP signaling in relation to presynaptic function in cultured rat hippocampal neurons using pharmacological tools and genetically encoded biosensors and sequestering probes of cGMP levels. Using the fluorescent cGMP biosensor cGull, we found that electrical stimulation-evoked NMDA-type glutamate receptor-independent presynaptic cGMP transients were inhibited 33.2% by isoflurane (0.51 mM) and 26.4% by sevoflurane (0.57 mM) ($p<0.0001$) compared to control stimulation without anesthetic. Stimulation-evoked cGMP transients were blocked by the nonselective inhibitor of nitric oxide synthase N-ω-nitro-L-arginine, but not by the selective neuronal nitric oxide synthase inhibitor N5-(1-imino-3-butenyl)-L-ornithine. Isoflurane and sevoflurane inhibition of stimulation-evoked increases in presynaptic Ca^{2+} concentration, measured with synaptophysin-GCaMP6f, and of synaptic vesicle exocytosis, measured with synaptophysin-pHlourin, was attenuated in neurons expressing the cGMP scavenger protein sponge (inhibition of exocytosis reduced by 54% for isoflurane and by 53% for sevoflurane). The anesthetic-induced reduction in presynaptic excitability was partially occluded by inhibition of HCN channels, a cGMP-modulated excitatory ion channel that can facilitate glutamate release. We propose that volatile anesthetics depress presynaptic cGMP signaling and downstream effectors like HCN channels that are essential to

[*]**Correspondence.** hchemmi@med.cornell.edu.

Author contributions

IAS and HCH conceptualized and designed the experiments. IAS, KP and VO conducted the experiments. IAS and HCH wrote the manuscript. IAS, VO, KP and HCH participated in manuscript review and editing.

Declaration of interest

HCH is editor-in-chief of the *British Journal of Anaesthesia,* and has received research funding from Instrumentation Laboratory and consulting fees from Elsevier unrelated to this work. IAS, KP and VO have no conflicts to declare.

Credit Author Statement

IAS and HCH conceptualized and designed the experiments. IAS, KP, and VO conducted the experiments. IAS, KP, and HCH wrote the manuscript. IAS, KP, VO, and HCH participated in review and editing.

Declaration of Interests Statement

Funding for the project was provided by US National Institutes of Health Grant R01 GM058055–21 (to HCH) and F31 GM133115 (to VO). IAS, KP, and VO have no competing interests to declare. HCH is editor-in-chief of the *British Journal of Anaesthesia* and has received consulting fees from Elsevier unrelated to this work.

presynaptic function and excitability. These findings identify novel mechanisms by which volatile anesthetics depress synaptic transmission via second messenger signaling involving the NO/cGMP pathway in hippocampal neurons.

Keywords

cyclic GMP; HCN channel; isoflurane; mechanisms of anesthesia; neurotransmitter release; nitric oxide; sevoflurane; synaptic transmission

Introduction

Presynaptic Ca^{2+} entry through voltage-gated calcium channels (Ca_v) is tightly coupled to fast depolarization-evoked neurotransmitter release[1]. This process is sensitive to general anesthetics, in particular the volatile anesthetics, which reversibly inhibit depolarization-evoked neurotransmitter release through neurotransmitter and cell subtype selective presynaptic mechanisms[2-4]. Various general anesthetics differentially affect presynaptic Ca^{2+} signaling via effects on ion channels or synaptic vesicle fusion mechanisms to inhibit exocytosis. Candidate presynaptic molecular targets for volatile anesthetics include voltage-gated Na^+, Ca^+, and K^+ channels[5-7], SNARE exocytotic proteins[8, 9] and presynaptic mitochondrial energy production[10]. Despite the identification of multiple presynaptic targets, none of these specific molecular sites of action fully explains presynaptic anesthetic effects on neurotransmitter release.

Nitric oxide (NO) is a gaseous messenger generated from L-arginine by nitric oxide synthase (NOS) in a Ca^{2+}-dependent manner [11]. The NO signaling system is widely expressed across many tissues and phyla, and is physiologically pleiotropic, with prominent roles in the nervous system that include several forms of synaptic modulation. The primary downstream effector of NO is soluble guanylyl cyclase (sGC), which cyclizes GTP to form the second messenger cyclic GMP (cGMP), which itself acts on multiple downstream targets including direct effects on ion channels and activation of downstream signaling proteins including cGMP-dependent protein kinase (PKG) [12]. Several lines of evidence indicate effects of volatile anesthetics on NO signaling,[13] although conflicting results have been reported. [14, 15]

Biochemical, pharmacological and genetic studies have implicated NO/cGMP signaling in the mechanisms of volatile anesthetic action.[3] Halothane, isoflurane, and sevoflurane all increase cerebral NO[16-18]and cGMP[19-21] concentrations. Consistent with these observations, genetic knockout of the NO target sGC-1α in mice reduced sensitivity to sevoflurane anesthesia as measured by both loss and return of righting reflex, suggesting that anesthetic-activated increases in sGC1α-derived cGMP promote loss of righting reflex, a surrogate endpoint for loss of consciousness in rodents[21]. However, anesthetic-induced reductions in NO and cGMP levels have also been reported[22], and pharmacological studies have shown that inhibition of NOS or sGC increases sensitivity to loss of righting reflex in rodents,[15, 23, 24] suggesting that reductions in NO signaling promote anesthetic-induced hypnosis. While acute administration of NOS inhibitors increase sensitivity to volatile anesthetics in most studies,[5,6] others have found no effect on volatile anesthetic sensitivity

(Adachi 1994, 1995), and knockout of neuronal NOS (nNOS) in mice did not alter sensitivity to isoflurane anesthesia.[4] Some of these disparate findings might be due to off-target effects of the inhibitors used, pleiotropic effects of manipulating nNOS signaling and/or compensatory or off-target effects that complicate interpretation of global gene knockout models. Thus, it is unclear whether changes in neuronal NO/cGMP signaling are critical to the production of anesthesia or are incidental to anesthetic effects in animal models.

While considerable evidence supports a role for NO signaling in mediating behavioral and biochemical responses to volatile anesthetics, the specific downstream neurophysiological targets of NO/cGMP signaling in the nervous system remain unsolved. Studies in isolated intact neurons have also yielded conflicting findings. For example, in cultured rat cortical neurons, isoflurane has been reported to inhibit[25, 26] or enhance[25, 26] glutamate agonist-induced increases in cGMP. Thus, the relationship between volatile anesthetic actions and cGMP production remains unclear. A technical barrier to elucidating NO/cGMP signaling pathways is that intracellular cGMP dynamics have been difficult to measure in intact cells. Previous studies of cGMP signaling have relied on static biochemical measurements of net changes in cell lysates at single timepoints, so anesthetic actions on time-resolved presynaptic cGMP dynamics are unknown.

While studies have implicated cGMP as a regulator of neurotransmitter release[12], none have measured changes in presynaptic cGMP levels directly. Given that volatile anesthetics inhibit neurotransmitter release via several mechanisms, we hypothesized that alterations in cGMP contribute to volatile anesthetic actions on presynaptic function. To investigate the potential role of NO/cGMP signaling in volatile anesthetic effects on neurotransmitter release, we took advantage of recently developed genetically encoded approaches to measure and manipulate levels of cGMP and its presynaptic actions in real time. We used a fluorescent cGMP biosensor,[27] a cGMP scavenger protein[28] and functional imaging of changes in presynaptic Ca^{2+} influx and synaptic vesicle exocytosis to probe cGMP actions and the effects of volatile anesthetics in an isolated hippocampal neuron model.[29] The current study was motivated by previous evidence that changes in NO/cGMP signaling alter anesthetic potency, and by the currently limited understanding of presynaptic cGMP mechanisms in particular, which we hypothesize contribute to the well-characterized presynaptic effects of volatile anesthetics.

Methods

All experimental procedures using animals were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee and conformed to National Institutes of Health (Bethesda, MD, USA) Guidelines for the Care and Use of Animals and adhered to ARRIVE guidelines where applicable.

Primary neuron culture and transfection:

Both hippocampi were dissected from postnatal rats (0–1 days old, both sexes). Cells were dissociated and plated onto coverslips as described[30]. Transfection was performed on day 6 or 7 *in vitro* (DIV6–7) using Ca^{2+} phosphate-mediated gene transfer to achieve sparce

transfection and allow selection of non-overlapping transfected neurons. Live-cell imaging was performed between DIV16–20. For each experiment, neurons were derived from at least three separate culture preparations.

Plasmids: Green cGull was a gift from Tetsuya Kitaguchi (Tokyo Institute of Technology, Kanagawa, Japan; Addgene plasmid # 86867). SponGee was a gift from Xavier Nicol (French National Centre for Scientific Research, Paris, France; pCX-SponGee-mRFP, Addgene plasmid # 134775). Synaptophysin-pHluorin (syn-pH) was a gift from Stephen Heinemann and Yongling Zhu (Northwestern University, Evanston, IL, USA; pcDNA3-SypHluorin 2x, Addgene plasmid # 37004). VAMP-mCherry and synaptophysin-GCaMP6f were gifts from Timothy Ryan (Weill Cornell Medicine, New York, NY, USA)[31].

Live-cell Imaging: Experiments were performed on a ZEISS Axio Observer Z1 widefield fluorescence microscope with filter cubes and LEDs for eGFP and RFP detection (Colibri 7 LED illumination modules 475 nm and 555 nm, with 38HE and 90HE filter cubes (Zeiss, Oberkochen, Germany) using an Andor iXon1 EMCCD camera (Belfast, Ireland). Synaptic vesicle (SV) exocytosis imaging was acquired at 10 Hz, Ca^{2+} levels at 20 Hz, and cGMP levels at 40 Hz. Coverslips were mounted in a closed field stimulation perfusion chamber with a total volume of 263 µl. Solutions were maintained at 37.0 ± 0.2°C by an in-line heater and imaging chamber heater, and were perfused at 1 ml/min using a custom system and a multi-barrel manifold (Warner Instruments, Hamden, CT, USA). The standard buffer was Tyrode's solution (119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES buffered to pH 7.4, 30 mM glucose) containing 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 µM D,L-2amino-5-phosphonovaleric acid (AP5) (both from Tocris, Bristol, UK) to block recurrent glutamatergic signaling.

Neurons expressing cGull were identified by resting green fluorescence, and co-labelled presynaptic boutons were identified by co-transfected VAMP-mCherry red fluorescence. Neurons co-expressing syn-pH or syn-GCaMP6f were identified by co-transfected mCherry- or mRFP-conjugated sponGee.

Action potentials (APs) were stimulated by trains of 20 stimuli generated by an electrical field pulse generator (Master-9, A.M.P.I., Jersalem, Israel) at 20 Hz, with a stimulus isolator (Model A385, World Precision Instruments, Sarasota, FL, USA) using platinum/iridium bath electrodes built into the imaging chamber yielding a field of 10 V/cm. A maximum of one imaging experiment was acquired per coverslip.

Volatile anesthetic delivery: An experimental solution of ~0.52 mM isoflurane dissolved in Tyrode's buffer was prepared daily from a saturated stock solution of 13 mM; this corresponds to ~1.5 MAC (minimum alveolar concentration; the EC_{50} for anesthesia defined as immobilization to a pain stimulus) in rat[32]. An experimental solution of ~0.72 mM sevoflurane was prepared from a saturated stock solution of 6 mM; this corresponds to ~1.5 MAC[32]. Isoflurane or sevoflurane solutions were focally perfused from gas-tight glass syringes into the imaging chamber for 5 min before imaging to allow equilibration. At the conclusion of each experiment, a perfusate sample was taken from the chamber for determination of delivered concentration by gas chromatography (Shimadzu GC-2010 Plus,

Kyoto, Japan) with external standard calibration. The reported values of 0.51 ± 0.09 mM isoflurane and 0.57 ± 0.13 mM sevoflurane reflect averaged measurements from all bath samples collected.

Drugs: Isobutylmethylxanthine (IBMX, 100 μM) was from Thermo Scientific (PHZ1124, Waltham, MA, USA). N-ω-nitro-L-arginine (L-NNA, 100 μM), and ivabradine hydrochloride (30 μM) were from Sigma-Aldrich (B1381, N5501, and SML0281, St. Louis, MO, USA). Spermine-NONOate (50 μM) was from Cayman Chemical (82150, Anne Arbor, MI, USA). N5-(1-Imino-3-butenyl)-L-ornithine (L-VNIO, 100 nM) was from Enzo Life Science (ALX-270–216-M005, Farmington, NY, USA). KT-5823 (500 nm) was from Tocris Bioscience (1289, Bristol, UK). ZD7288 (10 μM) was from Tocris Bioscience (1000, Bristol, UK). IBMX, ivabradine, KT-5823, and ZD7288 were perfused for 15 min prior to recordings. L-VNIO and L-NNA were perfused for 20 min prior to recordings[33].

Image and statistical analysis: Live-cell imaging recordings were analyzed in ImageJ (rsb.info.nih.gov/ij) using the plugin TimeSeries Analyzer (rsb.info.nih.gov/ij/plugins/timeseries.html) to measure fluorescence over time. For presynaptic Ca^{2+} measurements, fluorescence was analyzed within 2 μm diameter regions of interest (ROIs), each containing a visually identifiable synaptic bouton. ROIs were selected based on their peak response and latency to control stimulation in Tyrode's buffer. Time-series data were exported into MATLAB for further analysis with custom scripts. For each ROI, fluorescence was calculated as $\Delta F/F_0 = ((F-F_0)/ F_0)$. Baseline fluorescence (F_0) was averaged from the 10 frames immediately prior to stimulus onset, and peak fluorescence (F) was averaged from the three consecutive frames with the highest values following stimulation. ROIs with signal-to-noise ratio (SNR)>4 (where SNR= $\Delta F/\sigma$, and σ is the standard deviation of F_0) were pooled to create an ensemble single-coverslip average. Coverslips with washout (recovery) responses < 60% of control, indicating deteriorating cellular condition, were excluded from analysis. Values are shown as mean ± standard deviation (SD). ANOVA with Tukey's *post hoc* test, Student's unpaired t test, and 95% confidence intervals (CI) for differences between mean values were used to determine statistical significance, defined as $P<0.05$. Data were tested for normality using the Shapiro-Wilk test.

Results

Electrical stimulation increases presynaptic cGMP concentration

Presynaptic cGMP dynamics in response to electrical stimulation were determined using live-cell imaging of the cGMP biosensor green cGull. Green cGull is a chimeric protein of the fluorescent protein citrine and the cGMP-binding protein phosphodiesterase 5α that increases in fluorescence in response to micromolar cyclic GMP with 1000-fold selectivity over cyclic AMP.[27] Cultured rat hippocampal neurons were co-transfected with cGull and VAMP-mCherry, a chimera of red fluorescent protein and the synaptic vesicle protein VAMP (vesicle-associated membrane protein) as a marker of presynaptic boutons. Figure 1A shows basal and stimulation-evoked cGull fluorescence compared to VAMP-mCherry fluorescence. Activity-dependent changes in cGMP levels were identified by electrical stimulation, using buffer supplemented with the AMPA and NMDA receptor

antagonists CNQX and APV, respectively, to prevent indirect excitation and allow isolation of direct single neuron responses[34, 35].Fluorescence increased in all cellular compartments in response to electrical stimulation including in presynaptic boutons labelled by VAMP-mCherry (Figure 1B).

The presynaptic cGMP dynamics indicated by cGull showed a rapid increase with a rapid decay (Figure 1C). This presynaptic time course is consistent with the rapid sub-second kinetics of caged NO-stimulated cGMP generation reported in acutely isolated rat cerebellar cells[36]. Somatic cGMP did not change as markedly during electrical stimulation compared to presynaptic cGMP (Figure 1D). Presynaptic cGMP transients showed prolonged decay in the presence of the phosphodiesterase-5 inhibitor IBMX, which inhibits cGMP hydrolysis without affecting cGull[27], consistent with reduced cGMP hydrolysis (Figure 1E).

NOS is activated by elevated Ca^{2+}, and several forms of NOS-mediated synaptic plasticity require Ca^{2+} influx through NMDA-type glutamate receptors,[12] which are blocked by AP5 in our experiments. NMDA receptor-independent presynaptic cGMP generation has been implicated in the regulation of HCN1 currents but not measured directly[33]. Presynaptic cGull transient peak amplitude directly scaled with extracellular Ca^{2+} concentration in the presence of an NMDA receptor blocker (Figure 1F), consistent with an NMDA receptor-independent source of intracellular Ca^{2+} that activates NOS, NO synthesis, and sGC leading to cGMP synthesis.

The contributions of the endothelial and neuronal subtypes of NOS (eNOS and nNOS) to presynaptic cGMP transients were assessed using selective inhibitors (Figure 1G). The cGMP transients were insensitive to the nNOS selective inhibitor L-VNIO, but were completely suppressed by the dual eNOS and nNOS inhibitor L-NNA. Serial application of L-VNIO and L-NNA to the same cell showed that L-VNIO-resistant cGMP transients were completely blocked by L-NNA (Supplemental Figure 1).

Volatile anesthetics depress cGMP transients

The effects of the volatile anesthetics isoflurane and sevoflurane on electrically-evoked cGMP transients were examined using a pulse of 20 stimuli at 20 Hz with CNQX and AP5 included in the perfusate to isolate single cell presynaptic changes in the absence recurrent excitation (Figure 2A). Representative traces of presynaptic cGMP transients in the presence of isoflurane (0.51 ± 0.09 mM) or sevoflurane (0.57 ± 0.13 mM), equivalent to 1.5 and 1.2 MAC, respectively, are shown in Figure 2B. Both isoflurane and sevoflurane inhibited presynaptic increases in cGMP (Figure 2C). Peak cGMP transient amplitude was depressed by isoflurane (−33.2%; $p<0.0001$) or sevoflurane (−26.4%; $p<0.0001$) compared to control stimulation without anesthetic.

Sequestration of intracellular cGMP reduces isoflurane inhibition of presynaptic function

Since several cGMP effectors promote glutamatergic neurotransmission[12], we hypothesized that reduced cGMP signaling by volatile anesthetics leads to impaired synaptic vesicle (SV) exocytosis. We analyzed exocytosis and presynaptic Ca^{2+} concentration as measures of presynaptic function and excitability that are accessible to live-cell imaging of hippocampal neurons. We used the genetically encoded cGMP scavenger protein sponGee in combination

with fluorescent biosensors for presynaptic Ca^{2+} (syn-GCaMP6f) and SV exocytosis (syn-pH) (Figure 3A). sponGee is a recently developed chimeric protein of bovine cGMP-dependent protein kinase (PKG) 1α and 1β binding sites that has high affinity for cGMP but lacks the kinase domains for downstream effector activation[28]. Co-transfection of neurons with both cGull and sponGee (Figure 3B) abolished electrically-evoked presynaptic cGMP transients in all neurons observed (n=3), demonstrating that sponGee effectively scavenges electrically-evoked increases in presynaptic cGMP (Figure 3B-C).

In order to investigate the functional consequences of changes in presynaptic cGMP, we measured volatile anesthetic effects on Ca^{2+} influx and synaptic vesicle exocytosis using the paradigm shown in Figure 2A, with 20 AP at 20 Hz stimulation for imaging syn-GCaMP6f and 100 AP at 10 Hz stimulation for imaging syn-pH. Isoflurane reversibly inhibited synaptic vesicle exocytosis (Figure 3D) and Ca^{2+} entry (Figure 3E) evoked by electrical stimulation. Isoflurane inhibition of synaptic vesicle exocytosis was attenuated by co-expression of the cGMP scavenger sponGee (from 30% to 14% inhibition; p=0.0004, Figure 3F), representing a 53% contribution of cGMP signaling to the inhibitory effect. Isoflurane inhibition of presynaptic Ca^{2+} influx was also attenuated by co-expression of sponGee (from 22% to 15%; p=0.0016, Figure 3G), representing a 32% contribution of cGMP signaling to the inhibitory effect. Sevoflurane inhibition of Ca^{2+} influx was also attenuated by sponge co-expression (from 22% to 10% inhibition; p=0.018), representing a 54% contribution of cGMP signaling to the inhibitory effect. Because sponGee affected both synaptic vesicle exocytosis and Ca^{2+} influx, we attribute the reduced exocytosis to upstream effects on presynaptic excitability and Ca^{2+} entry rather than direct cGMP actions on vesicular exocytosis mechanisms.

Isoflurane effect on presynaptic Ca^{2+} is PKG-independent

Multiple downstream targets of cGMP can potentially regulate presynaptic excitability. Cyclic nucleotide-gated (CNG) channels and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are directly modulated by cGMP, which increases channel activation[12]. Other channels are regulated indirectly by cGMP-dependent protein kinase (PKG), including BK potassium channels and L-type voltage-gated Ca^{2+} channels ($Ca_v2.1$). If PKG mediates cGMP effects on presynaptic excitability, a PKG inhibitor should recapitulate the effect of cGMP sequestration by sponGee on presynaptic Ca^{2+} influx. We determined the effects of the membrane permeable PKG inhibitor KT-5823 on anesthetic inhibition of presynaptic Ca^{2+} influx (Figure 4A). KT-5823 had no effect on isoflurane inhibition of presynaptic Ca^{2+} (4% inhibition; p=0.801, Figure 4C), which suggests that PKG does not contribute to isoflurane inhibition of stimulation-evoked presynaptic Ca^{2+} influx.

Role of HCN channels in isoflurane effect on presynaptic Ca^{2+} influx

Having excluded a PKG-mediated mechanism in the effects of cGMP on the presynaptic actions of isoflurane, we analyzed the role of HCN channels, another direct target of cGMP. HCN channels modulate presynaptic excitability and activity-dependent presynaptic Ca^{2+} influx by conducting a depolarizing mixed cation current that increases glutamate release probability in hippocampal neurons. [22] Gating of HCN channels by cyclic nucleotides

including cGMP shifts their voltage dependence of activation in the hyperpolarized direction to allow channel opening at more negative potentials. We hypothesized that volatile anesthetic depression cGMP synthesis and cGMP signaling would blunt HCN-mediated enhancement of presynaptic excitability. Consistent with this hypothesis, the general HCN inhibitor ivabradine diminished isoflurane inhibition of presynaptic Ca^{2+} influx (from 22% to13% inhibition; p=0.0035, Figure 4B,C), which supports a contribution of HCN in regulating presynaptic excitability and Ca^{2+} influx in a cGMP-dependent manner that partially occludes the isoflurane effect by HCN inhibition. Ivabradine alone inhibited stimulation-evoked Ca^{2+} entry by ~25%, indicating a role for HCN in regulating presynaptic excitability, and indicating that the partial occlusion of the isoflurane effect overrode the direct inhibitory effect of ivabradine alone (Supplemental Figure 2). The specificity of this effect was confirmed with ZD7288, a structurally distinct HCN channel inhibitor, which like ivabradine inhibits multiple HCN channel subtypes[37]. ZD7288 also partially occluded the inhibitory effect of isoflurane on stimulation evoked Ca^{2+} entry (Supplemental Figure 4). ZD7288 alone inhibited stimulation evoked Ca^{2+} entry by ~17%, further supporting a role for HCN in regulating presynaptic excitability (Supplementary Figure 3).

Discussion

We found that in rat hippocampal neurons, the volatile anesthetics isoflurane and sevoflurane depress stimulus-evoked presynaptic cGMP signaling to inhibit presynaptic Ca^{2+} influx and synaptic vesicle exocytosis. The mechanism involves eNOS-generated NO signaling with a potential role for cGMP-mediated modulation of HCN channels, a downstream cGMP effector that regulates presynaptic excitability independent of PKG, mediated by anesthetic-induced inhibition of simulation-evoked generation of cGMP.

Depolarization-evoked stimulation of presynaptic cGMP transients

We used the novel genetically encoded cGMP indicator cGull to characterize presynaptic cGMP dynamics during electrical stimulation-evoked synaptic vesicle exocytosis from cultured rat hippocampal neurons. Live-cell imaging of second messenger dynamics provides real-time compartment-specific resolution of the balance of cGMP synthesis and degradation with superior temporal and spatial resolution compared to previous static biochemical methods[38].

We identified remarkably rapid cGMP transients in presynaptic boutons occurring on the time scale of exocytosis. These occurred despite blockade of NMDA receptors, a well-known mediator of Ca^{2+}-dependent NO-sGC activation coupled to nNOS activation. The external Ca^{2+} dependence of the cGMP transient amplitude implicates another Ca^{2+} entry pathway supporting NO/sGC/cGMP signaling, such as voltage-gated Ca^{2+} channels that are known to be coupled to synaptic vesicle exocytosis, or Ca^{2+} release form intracellular store (Osman et al., 2023, submitted). Further studies are necessary to identify the source of the increase in presynaptic Ca^{2+} concentration.

Our data using NOS inhibitors suggest that the NMDA receptor-independent increase in cGMP is derived from NO synthesis by eNOS, not by nNOS. This is consistent with electrophysiological experiments showing that basal cGMP modulates glutamatergic

signaling at a presynaptic site of action in an NMDA receptor-independent but eNOS-dependent manner[33]. As eNOS has been identified in dendritic spines of primary cortical neurons[39], one possibility is that dendritic NO generated by eNOS diffuses retrogradely to activate presynaptic sGC. In contrast, nNOS, which is abundantly expressed in select interneurons, does not appear to participate, despite the presence of interneurons in our primary cultures (Speigel et al interneuron paper). The nNOS-mediated form of NO signaling is NMDA receptor-dependent, as nNOS and NMDA receptor activation are spatially coupled by PSD95 tethering[40].

Reduction of presynaptic cGMP transients blunts anesthetic actions

The presynaptic effects of isoflurane and sevoflurane on Ca^{2+} influx and synaptic vesicle exocytosis were down-regulated by sequestering cGMP and thereby inhibiting downstream cGMP signaling, which was achieved experimentally by co-expression of the cGMP-binding protein sponGee[28]. Previous studies of anesthetic effects on NO/cGMP signaling have shown conflicting results, with both inhibition and facilitation of cGMP generation. This likely arises in part from differences in experimental paradigms to evoke neuronal activity and in methods to quantify cGMP, which are usually static and subject to limitations in time and spatial resolution, as well as degradation by phosphodiesterases.

Our observations are consistent with volatile anesthetic inhibition of cGMP in cultured cerebral neurons following NMDA application, even in the presence of the NMDA receptor blocker AP5[41]. In contrast, a recent study reported that sevoflurane increased cGMP in the forebrain of wild-type mice[21]. However, we specifically measured changes in presynaptic rather than whole brain cGMP levels, which has not been reported previously. Further work using the greater spatial and temporal resolution of the optogenetic methods described here is needed to resolve these apparently conflicting results, including imaging of somatic cGMP dynamics in live cells or *in vivo*.

Role of cGMP in depression of presynaptic excitability and neurotransmitter release

Previous studies have not identified the downstream consequences of volatile anesthetic inhibition of cGMP synthesis on neuronal function. Two findings motivated us to investigate the role of cGMP in presynaptic anesthetic actions: 1) sensitivity to sevoflurane anesthesia is decreased in mice with a congenital deletion of sGC-1α, the isoform associated with presynaptic terminals,[21] and 2) several cGMP effectors are known increase glutamate release[12, 42].

Neurotransmitter release by synaptic vesicle exocytosis is steeply dependent on presynaptic Ca^{2+} influx through voltage-gated Ca^{2+} channels, itself determined by depolarization of the presynaptic terminal[29]. Isoflurane inhibition of presynaptic Ca^{2+} influx and synaptic vesicle exocytosis could involve action potential depression[43] and diminished Ca^{2+} influx[3]. Previous work has identified presynaptic ion channel targets for volatile anesthetics including voltage-gated Na^{+} channels[30] and Ca^{2+} channels[44], and indirect effects of volatile anesthetics on second messenger signaling such as by α_{2A}-adrenergic receptors[45]. Our findings provide preliminary evidence that cyclic nucleotide-activated (HCN) channels act as

an indirect target, and are downregulated by volatile anesthetic actions to reduce presynaptic cGMP transients, thereby reducing presynaptic excitability.

HCN channels as presynaptic effectors for anesthetic effects on cGMP

Our preliminary pharmacologic findings suggest that cGMP/HCN signaling partially mediates volatile anesthetic actions on presynaptic function in hippocampal neurons. Previous studies have implicated HCN channels in anesthetic mechanisms in thalamic[46], thalamocortical[47], and forebrain neurons[48]. HCN channels can be gated by either cAMP or cGMP; our study is the first to link volatile anesthetic depression of cGMP production with reduced HCN function. This effect might have been masked previously by direct volatile anesthetic actions on channel function[49]. Sequestration of cGMP supports a cGMP-dependent mechanism, however our studies do not exclude parallel direct channel or cAMP-signaling effects in addition to the cGMP-mediated mechanism described here as modulators of HCN during volatile anesthetic exposure.

Our results are consistent with previous findings that presynaptic sGC1-mediated increases in cGMP facilitate glutamate release involving HCN channel activation in hippocampal glutamatergic neurons to increase presynaptic Ca^{2+} influx. [33] Our evidence for a role of HCN channels is indirect and depends on the limited specificity of the available channel inhibitors. Direct measurement of presynaptic HCN currents is necessary to link reduced cGMP production to reduced channel function, which is technically challenging due to the small size of presynaptic boutons. Additional studies are also necessary to identify the specific HCN channel subtype(s) involved[50, 51].

Within the hippocampus, HCN channel expression is best characterized in pyramidal neuron dendrites along a gradient with greatest enrichment in distal processes[52]. Axonal expression is not as well characterized, although HCN1 has been identified in axonal and presynaptic compartments of entorhinal neurons[53, 54]. Our experiments do not have the resolution to localize HCN1 or any particular HCN subtype in these cells to the active zone but do implicate their functional role in regulating presynaptic excitability and SV exocytosis.

Limitations and future experiments

The live-cell imaging approach has both strengths and limitations. While live-cell imaging of isolated cultured neurons enables optogenetic approaches to assessing presynaptic function at high temporal and spatial resolution, the necessary cell isolation disrupts the normal three-dimensional architecture of the hippocampus. Functional analysis is limited because cultures of dissociated neurons might not recapitulate the cellular properties and connectivity observed *in vivo*. Similarly, our focus on presynaptic function might exclude other mechanisms acting in intact neuronal circuits, including glial contributions. The cell culture methods used are also limited to a single brain region, and the neurotransmitter phenotype and neuron subtypes of the imaged cells are not identified, obscuring possible regional and cell type-specific heterogeneity in physiology and pharmacology. Possible sex-specific differences are also not resolved by our approach using mixed neonatal tissue harvest from both sexes.

Our proposed mechanism linking volatile anesthetic reductions in presynaptic cGMP levels, presynaptic excitability, HCN-mediated Ca^{2+} influx and synaptic vesicle exocytosis are limited by the absence of direct measurements of HCN channel function. We focused on presynaptic HCN channels because of their connections to presynaptic cGMP and Ca^{2+} dynamics related to exocytosis. However, this excludes the dendritic HCN channels, which likely contribute to synaptic excitability *in vivo*[30, 52, 55]. Further delineating the relationship between NO/cGMP signaling and anesthetic actions is challenging because of the complexity of overlapping signaling pathways within and between neurons. Several promising biosensors for NO might prove valuable in this regard [56, 57]. All our imaging experiments were conducted in the presence of glutamate receptor blockers to prevent recurrent excitation by field electrical stimulation, which precludes determination of the contributions of NMDA receptor and non-NMDA receptor involvement in Ca^{2+} entry to the presynaptic anesthetic actions observed.

Conclusions

We employed recently developed optogenetic fluorescent biosensors to study volatile anesthetic effects on cGMP signaling in isolated rat hippocampal neurons to describe subcellular and single-cell resolution of cGMP transients during neuronal activity and the effects of volatile anesthetics for the first time. Volatile anesthetics reduced presynaptic cGMP signaling to inhibit Ca^{2+} influx and synaptic vesicle exocytosis. Our novel characterization of presynaptic cGMP dynamics during neuronal activity will be valuable in understanding their roles in both presynaptic function and the presynaptic effects of anesthetics and other drugs in hippocampal and other neurons. The observed cGMP transients were independent of NMDA receptor activation and required eNOS. Building on these fundamental descriptions of presynaptic cGMP dynamics, we observed a novel role for cGMP and implicate HCN channels as mediators of volatile anesthetic modulation of presynaptic function. These findings are the first to identify robust presynaptic effects of volatile anesthetics on NO/cGMP signaling and to show that NO/cGMP signaling is involved in Ca^{2+} regulation and synaptic vesicle exocytosis such that anesthetic inhibition of cGMP signaling contributes to the inhibitory effects of anesthetics on neurotransmitter release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Hemmings laboratory for constructive comments.

Funding

US National Institutes of Health grants R01 GM058055-21 (to HCH) and 5F31 GM133115 (to VO).

References

1. Kim SH, Ryan TA. Balance of Calcineurin Aα and CDK5 Activities Sets Release Probability at Nerve Terminals. The Journal of Neuroscience 2013; 33: 8937–50 [PubMed: 23699505]
2. Hemmings HC Jr., Akabas MH, Goldstein PA, Trudell JR, Orser BA, Harrison NL. Emerging molecular mechanisms of general anesthetic action. Trends Pharmacol Sci 2005; 26: 503–10 [PubMed: 16126282]
3. Baumgart JP, Zhou ZY, Hara M, et al. Isoflurane inhibits synaptic vesicle exocytosis through reduced Ca2+ influx, not Ca2+-exocytosis coupling. Proc Natl Acad Sci U S A 2015; 112: 11959–64 [PubMed: 26351670]
4. MacIver MB. Anesthetic agent-specific effects on synaptic inhibition. Anesth Analg 2014; 119: 558–69 [PubMed: 24977633]
5. Study RE. Isoflurane inhibits multiple voltage-gated calcium currents in hippocampal pyramidal neurons. Anesthesiology 1994; 81: 104–16 [PubMed: 8042778]
6. Herold KF, Hemmings HC Jr. Sodium channels as targets for volatile anesthetics. Front Pharmacol 2012; 3: 50 [PubMed: 22479247]
7. Franks NP, Honoré E. The TREK K2P channels and their role in general anaesthesia and neuroprotection. Trends Pharmacol Sci 2004; 25: 601–8 [PubMed: 15491783]
8. Herring BE, Xie Z, Marks J, Fox AP. Isoflurane inhibits the neurotransmitter release machinery. J Neurophysiol 2009; 102: 1265–73 [PubMed: 19515956]
9. Nagele P, Mendel JB, Placzek WJ, Scott BA, D'Avignon DA, Crowder CM. Volatile anesthetics bind rat synaptic snare proteins. Anesthesiology 2005; 103: 768–78 [PubMed: 16192769]
10. Jung S, Zimin PI, Woods CB, et al. Isoflurane inhibition of endocytosis is an anesthetic mechanism of action. Curr Biol 2022; 32: 3016–32.e3 [PubMed: 35688155]
11. Vanhoutte PM, Zhao Y, Xu A, Leung SWS. Thirty Years of Saying NO. Circulation Research 2016; 119: 375–96 [PubMed: 27390338]
12. Hardingham N, Dachtler J, Fox K. The role of nitric oxide in pre-synaptic plasticity and homeostasis. Front Cell Neurosci 2013; 7: 190 [PubMed: 24198758]
13. Toda N, Toda H, Hatano Y. Nitric oxide: involvement in the effects of anesthetic agents. Anesthesiology 2007; 107: 822–42 [PubMed: 18073558]
14. Ichinose F, Huang PL, Zapol WM. Effects of targeted neuronal nitric oxide synthase gene disruption and nitroG-L-arginine methylester on the threshold for isoflurane anesthesia. Anesthesiology 1995; 83: 101–8 [PubMed: 7541615]
15. Engelhardt T, Lowe PR, Galley HF, Webster NR. Inhibition of neuronal nitric oxide synthase reduces isoflurane MAC and motor activity even in nNOS knockout mice. Br J Anaesth 2006; 96: 361–6 16 [PubMed: 16431879]
16. Sjakste N, Baumane L, Meirena D, Lauberte L, Dzintare M, Kalvins I. Drastic increase in nitric oxide content in rat brain under halothane anesthesia revealed by EPR method. Biochem Pharmacol 1999; 58: 1955–9 [PubMed: 10591150]
17. Berthiaume Y, Sapijaszko M, MacKenzie J, Walsh MP. Protein kinase C activation does not stimulate lung liquid clearance in anesthetized sheep. Am Rev Respir Dis 1991; 144: 1085–90 [PubMed: 1952436]
18. Sjakste N, Sjakste J, Boucher JL, et al. Putative role of nitric oxide synthase isoforms in the changes of nitric oxide concentration in rat brain cortex and cerebellum following sevoflurane and isoflurane anaesthesia. Eur J Pharmacol 2005; 513: 193–205 [PubMed: 15862801]
19. Nahrwold ML, Lust WD, Passonneau JV. Halothane-induced alterations of cyclic nucleotide concentrations in three regions of the mouse nervous system. Anesthesiology 1977; 47: 423–7 [PubMed: 20825]
20. Divakaran P, Rigor BM, Wiggins RC. Brain cyclic nucleotide responses to anesthesia with halothane delivered in air or purified oxygen. J Neurochem 1980; 35: 514–6 [PubMed: 6256490]
21. Nagasaka Y, Wepler M, Thoonen R, et al. Sensitivity to Sevoflurane anesthesia is decreased in mice with a congenital deletion of Guanylyl Cyclase-1 alpha. BMC Anesthesiol 2017; 17: 76 [PubMed: 28615047]

22. Galley HF, Le Cras AE, Logan SD, Webster NR. Differential nitric oxide synthase activity, cofactor availability and cGMP accumulation in the central nervous system during anaesthesia. Br J Anaesth 2001; 86: 388–94 [PubMed: 11573530]
23. Johns RA, Moscicki JC, DiFazio CA. Nitric oxide synthase inhibitor dose-dependently and reversibly reduces the threshold for halothane anesthesia. A role for nitric oxide in mediating consciousness? Anesthesiology 1992; 77: 779–84 [PubMed: 1384399]
24. Cechova S, Pajewski TN. The soluble guanylyl cyclase inhibitor ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, dose-dependently reduces the threshold for isoflurane anesthesia in rats. Anesth Analg 2004; 99: 752–7, table of contents [PubMed: 15333406]
25. Zuo Z, Tichotsky A, Johns RA. Inhibition of excitatory neurotransmitter-nitric oxide signaling pathway by inhalational anesthetics. Neuroscience 1999; 93: 1167–72 [PubMed: 10473281]
26. Loeb AL, Gonzales JM, Reichard PS. Isoflurane enhances glutamatergic agonist-stimulated nitric oxide synthesis in cultured neurons. Brain Res 1996; 734: 295–300 [PubMed: 8896837]
27. Matsuda S, Harada K, Ito M, et al. Generation of a cGMP Indicator with an Expanded Dynamic Range by Optimization of Amino Acid Linkers between a Fluorescent Protein and PDE5alpha. ACS Sens 2017; 2: 46–51 [PubMed: 28722423]
28. Ros O, Zagar Y, Ribes S, et al. SponGee: A Genetic Tool for Subcellular and Cell-Specific cGMP Manipulation. Cell Rep 2019; 27: 4003–12 e6 [PubMed: 31242429]
29. Dittman JS, Ryan TA. The control of release probability at nerve terminals. Nat Rev Neurosci 2019; 20: 177–86 [PubMed: 30647451]
30. Speigel IA, Hemmings HC Jr. Selective inhibition of gamma aminobutyric acid release from mouse hippocampal interneurone subtypes by the volatile anaesthetic isoflurane. Br J Anaesth 2021; 127: 587–99 [PubMed: 34384592]
31. Hoppa MB, Lana B, Margas W, Dolphin AC, Ryan TA. alpha2delta expression sets presynaptic calcium channel abundance and release probability. Nature 2012; 486: 122–5 [PubMed: 22678293]
32. Solt K, Eger EI 2nd, Raines DE. Differential modulation of human N-methyl-D-aspartate receptors by structurally diverse general anesthetics. Anesth Analg 2006; 102: 1407–11 [PubMed: 16632818]
33. Neitz A, Mergia E, Eysel UT, Koesling D, Mittmann T. Presynaptic nitric oxide/cGMP facilitates glutamate release via hyperpolarization-activated cyclic nucleotide-gated channels in the hippocampus. Eur J Neurosci 2011; 33: 1611–21 [PubMed: 21410795]
34. Andreasen M, Lambert JD, Jensen MS. Effects of new non-N-methyl-D-aspartate antagonists on synaptic transmission in the in vitro rat hippocampus. The Journal of Physiology 1989; 414: 317–36 [PubMed: 2575162]
35. Davies SN, Collingridge GL. Role of Excitatory Amino Acid Receptors in Synaptic Transmission in Area CA1 of Rat Hippocampus. Proceedings of the Royal Society of London Series B, Biological Sciences 1989; 236: 373–84 [PubMed: 2567518]
36. Bellamy TC, Garthwaite J. Sub-second kinetics of the nitric oxide receptor, soluble guanylyl cyclase, in intact cerebellar cells. J Biol Chem 2001; 276: 4287–92 [PubMed: 11073946]
37. Wu X, Liao L, Liu X, Luo F, Yang T, Li C. Is ZD7288 a selective blocker of hyperpolarization-activated cyclic nucleotide-gated channel currents? Channels (Austin) 2012; 6: 438–42 [PubMed: 22989944]
38. Sprenger JU, Nikolaev VO. Biophysical techniques for detection of cAMP and cGMP in living cells. Int J Mol Sci 2013; 14: 8025–46 [PubMed: 23584022]
39. Caviedes A, Varas-Godoy M, Lafourcade C, et al. Endothelial Nitric Oxide Synthase Is Present in Dendritic Spines of Neurons in Primary Cultures. Front Cell Neurosci 2017; 11: 180 [PubMed: 28725180]
40. Sattler R, Xiong Z, Lu WY, Hafner M, MacDonald JF, Tymianski M. Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science 1999; 284: 1845–8 [PubMed: 10364559]
41. Rengasamy A, Pajewski TN, Johns RA. Inhalational anesthetic effects on rat cerebellar nitric oxide and cyclic guanosine monophosphate production. Anesthesiology 1997; 86: 689–98 [PubMed: 9066336]

42. Wang Q, Mergia E, Koesling D, Mittmann T. Nitric oxide/cGMP signaling via guanylyl cyclase isoform 1 modulates glutamate and GABA release in somatosensory cortex of mice. Neuroscience 2017; 360: 180–9 [PubMed: 28782641]
43. Ouyang W, Hemmings HC Jr. Depression by isoflurane of the action potential and underlying voltage-gated ion currents in isolated rat neurohypophysial nerve terminals. J Pharmacol Exp Ther 2005; 312: 801–8 [PubMed: 15375177]
44. Koyanagi Y, Torturo CL, Cook DC, Zhou Z, Hemmings HC Jr. Role of specific presynaptic calcium channel subtypes in isoflurane inhibition of synaptic vesicle exocytosis in rat hippocampal neurones. Br J Anaesth 2019; 123: 219–27 [PubMed: 31056238]
45. Hara M, Zhou ZY, Hemmings HC Jr. alpha2-Adrenergic Receptor and Isoflurane Modulation of Presynaptic Ca2+ Influx and Exocytosis in Hippocampal Neurons. Anesthesiology 2016; 125: 535–46 [PubMed: 27337223]
46. Chen X, Sirois JE, Lei Q, Talley EM, Lynch C, 3rd, Bayliss DA. HCN subunit-specific and cAMP-modulated effects of anesthetics on neuronal pacemaker currents. J Neurosci 2005; 25: 5803–14 [PubMed: 15958747]
47. Schwerin S, Kopp C, Pircher E, et al. Attenuation of Native Hyperpolarization-Activated, Cyclic Nucleotide-Gated Channel Function by the Volatile Anesthetic Sevoflurane in Mouse Thalamocortical Relay Neurons. Front Cell Neurosci 2020; 14: 606687
48. Zhou C, Liang P, Liu J, et al. HCN1 Channels Contribute to the Effects of Amnesia and Hypnosis but not Immobility of Volatile Anesthetics. Anesth Analg 2015; 121: 661–6 [PubMed: 26287296]
49. Riegelhaupt PM, Tibbs GR, Goldstein PA. HCN and K2P Channels in Anesthetic Mechanisms Research. Methods Enzymol 2018; 602: 391–416 [PubMed: 29588040]
50. Shah MM. Cortical HCN channels: function, trafficking and plasticity. J Physiol 2014; 592: 2711–9 [PubMed: 24756635]
51. He C, Chen F, Li B, Hu Z. Neurophysiology of HCN channels: from cellular functions to multiple regulations. Prog Neurobiol 2014; 112: 1–23 [PubMed: 24184323]
52. Lorincz A, Notomi T, Tamas G, Shigemoto R, Nusser Z. Polarized and compartment-dependent distribution of HCN1 in pyramidal cell dendrites. Nat Neurosci 2002; 5: 1185–93 [PubMed: 12389030]
53. Bender RA, Kirschstein T, Kretz O, et al. Localization of HCN1 channels to presynaptic compartments: novel plasticity that may contribute to hippocampal maturation. J Neurosci 2007; 27: 4697–706 [PubMed: 17460082]
54. Huang Z, Li G, Aguado C, Lujan R, Shah MM. HCN1 channels reduce the rate of exocytosis from a subset of cortical synaptic terminals. Sci Rep 2017; 7: 40257 [PubMed: 28071723]
55. Ying SW, Jia F, Abbas SY, Hofmann F, Ludwig A, Goldstein PA. Dendritic HCN2 channels constrain glutamate-driven excitability in reticular thalamic neurons. J Neurosci 2007; 27: 8719–32 [PubMed: 17687049]
56. Eroglu E, Gottschalk B, Charoensin S, et al. Development of novel FP-based probes for live-cell imaging of nitric oxide dynamics. Nat Commun 2016; 7: 10623 [PubMed: 26842907]
57. Eroglu E, Charoensin S, Bischof H, et al. Genetic biosensors for imaging nitric oxide in single cells. Free Radic Biol Med 2018; 128: 50–8 [PubMed: 29398285]

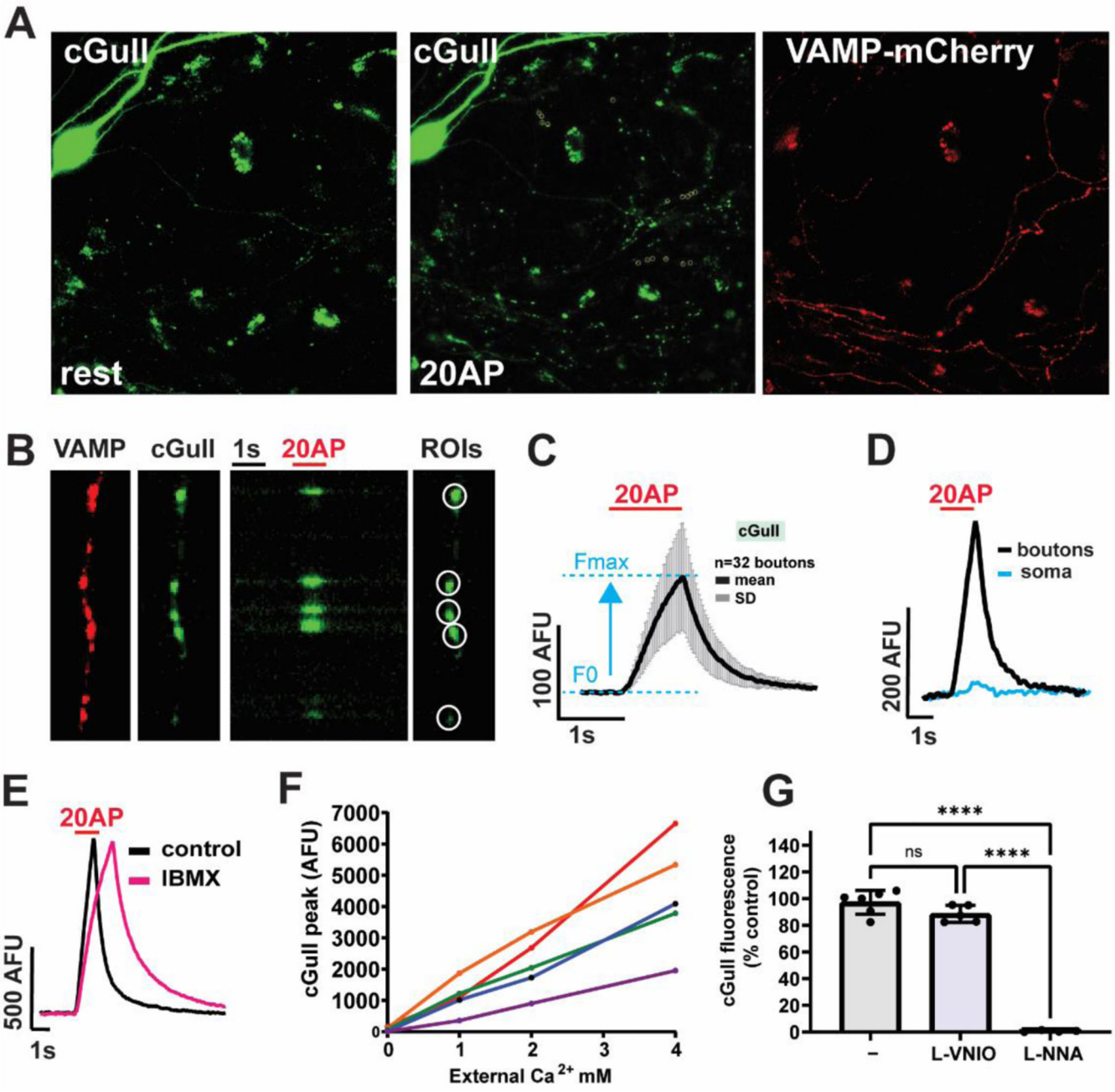

Figure 1. cGull reports basal and electrically-stimulated cGMP concentrations in hippocampal presynaptic boutons.

(A) At rest, cGull fluorescence is evident in somata and proximal dendrites (rest, left), and increases in all compartments following electrical stimulation (20 AP, middle), especially within presynaptic boutons co-expressing the presynaptic marker VAMP-mCherry (right). (**B**) Presynaptic boutons expressing VAMP-mCherry (VAMP) and cGull (far left and left), kymograph of evoked cGull fluorescence changes (middle), and regions of interest (ROIs) drawn over responsive boutons (right). (**C**) Boutons from one cell analyzed together as an ensemble averaged time series. Peak amplitude: $\Delta F = F_{max} - F_0$. Traces show mean over time per cell. (**D)** Representative cGMP transients from boutons measured using cGull

compared to signals from the soma. (**E**) Transient waveform is slowed and prolonged in the presence of the phosphodiesterase inhibitor IBMX. **(F)** Presynaptic cGull transient peak amplitudes increase with increases in external Ca^{2+} concentration. Each line represents data from a single cell (n=5). **(G)** Presynaptic cGMP transients are resistant to the selective nNOS inhibitor L-VNIO, and completely abolished by the combined eNOS/nNOS inhibitor L-NNA. Calculated as percent of a control stimulation: control= 97.2 ± 8.9%, n=6; L-VNIO = 88.6 ± 6.3%, n= 5, L-NNA = 0.64 ± 0.60%, n=4. One-way ANOVA $F(2, 12)= 272.3$, $p<0.0001$ with Tukey's *post hoc* test: control *vs* L-VNIO p=0.135, 95%CI [−2.41, 19.65], control *vs* L-NNA p<0.0001, 95%CI [84.8,108.3]; L-VNIO *vs* L-NNA p<0.0001, 95%CI [75.8, 100.2]. ns, not significant; ****, p<0.0001.

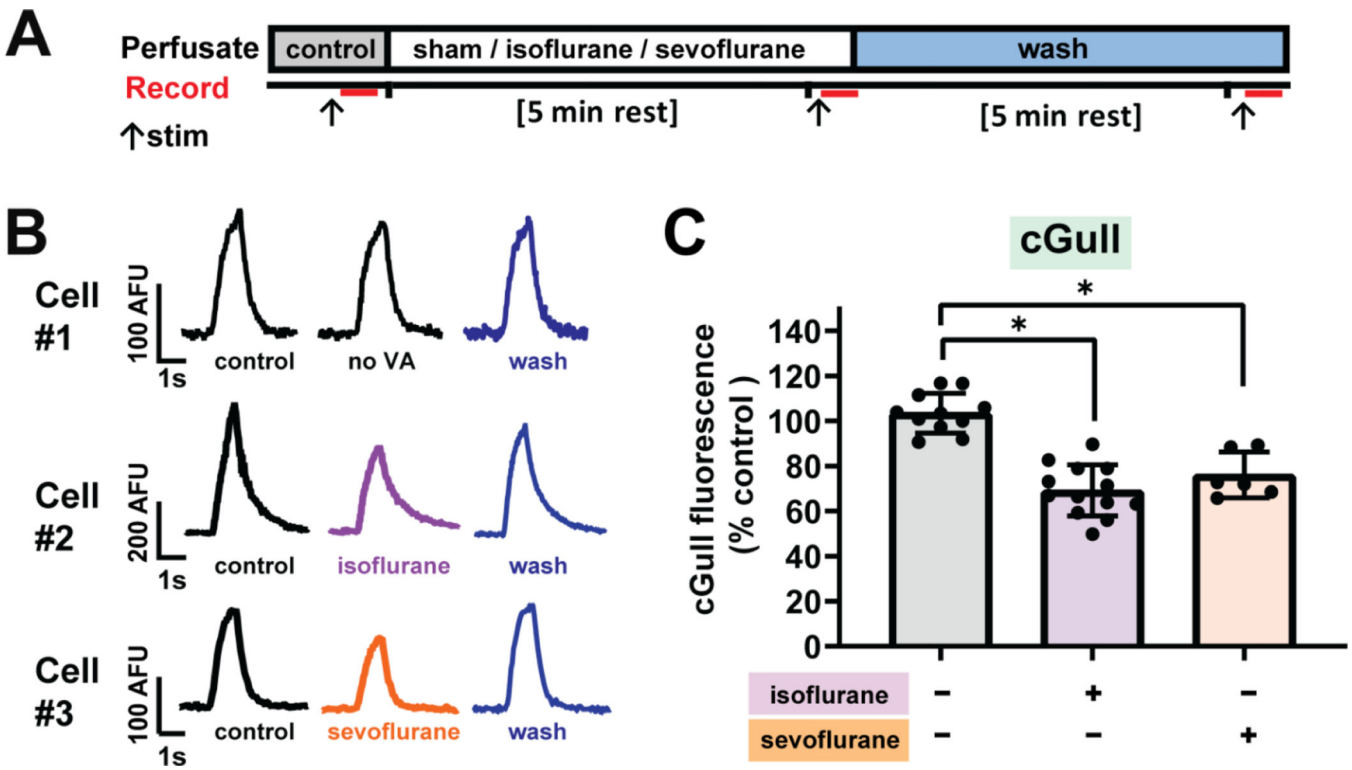

Figure 2: Volatile anesthetics inhibit stimulus-evoked presynaptic cGMP increases.

(A) Live-cell imaging and drug perfusion paradigm. **(B)** Representative traces of presynaptic cGull transients in the presence of isoflurane (0.51 ± 0.09 mM) or sevoflurane (0.57 ± 0.13 mM) compared to control without volatile anesthetic. **(C)** Isoflurane or sevoflurane inhibits the evoked increase in presynaptic cGMP. Peak cGMP transient amplitude was reduced by each anesthetic compared to stimulation without anesthetic. Calculated as a percentage of control stimulation: no anesthetic=103.6 ± 8.8%, n=11; isoflurane=69.2 ± 11.3%, n=13, sevoflurane = 76.2 ± 10.2%, n=6. One-way ANOVA $F(2, 27) = 35.3$, $p<0.0001$, with Tukey's *post hoc* test: no anesthetic *vs* isoflurane $p<0.0001$, 95%CI [23.9, 44.7], no anesthetic *vs* sevoflurane $p<0.0001$, 95%CI [14.5, 40.3]; isoflurane *vs* sevoflurane $p=0.373$, 95%CI [−19.4, −5.62]. *, $p<0.05$.

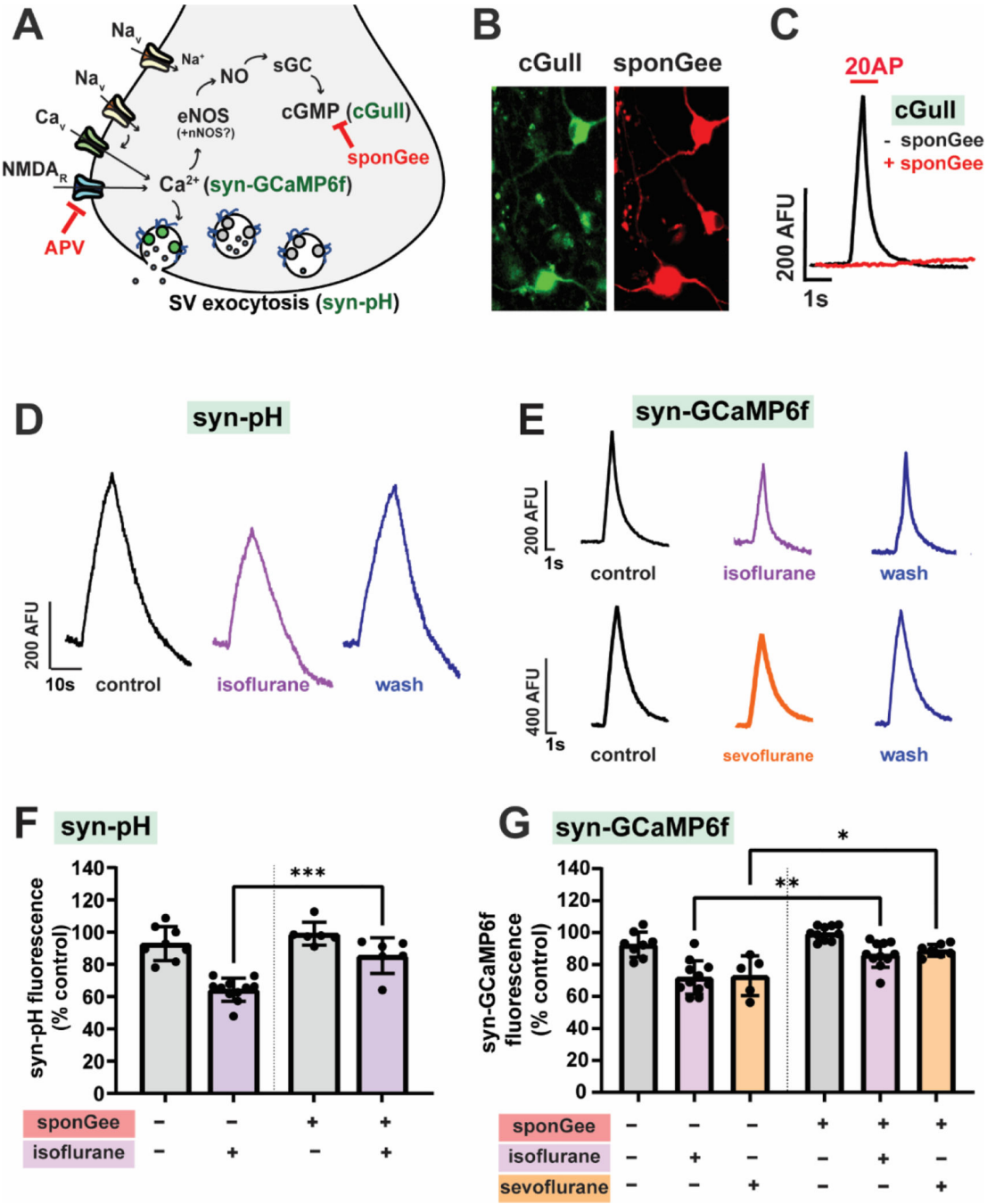

Figure 3: Sequestration of cGMP by sponGee blunts volatile anesthetic inhibition of presynaptic function.

(A) Schematic diagram showing probes used to investigate anesthetic effects on cGMP generation, Ca^{2+} influx, and synaptic vesicle (SV) exocytosis. NMDA receptors are blocked by APV included in the perfusion buffers to prevent recurrent excitation. NOS is shown in the bouton, but might also function as a retrograde messenger from postsynaptic neurons. Abbreviations: Ca_V (voltage-gated Ca^{2+} channel), cGMP (cyclic guanosine monophosphate), eNOS and nNOS (endothelial and neuronal nitric

oxide synthase), Na_v (voltage-gated Na^+ channel), NMDA (N-methyl-D-aspartate), NO (nitric oxide), sGC (soluble gyanylyl cyclase), SV (synaptic vesicle), syn-GCaMP6f (synaptophysin-GCaMP6f), syn-pH (synaptophysin-pHluorin). **(B)** Overlapping red and green somatic fluorescence confirms sponGee-mRFP and cGull co-expression. **(C)** Co-transfection of cGull with sponGee blocks activity-dependent cGMP increase with electrical stimulation. **(D)** Isoflurane reversibly inhibits (by −31.7%) synaptic vesicle exocytosis evoked by electrical stimulation as shown by representative traces from a syn-pH transfected neuron. **(E)** Isoflurane and sevoflurane reversibly inhibit presynaptic Ca^{2+} entry as shown by representative traces from syn-GCaMP6f transfected neurons (−23.3 and −21.9%, respectively). (**F**) Isoflurane inhibition of synaptic vesicle exocytosis is reduced by co-expression of syn-pH with the cGMP binding protein sponGee. Calculated as % control stimulation (left-right): syn-pH, 92.9 ± 10.6%, n=8; syn-pH plus isoflurane, 64.3 ± 7.1%, n=11; syn-pH and sponGee, 99.0 ± 7.2%, n=6; syn-pH and sponGee, plus isoflurane, 85.4 ± 11.1%. Two-way ANOVA $F(3,14)=12.87$, $p<0.0001$, with Tukey's *post hoc* test: syn-pH plus isoflurane *vs* syn-pH and sponGee plus isoflurane $p=0.0004$, 95%CI [13.33, 43.57]. (**G**) Inhibition by isoflurane (or sevoflurane; data not shown) of presynaptic Ca^{2+} influx was reduced by co-expressing syn-GCaMP6f with sponGee. Calculated as % control stimulation (left-right): syn-GCaMP6f = 92.4 ± 8.0%, n=8; syn-GCaMP6f plus isoflurane 71.8 ± 10.4%, n=11; syn-GCaMP6f plus sevoflurane 72.9 ± 12.4%, n=5; syn-GCaMP6f and sponGee 99.7 ± 4.5%, n=10; syn-GCaMP6f and sponGee plus isoflurane 86.3 ± 8.0%, n=11; syn-GCaMP6f and sponGee plus sevoflurane 89.9 ± 3.0%, n=7. Two-way ANOVA $F(5,33)=14.99$, $p<0.0001$: Isoflurane: syn-GCaMP6f *vs* syn-GCaMP6f plus sponGee; $p=0.0020$, 95%CI [4.74, 27.86]. Sevoflurane: syn-GCaMP6f vs syn-GCaMP6f plus sponGee $p=0.0311$, 95%CI [−30.45, −0.98]. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

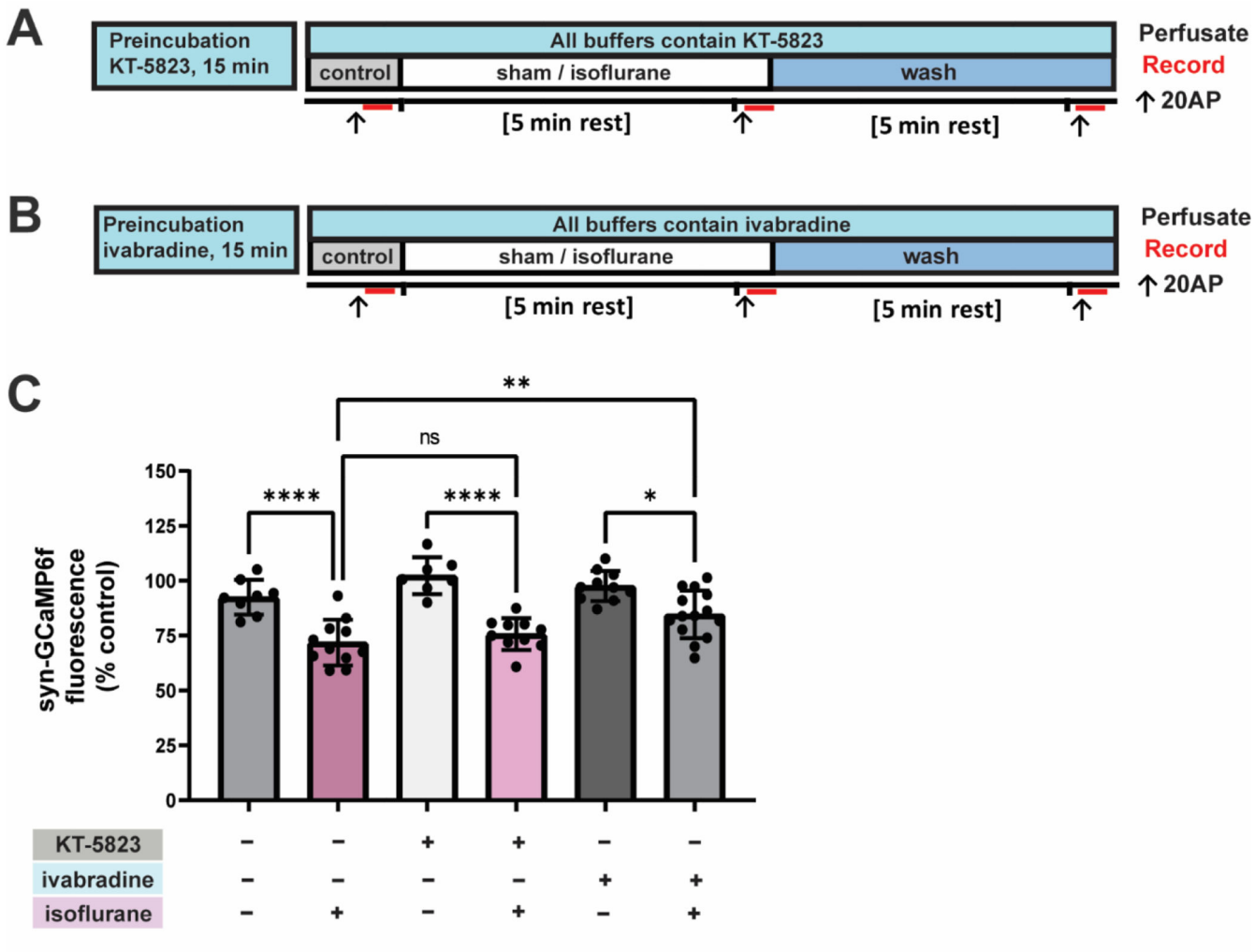

Figure 4: Isoflurane inhibits presynaptic Ca^{2+} influx through cGMP modulation of HCN channels independent of cGMP-dependent protein kinase.

(A) Live-cell imaging paradigm to identify the contribution of cGMP-dependent protein kinase (PKG) to isoflurane actions using the selective PKG inhibitor KT-5823. **(B)** Live-cell imaging paradigm to identify the contribution of HCN channels to isoflurane actions, using the HCN inhibitor ivabradine. **(C)** KT-5823 had no significant effect on isoflurane inhibition of presynaptic Ca^{2+} influx, while ivabradine diminished isoflurane inhibition of presynaptic Ca^{2+} influx Calculated as % control stimulation (left-right): control (no isoflurane), 92.4 ± 8.0%, n=8; 0.51 ± 0.09 mM isoflurane, 71.8 ± 10.4%, n=11; 500 nM KT-5823, no isoflurane, 102.3 ± 8.4%, n=7; KT-5823 plus isoflurane, 75.7 ± 7.2%, n=10; 30 μM ivabradine, no isoflurane, 97.6 ± 6.9%, n=10; ivabradine plus isoflurane, 84.6 ± 10.8%, n=14. Two-way ANOVA, $F(5,41)=18.76$, with Tukey's *post hoc* test: no isoflurane vs isoflurane, $p=0.00002$, 95%CI [10.54, 35.74]; isoflurane *vs* isoflurane plus KT-5823, $p=0.732$, 95%CI [−16.6, 6.72]; no isoflurane plus ivabradine *vs* isoflurane plus ivabradine, $p=0.03$, 95%CI [0.61, 23.3]; isoflurane *vs* isoflurane plus ivabradine, $p=0.03$, 95% CI [−25.4, −3.6]. ns, not significant; *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$.