Syntaxin1A-mediated Resistance and Hypersensitivity to Isoflurane in *Drosophila melanogaster*

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ABSTRACT

Background: Recent evidence suggests that general anesthetics activate endogenous sleep pathways, yet this mechanism cannot explain the entirety of general anesthesia. General anesthetics could disrupt synaptic release processes, as previous work in *Caenorhabditis elegans* and *in vitro* cell preparations suggested a role for the soluble NSF attachment protein receptor protein, syntaxin1A, in mediating resistance to several general anesthetics. The authors questioned whether the syntaxin1A-mediated effects found in these reductionist systems reflected a common anesthetic mechanism distinct from sleep-related processes.

Methods: Using the fruit fly model, *Drosophila melanogaster*, the authors investigated the relevance of syntaxin1A manipulations to general anesthesia. The authors used different behavioral and electrophysiological endpoints to test the effect of syntaxin1A mutations on sensitivity to isoflurane.

Results: The authors found two syntaxin1A mutations that confer opposite general anesthesia phenotypes: *syxH3-C*, a 14-amino acid deletion mutant, is resistant to isoflurane (n = 40 flies), and *syxKARRAA*, a strain with two amino acid substitutions, is hypersensitive to the drug (n = 40 flies). Crucially, these opposing effects are maintained across different behavioral endpoints and life stages. The authors determined the isoflurane sensitivity of *syxH3-C* at the larval neuromuscular junction to assess effects on synaptic release. The authors find that although isoflurane slightly attenuates synaptic release in wild-type animals (n = 8), *syxH3-C* preserves synaptic release in the presence of isoflurane (n = 8).

Conclusion: The study results are evidence that volatile general anesthetics target synaptic release mechanisms; in addition to first activating sleep pathways, a major consequence of these drugs may be to decrease the efficacy of neurotransmission.
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ELUCIDATING how general anesthetics work has been an ongoing debate in medical research.[1] Why has uncovering the mechanism of general anesthesia remained problematic even though some molecular targets have now been identified[2]? One reason perhaps relates to teasing apart the plethora of effects that general anesthetics have on various targets in the brain, both at the molecular and circuit levels.[2] Recent evidence suggests that there is a relation between endogenous sleep pathways and general anesthesia. General anesthetics may take effect through arousal pathways *via* disinhibition (or activation) of sleep-promoting circuits.[2–8] Consistent with this idea is evidence that general anesthetics, particularly intravenous anesthetics such as propofol, can potentiate γ-aminobutyric acid (GABA) type A receptors,[9–12] and the arousal centers in the brain are inhibited by GABAergic input from sleep-promoting centers.[6] Yet, not all animals sleep[13] but all animals can be rendered unresponsive by general anesthetics.[14] This suggests that other anesthetic mechanisms might exist in addition to those targeting GABAergic sleep–arousal pathways. Also, general anesthetics promote a more profound loss of responsiveness than can ever be achieved by sleep, so other mechanisms clearly must be involved.

What We Already Know about This Topic

- Volatile anesthetics impair transmitter release from glutamatergic synapses
- In nematodes, mutations in syntaxin1A, a protein involved in the synaptic transmitter release machinery, resulted in resistance and hypersensitivity to general anesthetics

What This Article Tells Us That Is New

- Isoflurane targets synaptic release mechanisms in addition to sleep pathways in flies
- Different mutations in syntaxin1A confer resistance and hypersensitivity across multiple behavioral and electrophysiological endpoints in flies

Another mechanism through which general anesthetics could act is by disrupting neuronal communication, by directly targeting synaptic transmission. In the mouse hippocampus, the volatile anesthetic halothane has been shown to

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impair transmitter release from glutamatergic synapses.[15] This decrease in glutamate release is independent of any increase in GABAergic transmission,[16] indicating that anesthetics are affecting glutamate release directly. Such decreased release could reflect anesthetic action on the transmitter release machinery. Indeed, a genetic screen for anesthetic sensitivity in the nematode model *Caenorhabditis elegans* identified specific mutations in the synaptic protein syntaxin1A that produced animals that were either resistant or hypersensitive to volatile anesthetics,[17] suggesting that volatile general anesthetics may directly target the neurotransmitter release machinery. In particular, a syntaxin1A isoform containing a deletion in the H3 domain of the protein was found to confer a high level of resistance to isoflurane and halothane.[17] An equivalent mutation engineered in mammalian cell lines was found to reduce the effects of isoflurane[18] and propofol[19] on transmitter release, suggesting that syntaxin1A is central to general anesthetic mechanisms. Because the H3 domain of syntaxin1A is extremely conserved across all animals,[20] we hypothesized that these anesthetic resistance effects on the transmitter release machinery are likely to be preserved across species, representing a conserved target mechanism for general anesthesia, in addition to sleep-related mechanisms.

General anesthetics first produce unconsciousness by activating endogenous sleep pathways. Accordingly, in previous work, we have identified a sleep–wake pathway that controls sensitivity to isoflurane in *Drosophila melanogaster.*[3] In this study, we investigate synaptic release as an alternate anesthetic mechanism, by assaying the effects of two mutations in the H3 domain of syntaxin1A, across different behavioral and electrophysiological endpoints. We found that these syntaxin1A mutations produced both resistance and hypersensitivity, mirroring the nematode results. Remarkably, syntaxin1A-induced resistance to isoflurane was observed across all endpoints, ranging from behaviors in adults and larvae to effects at the larval neuromuscular junction (NMJ). Our results suggest that syntaxin1A-mediated neurotransmitter release represents a parallel target process of general anesthetics that is independent of species-specific sleep circuitry.

Materials and Methods

Fly Stocks

D. melanogaster were cultured on a yeast–sugar–agar medium in vials at 25°C on a 12-h light–dark cycle. Female flies (3 to 5 days old) were selected at random for behavioral experiments by brief carbon dioxide exposure and kept in food vials overnight before experiments. The control strains used in this study were wild-type *Canton-S* strain (*CS*) (Bloomington Stock Center, USA) and *isoCJ1*.[21,22] The syntaxin1A mutants used in this study have been described previously: the deletion mutant, *syxH3-C*[23] and *syxKARRAA*.[24] These strains express a syntaxin1A-mutant protein in a heterozygous null *syx*^{229} background: *syxH3*-C, genotype: yw; P(syx[H3-C]); syx1A$^{\Delta 229}$/TM6 (gift from Hugo Bellen, Ph.D., Department of Molecular and Human Genetics and Neuroscience, Howard Hughes Medical Institute, Houston, Texas); *syxKARRAA*, genotype: yw;P[CaryP]attP40 (HA-syx1A^{KARRAA}); syx1A$^{\Delta 229}$/TM6b. The genetic control strain for *syxKARRAA* is *syxWT*: yw;P[CaryP]attP40 (HA-syx1A^{WT}); syx1A$^{\Delta 229}$/TM6b (gifts from Patrik Verstreken, Ph.D., V.I.B. Center for the Biology of Disease, Leuven, Belgium). Syntaxin1A mutations were also placed on a common wild-type background (isolated from the null background) by outcrossing for five generations to *isoCJ1*.

Western Blots

Flies were frozen in dry ice, and their heads collected with a sieve (no. 25 and no. 40; Thermo Fisher, Australia). Heads were homogenized in sample buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 12.5 mM EDTA, 1 mM dithiothreitol, plus protease inhibitor cocktail [Roche, Australia]). Proteins were added to commercial sample buffer before sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (NuPage LDS Sample Buffer; Life Technologies, Australia). Proteins were separated using a 12% NuPage polyacrylamide gel (Life Technologies) with buffers prepared and protocol followed as per the manufacturer's recommendations. After separation, the proteins were transferred to a polyvinylidine fluoride membrane (Merck Millipore, Australia) in transfer buffer (25 mM Tris, 0.2 M glycine, and 20% v/v methanol). The transfer apparatus was assembled to allow the proteins to transfer to the polyvinylidene fluoride membrane using settings recommended by the manufacturer. Proteins were detected using a syntaxin antibody (mouse anti-8c3, 1:1,000; Developmental Studies Hybridoma Bank, USA).

Behavioral Assays

Coordination Assay. The fly coordination assay was developed from previous *Drosophila* general anesthesia assays.[25,26] Approximately 10 flies are loaded into 100 ml cylindrical glass tubes (length 20 cm and diameter 2.5 cm) with a side arm (Pyrex, USA), custom fit with a rubber stopper. Into the side arm of the tube, 3 μl isoflurane (Attane; Baxter Healthcare, Australia) was added using a Hamilton syringe (Hamilton Company, USA), which corresponds to 0.15 vol% isoflurane as quantified by gas chromatography (table 1). Experiments with halothane (Sigma-Aldrich, Australia),

Table 1. Gas Chromatography Concentrations for Isoflurane (Volume % Atmospheres) in Fly Coordination Assay and Larval Anesthesia Assay

Microliter Volume	Concentration ± SEM
1	0.02 vol% ± 0.01
2	0.09 vol% ± 0.01
3	0.15 vol% ± 0.004
3.5	0.56 vol% ± 0.02
4	0.9 vol% ± 0.028
5	1.5 vol% ± 0.002

which is more potent than isoflurane, were performed with 2 μl injected as a liquid. Immediately after anesthetic injection, the number of flies in the bottom 2 cm of the tube (and therefore unable to hold on to or climb onto the sides) was counted every 10 s. This was repeated until the last fly had dropped to the bottom of the tube.

Startle Assay. The startle-induced locomotion assay assesses flies' movement response to a startle-inducing vibration stimulus and has been described previously.[3] Flies are loaded into individual glass tubes (Trikinetics, USA; length 65 mm and diameter 3 mm), with paper and cotton rolled together on either end of the tubes to allow anesthetic gas to reach the fly. Tubes are placed on two custom-made scaffolds set into a closed chamber at least 15 min before the beginning of an experiment. Each scaffold apparatus holds 20 tubes, enabling comparison of a total of 40 flies per experiment. Startle stimuli were delivered using four shaft-less vibrating motors (model 312-101, Precision Microdrives, United Kingdom). Stimulus intensities were controlled using a custom MATLAB program (Mathworks, USA) (*Drosophila* Arousal Tracking[27]) interfacing with the analog output channels of a USB data acquisition device (Measurement Computing, USA). The startling stimulus used in this study was a 5 × 200 ms vibration set at 1.3 g, delivered every 1 s. The startle stimulus amplitude and sequence chosen was optimized for the common *isoCJ1* genetic background strain (see Supplemental Digital Content 1, fig. 1, http://links.lww.com/ALN/B139, for responses of flies to startle stimulus presentations without isoflurane gas).

Fly activity is filmed continuously using a webcam (5 frames per second; Logitech, Australia) all throughout the duration of an experiment (approximately 70 min). The experimental protocol consists of baseline and startle behavioral metrics, which are both measured as the velocity of the flies (mm/s) during a 1-min period immediately before and after the stimulus respectively. Baseline reflects the general locomotion capabilities of the fly (in air and at different drug concentrations), and the startle is the movement of the flies after the vibration stimulus. During each anesthesia trial, baseline is defined as the 1 min before the vibration stimulus, and the startle response is defined as the 1 min of fly activity immediately after the vibration stimulus. After this 1 min of locomotion activity, the concentration of isoflurane gas is increased until the concentration reaches 1 vol% isoflurane. For each experiment, the concentrations used were 0, 0.12, 0.25, 0.37, 0.5, 0.75, and 1 vol% isoflurane. The startle stimulus is delivered automatically every 10 min until the end of the experiment. Data were extracted and analyzed using custom MATLAB software.[27]

Larval Anesthesia Assay. Nine third-instar larvae were brushed from food vials and pipetted into the bottom of 100 ml cylindrical glass tubes (length 20 cm and diameter 2.5 cm; Pyrex). Larvae were allowed to recover for 1 min after this manipulation, during which time they typically began crawling up the sides of the tube. After this 1-min recovery period, a set volume of volatile anesthetic (isoflurane or halothane) was added into the side arm of the tube using a Hamilton syringe (Hamilton Company). After 4 min of anesthesia equilibration time, the larvae's position was circled with a marker on the outside of the glass. After a further 1 min, the number of larvae that had moved at least one-body-length outside the marked circle was noted, and data converted to express the number of larvae that moved as a proportion of the total.

Sleep Analysis and Arousal Probing. Locomotor activity during several days was monitored using similar materials as per the startle-induced locomotion assay (see Materials and Methods, Startle Assay). Flies were loaded into individual tubes and placed into behavioral scaffolds holding 17 tubes per apparatus. Tubes were sealed with food capped with wax and rolled cotton. The behavioral apparatus was placed into a temperature-controlled incubator set to 24°C with 12-h light–dark cycle to study fly activity during 3 consecutive days. Fly activity and responsiveness to startle vibration stimuli is monitored as described for the startle-induced locomotion assay. In brief, fly activity is filmed continuously with a webcam (Logitech). Custom MATLAB software was used to deliver a 5 × 200 ms vibration pulse to the flies every hour across the experiment duration. The same software package[27] was used to analyze sleep duration metrics and fly responsiveness to the periodic vibration stimuli. Sleep was determined as 5 min or more without activity,[28] allowing for cumulative sleep bouts to be tallied across multiple days and nights. The responsiveness to vibration stimuli was characterized as an average velocity curve, normalized to baseline locomotion.[27]

Isoflurane Delivery and Quantification. For the startle assay, humidified isoflurane gas was delivered to the sealed chamber by an isoflurane evaporator (Mediquip, Australia) under a constant flow of 2.5 l/min, and gas was vacuumed out of the chamber to ensure a constant gas flow and pressure. Isoflurane should equilibrate within fly tissue in less than 1 min.[29] The concentration delivered into the behavioral chamber from the evaporator was verified using gas chromatography as described previously.[3]

Isoflurane concentrations in saline at the NMJ was determined by gas chromatographic headspace analysis (PerkinElmer Clarus 680 GC-FID; Perkin Elmer, USA), performed as described previously.[30] In brief, 1 ml of perfusate was placed into 10 ml headspace vials and sealed immediately with lids containing a polytetrafluoroethylene septum. Samples were heated to 60°C, and 1 ml of headspace gas was injected into the gas chromatograph *via* an autosampler. All samples were analyzed in duplicate. The concentration of isoflurane was determined by comparing to a saturated isoflurane solution.

Electrophysiology. Sharp intracellular recordings were made from the larval NMJ as described previously.[31] Wandering third-instar larvae were dissected in ice-cold Schneider insect medium (Sigma-Aldrich) and pinned onto glass dissection plates to expose the body wall muscles. Intracellular electrodes (50 to 80 MΩ) were filled with a 2:1 mixture

of 3 M potassium chloride and 3 M potassium acetate. Recordings were conducted at room temperature in HL3 hemolymph-like solution[32,33] with $[Ca^{2+}]$ = 0.7 mM and $[Mg^{2+}]$ = 20 mM, from muscle 6, abdominal segment A3. Analysis was performed on recordings with membrane potentials lower than −65 mV. Data for calcium dependence of transmitter release included experiments performed in $[Ca^{2+}]$ of 0.5 and 0.6 mM.

Signals from intracellular recordings were amplified using an Axoclamp2B amplifier (Molecular Devices, USA) in bridge mode. Signals were captured and stored using the Chart software (v.5.5.4; 2-kHz sampling rate) and hardware incorporated with the PowerLab/4s data acquisition system (ADInstruments, Australia).

Isoflurane solutions were prepared as described previously.[30] HL3 saline was placed into a 20 ml vial, and a set volume of isoflurane was added to the saline using a Hamilton syringe (Hamilton Company) and immediately vortexed for 1 min. Saline was placed into a syringe and perfused onto the dissected larvae using a syringe pump (KD Scientific, USA) at a rate of approximately 1 ml/min with Teflon tubing (2 mm inner diameter; Gecko Optical, Australia). Recordings begin with 3 min of baseline excitatory junctional potentials (EJPs), stimulated at a frequency of 1 Hz. Isoflurane perfusion is then initiated and continues until the recording has lasted 10 min or the muscles start contracting[30] and the impalement is lost, whichever occurs first.

Statistical Analyses

All statistical comparisons were performed using Prism (GraphPad, USA). It was not feasible to blind experimenters to fly genotypes. Sample sizes for all assays were selected based on previous experience.[3] The number of reported animals was the same as the number tested for all experiments.

Coordination Assay. Data were converted to express the number of flies in the bottom of the tube as a proportion and tested for normality using the Lilliefors test.[34] The mean time for 50% of the flies to fall to the bottom was compared across genotypes. Normally distributed data was tested for significance ($P < 0.05$) by two-tailed t test comparing means. Otherwise, for the nonparametric data, a Mann–Whitney test for unpaired comparisons was used.

Startle Assay. Velocity data for the flies were normalized to the behavior at 0 vol% isoflurane to enable comparisons across experiments. Data were fit by nonlinear regression (Prism 6; GraphPad) to estimate an EC_{50} and standard error of the estimate. The following logistic equation was used for curve fitting: $Y = \text{Min} + (\text{Max} - \text{Min}) / (1 + 10^{((\text{LogEC50} - X) \times \text{HillSlope})})$, with Y representing the behavioral response and X the gas concentration. The EC_{50} represents the isoflurane concentration at which the behavior is half-maximal and was calculated using the best-fit parameters for each genotype to give the lowest standard error of the estimate. This was obtained by constraining the maximum value to 1 and the minimum value to 0. Separate curves were compared for significant differences by simultaneous curve fitting, where all data are fit together while constraining the EC_{50} to be shared,[35] with significance indicating rejection of the null hypothesis that both datasets share a common EC_{50} ($P < 0.05$). EC_{50} data represent isoflurane volume % atmospheres mean ± standard error of the estimate with 95% CIs reported.

Larval Anesthesia Assay. Data were tested for normality using the Lilliefors test.[34] A two-tailed, unpaired t test was used to compare experimental and control strains with normal distributions. Otherwise, for nonparametric comparisons, the Mann–Whitney test was used for unpaired comparisons, with significance thresholds set at P value less than 0.05.

NMJ Analyses. Recordings were processed in Axograph X (version 1.5.4; Axon Instruments, Inc., USA) to obtain the amplitude and baseline offset of EJPs and spontaneous miniature EJPs (mEJP). To process recordings, a template function was created following instructions in the Axograph manual. This template was used to process all recordings. The signal-to-noise ratio was set to 3.5, and this value was determined by comparing Axograph measures to those obtained with manual measures obtained in Chart (ADInstruments, cursor comments function). Values obtained from the two different analyses were not significantly different. Quantal content was calculated by dividing the mean EJP amplitude by the mean mEJP amplitude. Evoked responses were corrected for nonlinear summation[36] before calculations. Tests for significant differences between control and isoflurane perfusion were conducted using one-way ANOVA with Dunnett multiple comparisons test. To test for significant differences between genotypes under isoflurane perfusion, two-way ANOVA with Sidak multiple comparison test was used. Differences were considered significant at P value less than 0.05. To analyze the calcium dependence of transmitter release, quantal content and calcium concentrations were plotted on log–log scales, with slopes of regression fitted to these points.

Results

Syntaxin1A Mutations Produce Isoflurane Resistance and Hypersensitivity in Adult Flies

Evidence from both nematodes[17] and mammalian cell lines[18] suggests that a target site for volatile general anesthetics might involve the transmitter release machinery, in particular, the H3 domain of the protein syntaxin1A (fig. 1A). We investigated the relevance of syntaxin1A to anesthetic sensitivity in the fruit fly, *D. melanogaster*. Two mutant strains were acquired in which regions of the H3 domain of syntaxin1A were modified: *syxH3-C*,[23] which contains a deletion of 14 amino acids in the C-terminal region of the H3 domain, and *syxKARRAA*,[24] a strain with two amino acid substitutions in the H3 domain (fig. 1, A and B). These strains therefore express both mutant syntaxin1A protein and wild-type syntaxin1A protein. To verify the expression of the syntaxin1A mutations in these fly strains, we performed Western blot in adult animals (fig. 1A). *syxH3-C* mutants expressed

wild-type syntaxin1A protein (33 kDa) and a syntaxin1A protein of smaller size, consistent with predicted size of the deletion protein. In previous *C. elegans* and cell culture studies, coexpression of mutant syntaxin1A containing H3 deletions resulted in anesthesia resistance,[17,18] whereas amino acid substitutions in the H3 domain produced anesthesia hypersensitivity in *C. elegans*.[17]

To investigate whether syntaxin1A mutations might modulate general anesthesia in *Drosophila*, we tested flies in the startle-induced locomotion assay[3] (fig. 1C). Two anesthesia endpoints were derived from this assay: baseline locomotion and startle-induced locomotion (fig. 1D). In air and under low concentrations of isoflurane (>0.25 vol %), wild-type (*CS*) flies walked significantly faster after the vibration stimulus compared with before the stimulus ($P < 0.0001$, *t* test, see Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/B139, for raw data baseline and startle behavioral responses in wild-type flies). This significant increase in locomotion after the vibration stimulus is lost at 0.37 vol% isoflurane (see Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/B139). The startle-induced locomotion endpoint EC_{50} is 0.30 ± 0.005 (95% CI, 0.29 to 0.31; n = 60 flies) and is more sensitive to the effects of isoflurane than baseline locomotion (EC_{50} = 0.35 ± 0.01; 95% CI, 0.32 to 0.38; n = 60 flies). The EC_{50} for startle-induced locomotion is significantly lower ($P < 0.01$; fig. 1D), indicating that this form of behavioral responsiveness is a more sensitive anesthesia endpoint than baseline locomotion.

We used the startle-induced locomotion endpoint to characterize the isoflurane sensitivity of the syntaxin1A-mutant strains *syxH3-C* and *syxKARRAA*. We found that *syxH3-C* was resistant to isoflurane compared with the common genetic background (syx^{229}/+, a syntaxin1A null mutation[37]) (EC_{50} = 0.33 ± 0.01; 95% CI, 0.30 to 0.37; n = 40; $P < 0.001$; fig. 1E). In contrast, *syxKARRAA* was found to be hypersensitive compared in the same genetic background (EC_{50} = 0.24 ± 0.005; 95% CI, 0.22 to 0.26; n = 40; $P < 0.001$; fig. 1E).

To exclude any effects of the syx^{229}/+ genetic background, we outcrossed the two different syntaxin1A-mutant strains to a common wild-type genetic background (*isoCJ1*) and tested these flies for isoflurane sensitivity in the startle-induced locomotion assay. We found a consistency of general anesthetic effects in the *isoCJ1* background for the startle endpoint, with *syxH3-C* still showing resistance to isoflurane (EC_{50} = 0.28 ± 0.004; 95% CI, 0.27 to 0.30; n = 40; $P < 0.0001$; fig. 1F) and *syxKARRAA* still being hypersensitive compared with the *isoCJ1* control strain (EC_{50} = 0.24 ± 0.008; 95% CI, 0.20 to 0.27; n = 40; $P < 0.01$; fig. 1F). This confirms that the isoflurane resistance and hypersensitivity phenotypes observed in these strains are respectively attributable to the coexpressed syntaxin1A mutations, *syxH3-C* and *syxKARRAA*. When testing the overall responsiveness of either strain to the startle stimulus, we find both mutants are strongly responsive to the vibration stimulus (fig. 1, G and H). The isoflurane hypersensitive strain, *syxKARRAA*, is actually even more responsive than the resistant strain *syxH3-C* ($P < 0.01$, *t* test; fig. 1H). This excludes the possibility that the resistance or hypersensitivity effects stem from different locomotion or responsiveness levels in the absence of the drug. Rather, these are anesthesia-specific effects.

We next addressed whether the syntaxin1A-mediated resistance and hypersensitivity effects were specific to the startle endpoint (thereby indicating behavioral responsiveness circuits[38] as possible targets), or if these same anesthesia phenotypes were present for baseline locomotion. We found *syxH3-C* was also resistant to isoflurane for baseline locomotion compared with *isoCJ1* (EC_{50} = 0.35 ± 0.01; 95% CI, 0.32 to 0.39; n = 40; $P < 0.001$; fig. 2A) and *syxKARRAA* was also hypersensitive for baseline locomotion compared in the *isoCJ1* background (EC_{50} = 0.30 ± 0.01; 95% CI, 0.27 to 0.33; n = 40; $P < 0.01$; fig. 2A). This suggests that the syntaxin1A effects on isoflurane sensitivity are not restricted to a circuit relating only to behavioral responsiveness.

To further explore baseline behavioral capabilities of flies under general anesthesia, we devised a fly coordination assay (fig. 2B). This simple general anesthesia assay assessed a fly's capability to display negative geotaxis and move upwards against gravity, displaying climbing behavior.[25] This assay again confirmed our anesthesia phenotypes found for the other endpoints: in the *isoCJ1* background, flies expressing the *syxH3-C* protein were resistant to isoflurane (mean time for 50% to fall to bottom: 113 ± 71 s SD) and halothane (mean time for 50% to fall to bottom: 116 ± 85 s SD), with these flies taking significantly longer to fall to the bottom of the tube ($P < 0.05$, *t* test; fig. 2, C and D). In contrast, *syxKARRAA* are hypersensitive to isoflurane compared with the *isoCJ1* control (mean time for 50% to fall to bottom: 26 ± 13 s SD, $P < 0.05$, *t* test; fig. 2C). *syxKARRAA* shows no change in sensitivity to the more potent volatile anesthetic, halothane, compared with controls ($P = 0.78$, *t* test; fig. 2D). Taken together, our general anesthesia results suggest that the syntaxin1A mutations modulate different behavioral capabilities under general anesthesia rather than any specific behavior.

We wondered whether the opposing anesthesia phenotypes in *syxH3-C* and *syxKARRAA* reflected indirect effects on the sleep circuitry. In previous work, we found a negative correlation between sleep and general anesthesia: flies that were resistant to isoflurane slept less during several days and nights, and flies that were hypersensitive to isoflurane slept more.[3] This strong correlation was found in strains that carried specific genetic manipulations directly targeting particular circuits within the fly brain, including the sleep–wake circuitry. We questioned whether the syntaxin1A mutants would also show a negative correlation between sleep and general anesthesia because these strains expressed syntaxin1A mutations not only in the sleep–wake circuitry but also in all neurons. Consistent with the correlation,[3] we found that *syxH3-C* which is resistant to isoflurane slept less than the

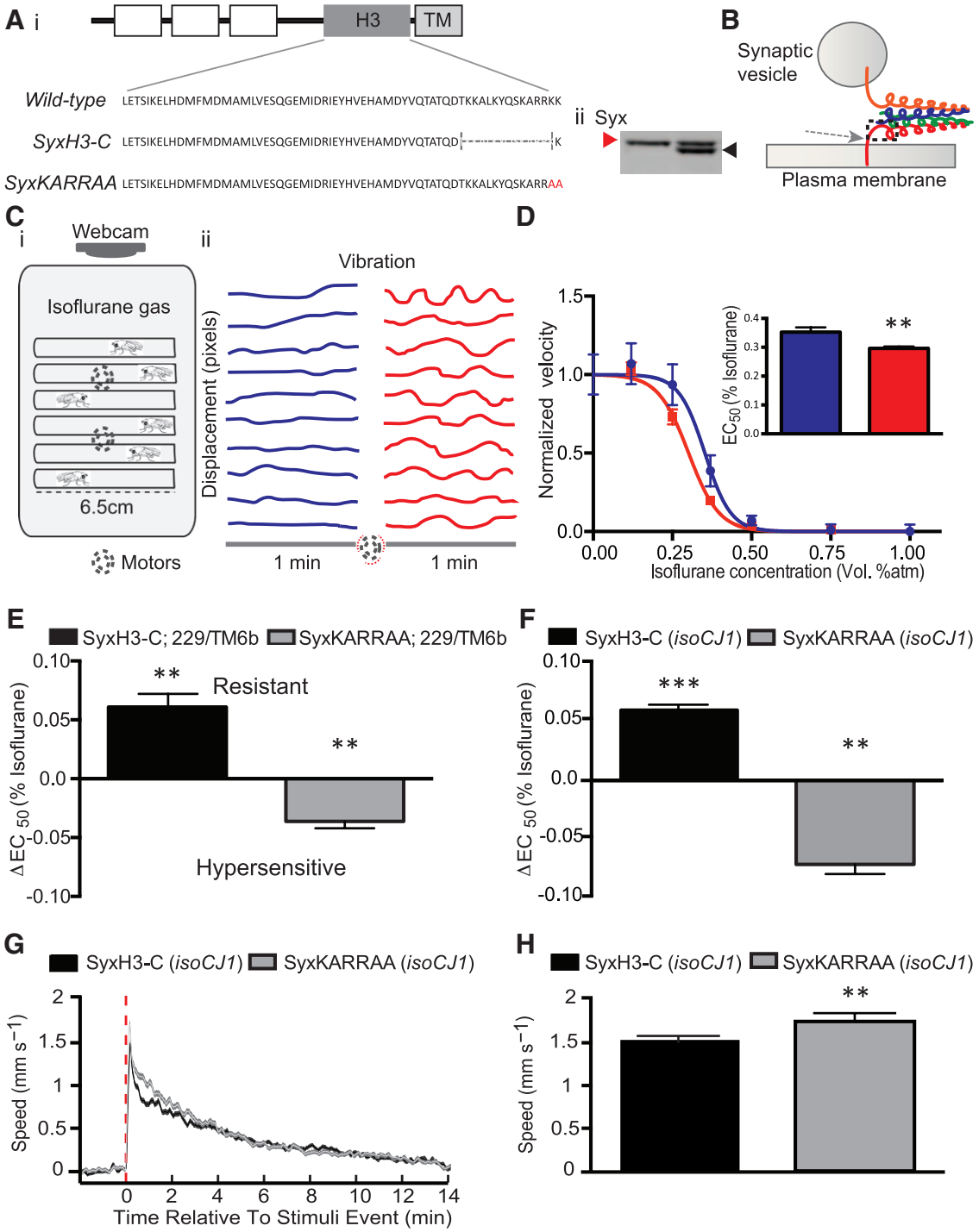

Fig. 1. Syntaxin1A mutations produce isoflurane resistance and hypersensitivity in *Drosophila*. (*A*) i: Syntaxin1A protein domains, with H3 domain expanded below showing amino acid residues in wild-type, *syxH3-C*, and *syxKARRAA*. These strains coexpress mutant syntaxin1A protein in addition to the wild-type protein. *syxH3-C* is a deletion of 14 amino acid residues.[23] *syxKARRAA* makes two amino acid substitutions from lysine to alanine, in *red*.[24] ii: Western blot of syntaxin1A protein expression in wild-type (*Canton-S, [CS], first lane*) and *syxH3-C* (*second lane*), denoting endogenous syntaxin1A protein (*red arrowhead*) and a smaller deletion protein produced in *syxH3-C* (*black arrowhead*). (*B*) Schematic of synaptic vesicle and proteins required to form a core complex for synaptic release. *Red* = syntaxin1A, *blue/green* = SNAP-25 (synaptosomal-associated protein, 25 kDa), *orange* = synaptobrevin. *Black dashed box* denotes approximate position of the *syxH3-C* deletion strain, and *gray dashed arrow* denotes position of amino acid substitutions in *syxKARRAA*. (*C*) i: Schematic of isoflurane anesthesia apparatus. Flies placed in individual glass tubes are presented with vibration stimuli delivered by motors (*dashed circles*) underneath the behavioral scaffold, which is enclosed in a chamber. Fly locomotion is monitored with a webcam. ii: A representative trace of fly locomotion before (*blue*) and after (*red*) the vibration stimulus (y-axis denotes horizontal displacement in the tube (pixels) and x-axis denotes time).

control strain (*isoCJ1)* (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/B139, for sleep profiles of *isoCJ1* and *syxH3-C* and quantification of time spent asleep). Unexpectedly, sleep duration was not increased in the hypersensitive *syxKARRAA* strain compared with controls (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/B139, for sleep profile of *syxKARRAA* and quantification of time spent asleep). This suggests that syntaxin1A-mediated effects on isoflurane sensitivity do not fit the model predicted by sleep–wake circuits on general anesthesia although the resistance effects still match the prediction.

Both *syxH3-C* and *syxKARRAA* modulate isoflurane sensitivity in a wild-type background (*isoCJ1*), suggesting a gain of function (or neomorphic) effect. We determined whether one copy of the mutant syntaxin1A protein was therefore sufficient to produce resistance or hypersensitivity to isoflurane in adult flies. After verifying that these flies still expressed the syntaxin1A deletion protein through Western blot (fig. 3A), we found that flies expressing only one copy of *syxH3-C* were as resistant to isoflurane as the homozygous strain (EC_{50} = 0.28 ± 0.007; 95% CI, 0.26 to 0.30; n = 40; $P < 0.001$; fig. 3B). Interestingly, *syxKARRAA* is also dominant, with heterozygotes remaining as hypersensitive compared with genetic controls (EC_{50} = 0.25 ± 0.01; 95% CI, 0.24 to 0.26; n = 40; $P < 0.01$; fig. 3B). These results show that both syntaxin1A lesions are dominant and that half the amount of either mutant protein is sufficient to confer either opposing phenotype.

Given both syntaxin1A lesions act dominantly, this raises the question: which general anesthesia phenotype will predominate if both resistant and hypersensitive syntaxin1A alleles are combined? We tested flies carrying both syntaxin1A mutations as a transheterozygote (on an *isoCJ1* wild-type background). We found that flies carrying both *syxH3-C* and *syxKARRAA* are resistant, showing no significant difference compared with *syxH3-C*/+ (EC_{50} = 0.28 ± 0.008; 95% CI, 0.25 to 0.30; n = 40; $P = 0.719$; fig. 3B).

Syntaxin1A Mutations Modulate Sensitivity to General Anesthetics in Larvae

Larvae are an immature stage of the flies' lifecycle, with a different central nervous system compared with the adult fly[39,40] and probably lacking the sleep-promoting circuits found in adults.[41] We questioned whether the syntaxin1A mutations would also confer general anesthetic resistance and hypersensitivity as larvae. The syntaxin1A deletion protein is expressed in larvae (fig. 4A), so effects at this early stage might indicate a common mechanism of action for general anesthetics that does not encompass a developed sleep circuitry.[3,21,42]

We devised a coordination assay to test larval behavior under general anesthesia (fig. 4A, and see Materials and Methods). Under increasing concentrations of isoflurane, the proportion of wild-type (*isoCJ1*) larvae that can display coordinated movement decreased significantly ($P < 0.01$, *t* test; fig. 4B). We found that *syxH3-C* were also resistant as larvae: a greater proportion of *syxH3-C* larvae (on the *isoCJ1* background) were capable of coordinated movement compared with the *isoCJ1* controls ($P < 0.01$, *t* test; fig. 4C). Also, consistent with our observations in adult flies, *syxKARRAA* larvae remain hypersensitive to isoflurane compared with *isoCJ1* controls ($P < 0.01$, *t* test; fig. 4C). These experiments show that the syntaxin1A-mediated effects are independent of the life stage, and therefore, do not require adult-specific circuitry, such as sleep–wake promoting pathways.[3,21,42] Importantly, resistance and hypersensitivity also were evident when these syntaxin1A mutants were exposed to another volatile anesthetic, halothane ($P < 0.05$, *t* test; fig. 4D). Thus, our behavioral analysis shows that syntaxin1A mutations produce consistent anesthesia phenotypes across the *Drosophila* life cycle in animals with vastly different brains and sleep requirements. This suggests that the syntaxin1A-mediated effects on isoflurane sensitivity are unlikely to involve interactions with the sleep–wake pathway that has been identified in adult flies.[3,21,42] Instead, a process common to adult and larval nervous systems must be involved.

Isoflurane Decreases Transmitter Release from Wild-type Synapses

To investigate whether the decrease in larval coordination under isoflurane reflected a change in transmitter release,

Fig. 1. (*Continued*) (*D*) Nonlinear regression of normalized baseline (*blue*) and startle-induced velocity (*red*) ± SEM under increasing isoflurane concentrations (vol% atm). *Inset*: estimated EC_{50} ± standard error of the estimate (SEE) for baseline (*blue*) and startle-induced velocity (*red*) in wild-type female flies (n = 60). The EC_{50} represents the concentration at which the velocity of the flies is half-maximal. The startle endpoint is significantly lower than the baseline endpoint. $^{**}P < 0.01$, calculated by extra sum-of-squares *F* test between estimated EC_{50} (n = 60). (*E*) ΔEC_{50} ± SEE (isoflurane vol% atm) for the startle-induced velocity endpoint in *syxH3-C* (*black*) and *syxKARRAA* (*gray*) in a heterozygous null syx^{229} background ($syx1A^{\Delta 229}$/*TM6b*). ΔEC_{50} is calculated by subtracting the genetic background EC_{50} ($syx1A^{\Delta 229}$/*TM6b*) from the experimental syntaxin1A strain.[3] *syxKARRAA* genetic control is *syxWT* (see Materials and Methods) in the syx^{229} background. $^{**}P < 0.01$, calculated by extra sum-of-squares *F* test between estimated EC_{50} (n = 40 flies per genotype). (*F*) ΔEC_{50} ± SEE (isoflurane vol% atm) for the startle-induced velocity endpoint in *syxH3-C* (*black*) and *syxKARRAA* (*gray*) in a wild-type background (*isoCJ1*). ΔEC_{50} is calculated by subtracting the genetic background EC_{50} (*isoCJ1*) from the experimental syntaxin1A strain. *syxKARRAA* genetic control is *syxWT* in the *isoCJ1* background. $^{**}P < 0.01$, $^{***}P < 0.001$, calculated by extra sum-of-squares *F* test between estimated EC_{50} (n = 40 flies per genotype). (*G*) Stimulus response plot showing the average speed (mm/s ± SEM) of *syxH3-C* (*black*) and *syxKARRAA* (*gray*) after the vibration stimulus is delivered (*vertical red dashed line*) (n = 34 flies per genotype). *Shaded area* denotes SEM. (*H*) Average amplitude of the stimulus response (mm/s ± SEM) of *syxH3-C* (*black*) and *syxKARRAA* (*gray*) after the startle vibration stimulus. $^{**}P < 0.01$, *t* test comparing means (n = 34 flies per genotype).

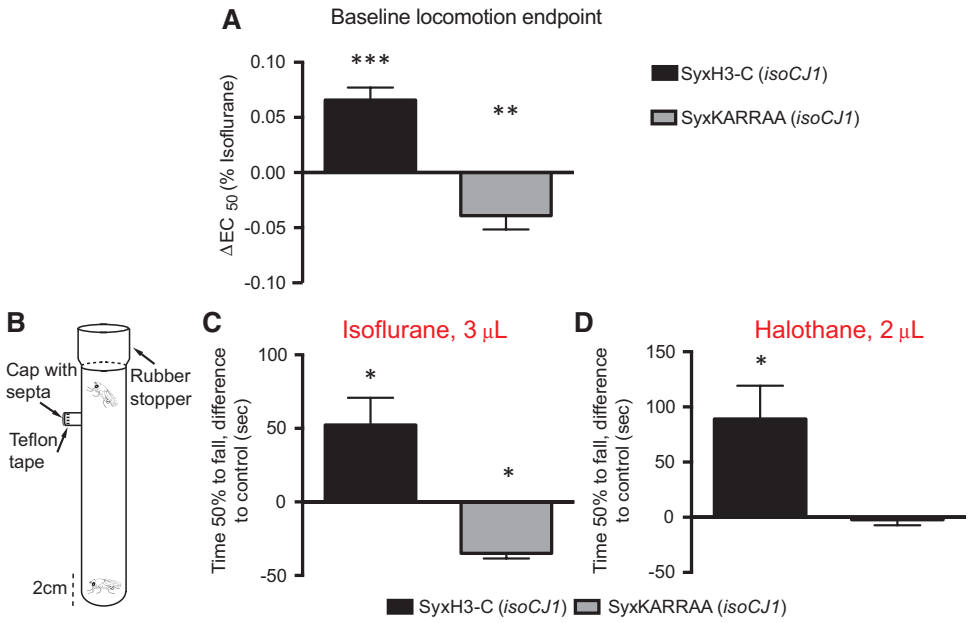

Fig. 2. Different behavioral endpoints reveal syntaxin1A-mediated resistance and hypersensitivity in adult *Drosophila.* (*A*) Baseline locomotion endpoint from the startle-induced locomotion assay showing ΔEC_{50} ± standard error of the estimate (isoflurane vol% atm) for the baseline endpoint in *syxH3-C* (*black*) and *syxKARRAA* (*gray*) in a wild-type background (*isoCJ1*). ΔEC_{50} is calculated by subtracting the genetic background EC_{50} (*isoCJ1*) from the experimental syntaxin1A strain (n = 40 flies per genotype). *syxKARRAA* genetic control is *syxWT* in the *isoCJ1* background. $^{**}P < 0.01$, $^{***}P < 0.001$, calculated by extra sum-of-squares *F* test between estimated EC_{50}. (*B*) Schematic of the fly coordination assay is shown (flies are not shown to scale). The tubes are commercially available glass tubes, 100 ml volume. The tubes were sealed with a rubber stopper, and the side arm of the tube was sealed with Teflon tape, and a small cap with septa which would facilitate the insertion of a syringe. After anesthetic injection, the number of flies in the bottom of the tube is counted every 10 s. Data shown are the mean time for 50% of the flies to become anesthetized, *error bars* represent the SEM; n >10 experiments per genotype. (*C*) The mean time for 50% of *syxH3-C* (*black*) and *syxKARRAA* (*gray*) to become anesthetized to 3 μl isoflurane in a wild-type background (*isoCJ1*) is shown, subtracting the experimental syntaxin1A strain from the time taken for 50% of the genetic control (*isoCJ1*) to become anesthetized. $^{*}P < 0.05$, *t* test comparing means. (*D*) The mean time for 50% of *syxH3-C* (*black*) and *syxKARRAA* (*gray*) to become anesthetized to 2 μl halothane in a wild-type background (*isoCJ1*) is shown, subtracting the experimental syntaxin1A strain from the time taken for 50% of the genetic control (*isoCJ1*) to become anesthetized. $^{*}P < 0.05$, nonparametric *t* test comparing means.

we examined synaptic transmission at the larval NMJ. The larval NMJ is a model glutamatergic synapse, sharing many similarities with mammalian glutamatergic synapses.[43] To characterize the general anesthetic effects on synaptic transmission, we recorded mEJPs and EJPs in wild-type NMJs (fig. 5, A and B) before and during exposure to isoflurane (fig. 5, C and D). A dose–response characterization of the effects of isoflurane on synaptic transmission in *Drosophila* larvae has been previously reported, using a slightly different recording technique.[30] We focused our investigations on isoflurane concentrations around the reported EC_{50} for larval locomotion: 0.4 vol% isoflurane for wild-type (*CS*) larvae, which closely corresponded with the EC_{50} of 0.17 mM isoflurane that decreased EJP amplitudes.[30]

At wild-type NMJs, we found isoflurane significantly decreases the amplitude of evoked responses ($P < 0.05$, one-way ANOVA; fig. 5, C and E; see Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/B139, for electrophysiology response measures over time). In contrast, isoflurane does not affect miniature endplate potential amplitudes ($P = 0.06$, one-way ANOVA; fig. 5, D and F). Quantal content (average number of vesicles released per action potential) was significantly decreased by isoflurane ($P < 0.05$, one-way ANOVA; fig. 5G). This suggests that a decrease in transmitter release could underlie the coordination defects observed in wild-type larvae under isoflurane exposure (fig. 4, A and B).

The syxH3-C Mutation Confers Resistance to Isoflurane at the Level of Transmitter Release

We showed earlier that *syxH3-C* larvae were resistant to the effects of general anesthetics, being capable of coordinated movement at isoflurane concentrations where control larvae had stopped moving (fig. 4, C and D). To investigate whether these resistance effects in *syxH3-C* reflected altered transmitter release, we analyzed quantal content in *syxH3-C*-mutant and control synapses. As in wild-type *CS* larvae, quantal content was significantly decreased in *isoCJ1* after isoflurane perfusion compared with before perfusion ($P < 0.05$, one-way ANOVA; fig. 5G). In contrast, quantal content in *syxH3-C* was not significantly decreased at the same perfusion time point ($P = 0.16$, one-way ANOVA;

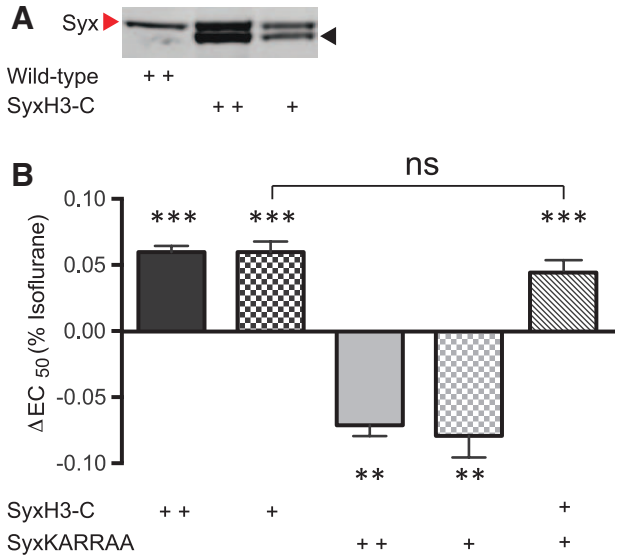

Fig. 3. Syntaxin1A mutations are dominant for general anesthesia phenotypes. (*A*) Western blot of syntaxin1A protein expression in wild-type (*isoCJ1*, *first lane*), *syxH3-C* (*middle lane*), and *syxH3-C*/+ (*third lane*). Endogenous syntaxin1A protein is shown (*red arrowhead*) and a smaller deletion protein produced in *syxH3-C* (*black arrowhead*). +Denotes gene copy number. (*B*) ΔEC_{50} ± standard error of the estimate (isoflurane vol% atm) for fly strains carrying one copy of the syntaxin1A lesions for the startle endpoint: *syxH3-C* (*black*, *checkered*) and *syxKARRAA* (*gray*, *checkered*) in wild-type background (*isoCJ1*). Fly strains carrying one copy of each syntaxin1A lesion is also shown: *syxH3-C*/*syxKARRAA* (*black*, *stripes*). There is no significant difference between the transheterozygous syntaxin1A strain and the heterozygous *syxH3-C* strain (*syxH3-C*/+, $P = 0.719$, calculated by extra sum-of-squares *F* test between estimated EC_{50}). ΔEC_{50} is calculated by subtracting the genetic background EC_{50} (*isoCJ1*) from the experimental syntaxin1A strain. *syxKARRAA* genetic control is *syxWT* (see Materials and Methods) in the *isoCJ1* background. The homozygous strains of both syntaxin1A strains are shown for comparison: *syxH3-C* (*black*) and *syxKARRAA* (*gray*). +Denotes gene copy number. $^{**}P < 0.01$, $^{***}P < 0.001$, calculated by extra sum-of-squares *F* test between estimated EC_{50} (n = 40 flies per genotype). ns = not significant.

fig. 5G) and is significantly higher than in *isoCJ1* ($P < 0.05$, two-way ANOVA; fig. 5G). In addition, in the presence of isoflurane, the percentage of failures in nerve-evoked transmitter release was lower at *syxH3-C* NMJs compared with control NMJs ($P < 0.05$, *t* test; fig. 6, A and B). Accordingly, we found no change in quantal content in *syxH3-C* with isoflurane, whereas transmitter release has decreased in *isoCJ1* (fig. 5G). These observations suggest that at *syxH3-C* NMJs, transmitter release is partially protected from the inhibitory effects of isoflurane.

Evoked transmitter release is calcium dependent. We explored whether isoflurane impacted transmitter release by changing the calcium dependence of transmitter release (calcium sensitivity and/or cooperativity) at wild-type and *syxH3-C* synapses. By constructing log–log plots of quantal content *versus* the extracellular calcium concentration (fig. 6, C–F), we investigated whether changes in transmitter release were produced through changes in calcium dependence.[44,45] Changes in calcium cooperativity (linear slope of the log–log plot) indicate changes in the number of calcium ions binding with the calcium sensor(s) to evoke transmitter release, whereas changes in the calcium sensitivity (left or right shift of the line) indicate changes in the amount of calcium required to evoke neurotransmitter release.[44,45] Before isoflurane perfusion, we found that calcium dependence was not different at *isoCJ1* synapses and *syxH3-C* synapses ($P = 0.294$; fig. 6C), indicating similar transmitter release kinematics in both strains in the absence of isoflurane. After isoflurane perfusion, the calcium cooperativity increased in both strains ($P < 0.05$; fig. 6, D and E), as evident from changes in the slope of the relation, although to a greater extent in *isoCJ1* (7.6; fig. 6D) compared with *syxH3-C* (4.4; fig. 6E). The right shift of the lines under isoflurane (fig. 6, D–F) indicated changes in calcium sensitivity, where more calcium was required to evoke similar levels of transmitter release than before isoflurane perfusion. These results confirm a presynaptic mechanism for isoflurane, indicating that with isoflurane, the dependence on calcium is greater to achieve a similar level of transmitter release ($P < 0.05$; fig. 6F). However, it is also clear that the calcium dependence of transmitter release is less in *syxH3-C* under isoflurane perfusion (fig. 6F), even though calcium dependence is similar without isoflurane (fig. 6C). This suggests that the mutant syntaxin1A protein is specifically interfering with the action of isoflurane rather than merely increasing synaptic efficacy in general.

Discussion

Our understanding of general anesthesia has mirrored our evolving appreciation of how the brain works, so it is not entirely surprising that the mechanism of general anesthesia remains somewhat of a mystery. Early work on the structure and excitability of neurons pointed to a nonspecific role for these drugs disrupting cellular excitability by interfering with the lipid membrane of neurons.[46] A subsequent elucidation of the roles of proteins embedded in the membrane, notably ion channels, led to the realization that inhibitory GABA receptors are the most likely target for these drugs.[2,11] More recent studies have highlighted the involvement of the sleep–wake circuitry in the mammalian and fly brain, showing that endogenous sleep pathways are disinhibited by some general anesthetics.[2–6,8] Moving beyond dedicated circuits, a new systems-level view of whole-brain dynamics now proposes that these drugs disrupt long-range communication, coherence, and integration across the brain.[47–49]

We have proposed recently that general anesthesia may primarily be a two-step process, whereby sleep-promoting pathways are activated first at low drug concentrations (thereby producing a "gentle" loss of consciousness for GABAergic drugs such as isoflurane), and synaptic mechanisms are attenuated

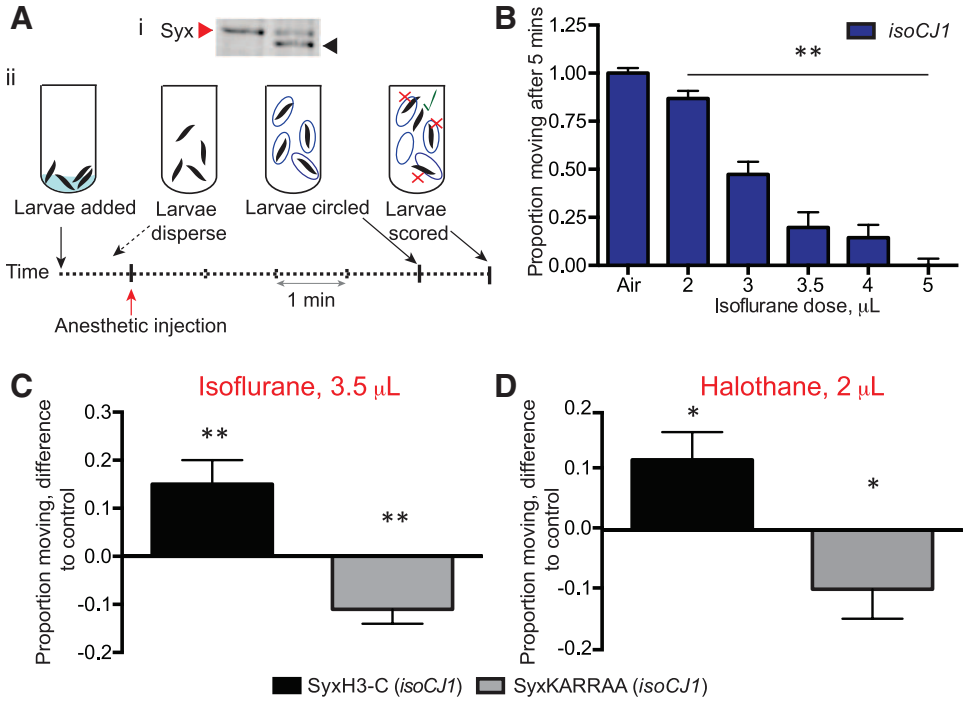

Fig. 4. Syntaxin1A mutations produce general anesthesia resistance and hypersensitivity in larvae. (*A*) i: Western blot of syntaxin1A protein expression in wild-type (*isoCJ1*, *first lane*) and *syxH3-C* (*second lane*) larvae, indicating endogenous syntaxin1A protein (*red arrowhead*) and a smaller deletion protein produced in *syxH3-C* (*black arrowhead*). ii: Schematic and timeline of larval anesthesia experiments (see Materials and Methods). After general anesthetic exposure, the position of the larvae is traced on the outside of the glass (*blue circles*). Larvae that have moved at least one-body-length outside the marked circle are noted (*green tick*, see Materials and Methods). (*B*) The proportion of wild-type larvae (*isoCJ1*) moving after 5 min of anesthetic exposure as a function increasing isoflurane dose is shown. Isoflurane concentrations are shown in µl volumes; see table 1 for corresponding vol% atm. $^{**}P < 0.01$, *t* test comparing means to air control (n = 12 experiments per concentration). (*C*) Average proportion of larvae moving (±SEM) at 3.5 µl isoflurane compared with genetic control (*isoCJ1*) for *syxH3-C* (*black*) and *syxKARRAA* (*gray*); *syxKARRAA* genetic control is *syxWT* (see Materials and Methods) in the *isoCJ1* background. $^{**}P < 0.01$, *t* test comparing means (n = 12 experiments per genotype). (*D*) Average proportion (±SEM) of larvae moving at 2 µl halothane compared with genetic control (*isoCJ1*) for *syxH3-C* (*black*) and *syxKARRAA* (*gray*); *syxKARRAA* genetic control is *syxWT* (see Materials and Methods) in the *isoCJ1* background. $^{*}P < 0.05$, *t* test comparing means (n = 12 experiments per genotype).

at the higher drug concentrations required for surgery.[7] In a previous report, we have uncovered a sleep–wake circuit supporting the first step in this process.[3] In this study, we examine syntaxin1A mutants in *Drosophila* to explore the second step, synaptic release mechanisms.

We characterized the general anesthesia phenotypes of two *Drosophila* strains containing modifications to the H3 domain of syntaxin1A: *syxH3-C*, which expresses a syntaxin1A protein with 14 amino acids deleted within the H3 domain,[23] and *syxKARRAA*, a strain with two amino acid substitutions (lysine to alanine) in the H3 domain adjoining the transmembrane portion of the protein.[24] We found that *syxH3-C* is resistant to isoflurane and *syxKARRAA* is hypersensitive to isoflurane across a variety of behavioral endpoints in both adults and larvae (figs. 1–4). These findings provide two important conclusions. First, neomorphic modifications in one synaptic protein can produce both hypersensitivity and resistance to isoflurane. Interestingly, anesthesia effects were not consistent with another volatile anesthetic, halothane, with *syxH3-C* being resistant but *syxKARRAA* showing hypersensitivity only as an immature larva. This suggests that the *syxH3-C* mutation more strongly affects anesthetic sensitivity than *syxKARRAA*. Indeed, when both mutations are placed together as a transheterozyote, the prevailing anesthesia phenotype is resistance (fig. 3B). This suggests a central role for syntaxin1A function in mediating general anesthesia. Second, the conservation of resistance and hypersensitivity effects for isoflurane across behavioral endpoints and life stages suggest that these are general mechanisms that are not necessarily linked to any specific brain circuitry, such as arousal pathways in the adult brain.

What could account for the general anesthesia phenotypes we have uncovered in the syntaxin1A mutants? For either strain, it is unlikely that the differences in anesthetic sensitivities we have found stem from altered anesthetic uptake, as behaviors were assayed after the anesthetics had equilibrated for several minutes. More specifically, *syxKARRAA* was created to study the effects of electrostatic interactions in syntaxin1A clustering. Changing two positive lysine residues to neutral alanine within the juxtamembrane region

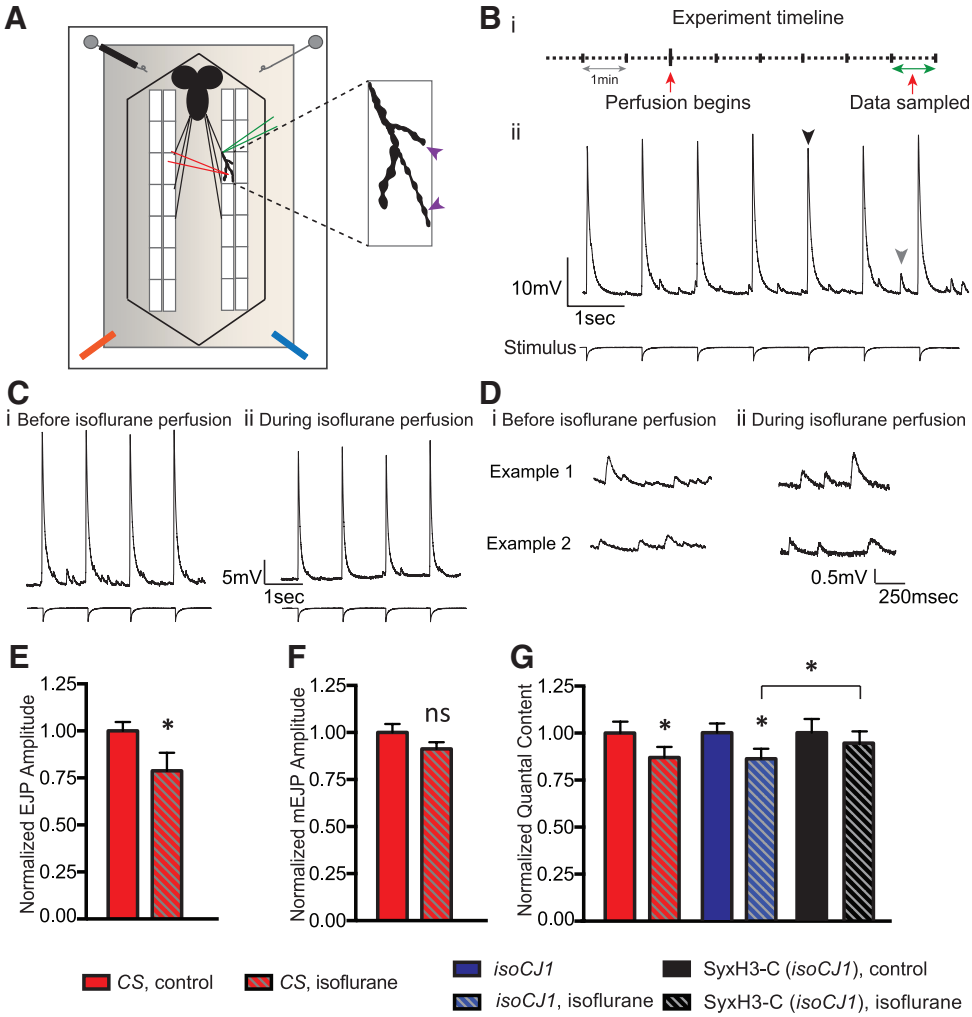

Fig. 5. Isoflurane decreases transmitter release. (*A*) A schematic of the *Drosophila* larval neuromuscular junction (NMJ) recording preparation is shown. Ground for stimulation wire (*gray*) and recording ground wire shielded with nail polish (*black*) plus perfusion set up (*blue*) with the vacuum (*orange*) is shown. The recording electrode (*red*) is impaled into muscle 6 of segment 3 of dissected third-instar larvae. A stimulating electrode (*green*) encases the nerve, which innervates the muscle. *Dashed lines* represent a more detailed schematic of muscle 6 synaptic boutons (*purple arrowheads*) which release glutamate onto the muscle. Not all muscles or nerves are shown for simplicity, and the brain is shown in this schematic for illustration purposes only (brain is removed when recording). (*B*) i: Time course of NMJ experiment, showing duration of the recording (in min) with *red arrow* denoting when isoflurane perfusion begins, and *green arrow* denoting time window when isoflurane perfusion data was sampled. ii: Example recording trace, showing evoked excitatory junctional potentials (EJPs, *black arrowhead*) which are recorded when the stimulus is presented (stimulus trace shown below recording). Spontaneous miniature EJPs (mEJPs, *gray arrowhead*) are also shown. (*C*) Example recording traces from wild-type (*Canton-S,* [*CS*]) NMJs showing EJPs before isoflurane perfusion (i) and during isoflurane perfusion (ii), with stimulus traces shown. (*D*) Two example recording traces from wild-type (*CS*) NMJs showing mEJPs before isoflurane perfusion (i) and during isoflurane perfusion (ii). (*E*) Normalized EJP amplitude in wild-type (*CS*) before (*solid bar*) and after 6 min of isoflurane perfusion (*shaded bar*). **P* < 0.05, one-way ANOVA with Dunnett multiple comparisons test. (*F*) Normalized mEJP amplitude in wild-type (*CS*) before (*solid bar*) and after 6 min of isoflurane perfusion (shaded bar). Data represent >700 individual measures. ns = not significant. (*G*) Normalized quantal content before (*solid bars*) and after 6 min of isoflurane perfusion (*shaded bars*) in *CS* (*red*), *isoCJ1* (*blue*), and *syxH3-C* (*black*). Quantal content is calculated by dividing the mean EJP amplitude by the mean mEJP amplitude. **P* < 0.05, one-way ANOVA with Dunnett multiple comparisons test. Quantal content with isoflurane perfusion is significantly higher in *syxH3-C* compared with *isoCJ1*. **P* < 0.05, two-way ANOVA with Sidak multiple comparison test. Data in *E* to *G* represent the mean ± SEM from: *CS* (n = 12), *isoCJ1* (n = 8), and *syxH3-C* (n = 8).

of syntaxin1A abolishes the ability of the phosphoinositide $PI(3,4,5)P_3$ to cluster syntaxin1A.[24] This lack of syntaxin1A clustering compromises transmitter release, with evoked release significantly decreased in *syxKARRAA* compared with control.[24] A decrease in transmitter release is therefore a likely explanation for the general anesthetic hypersensitivity in *syxKARRAA*. Interestingly, the anesthesia phenotype of *syxKARRAA* is dominant, suggesting endogenous syntaxin1A cannot compensate for the compromised transmitter release, which is consistent with the electrophysiology data.[24]

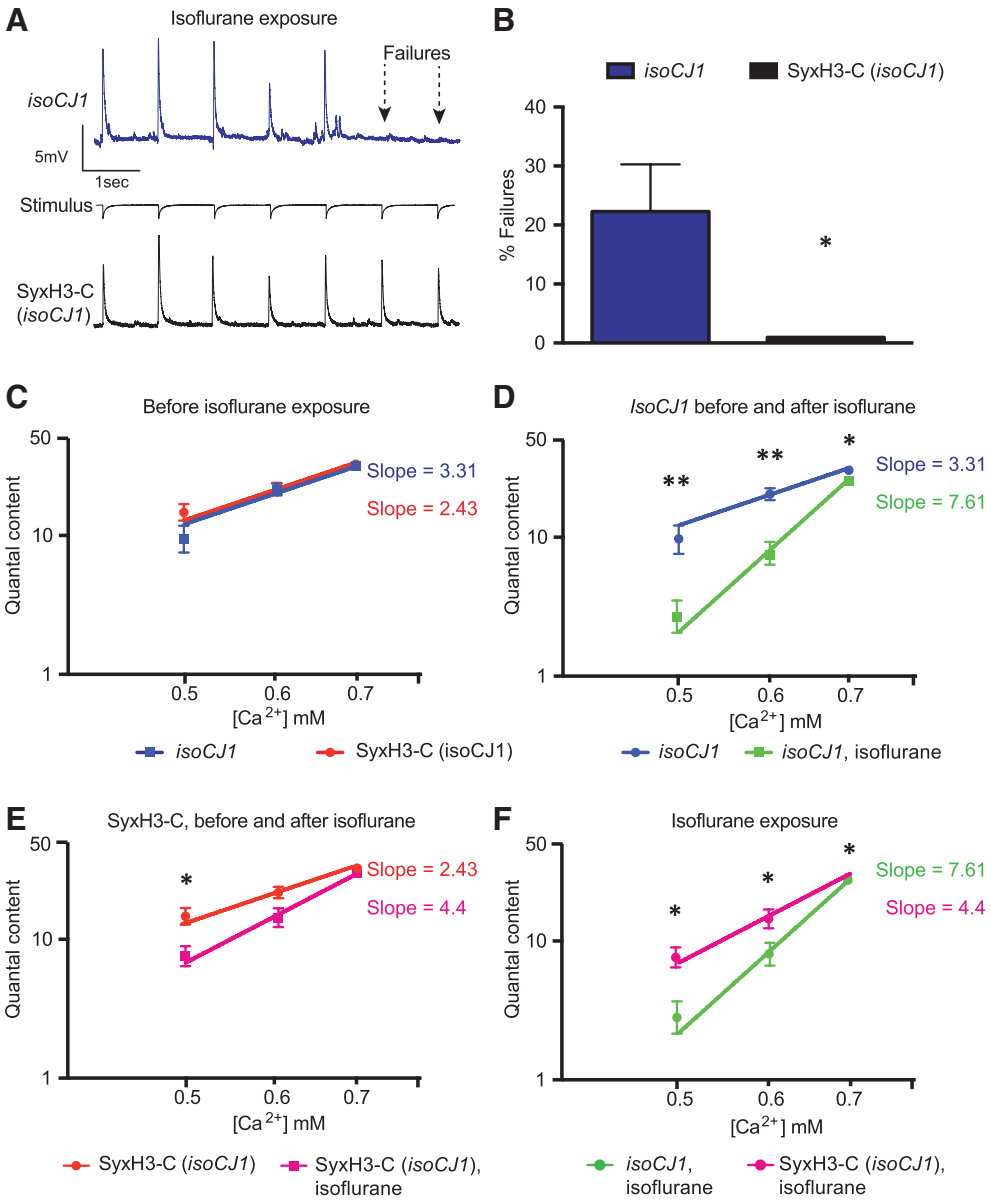

Fig. 6. Isoflurane increases calcium dependence of transmitter release. (*A*) Example recording trace in *isoCJ1* (*blue*) and *syxH3-C* (*black*) with failures denoted by *dashed arrows*. (*B*) Quantification of percentage of failures of evoked response after nerve stimulation in 0.5 mM calcium under isoflurane perfusion in *isoCJ1* (*blue*) and *syxH3-C* (*black*). **P* < 0.05, *t* test comparing means. Data in *C* to *F* show log–log plots of quantal content with increasing calcium concentrations: 0.5, 0.6, and 0.7 mM. Each point represents the mean ± SEM from more than eight separate recordings per genotype, with data averaged during a 1-min period before and after 6 min of isoflurane perfusion. *Lines* were fitted with linear regression for each plot. (*C*) Quantal content–calcium relation for *isoCJ1* (*blue*; slope = 3.31, *r* = 0.99) and *syxH3-C* (*red*; slope = 2.43, *r* = 0.99) before isoflurane (*P* = 0.29). (*D*) Quantal content–calcium relation in *isoCJ1* before (*blue*; slope = 3.31, *r* = 0.99) and after isoflurane perfusion (*green*; slope = 7.6, *r* = 0.99) (*P* < 0.05). (*E*) Quantal content–calcium relation in *syxH3-C* before (*red*; slope = 2.43, *r* = 0.99) and after isoflurane perfusion (*magenta*; slope = 4.4, *r* = 0.99) (*P* < 0.05). (*F*) Quantal content–calcium relation after isoflurane perfusion for *isoCJ1* (*green*; slope = 7.6, *r* = 0.99) and *syxH3-C* (*magenta*; slope = 4.4, *r* = 0.99) (*P* < 0.05). **P* < 0.05, ***P* < 0.01, two-way ANOVA with Sidak multiple comparison test.

In contrast, the general anesthesia resistance effects of *syxH3-C* are likely to stem from altered interactions with soluble NSF attachment protein receptor (SNARE)–binding partners. Indeed, *syxH3-C* was originally created to identify putative syntaxin1A binding partners. Wu *et al.*[23] showed that this syntaxin1A deletion spans the calcium effector domain and synprint binding site and that *syxH3-C* has defects in binding other SNARE proteins, yet the core complex can still form in these mutants. The fidelity of calcium-triggered transmitter release in this mutant was severely affected when assayed as embryos in a null homozygous background (these animals do not survive beyond the embryonic stage).[23] In contrast, we see

that when the mutant is coexpressed together with wild-type syntaxin1A, calcium-triggered release is normal (fig. 6C), and instead a phenotype only emerges under isoflurane perfusion (fig. 6F). This suggests that the syntaxin1A deletion protein confers an isoflurane resistance–promoting effect on the drug's target mechanism, specifically. Exactly how this resistance effect is mediated requires further elucidation.

Our work suggests that syntaxin1A represents a conserved target of general anesthetics across animals. Interestingly, the resistance effects from *syxH3-C* in *Drosophila* are modest compared with the original *C. elegans md130* truncation.[17] One reason for this may relate to the lack of conserved sleep-promoting circuitry in adult nematodes.[7] If the entry into general anesthesia involves activation of sleep pathways followed by global attenuation of synaptic release,[7] animals that lack sleep circuitry will unmask other relevant targets of general anesthetics. Consequently, mutations in synaptic release proteins will produce greater effects on general anesthetic sensitivity in animals that do not sleep.

There are only three previous reports on the effects of general anesthetics on transmitter release properties at the larval NMJ, one with halothane,[50] and two isoflurane studies.[30,51] For wild-type fly larvae, consistent with the previous reports, we found that evoked responses decrease after isoflurane exposure (fig. 5E), whereas the amplitude of miniature endplate potentials is unaffected (fig. 5F). We report here for the first time in *Drosophila* that isoflurane decreases quantal content (fig. 5G). Interestingly, we found preserved quantal content in *syxH3-C* after isoflurane exposure (fig. 5G). The lack of isoflurane inhibition of transmitter release may account for why *syxH3-C* larvae are capable of coordinated movement under general anesthesia compared with genetic controls (fig. 4, C and D), but also why *syxH3-C* adult flies are resistant to isoflurane across a variety of behavioral endpoints (fig. 1–3).

Previous work by others indicates that isoflurane decreases transmitter release by decreasing release probability (the chance of a vesicle undergoing exocytosis after an action potential).[30,52] Release probability is calcium dependent.[53,54] Therefore, to further understand the changes in quantal content with isoflurane, we investigated the calcium dependence of transmitter release (fig. 6). At control synapses, our results indicate that transmitter release is more calcium dependent after isoflurane exposure than before exposure (fig. 6, C–F). *syxH3-C* synapses also showed this calcium dependency, however, to a lesser extent under isoflurane perfusion than control synapses. Because *syxH3-C* fails to bind the calcium sensor synaptotagmin[55] while still forming part of the core SNARE complex,[23] it is possible the mutant syntaxin1A protein might be less dependent on calcium for transmitter release under isoflurane. Alternatively, the deletion protein might be accomplishing another SNARE-related function such as preserving SNARE clustering[56,57] on the plasma membrane under isoflurane anesthesia.

One reason why *syxH3-C* is less dependent on calcium may lie with how isoflurane interacts with the transmitter release machinery. A recent study suggests that synaptotagmin1 and syntaxin1A together form binding pockets for isoflurane.[58] Synapses where the synaptotagmin and syntaxin interaction has been modified display altered calcium cooperativity[59,60] even in the absence of general anesthetics. Our results therefore suggest that volatile anesthetics such as isoflurane may partially disable interactions between syntaxin1A and the calcium sensor(s) although we cannot exclude the possibility that other syntaxin1A-interacting proteins, such as UNC13,[61] may also be involved.

Our combined results point to a mechanism of general anesthesia that is distinct from the better-understood GABAergic sleep pathway.[2–4,6,8] We propose that general anesthetics such as isoflurane and halothane indeed target the sleep pathway as proposed,[3,5] thereby producing unconsciousness in all animals that sleep, but that these drugs also target synaptic release mechanisms in general, as is now evident from the *Drosophila* model as well as a number of other studies in simpler systems.[16–19] Anesthetic effects on synaptic release are likely to impair information processing across the brain, even if the effect at each individual synapse may be small.

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Competing Interests

The authors declare no competing interests.

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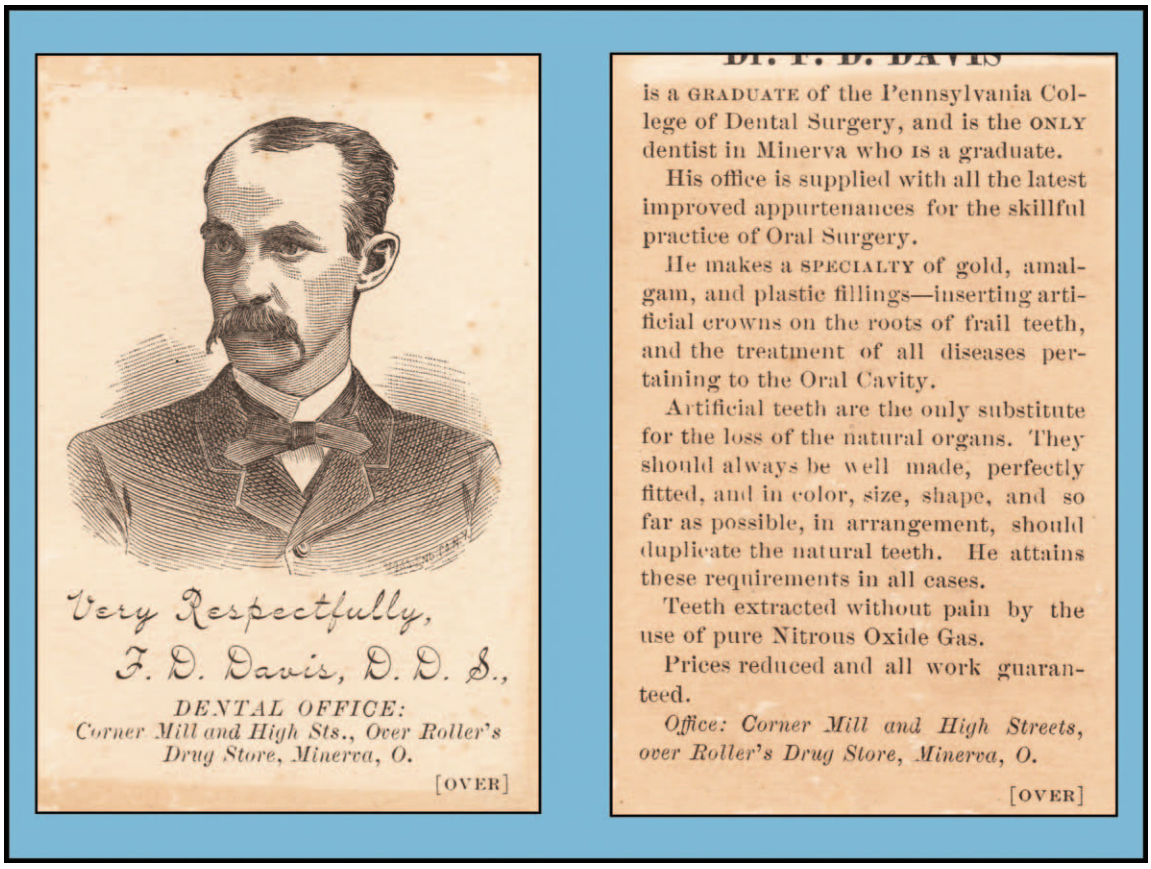

From the town of Minerva, Ohio, Fremont D. Davis, D.D.S. (*left*), advertised painless dentistry "by the use of pure Nitrous Oxide Gas" (*right*). Sadly, his personal health was hardly pain free. While driving back into Minerva, Dr. Davis's buggy was struck after a railroad flatcar bumped into a switch. As his horse bolted, the hapless dentist was "dragged out over the dashboard and severely injured." This trade card is part of the Wood Library-Museum's Ben Z. Swanson Collection. (Copyright © the American Society of Anesthesiologists, Inc.)

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