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Laboratory Investigation

LABORATORY INVESTIGATION

Role of specific presynaptic calcium channel subtypes in isoflurane inhibition of synaptic vesicle exocytosis in rat hippocampal neurones

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Abstract

Background: P/Q- and N-type voltage-gated calcium channels (VGCC) are the principal subtypes mediating synaptic vesicle (SV) exocytosis. Both the degree of isoflurane inhibition of SV exocytosis and VGCC subtype expression vary between brain regions and neurotransmitter phenotype. We hypothesised that differences in VGCC subtype expression contribute to synapse-selective presynaptic effects of isoflurane.

Methods: We used quantitative live-cell imaging to measure exocytosis in cultured rat hippocampal neurones after transfection of the fluorescent biosensor vGlut1-pHluorin. Selective inhibitors of P/Q- and N-type VGCCs were used to isolate subtype-specific effects of isoflurane.

Results: Inhibition of N-type channels by 1 μM ω-conotoxin GVIA reduced SV exocytosis to 81±5% of control (n=10). Residual exocytosis mediated by P/Q-type channels was further inhibited by isoflurane to 42±4% of control (n=10). The P/Q-type channel inhibitor ω-agatoxin IVA at 0.4 μM inhibited SV exocytosis to 29±3% of control (n=10). Residual exocytosis mediated by N-type channels was further inhibited by isoflurane to 17±3% of control (n=10). Analysis of isoflurane effects at the level of individual boutons revealed no difference in sensitivity to isoflurane between P/Q- or N-type channel-mediated SV exocytosis (P=0.35). There was no correlation between the effect of agatoxin (P=0.91) or conotoxin (P=0.15) and the effect of isoflurane on exocytosis.

Conclusions: Sensitivity of SV exocytosis to isoflurane in rat hippocampal neurones is independent of the specific VGCC subtype coupled to exocytosis. The differential sensitivity of VGCC subtypes to isoflurane does not explain the observed neurotransmitter-selective effects of isoflurane in hippocampus.

Keywords: calcium channel; isoflurane; mechanisms of anaesthesia; optical imaging; presynaptic terminal; synaptic vesicle; vGlut1-pHluorin

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Editor's key points

- Isoflurane inhibits synaptic release of the excitatory neurotransmitter glutamate to a greater extent than of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) by unclear mechanisms.
- The authors' hypothesis that this difference derives from selective inhibition of specific presynaptic voltage-gated calcium channels was investigated using genetically encoded biosensors for exocytosis from hippocampal neurones in culture.
- Synaptic vesicle exocytosis mediated by either N-type or P/Q-type calcium channels, which together mediated all exocytosis, was inhibited by isoflurane with similar potencies.
- The differential sensitivity to isoflurane of presynaptic glutamate and GABA release is not explained by differences in the sensitivity of the major presynaptic calcium channels to isoflurane, and must therefore involve other presynaptic determinants of exocytosis.

The volatile anaesthetic isoflurane decreases excitatory synaptic transmission primarily by presynaptic actions.[1–3] Isoflurane selectively inhibits action potential (AP)-evoked synaptic vesicle (SV) exocytosis from glutamatergic hippocampal neurones to a greater degree than from GABAergic neurones.[4] However, the molecular basis for this differential inhibition and synaptic selectivity is unclear.

Voltage-gated Ca^{2+} channels (VGCCs) play a critical role in neurotransmission by mediating the Ca^{2+} influx essential to triggering SV exocytosis.[5,6] Because isoflurane does not alter the Ca^{2+}-exocytosis coupling relationship in hippocampal neurones,[4] its presynaptic selectivity must reside upstream of Ca^{2+} influx; possible mechanisms include effects on voltage-gated Na^+ channels, K^+ channels, and VGCCs. Neurotransmitter phenotype-specific differences in expression, or anaesthetic sensitivity of presynaptic VGCC subtypes, or both could contribute to the observed selective inhibition of glutamatergic *vs* GABAergic SV exocytosis by isoflurane. We used live-cell fluorescence microscopy to examine the isoflurane sensitivity of P/Q-type and N-type VGCCs, which are the principal subtypes coupled to SV exocytosis in the central nervous system, at individual hippocampal synapses.[7,8] Previous studies examining the effects of isoflurane on VGCCs have led to discordant results.[9–13] We tested the hypothesis that differential inhibition of specific presynaptic VGCC subtypes underlies the neurotransmitter phenotype selectivity observed for isoflurane inhibition of SV exocytosis.[4]

Methods

Cell culture and transfection

All experiments were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee (New York, NY, USA) and conformed to National Institutes of Health Guidelines for the Care and Use of Animals. The techniques for preparing rat hippocampal mixed neuronal cultures and characterisation of the cultures have been described.[4,14–18] After 7 days *in vitro* (DIV), cultured neurones were transfected with the vesicular glutamate transporter 1-pHluorin (vGlut1-pH), a vesicular fusion biosensor.[19] A modified calcium phosphate precipitation protocol was used to ensure low transfection efficiency so recorded boutons within a coverslip could be confidently attributed to a single neurone. Live-cell imaging was performed at 16–17 DIV (corresponding to postnatal days 17–18) to minimise variability resulting from a possible developmental shift in the ratio of VGCC subtype expression. We co-transfected mCherry to see vGlut1-pH transfected neurones. The number of vGlut1-pH/mCherry transfected neurones in each coverslip was 3.0 (0.9) (n=7). The efficiency of vGlut1-pH transfection was 0.23 (0.08)%.

Synaptic vesicle exocytosis

Measurement of SV exocytosis was performed as previously described.[4,14] Briefly, one vGlut1-pH positive neurone in each coverslip was chosen for analysis. APs were evoked by passing 1 ms current pulses via platinum–iridium electrodes incorporated into the chamber, yielding fields of ~10 V cm^{-1}, at 10 Hz for 10 s. Fluorescence images were collected with an iXon+ camera (DU-897E-BV; Andor, Belfast, UK) using a solid-state, diode-pumped 488 nm laser. Fluorescent boutons were imaged with a 40×, 1.3 numerical aperture objective (Fluar; Carl Zeiss, Oberkochen, Germany) and a 1.0× Optovar. Imaging was conducted at 29–30°C with continuous perfusion at 0.75 ml min^{-1} of Tyrode's solution containing (in mM): 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffered to pH 7.4, 30 D-glucose, with 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM D,L-2-amino-5-phosphonovaleric acid (AP5) to block glutamatergic excitation. ω-Conotoxin GVIA[20] (conotoxin; Alomone, Jerusalem, Israel) at 1 μM and ω-agatoxin IVA[21] (agatoxin; Alomone) at 0.4 μM were included in the perfusate to completely and selectively block N- or P/Q-type VGCCs, respectively.[14 15] Isoflurane-saturated Tyrode's solution (~12 mM) was prepared and diluted daily into gastight glass syringes, from which a sample was obtained for determination of isoflurane concentration by gas chromatography (Shimadzu GC-2010 Plus; Shimadzu, Kyoto, Japan) after extraction into octane.[22] The final isoflurane concentration used (0.51 [0.02] mM) corresponds to 1.6 (0.1) times the minimum alveolar concentration (MAC) for isoflurane in rats, a clinically relevant concentration equivalent to ~1.6 times the ED_{50} corrected to 30°C,[23] which corresponds to ~0.7 times a maximally effective dose (ED_{95}, ~2.3 times the ED_{50}).[24] Total pool (TP) of SVs was determined by alkalisation of vesicles at the end of each experiment by perfusing 50 mM NH_4Cl solution (substituted for 50 mM NaCl in Tyrode's solution and buffered to pH 7.4).

Data analysis

Images were analysed with ImageJ (http://rsb.info.nih.gov/ij/) using a custom plugin (http://rsb.info.nih.gov/ij/plugins/time-series.html). Boutons (~20–50 boutons per neurone) were chosen from control images before drug application, and were defined as 2 μm diameter circular regions of interest (ROIs). Exocytosis was defined as the ΔF of vGlut1-pH determined by the difference in fluorescence between baseline (average of the 10 frames before stimulation) and peak (average of the 10 highest frames after completion of the stimulus train) acquired at 10 Hz. Boutons with signal/noise ratio (SNR) of <5 in response to 100 APs in control or after VGCC toxin application were excluded from analysis. For each bouton, SNR was

defined as SNR=ΔF/σ, where σ is the standard deviation (SD) of the fluorescence measured for 10 frames before stimulation. Signals at each bouton were normalised to TP ($\Delta F/F_{TP}$). Applying these criteria, we included 329 boutons from 10 experiments using the N-type VGCC blocker conotoxin (87% of all boutons) and 135 boutons from nine experiments using the P/Q-type VGCC blocker agatoxin (29% of all boutons). Greater inhibition of SV exocytosis by the P/Q-type blocker compared with the N-type blocker meant that fewer boutons met the criteria for analysis using agatoxin.

Statistical analysis

Data on the population effects of VGCC blockers and isoflurane are shown as mean (standard error of the mean, SEM) unless stated otherwise, and were analysed by one-way repeated-measures analysis of variance (ANOVA) with *post hoc* Bonferroni analysis. Normality was determined using the Shapiro–Wilk test, using α=0.05 as a cut-off. Data for individual boutons were not normally distributed, and are presented as median (inter-quartile range), and analysed using the Mann–Whitney *U*-test and two-sample Kolmogorov–Smirnov test. The multimodality of distribution histograms was estimated using Hartigan's dip test. Correlation coefficients were analysed against the null hypothesis μ=0 by two-tailed one-sample *t*-test. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and Stata SE (version 15; StataCorp, College Station, TX, USA), with $P<0.05$ defined as significant. The number of recorded neurones is presented as *n*. Data for multiple boutons were acquired from only one neurone per coverslip to avoid contaminating and potentially irreversible effects of multiple stimulations and drug treatments. Each experimental group contained coverslips from two different preparations of primary neuronal cultures to minimise artifacts because of differing culture conditions.

Results

Isoflurane inhibits SV exocytosis mediated by N- or P/Q-type voltage-gated calcium channels

vGlut1-pH was used as an optical biosensor of SV exocytosis (Fig 1).[15 19 25] Changes in fluorescence (ΔF) during electrical stimulation with 100 APs at 10 Hz reflect the alkalisation of vGlut1-pH because of SV exocytosis, whereas changes during the post-stimulus period reflect re-acidification after vesicle endocytosis.[26] We examined the contributions of the principal VGCC subtypes known to control SV exocytosis using the specific blockers conotoxin for N-type and agatoxin for P/Q-type VGCCs. Together conotoxin (1 μM) and agatoxin (0.4 μM) inhibited exocytosis measured as mean $\Delta F/F_{TP}$ by 96.9 (0.2)% (control 0.37 [0.04], plus conotoxin + agatoxin 0.01 [0.00], *n*=3). Thus, SV exocytosis was almost entirely mediated by N-type and P/Q-type VGCCs in cultured rat hippocampal neurones.[27]

Isoflurane has been shown to inhibit hippocampal SV exocytosis elicited by electrical stimulation.[4] We examined the inhibitory effects of isoflurane on SV exocytosis mediated by either N-type or P/Q-type VGCCs using conotoxin and agatoxin to isolate each channel subtype selectively (Figs 2 and 3). Representative effects of conotoxin and isoflurane on exocytosis in a single neurone are shown in Fig 2b. On average, conotoxin reduced $\Delta F/F_{TP}$ from 0.17 (0.03) to 0.13 (0.02) (80.5 [5.0]% of control, P=0.025; n=10; Fig 2c). Treatment with isoflurane further inhibited $\Delta F/F_{TP}$ to 0.06 (0.01) (41.8 [3.5]% of control, P=0.018). Agatoxin inhibited SV exocytosis more than conotoxin (Fig 3b): agatoxin reduced $\Delta F/F_{TP}$ from 0.25 (0.03) to 0.06 (0.01) (28.9 [3.4]% of control, $P<0.0001$; n=10, Fig. 3c). In the presence of agatoxin, isoflurane further reduced $\Delta F/F_{TP}$ to 0.04 (0.01) (16.6 [2.5]%, $P<0.0001$). Isoflurane therefore inhibits SV exocytosis mediated by either P/Q- or N-type VGCCs.

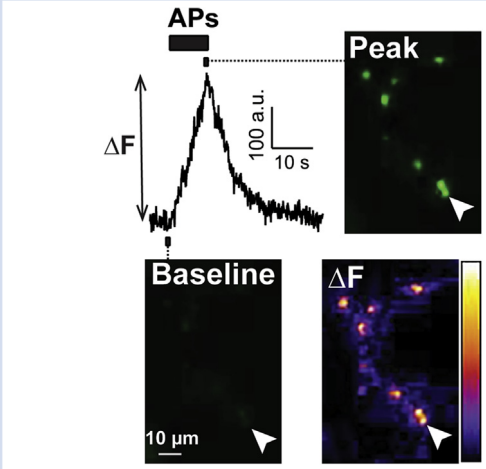

Fig. 1. Measurement of synaptic vesicle exocytosis using vGlut1-pH. Representative trace of vGlut1-pH response to 100 action potential (AP) stimulation at 10 Hz in a single bouton (upper left). Live-cell image of a vGlut1-pH transfected neurone at baseline (lower left), after stimulation (upper right), and the resulting difference in fluorescence from the stimulation (lower right). Arrowheads indicate the single bouton that is represented in the raw trace.

P/Q-type channels contribute more to synaptic vesicle exocytosis than N-type channels

VGCC subtypes are differentially expressed in presynaptic terminals according not only to the neurone phenotype, but also to the type of postsynaptic cells with which terminals synapse,[28 29] such that expression of VGCC subtypes can differ between individual boutons of the same neurone.[30] To investigate the relative contributions of VGCC subtypes to SV exocytosis by bouton, we compared the effects of selective inhibitors at individual boutons, pooling all boutons from 10 individual neurones for analysis (Fig. 4). Selective block of P/Q-type VGCCs with agatoxin caused a greater reduction in SV exocytosis at individual boutons (71 [1]% inhibition, n=460 boutons from 10 neurones) than selective block of N-type VGCCs with conotoxin (22 [1]% inhibition, n=380 boutons from 10 neurones; $P<0.0001$, Mann–Whitney *U*-test). There was a high degree of variability in the effects of VGCC subtype-specific inhibitors between boutons, particularly for conotoxin (SD=24.0) compared with agatoxin (SD=18.4). P/Q-type channels were the major VGCC subtype mediating SV exocytosis in cultured hippocampal neurones.

Calcium channel subtypes coupled to exocytosis have comparable sensitivities to inhibition by isoflurane

We investigated the contribution of VGCC subtypes to inhibition of SV exocytosis by isoflurane, first by determining

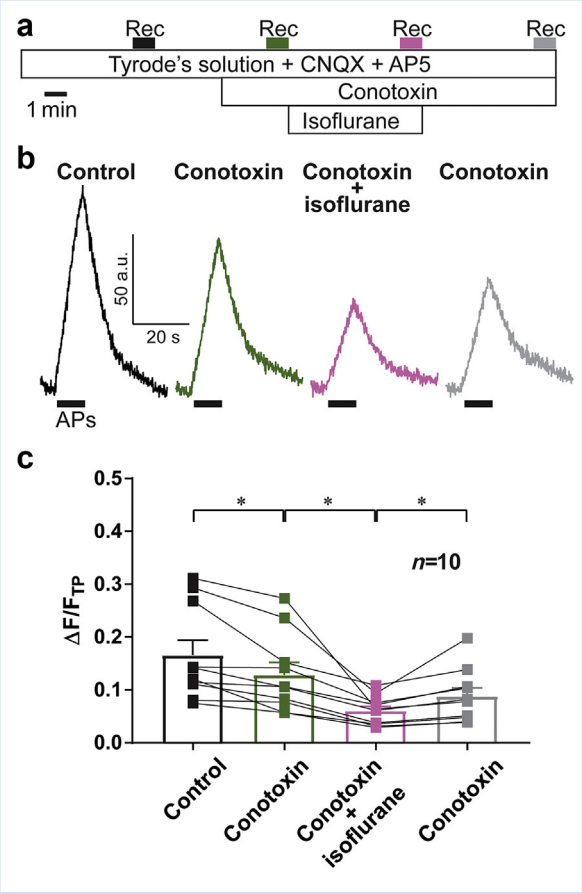

Fig. 2. Inhibition of synaptic vesicle exocytosis by the N-type channel blocker conotoxin and isoflurane. (a) Schematic diagram of the protocol. During each recording (Rec), electrical stimulation of 100 APs at 10 Hz (10 s of stimulation) was applied. (b) Ensemble average traces of vGlut1-pH responses to 100 AP stimulation in a single representative neurone for control (black), conotoxin (green), conotoxin plus isoflurane (pink), and conotoxin after wash out of isoflurane (grey). (c) The effects of conotoxin and isoflurane on exocytosis are shown (*$P<0.05$, one-way analysis of variance [ANOVA] with Bonferroni's *post hoc* test). Signals were normalised for each bouton to the total pool ($\Delta F/F_{TP}$). Each point represents the average $\Delta F/F_{TP}$ from all of the boutons in each neurone tested.

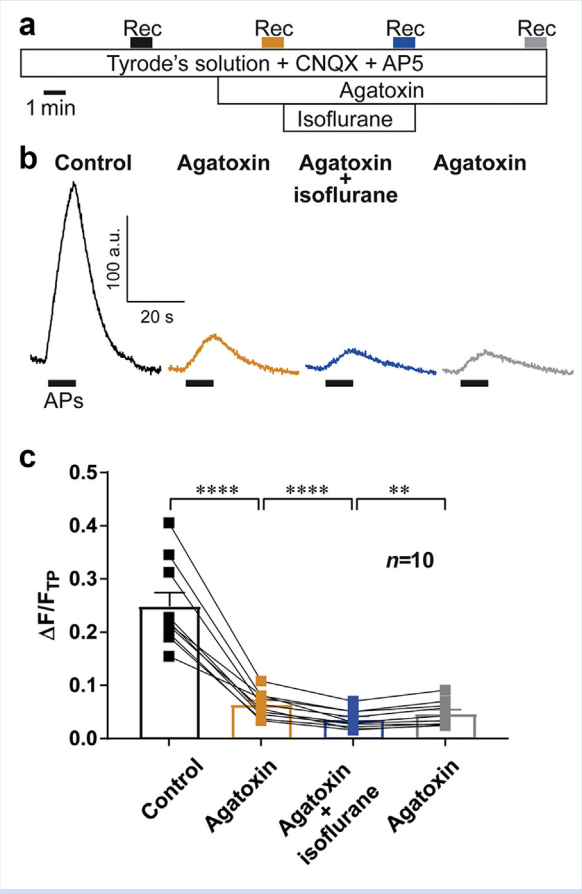

Fig. 3. Inhibition of synaptic vesicle exocytosis by the P/Q-type channel blocker agatoxin and isoflurane. (a) Schematic diagram of the protocol. During each recording (Rec), electrical stimulation of 100 APs at 10 Hz (10 s of stimulation) was applied. (b) Ensemble average traces of vGlut1-pH responses to 100 AP stimulation in a single representative neurone for control (black), agatoxin (gold), agatoxin plus isoflurane (blue), and agatoxin after wash out of isoflurane (grey). (c) The effects of agatoxin and isoflurane on exocytosis are shown (**$P<0.01$, ****$P<0.0001$, one-way analysis of variance [ANOVA] with repeated measures and Bonferroni post hoc test). Signals were normalised for each bouton to the total pool ($\Delta F/F_{TP}$). Each point represents the average $\Delta F/F_{TP}$ from all of the boutons in each neurone tested. AP, action potential.

whether isoflurane differentially inhibits SV exocytosis depending on the VGCC subtype involved, and second by analysing the correlation between inhibition by isoflurane and contribution to exocytosis of each VGCC subtype between individual boutons. Figure 5 shows the distribution histograms for isoflurane inhibition of SV exocytosis mediated either by P/Q- or N-type VGCCs across all boutons with SNR >5. The effect of isoflurane on P/Q- or N-type VGCC-mediated SV exocytosis showed a unimodal distribution (Hartigan's dip test for P/Q-type VGCC-mediated exocytosis=0.01, $P=1.00$; for N-type VGCC-mediated exocytosis=0.02, $P=0.94$). Isoflurane reduced SV exocytosis mediated by P/Q-type VGCCs by 46.7 (1.2)%, and SV exocytosis mediated by N-type VGCCs by 49.4 (1.9)%. These results indicate that presynaptic P/Q-type and N-type VGCCs coupled to SV exocytosis have similar sensitivities to isoflurane ($P=0.35$, Mann–Whitney *U*-test).

We also analysed correlations between the effects of isoflurane and of conotoxin or agatoxin on SV exocytosis (Fig. 6). Mean correlation coefficients between the effects of isoflurane and conotoxin were not significantly different from 0, indicating no correlation between the effect of isoflurane and the contribution of N-type channels to exocytosis (one-sample *t*-test against null hypothesis $\mu=0$, $P=0.15$, $t=1.56$; Fig. 6b). Similarly, mean correlation coefficients between the effects of isoflurane and agatoxin were not significantly different from 0, indicating no correlation between the effect of isoflurane and contribution of P/Q-type channels to exocytosis (one-sample *t*-

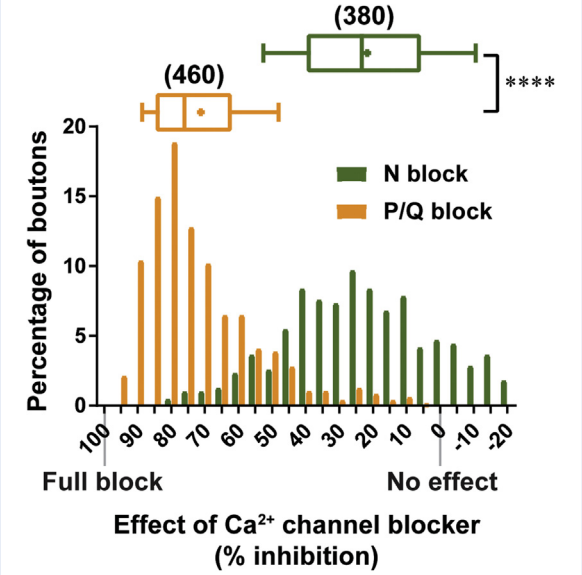

Fig. 4. P/Q-type channels contribute to synaptic vesicle exocytosis to a greater degree than N-type channels at individual boutons. Distribution histogram shows the effects of VGCC blockers in individual boutons from all neurones tested. Boutons with −20% to −60% inhibition were excluded for convenience of presentation. Box whisker plots show the median (line), mean (cross), 25–75 percentile (box), and 10–90 percentile (whisker) ranges for the effects of VGCC blockers across all boutons ($P<0.0001$, Mann–Whitney U-test). VGCC, voltage-gated calcium channel.

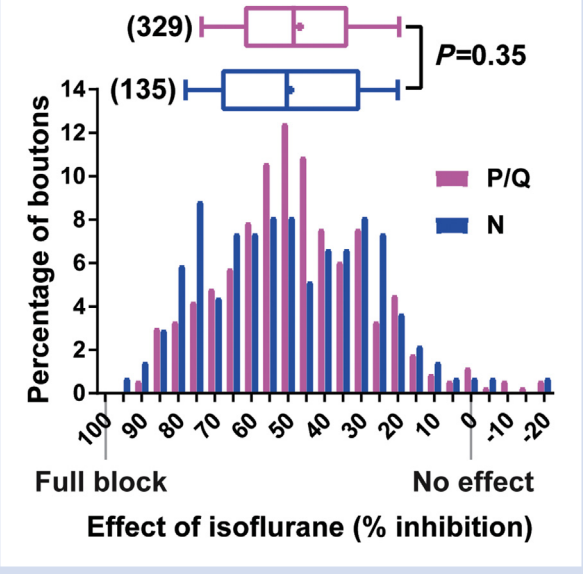

Fig. 5. Isoflurane effects on synaptic vesicle exocytosis mediated by P/Q-type and N-type channels do not differ at the level of individual boutons. Distribution histogram shows the effects of isoflurane after application of P/Q-type or N-type blockers. Box whisker plots show the median (line), mean (cross), 25–75 percentile (box), and 10–90 percentile (whisker) ranges for the effects of isoflurane on SV exocytosis mediated by P/Q- and N-type VGCCs across all boutons (Mann–Whitney U-test). SV, synaptic vesicle; VGCCs, voltage-gated calcium channels.

test against null hypothesis $\mu=0$, $P=0.91$, $t=0.11$; Fig. 6d). Thus, isoflurane inhibition of SV exocytosis in presynaptic hippocampal boutons is independent of expression of the principal presynaptic VGCC subtypes.

Discussion

SV exocytosis from excitatory boutons is more sensitive to inhibition by isoflurane than exocytosis from inhibitory boutons,[4 31] but the mechanism(s) of this synaptic selectivity is unknown. Although P/Q-type VGCCs contributed quantitatively more to SV exocytosis than did N-type VGCCs in rat hippocampal neurones, there was no significant difference in their functional sensitivities to isoflurane. These results indicate that differential expression, or coupling of presynaptic VGCC subtypes to SV exocytosis, or both are not the basis for the synaptic selectivity of isoflurane in hippocampus, which likely involves targets upstream of Ca^{2+} entry.

The greater contribution of P/Q-type VGCCs to SV exocytosis in hippocampal excitatory neurones, evident in the greater degree of inhibition by the specific toxin agatoxin than conotoxin, has been reported by others,[14] although we observed a greater contribution of P/Q-type VGCCs. Electrophysiological studies also show a dominant contribution of P/Q-type VGCCs to neurotransmission in hippocampus.[27 29] In contrast, greater inhibition of SV exocytosis by conotoxin has been reported in cultured neurones from the rat hippocampal CA3–CA1 subregion,[15 32] which might be attributable to subregion-specific differences or days in culture. In immature hippocampal neurones (e.g., before 15 DIV corresponding to postnatal day 10), conotoxin inhibits synaptic transmission more than agatoxin because of a relative predominance of N-type VGCCs. In contrast, later in development (e.g., after 21 DIV corresponding to postnatal day 16), agatoxin leads to greater inhibition of exocytosis as P/Q-type channels predominate.[33 34] Our results are consistent with these findings, although the age, in days, of the rats used to produce the cultures is different. Taken together, these data indicate that expression of VGCC subtypes at synaptic boutons varies depending on factors including neuronal phenotype and culture age.

The basis of the greater sensitivity to inhibition by isoflurane of glutamate release compared with GABA release is unclear.[4 35 36] One hypothesis is that differential expression of critical ion channels with greater anaesthetic sensitivity leads to such differences between synapses. However, our results indicate that differential presynaptic VGCC subtype expression does not contribute to differences in sensitivity of SV exocytosis to isoflurane in hippocampal neurones. These results are consistent with previous electrophysiological studies showing that isoflurane inhibits P/Q- and N-type Ca^{2+} currents to a similar extent in rat hippocampal pyramidal neurones,[9] cultured cerebellar granule neurones,[10] and *Xenopus* oocytes heterologously expressing mammalian N- or P/Q-type channels.[37] In contrast, isoflurane was reported to inhibit N-type currents to a greater extent than P/Q-type currents in rat dorsal root ganglion neurones.[11] The reason for this difference in sensitivity to isoflurane is not clear, although it may reflect differences in channel subunit compositions. The auxiliary

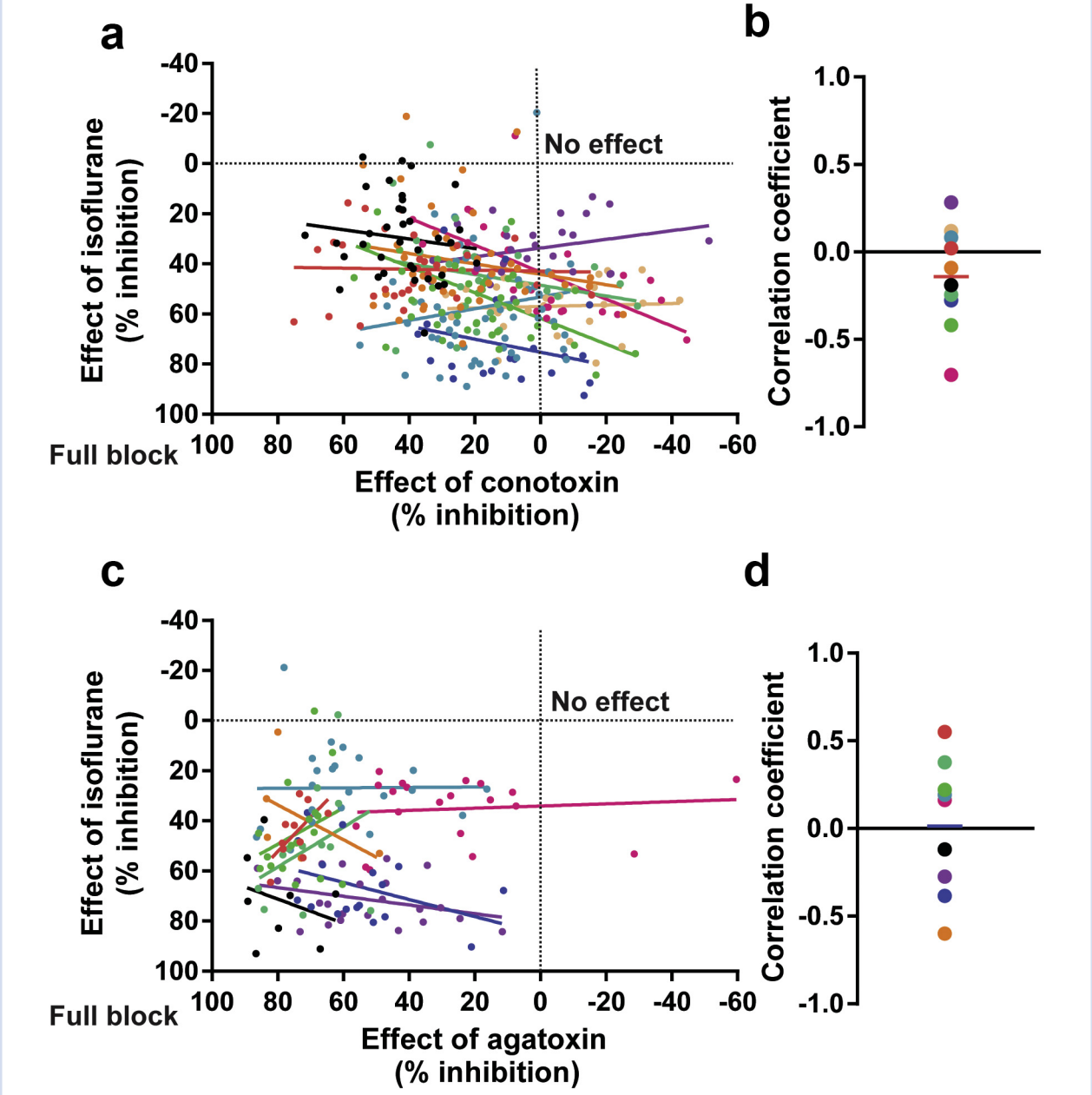

Fig. 6. The effects of isoflurane on synaptic vesicle exocytosis are independent of VGCC subtype distribution. (a) Correlation between the effects of isoflurane and the effects of conotoxin on SV exocytosis. Different colours represent individual neurones, with each dot representing a single bouton. Lines represent best fits for each neurone. (b) Correlation coefficients of the effects of isoflurane with the effects of the N-type VGCC blocker conotoxin were not significantly different from 0 (one-sample *t*-test against null hypothesis $\mu=0$, $P=0.15$, $t=1.56$). Each dot represents the value of the correlation coefficient for each neurone shown in (a), coloured accordingly. The red line shows the average correlation coefficient across all neurones. (c) Correlation between the effects of isoflurane and the effects of agatoxin on SV exocytosis. Different colours represent individual neurones, whereas each dot represents a single bouton. Lines represent best fits for each neurone. (d) Correlation coefficients of the effects of isoflurane with the effects of the P/Q-type VGCC blocker agatoxin were not significantly different from 0 (one-sample *t*-test against null hypothesis $\mu=0$, $P=0.91$, $t=0.11$). Each dot represents the value of the correlation coefficient for each neurone shown in (c), coloured accordingly. The red line shows the average correlation coefficient across all of neurones. VGCC, voltage-gated calcium channel.

subunits of VGCCs are differentially distributed amongst brain regions,[38 39] such that pharmacological properties might vary between brain regions.

Several limitations must be considered in interpreting our results. Experiments were performed at 29–30°C to facilitate electrophysiological recording stability and volatile

anaesthetic use. The kinetics of SV exocytosis and endocytosis are faster at higher physiological temperatures, but exhibit similar fundamental properties at 30°C.[40] Use of SV-targeted pHluorins such as vGlut1-pHlourin is an established and validated method for measuring vesicular exocytosis and endocytosis in live cells.[4 15 25 32] Although this approach does not directly see membrane fusion, as in the classic fast freeze electron micrographs of Heuser and Reese,[41 42] membrane fusion is necessary to neutralise the vesicle lumen to induce the fluorescence change. Live-cell imaging also has the advantage of providing dynamic rather than static imaging of vesicle fusion and re-endocytosis. To determine isoflurane effects, we analysed boutons with SNR $\geq$ 5 in response to 100 APs in the presence of VGCC blockers, which biases data to boutons with larger fluorescent signals. We used a primary hippocampal neurone culture system in which functional synapses are formed.[16–18] Given that expression of VGCC subtypes in individual boutons is known to be determined by factors including the type of postsynaptic cell,[28–30] differential contributions of P/Q- and N-type VGCCs to SV exocytosis between different boutons of the same transfected neurone as observed in this study may result from differences in their postsynaptic targets. Cultured hippocampal neurones consist of various phenotypes,[28 29 43] so the isoflurane effects observed represent a heterogeneous neuronal population. The effects of isoflurane on SV exocytosis in other brain regions with different properties may differ from those observed in hippocampus. Our studies focus on the hippocampus as a model given the large body of data available on its fundamental neurophysiology, sensitivity to anaesthetics, and critical role as a target for anaesthetics.[44 45] Finally, we used a relatively high, but clinically relevant, concentration of isoflurane for these mechanistic studies to maximise detection of possible presynaptic inhibitory effects. It is possible that the lack of inhibition reported by Hall and colleagues[13] was because of the lower concentration they used, but also to the fact that they measured electrophysiological effects on somatic, rather than presynaptic, channels.

Previous studies have shown that inhibition of voltage-gated Na^+ channels has a reduced effect on GABAergic boutons compared with other neurotransmitter phenotypes,[46 47] and voltage-gated Na^+ channel subtypes are differentially expressed in hippocampus.[48] In addition, SV exocytosis evoked by elevated extracellular K^+, which bypasses Na^+ channels activation and activates VGCCs directly,[49] is relatively insensitive to isoflurane.[31 50] Taking these findings into account, voltage-gated Na^+ channel activation upstream of VGCC activation is a plausible mechanism for isoflurane inhibition of SV exocytosis.[51–53] Isoflurane inhibition of the mitochondrial respiratory chain has also been implicated in its presynaptic effects. Isoflurane directly inhibits mitochondrial complex I to limit synaptic ATP production in the excitatory pre-synapse which, in turn, inhibits excitatory SV endocytosis and exocytosis.[54] Given that GABAergic neurone-specific complex I defects do not alter the sensitivity of isoflurane,[55] the role of complex I function in vesicle cycling might be different between excitatory and inhibitory neurones. Future studies of the contributions of voltage-gated Na^+ channels, mitochondrial complex I, or other targets to isoflurane-induced inhibition of SV exocytosis are necessary to clarify further the bouton selectivity of presynaptic isoflurane action.

In conclusion, we show that SV exocytosis linked to N-type and P/Q-type Ca^{2+} channels, the principal neuronal VGCC subtypes that mediate the Ca^{2+} entry coupled to SV exocytosis in the hippocampus, have similar sensitivities to isoflurane. As presynaptic inhibition of SV exocytosis by isoflurane was independent of relative VGCC subtype expression in hippocampal synaptic boutons, we conclude that VGCCs are unlikely to determine the selective sensitivity to inhibition of SV exocytosis by isoflurane in specific neuronal phenotypes.

Authors' contributions

Study supervision: HCH.
Study design/planning: YK, DCC, ZZ, HCH.
Study conduct: YK, DCC, ZZ.
Data analysis: YK, CLT, DCC, HCH.
Writing manuscript: YK, CLT, HCH.
All authors participated in the revision and approved the final version of the manuscript.

Declarations of interests

HCH is the editor-in-chief of the *British Journal of Anaesthesia*. The other authors have no competing interests.

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References

1. MacIver MB. Anesthetic agent-specific effects on synaptic inhibition. *Anesth Analg* 2014; **119**: 558–69
2. Winegar BD, MacIver MB. Isoflurane depresses hippocampal CA1 glutamate nerve terminals without inhibiting fiber volleys. *BMC Neurosci* 2006; **7**: 1–9
3. MacIver MB, Mikulec AA, Amagasu SM, Monroe FA. Volatile anesthetics depress glutamate transmission via presynaptic actions. *Anesthesiology* 1996; **85**: 823–34
4. Baumgart JP, Zhou Z-Y, Hara M, et al. Isoflurane inhibits synaptic vesicle exocytosis through reduced Ca^{2+} influx, not Ca^{2+}–exocytosis coupling. *Proc Natl Acad Sci* 2015; **112**: 11959–64
5. Catterall WA, Few AP. Calcium channel regulation and presynaptic plasticity. *Neuron* 2008; **59**: 882–901
6. Olivera BM, Miljanich GP, Ramachandran J, Adams ME. Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. *Annu Rev Biochem* 1994; **63**: 823–67
7. Wu LG, Saggau P. Pharmacological identification of two types of presynaptic voltage-dependent calcium channels at CA3–CA1 synapses of the hippocampus. *J Neurosci* 1994; **14**: 5613–22
8. Wheeler DB, Randall A, Tsien RW. Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science* 1994; **264**: 107–11
9. Study RE. Isoflurane inhibits multiple voltage-gated calcium currents in hippocampal pyramidal neurons. *Anesthesiology* 1994; **81**: 104–16

10. Lynch III C, Miao N, Nagao K, Pancrazio J. Volatile anesthetic depression of Ca^{2+} entry into and glutamate release from cultured cerebellar granule neurons. *J Anesth Perioper Med* 2017; **4**: 45–59
11. Kameyama K, Aono K, Kitamura K. Isoflurane inhibits neuronal Ca^{2+} channels through enhancement of current inactivation. *Br J Anaesth* 1999; **82**: 402–11
12. White IL, Franks NP, Dickinson R. Effects of isoflurane and xenon on Ba^{2+}-currents mediated by N-type calcium channels. *Br J Anaesth* 2005; **94**: 784–90
13. Hall AC, Lieb WR, Franks NP. Insensitivity of P-type calcium channels to inhalational and intravenous general anesthetics. *Anesthesiology* 1994; **81**: 117–23
14. Hara M, Zhou Z, Hemmings HC. α_2-Adrenergic receptor and isoflurane modulation of presynaptic Ca^{2+} influx and exocytosis in hippocampal neurons. *Anesthesiology* 2016; **125**: 535–46
15. Ariel P, Hoppa MB, Ryan TA. Intrinsic variability in Pv, RRP size, Ca^{2+} channel repertoire, and presynaptic potentiation in individual synaptic boutons. *Front Synaptic Neurosci* 2013; **5**: 1–18
16. Ryan TA. Inhibitors of Myosin light chain kinase block synaptic vesicle pool mobilization during action potential firing. *J Neurosci* 1999; **19**: 1317–23
17. Ryan TA, Smith SJ. Vesicle pool mobilization during action potential firing at hippocampal synapses. *Neuron* 1995; **14**: 983–9
18. Ryan TA, Reuter H, Wendland B, Schweizer FE, Tsien RW, Smith SJ. The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron* 1993; **11**: 713–24
19. Voglmaier SM, Kam K, Yang H, et al. Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. *Neuron* 2006; **51**: 71–84
20. Boland LM, Morrill JA, Bean BP. ω-Conotoxin block of N-type calcium channels in frog and rat sympathetic neurons. *J Neurosci* 1994; **14**: 5011–27
21. Mintz IM, Venema VJ, Swiderek KM, Lee TD, Bean BP, Adams ME. P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature* 1992; **355**: 827–9
22. Ratnakumari L, Hemmings HC. Inhibition of presynaptic sodium channels by halothane. *Anesthesiology* 1998; **88**: 1043–54
23. Franks NP, Lieb WR. Selective actions of volatile general anesthetics at molecular and cellular levels. *Br J Anaesth* 1993; **71**: 65–76
24. Iwai T, Kihara H, Imai K, Uchida M. Dose–response curve for anaesthetics based on the Monod–Wyman–Changeux model. *Br J Anaesth* 1996; **77**: 517–21
25. Ariel P, Ryan TA. Optical mapping of release properties in synapses. *Front Neural Circuits* 2010; **4**: 1–10
26. Kavalali ET, Jorgensen EM. Visualizing presynaptic function. *Nat Neurosci* 2014; **17**: 10–6
27. Takahashi T, Momiyama A. Different types of calcium channels mediate central synaptic transmission. *Nature* 1993; **366**: 156–8
28. Éltes T, Kirizs T, Nusser Z, Holderith N. Target cell type-dependent differences in Ca^{2+} channel function underlie distinct release probabilities at hippocampal glutamatergic terminals. *J Neurosci* 2017; **37**: 1910–24
29. Poncer JC, McKinney RA, Gähwiler BH, Thompson SM. Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. *Neuron* 1997; **18**: 463–72
30. Yamamoto K, Kobayashi M. Opposite roles in short-term plasticity for N-type and P/Q-type voltage-dependent calcium channels in GABAergic neuronal connections in the rat cerebral cortex. *J Neurosci* 2018; **38**: 9814–28
31. Westphalen RI, Kwak NB, Daniels K, Hemmings HC. Regional differences in the effects of isoflurane on neurotransmitter release. *Neuropharmacology* 2011; **61**: 699–706
32. Kim SH, Ryan TA. Balance of calcineurin A and CDK5 activities sets release probability at nerve terminals. *J Neurosci* 2013; **33**: 8937–50
33. Scholz KP, Miller RJ. Presynaptic inhibition at excitatory hippocampal synapses: development and role of presynaptic Ca^{2+} Channels. *J Neurophysiol* 1996; **76**: 39–46
34. Scholz KP, Miller RJ. Developmental changes in presynaptic calcium channels coupled to glutamate release in cultured rat hippocampal neurons. *J Neurosci* 1995; **15**: 4612–7
35. Westphalen RI, Hemmings HC. Volatile anesthetic effects on glutamate versus GABA release from isolated rat cortical nerve terminals: 4-aminopyridine-evoked release. *J Pharmacol Exp Ther* 2006; **316**: 216–23
36. Westphalen RI, Hemmings HC. Selective depression by general anesthetics of glutamate versus GABA release from isolated cortical nerve terminals. *J Pharmacol Exp Ther* 2003; **304**: 1188–96
37. Kamatchi GL, Chan CK, Snutch T, Durieux ME, Lynch C. Volatile anesthetic inhibition of neuronal Ca channel currents expressed in *Xenopus* oocytes. *Brain Res* 1999; **831**: 85–96
38. Buraei Z, Yang J. The beta subunit of voltage-gated calcium channels. *Physiol Rev* 2010; **90**: 1461–506
39. Cole RL, Lechner SM, Williams ME, et al. Differential distribution of voltage-gated calcium channel alpha-2 delta ($\alpha_2\delta$) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia. *J Comp Neurol* 2005; **491**: 246–69
40. Balaji J, Ryan TA. Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. *Proc Natl Acad Sci* 2007; **104**: 20576–81
41. Heuser JE, Reese TS. Structural changes after transmitter release at the frog neuromuscular junction. *J Cell Biol* 1981; **88**: 564–80
42. Heuser JE, Reese TS. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J Cell Biol* 1973; **57**: 315–44
43. Senzai Y, Buzsáki G. Physiological properties and behavioral correlates of hippocampal granule cells and mossy cells. *Neuron* 2017; **93**: 691–704
44. Wang D-S, Orser BA. Inhibition of learning and memory by general anesthetics. *Can J Anaesth* 2011; **58**: 167–77
45. Rudolph U, Antkowiak B. Molecular and neuronal substrates for general anaesthetics. *Nat Rev Neurosci* 2004; **5**: 709–20
46. He Y, Zorumski CF, Mennerick S. Contribution of presynaptic Na^+ channel inactivation to paired-pulse synaptic depression in cultured hippocampal neurons. *J Neurophysiol* 2002; **87**: 925–36
47. Meeks JP, Mennerick S. Selective effects of potassium elevations on glutamate signaling and action potential conduction in hippocampus. *J Neurosci* 2004; **24**: 197–206

48. Johnson KW, Herold KF, Milner TA, Hemmings HC, Platholi J. Sodium channel subtypes are differentially localized to pre- and post-synaptic sites in rat hippocampus. *J Comp Neurol* 2017; **525**: 3563–78
49. Tibbs GR, Barrie AP, Van Mieghem FJE, McMahon HT, Nicholls DG. Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: effects on cytosolic free Ca^{2+} and glutamate release. *J Neurochem* 1989; **53**: 1693–9
50. Hemmings HC, Yan W, Westphalen RI, Ryan TA. The general anesthetic isoflurane depresses synaptic vesicle exocytosis. *Mol Pharmacol* 2005; **67**: 1591–9
51. Herold KF, Andersen OS, Hemmings HC. Divergent effects of anesthetics on lipid bilayer properties and sodium channel function. *Eur Biophys J* 2017; **46**: 617–26
52. Herold KF, Sanford RL, Lee W, et al. Volatile anesthetics inhibit sodium channels without altering bulk lipid bilayer properties. *J Gen Physiol* 2014; **144**: 545–60
53. Herold KF, Hemmings HC. Sodium channels as targets for volatile anesthetics. *Front Pharmacol* 2012; **3**: 1–7
54. Zimin PI, Woods CB, Kayser EB, Ramirez JM, Morgan PG, Sedensky MM. Isoflurane disrupts excitatory neurotransmitter dynamics via inhibition of mitochondrial complex I. *Br J Anaesth* 2018; **120**: 1019–32
55. Zimin PI, Woods CB, Quintana A, Ramirez JM, Morgan PG, Sedensky MM. Glutamatergic neurotransmission links sensitivity to volatile anesthetics with mitochondrial function. *Curr Biol* 2013; **26**: 2194–201

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