ANESTHESIOLOGY

Syntaxin1A Neomorphic Mutations Promote Rapid Recovery from Isoflurane Anesthesia in *Drosophila melanogaster*

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Syntaxin1A is a presynaptic molecule that plays a key role in vesicular neurotransmitter release
- Mutations of syntaxin1A result in resistance to both volatile and intravenous anesthetics
- Truncated syntaxin1A isoforms confer drug resistance in cell culture and nematode models of anesthesia

What This Article Tells Us That Is New

- Resistance to isoflurane anesthesia can be produced by transiently expressing truncated syntaxin1A proteins in adult Drosophila flies
- Electrophysiologic and behavioral studies in Drosophila show that mutations in syntaxin1A facilitate recovery from isoflurane anesthesia
- These observations suggest that presynaptic mechanisms, *via* syntaxin1A-mediated regulation of neurotransmitter release, are involved in general anesthesia maintenance and recovery

ABSTRACT

Background: Mutations in the presynaptic protein syntaxin1A modulate general anesthetic effects *in vitro* and *in vivo*. Coexpression of a truncated syntaxin1A protein confers resistance to volatile and intravenous anesthetics, suggesting a target mechanism distinct from postsynaptic inhibitory receptor processes. Hypothesizing that recovery from anesthesia may involve a presynaptic component, the authors tested whether syntaxin1A mutations facilitated recovery from isoflurane anesthesia in *Drosophila melanogaster*.

Methods: A truncated syntaxin1A construct was expressed in *Drosophila* neurons. The authors compared effects on isoflurane induction *versus* recovery in syntaxin1A mutant animals by probing behavioral responses to mechanical stimuli. The authors also measured synaptic responses from the larval neuromuscular junction using sharp intracellular recordings, and performed Western blots to determine whether the truncated syntaxin1A is associated with presynaptic core complexes.

Results: *Drosophila* expressing a truncated syntaxin1A (syx^{227}, n = 40) were resistant to isoflurane induction for a behavioral responsiveness endpoint (ED50 0.30 ± 0.01% isoflurane, $P < 0.001$) compared with control (0.240 ± 0.002% isoflurane, n = 40). Recovery from isoflurane anesthesia was also faster, with syx^{227}-expressing flies showing greater levels of responsiveness earlier in recovery (reaction proportion 0.66 ± 0.48, $P < 0.001$, n = 68) than controls (0.22 ± 0.42, n = 68 and 0.33 ± 0.48, n = 66). Measuring excitatory junction potentials of larvae coexpressing the truncated syntaxin1A protein showed a greater recovery of synaptic function, compared with controls (17.39 ± 3.19 mV and 10.29 ± 4.88 mV, $P = 0.014$, n = 8 for both). The resistance-promoting truncated syntaxin1A was not associated with presynaptic core complexes, in the presence or absence of isoflurane anesthesia.

Conclusions: The same neomorphic syntaxin1A mutation that confers isoflurane resistance in cell culture and nematodes also produces isoflurane resistance in *Drosophila*. Resistance in *Drosophila* is, however, most evident at the level of recovery from anesthesia, suggesting that the syntaxin1A target affects anesthesia maintenance and recovery processes rather than induction. The absence of truncated syntaxin1A from the presynaptic complex suggests that the resistance-promoting effect of this molecule occurs before core complex formation.

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There is growing evidence that general anesthetics target presynaptic mechanisms in addition to postsynaptic receptors.[1–6] For example, clinical concentrations of both intravenous and volatile anesthetics have been found to impair neurotransmission.[3,4,7] Syntaxin1A plays a key role in neurotransmission,[8] presenting a crucial endpoint for synaptic vesicle release, without which neurotransmission could not occur.[9] Mutations in this protein produce both hypersensitivity and resistance to volatile anesthetics in nematode worms[10] and *Drosophila* flies,[6] suggesting that the protein may be proximal to a presynaptic target for these drugs.[11] Coexpression of a truncated syntaxin1A protein has been shown to produce resistance to volatile anesthetics in nematode worms[10] and mammalian neurosecretory cells,[4]

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as well as resistance to the intravenous anesthetic propofol in mammalian cells.[3] How exactly this neomorphic syntaxin1A protein protects synaptic release from the effect of general anesthetics remains unclear, although an interaction with other presynaptic-related proteins seems likely.[1]

Most explanations of general anesthesia relate to postsynaptic targets.[12,13] However, should general anesthesia comprise at least two distinct target domains, one postsynaptic and one presynaptic,[11] this is likely to be reflected in the different kinetics of anesthesia induction and recovery. Many general anesthetics have rapid induction and slower recovery kinetics.[14] This recovery inertia has been proposed as evidence that different processes might be involved during induction and recovery.[15] One idea that has been proposed is that induction is rapid because it primarily reflects the sedative properties of these drugs,[12] and the loss of consciousness associated with sleep is a rapid process.[16] However, recovery time can vary significantly,[17] and some patients report incomplete recovery for days or even months after the procedure.[18] We have proposed that recovery inertia reflects in part presynaptic processes, in contrast to the rapid induction kinetics which are understood to reflect postsynaptic processes.[11] Therefore, manipulations of presynaptic proteins that preserve neurotransmission under isoflurane anesthesia *in vitro*[3,4] should reduce the recovery time required after the procedure, *in vivo*. We decided to test this in an animal model, *Drosophila melanogaster.*

General anesthesia is fundamentally a behavioral endpoint, and understanding its mechanisms of action requires methods to probe behavioral responsiveness in behaving animals.[19] Because the sedative component of general anesthesia most likely engages sleep-promoting pathways in the brain,[12] we decided to use *Drosophila*, which have sleep-promoting neurons[20,21] that have been found to be involved in isoflurane anesthesia.[22] *Drosophila* is an established model to study general anesthesia.[23–25] Assays for probing sleep intensity or behavioral responsiveness are also well developed for *Drosophila*,[26] providing an effective way of assessing the role of syntaxin1A in isoflurane induction and recovery. We designed a tagged, truncated version of syntaxin1A which we could express in fly neurons, to determine how this affected isoflurane induction and recovery for behavioral endpoints as well as for neurotransmission. We hypothesized that syntaxin1A effects on recovery from isoflurane anesthesia would be reflected across these different levels of investigation.

Materials and Methods

Fly Stocks and Rearing Conditions

Drosophila melanogaster flies were grown 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 collected for behavioral experiments by brief carbon dioxide exposure and kept in food vials overnight before experiments, which were performed between 9:00 AM and 5:00 PM. Fly stocks were sourced from the Bloomington Stock Center (USA): control wild-type *Canton-S* strain, Elav^{C155}-Gal4, and the tubulin-gal80^{TS} strain was a gift from Mani Ramaswami (Trinity College Dublin, Ireland). The syntaxin1A deletion strains used in this study have been described previously: the deletion mutants, *syxH3-C* and *syxH3-N*,[27] were both outcrossed for 5 generations to *isoCJ1*.[28,29] *syxH3-C* and *syxH3-N* flies coexpress mutant syntaxin1A proteins alongside wild-type syntaxin1A. For experiments using the temperature-sensitive the tubulin-gal80^{TS} strain, flies were kept in a food vial overnight (12 to 14 h) in an incubator at 32°C then immediately loaded into the behavioral chamber. After experiments flies were euthanized by submersion in canola oil.

Construction of a Truncated Syntaxin1A

Syx^{227} was cloned as described in Bademosi *et al.*[1] Briefly, syx^{227} was generated by site-directed mutagenesis (Change-IT, USB Corporation, USA) to place a stop codon at the desired site (primer sequence 5'-TCTAGAATGCGATCG ATCATCT CGCC TTACTGCG ACTCCACCAGCAT-3'). The generation of human influenza hemagglutinin (HA)-tagged HA-Syx^{227} and wild-type syntaxin1A (HA-Syx^{FL}) was described previously.[1] To enable expression of HA-syx^{FL} and HA-syx^{227} in HEK cells, constructs were subcloned into the pRK5-HA vector as reported previously.[1] Sequence-verified constructs were stably integrated into the attb sites on the second chromosome of *D. melanogaster* (BestGene, USA). HA-syx^{FL} and HA-syx^{227} both survived as larvae, although some lethality was evident in HA-syx^{FL}. Only HA-syx^{227} survived to adulthood.

Western Blots

Protein samples were analyzed by Western blot as previously described.[6,30] For detection of soluble n-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complexes, total brain extracts were prepared by homogenizing frozen fly heads in ice-cold cell lysis buffer (1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na-pyrophosphate in phosphate-buffered saline) supplemented with EDTA-free complete protease inhibitor cocktails (Roche, Australia). The homogenates were centrifuged at 14,000 rpm for 20 min at 4°C. Soluble lysates were then mixed with the sodium dodecyl sulfate sample buffer and further incubated at 37°C or 100°C for 10 min. Each sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting with anti-HA (rabbit anti-HA [C29F4], 1:3,000, Cell Signaling Technology, USA) and anti-syntaxin (mouse anti-8c3, 1:1,000, Developmental Studies Hybridoma Bank, USA) antibodies.

Anesthesia Induction and Recovery: Adult Behavioral Assays

The startle-induced locomotion assay assesses flies' movement response to a startle-inducing vibration stimulus, and

has been described previously.[6,22,30] In this study, responsiveness was probed using five 2.4-g 0.2-s pulses. To determine isoflurane sensitivity, fly activity was recorded for 30 s both before (baseline locomotion endpoint) and after (responsiveness endpoint) the stimulus was delivered at intervals of 10 min. After each interval, isoflurane concentration in air was increased starting from 0% in increments of 0.12, 0.25, 0.37, 0.5, 0.75, and 1% for a total of seven trials. 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.[15] The concentration delivered into the behavioral chamber from the evaporator was verified using gas chromatography as described previously.[22]

To assess recovery from isoflurane anesthesia, flies were placed in the same behavioral apparatus used for induction. A baseline mechanical vibration stimulus (5× 0.2-s 2.4-g pulses) was delivered before anesthesia. Isoflurane (1%) was then delivered for 10 min. Isoflurane delivery was then stopped and a stimulus was delivered immediately. Over the next 2 h, a stimulus was delivered every 10 min. To determine stimuli responsiveness, flies were defined as responding when their movement over the 10 s after the stimulus was greater than 3 mm (an approximate body length movement). To determine the time taken until first spontaneous movement, fly activity was monitored after isoflurane delivery was stopped and the time point for each fly at which movement exceeded 4 mm within a 10-s moving window was averaged across a genotype.

Larval Anesthesia Assay

Larvae were tested for their ability to display coordinated movement under isoflurane anesthesia as described previously[6,30] with minor modifications. Recovery from isoflurane anesthesia was analyzed after 5 min of isoflurane exposure. The rubber stopper on the behavioral chamber was removed and larvae were allowed to recover for 5 min. The number of larvae that had moved during this recovery period was noted.

Electrophysiology

Sharp intracellular recordings were made from the larval neuromuscular junction as described previously.[6,30,31] Briefly, wandering third instar larvae were dissected in ice-cold Schneider's insect medium (Sigma, Australia), and pinned onto glass dissection plates. Recordings were conducted at room temperature in HL3 hemolymph-like solution[32] using $[Ca^{2+}]_e = 0.7$ mM. Analysis was performed on recordings with membrane potentials exhibiting values lower than −65mV.

Isoflurane solutions were prepared and perfused onto the larvae as previously described.[6,30,33] After isoflurane perfusion, the preparation was then washed extensively for several minutes and recordings reinitiated to obtain recovery measures. Isoflurane concentrations in the saline perfusing the neuromuscular junction preparation was determined by gas chromatographic headspace analysis (PerkinElmer Clarus 680 GC-FID), performed as described previously.[33] Briefly, 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 with a saturated isoflurane solution.

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

Statistical Analysis

All statistical comparisons were performed using Prism (GraphPad Software, USA). It was not feasible to blind experimenters to fly genotypes. Flies were assigned to experimental groups defined by their genotype. For adult behavioral experiments, genotypes were tested simultaneously in each experiment. For larval experiments genotypes were tested sequentially. Sample sizes for all assays were selected based on standards in the field and no statistical power calculation was conducted before experiments. All n values refer to the number of individual flies. Data was checked for normality using the D'Agostino–Pearson test. Data that was not normally distributed was compared using a Kruskal–Wallis test with Dunn's multiple comparison test. Outliers were assessed using established methods,[34] but no further action was necessary.

Comparison of ED50 values for induction behavioral experiments has been described previously.[6,22,30] Briefly, mean fly velocity was taken for each of the seven trials and normalized to baseline (0% isoflurane) activity. ED50 values were generated by fitting a nonlinear regression with the minimum constrained to 0. Genotypes were compared with an extra sum-of-squares F test with significance threshold of $P < 0.05$. ED50 data represent isoflurane volume % atmospheres mean ± standard error of the estimate.

For larval anesthesia assays, data were converted to express the number of larvae that moved as a proportion of the total. A two-tailed, unpaired independent t test was used to compare experimental and control strains with normal distributions, with significance thresholds set at $P < 0.05$.

Neuromuscular junction data were processed in Axograph as described previously.[6,30] The amplitude and baseline offset of excitatory junction potentials and miniature excitatory junction potentials was obtained. Quantal content was calculated by dividing the mean excitatory junction potential amplitude by the mean miniature excitatory junction

potential amplitude. Evoked responses were corrected for nonlinear summation[35] before calculations. Tests for significant differences between control and isoflurane perfusion were conducted using two-way ANOVA with Dunnett's multiple comparisons test. Comparison of genotypes under isoflurane perfusion was conducted with 2-way ANOVA with Sidak's multiple comparison test. Differences were considered significant at $P < 0.05$.

Results

Syntaxin1A H3 Domain Deletions Promote a More Complete and Rapid Recovery from Isoflurane Anesthesia

General anesthesia induction sensitivity was assessed in adult female *Drosophila* by video-tracking the behavior of multiple flies exposed to increasing concentrations of the volatile gas isoflurane (fig. 1A). Flies were contained individually in glass tubes on a platform inside an airtight chamber equilibrated with isoflurane.[6,22,30] Mechanical vibrations delivered by motors attached to the platforms probed behavioral responsiveness of the flies during the anesthesia experiments. Two behavioral metrics were assessed from each experiment: baseline locomotion, and responsiveness to the vibration stimulus (fig. 1B). The concentration producing half-maximal behavior, measured as the ED50, can be calculated for normalized data for both behavioral endpoints, as in previous studies (fig. 1C shows example dose–response curves).[22] Generally, behavioral responsiveness is a more sensitive isoflurane endpoint than baseline locomotion.[19,22,30] To better understand the effects of syntaxin1A mutations on isoflurane anesthesia, we first

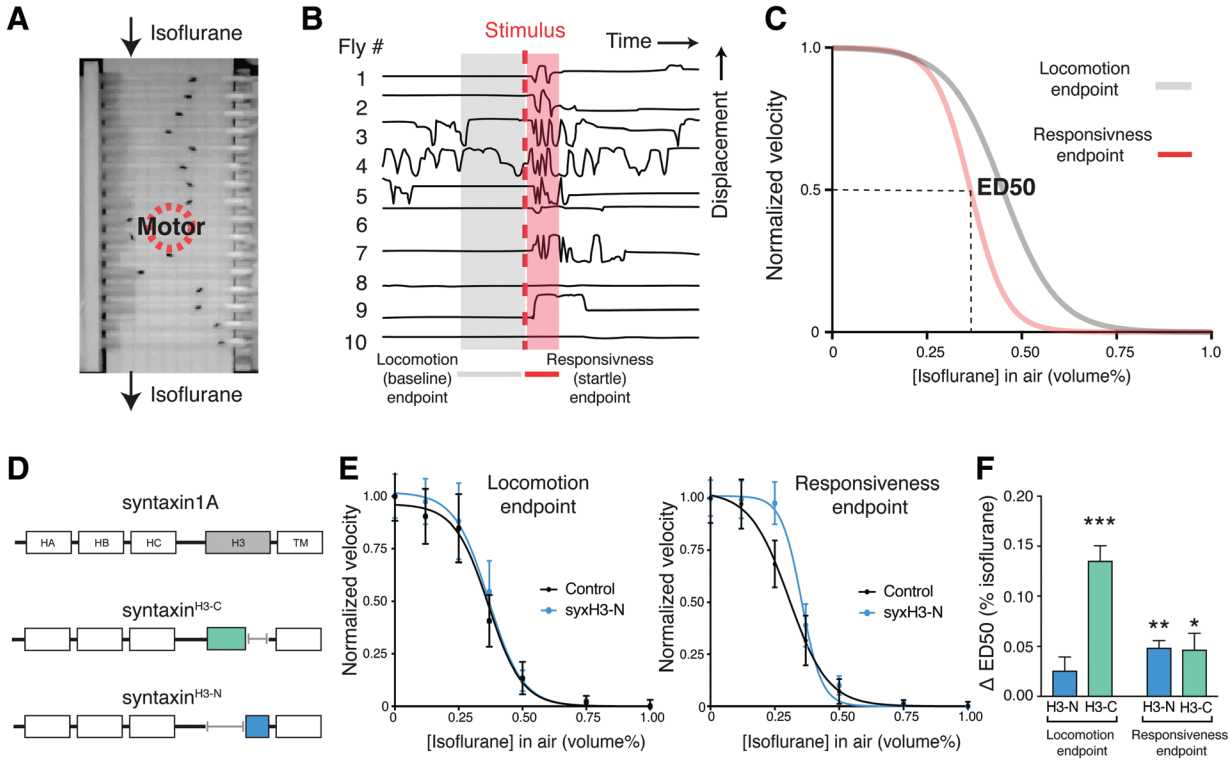

Fig. 1. Isoflurane resistance effects. (*A*) Flies housed in glass tubes with their activity tracked from above with a camera. Motors underneath provided a mechanical stimulus. A sealed chamber allowed a controlled concentration of isoflurane to be delivered to the flies. (*B*) Two endpoints were measured in this assay. Before a stimulus, baseline locomotor fly activity (*grey*) was measured for 60 s. After a stimulus (*red dashed line*), flies increased their locomotion in a startle behavior and this is captured as a responsiveness endpoint. (*C*) Two example dose–response curves. As isoflurane concentration increases, baseline fly locomotion decreases (*grey*). Behavioral responsiveness to mechanical stimuli is also affected in a dose–response manner, and this endpoint is more sensitive to isoflurane. This is reflected by a lower ED50 value, the concentration at which the behavior becomes half-maximal. (*D*) Schema of syntaxin1A protein showing the 3 HABC, H3 and transmembrane domains. *Drosophila* mutants coexpress syntaxin1A with deletions in the N terminus (H3-N) and C terminus (H3-C) domains along with wild-type protein in all neurons. (*E*) Nonlinear regression of normalized locomotion velocity for locomotion (*left*) and responsiveness (*right*) endpoints under increasing isoflurane concentrations for syxH3-N flies (*blue*, n = 51) compared to genetic background control (*isoCJ1*, n = 42). *Error bars* show 95% CI. (*F*) Increases in ED50 values for syntaxinH3-C (*green*, n = 51) and syxH3-N (*blue*, n = 51) flies compared with genetic background controls (n = 42 H3N, n = 50 H3C) for locomotion (*left*) and responsiveness (*right*) behavioral endpoints. *Error bars* show ± standard error of the estimate. Extra sum-of-squares *F* test, *asterisks* represent ED50 tested against control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

examined two different syntaxin1A deletion constructs (syxH3-C and syxH3-N; fig. 1D) that are coexpressed alongside wild-type syntaxin1A,[6,27] for either endpoint (fig. 1, E and F). Both mutants were resistant for the responsiveness endpoint (H3-C ED50 = 0.324 ± 0.016% isoflurane *vs.* control 0.277 ± 0.010% isolfurane, $P = 0.03$ and H3-N ED50 = 0.354 ± 0.007% isoflurane *vs.* control 0.305 ± 0.014% isoflurane, $P = 0.001$, extra sum-of-squares *F* test; we confirmed here the results for syxH3-C, which have been reported previously,[6] Supplemental Digital Content 1, http://links.lww.com/ALN/B984), but only syxH3-C was resistant for baseline locomotion (fig. 1F; H3-C ED50 = 0.430 ± 0.014% isoflurane *vs.* control 0.294 ± 0.023% isoflurane, $P < 0.001$ and H3-N ED50 = 0.380 ± 0.013% isoflurane *vs.* control 0.354 ± 0.045% isoflurane, $P = 0.209$, extra sum-of-squares *F* test). Therefore, the responsiveness endpoint seems to better convey the resistance-promoting effect in both mutants than baseline locomotion, and deleting either portion of the H3 domain (fig. 1D) appears to be sufficient to promote this isoflurane resistance effect.

The ED50 mostly captures anesthesia induction effects. However, general anesthesia induction and recovery could involve different processes,[11,14,15] so we questioned whether the effects of the syntaxin1A mutations might be more evident for recovery rather than induction. To test anesthesia recovery in our fly model, we exposed animals to 1% (vol/vol) isoflurane for 10 min and then probed their behavioral responsiveness every 10 min, for the next 2 h (fig. 2A). Wild-type *isoCJ1* flies were rapidly rendered immobile and unresponsive at this clinically-relevant concentration, although we observed a period of hyperexcitability before induction, which has been observed before in other animals (fig. 2A).[36] Recovery from isoflurane anesthesia was prolonged in wild-type control flies, with responsiveness levels never returning to baseline levels even after 2 h (fig. 2, A and B). The syntaxin1A mutants were just as effectively anesthetized using this approach; however, their recovery was significantly faster than wild-type (fig. 2, A–C). The syxH3-N mutant recovered especially quickly, responding immediately at higher levels to the first stimulus (10 min postisoflurane) compared with control flies (proportion responding 0.69 ± 0.47 *vs.* control proportion responding 0.29 ± 0.46 $P < 0.001$, Kruskal–Wallis), and returning to baseline responsiveness levels 30 min after the procedure (fig. 2C). However, mean time to first movement (locomotion irrespective of a stimulus, see Materials and Methods) was not significantly different among the strains (fig. 2D, control 398 ± 181s; H3-C 335 ± 41s $P = 0.061$; H3-N 365 ± 157s $P = 0.463$, one-way ANOVA). This suggests that the syntaxin1A mutations are specifically involved in facilitating the ability of animals to recover their capacity to respond behaviorally to stimuli after exposure to a clinically-relevant dose of isoflurane.

Expressing a Truncated Syntaxin1A Protein Promotes a Faster Recovery from Isoflurane Anesthesia

To better understand the effect of syntaxin1A deletion proteins on isoflurane anesthesia, we designed a truncated syntaxin1A construct (syx^{227}) for which expression levels could be controlled in *Drosophila* neurons, by using the upstream activation sequence (UAS)/Gal4 system.[37] This syntaxin1A mutant has a large deletion of most of the H3 SNARE interaction domain, as well as the transmembrane domain (fig. 3A). Previous work in nematodes[10] and mammalian cell cultures[3,4] has shown that coexpression of a similar syntaxin1A H3 domain truncation protein (syx^{md130}, fig. 3A) alongside endogenous syntaxin1A confers a high level of resistance to diverse general anesthetics, including isoflurane. We also generated another strain that expresses full-length syntaxin1A protein (syx^{FL}) under the control of the UAS/Gal4 system as a control for our study (fig. 3A). Both constructs (syx^{227} and syx^{FL}) contained an HA tag at the N terminus which allow for biochemical detection of these exogenous syntaxin1A proteins (fig. 3B, green). We used a pan-neuronal Gal4 driver to express both constructs in the fly nervous system, and discovered that overexpressing the full-length protein (syx^{FL}) resulted in high rates of lethality in adults (see Materials and Methods), and surviving flies were sick, so interpretation of behavioral data in adults for this strain was difficult. In contrast, coexpressing the truncated protein (syx^{227}) throughout the fly nervous system had no deleterious effect on survival, and animals proved resistant to both the locomotion (ED50 = 0.348 ± 0.019% isolfurane, *vs.* control 0.280 ± 0.010% isolfurane $P = 0.009$ extra sum-of-squares *F* test) and responsiveness (ED50 = 0.30 ± 0.01% isolfurane *vs.* control 0.240 ± 0.002% isolfurane, $P < 0.001$ extra sum-of-squares *F* test) endpoint (fig. 3C). This shows that a similar truncated syntaxin1A construct that confers isoflurane resistance in mammalian neurosecretory cells[3,4] and nematode worms[10] also produces resistance in adult *Drosophila*.

One advantage of expressing proteins *via* the UAS/Gal4 system in the *Drosophila* model is that expression levels can be controlled temporally as well as spatially.[38] To enable this, we added a temperature-sensitive tubulin-$Gal80^{TS}$ construct to our strains. $Gal80^{TS}$ suppresses Gal4 at room temperature (25°), thereby preventing expression of the UAS-associated construct, until flies are transiently exposed to a higher temperature (32°), which inactivates $Gal80^{TS}$ and thereby allows expression of the Gal4-driven molecule (fig. 3D). In this way, we were able to drive expression of the truncated syntaxin1A construct only in adults, thereby determining whether resistance to isoflurane could be effectively turned on in adult animals. Importantly, this method also eliminates any confounds that might be associated with the effects of syx^{227} overexpression during development. We first confirmed that this protocol effectively generated a truncated syntaxin1A protein only after the heat treatment (fig. 3E). Behavioral experiments on these animals showed that statistically significant isoflurane resistance

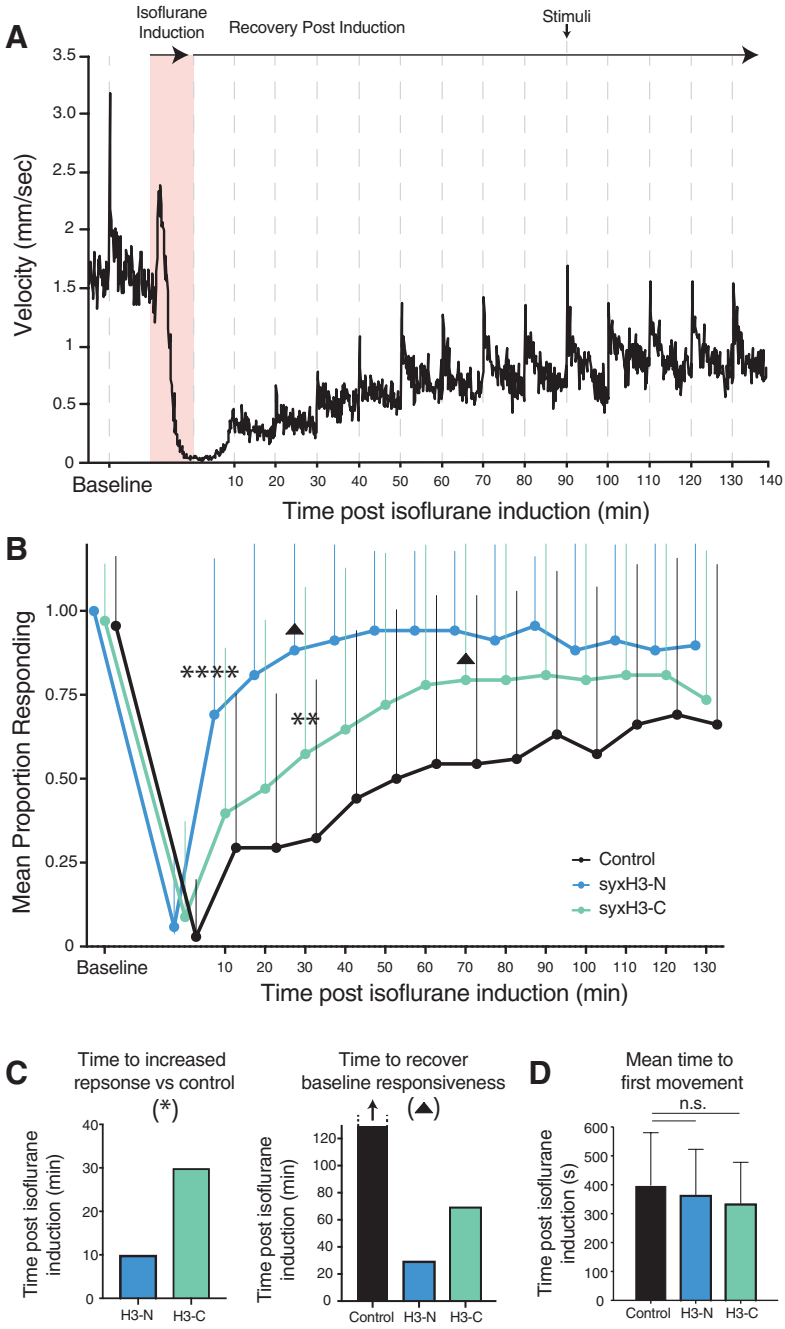

Fig. 2. Faster recovery in syntaxin1A mutants. (*A*) Velocity (mm/sec) of wild-type *isoCJ1* flies (n = 68) during an isoflurane anesthesia recovery experiment. Red shading indicates delivery of 1% isoflurane for 10 min. *Grey dotted lines* show mechanical vibration stimulus. Baseline is the stimulus occurring before induction. (*B*) Mean proportion of flies responding to each stimulus event. Flies show nearly complete responsiveness at baseline and no responsiveness at the end of the induction period. *Error bars* show ± SD. *Asterisks* indicate the first stimulus where responses of syxH3-N (*blue*, n = 68) and syxH3-C (*green*, n = 68) were significantly different from control flies (*black*, n = 68). Kruskal–Wallis corrected for multiple comparisons (Dunn). $^{****}P < 0.001$, $^{**}P < 0.01$. *Triangles* indicate stimulus at which response is no longer significantly different from baseline. Time points on the *x* axis show discrete stimulus events every 10 min. Data points show reaction proportion for each genotype at each stimulus event. (*C*) *Left*: syxH3-N flies (*blue*) respond significantly more than control flies after 10 min, whereas syxH3-C (*green*) take 20 min (*asterisks* in *B*). *Right*: Time taken to recover baseline responsiveness as measured by the stimulus at which response is no longer significantly different from baseline (*triangles* in *B*). Control flies (*black*) never fully return to baseline levels even after 120 min. (*D*) Mean time taken for flies to first initiate locomotion after anesthesia induction. *Error bars* show ± SD. One-way ANOVA (between-subjects), n.s., not significant.

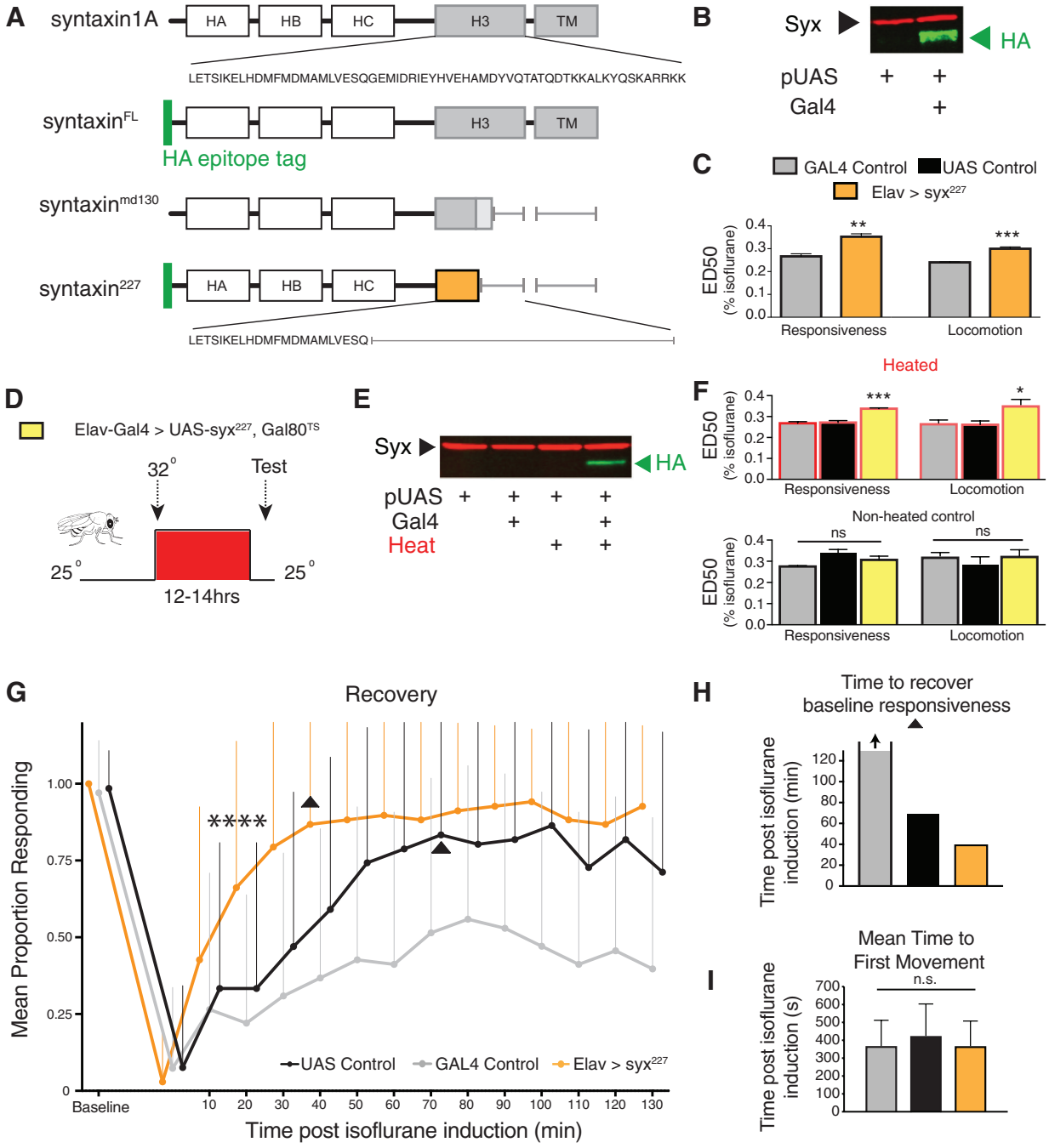

Fig. 3. Acute coexpression of a truncated syntaxin1A produces resistance. (*A*) The syntaxin1A protein shown with 3 HABC, H3 (with amino acid sequence), and transmembrane domains. Upstream activation sequence (UAS)-driven constructs with human influenza hemagglutinin (HA) epitope tags were created to drive overexpression of full-length wild-type syntaxin1A (syx^{FL}) and syx^{227}, a mutant truncated in a similar position to the *md130* mutation that confers resistance to isoflurane in nematodes.[10] (*B*) Western blot of syntaxin1A protein expression in flies expressing the UAS element alone (UAS-syx^{227}/+, *left*) and UAS and Gal4 elements together (UAS-syx^{227}/+; Actin-Gal4/+, *right*). Endogenous syntaxin1A protein is shown in red (*black arrowhead*) and the truncated protein as labeled with HA antibody is shown in green (*green arrowhead*). (*C*) ED50 values for flies expressing syx^{227} in all neurons (orange, Elav-Gal4/+; UAS-syx^{227}, n = 40) for responsiveness and baseline locomotion endpoints compared to genetic control (grey, Gal4 Control, Elav-Gal4/+, n = 40). *Error bars* show ± standard error of the estimate. Extra sum-of-squares *F* test, $^{**}P < 0.01$, $^{***}P < 0.001$. (*D*) Schematic of Gal80^{TS} experimental protocol to express syx^{227} transiently in adult flies. Flies are housed at 25° then moved to 32° overnight (more than12 h) to remove Gal80^{TS} suppression of Gal4 expression.

(*Continued*)

Fig. 3. (*Continued*). (*E*) Western blot of endogenous syntaxin1A protein (*red*) and HA tag (*green*) to verify the heat treatment of flies induced expression of syx^{227}. Flies expressing the UAS element alone (UAS-syx^{227}/+) and UAS and Gal4-Gal80^{TS} elements together (Elav-Gal4/+; UAS-syx^{227}/+; tubulin-Gal80^{TS}/+) were left at room temperature (first two lanes) or heated overnight. HA protein expression is only present after flies have been heated. (*F*) Responsiveness and locomotion ED50 values for heated flies (top) expressing syx^{227} in all neurons (*yellow*, Elav-Gal4/+; UAS-syx^{227}, n = 40) following removal of GAL80^{TS} suppression compared to Gal4 (*grey*, Gal4 Control, Elav-Gal4/+, n = 40) and UAS (*black*, UAS Control, UAS-syx^{227}/+; tubulin-Gal80^{TS}/+, n = 40) genetic controls. Bottom is a nonheated control. *Error bars* show standard error of the estimate. Extra sum-of-squares F test, *$P < 0.05$, ***$P < 0.001$. (*G*) Recovery from isoflurane anesthesia (mean proportion responding) in flies expressing syx^{227} in all neurons (*orange*, Elav-Gal4/+; UAS-syx^{227}, n = 68) compared with Gal4 (*grey*, Gal4 Control, Elav-Gal4/+, n = 68) and UAS (*black*, UAS Control, UAS-syx^{227}/+, n = 68) genetic controls. *Error bars* show ± SD. *Asterisks* indicate the first stimulus where responses of Elav-Gal4 > syx^{227} flies were significantly different from control flies. Kruskal–Wallis corrected for multiple comparisons (Dunn). ****$P < 0.001$. Time points on the *x* axis show discrete stimulus events every 10 min. Data points show reaction proportion for each genotype at each stimulus event. (*H*) Triangles in *G* indicate stimulus at which response is no longer statistically significantly different from baseline. (*I*) Mean time taken to first initiate locomotion following anesthesia induction. *Error bars* show ± SD. One-way ANOVA (between-subjects), n.s., not significant.

could be produced for both the responsiveness (ED50 = 0.338 ± 0.004% isoflurane, $P < 0.001$ extra sum-of-squares F test) and the baseline locomotion endpoint (ED50 = 0.348 ± 0.027% isoflurane, $P = 0.019$ extra sum-of-squares F test), compared with genetic controls that did not express the truncated syntaxin1A but instead only expressed the Gal4 protein (Elav-Gal4/+, grey; responsiveness ED50 = 0.268 ± 0.027% isoflurane, baseline ED50 = 0.271 ±0.025% isoflurane) or contained the UAS construct (syx^{227}/+, black; responsiveness ED50 = 0.272 ± 0.010% isoflurane, baseline ED50 = 0.261 ± 0.022% isoflurane) and had also been transiently exposed to heat (fig. 3F, top). There were no differences among the strains in experimental animals that were not exposed to 32° (fig. 3F, bottom, responsiveness $P = 0.092$, locomotion $P = 0.165$, extra sum-of-squares F test). This confirms that the resistance-inducing effect of the coexpressed, truncated syntaxin1A protein (syx^{227}) is not a consequence of altered neural development, but rather an acute effect on presynaptic mechanisms. Similar to the syxH3-C and syxH3-N mutants, coexpression of syx^{227} in the fly nervous system promoted a significantly faster recovery from isoflurane anesthesia (fig. 3, G and H; proportion responding after 20 min 0.62 ± 0.48, $P < 0.001$, Kruskal–Wallis), compared with genetic controls (proportion responding 0.22 ± 0.42 and proportion responding 0.33 ± 0.48). We noted that recovery was faster for one of the genetic controls, compared with the other control (UAS-syx^{227}/+, black line compared with gray line in fig. 3G), which could be an effect of (currently undetectable) leaky expression or genetic background.[6,30] The time to first movement was not affected by expression of syx^{227} (fig. 3I; 366 ± 140 s; UAS-syx^{227}/+ 424 ± 179 s, $P = 0.059$; Elav-Gal4/+ 367 ± 144 s, $P = 0.999$, one-way ANOVA), in line with the other neomorphic syntaxin1A mutants.

Drosophila exist as two distinct life stages, adult flies and larval maggots. We adapted our larval general anesthesia assay[6,30] to monitor both induction and recovery from isoflurane anesthesia (fig. 4A). We found that syx^{227} also produced behavioral resistance to isoflurane in larvae for both induction (proportion moving 0.56 ± 0.23 *vs.* control proportion moving 0.28 ± 0.13, $P = 0.012$, t test) and recovery

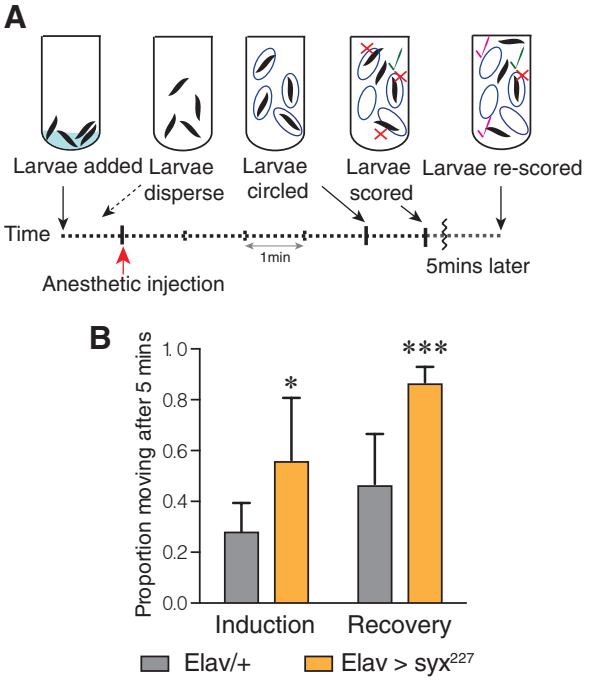

Fig. 4. Isoflurane resistance in larvae. (*A*) Schematic and timeline of larval anesthesia experiments. After isoflurane (0.56%) exposure, the position of the larvae is traced on the outside of the glass (*blue circles*). Larvae that have moved at least one-body-length outside the marked circle are noted (*green tick*). Larvae that have not moved are also scored (*red cross*). To quantify recovery from anesthesia, the behavioral apparatus is then opened to allow anesthetic gas to escape. After a further 5 min, larvae are rescored (*pink ticks*), using same criteria to quantify recovery as per induction: larvae that had been marked with a cross must have moved at least one-body-length outside the marked circle to qualify for recovery. (*B*) The proportion of larvae moving after isoflurane exposure (induction) and upon removal of isoflurane gas (recovery) for Elav-Gal4/+; upstream activation sequence-syx^{227} flies (*orange*) compared with Elav-Gal4/+ controls larvae (*grey*). *$P < 0.05$, ***$P < 0.001$, t test (n = 8 experiments per genotype). *Error bars* show ± SD.

(proportion moving 0.86 ± 0.05 *vs.* control proportion moving 0.46 ± 0.19, $P < 0.001$, *t* test) from isoflurane anesthesia (fig. 4B). Larvae provide an ideal platform for investigating electrophysiologic properties of synapses, which we examined next.

Expressing a Truncated Syntaxin1A Protein Promotes Recovery of Neurotransmitter Release in Larval Fly Synapses

A reason why syx^{227} expression affords resistance to isoflurane could relate to presynaptic mechanisms, because syntaxin1A is a presynaptic protein involved in neurotransmitter release.[8] To address how syx^{227} might be producing this resistance effect at a synaptic level, we turned to electrophysiologic recordings at the glutamatergic larval neuromuscular junction (fig. 5A).[39] Experiments were performed on both syx^{FL} and syx^{227} strains, where we recorded excitatory junction potentials and miniature excitatory junction potentials (fig. 5A), from which quantal content could be calculated.[6,30,39] As previously, the preparations were perfused with a saturated isoflurane solution (0.19 mM[6]; fig. 5B). We investigated the effects of isoflurane during induction as well as during recovery, after the drug had been washed away (fig. 5B). In both strains, excitatory junction potential amplitudes were significantly decreased with isoflurane perfusion (fig. 5C, after 2 min of perfusion for syx^{FL} $P = 0.036$, and after 4 min of perfusion for syx^{227} $P = 0.025$, both statistics two-way ANOVA with Dunnett's multiple comparisons test). Within 5 min after washout of the anesthetic, excitatory junction potential amplitudes remained decreased in syx^{FL} whereas they returned to baseline levels in syx^{227} larvae (fig. 5D, $P = 0.014$ two-way ANOVA with Sidak's multiple comparisons test). In contrast to evoked responses, miniature excitatory junction potential amplitudes were unaffected in either strain (fig. 5, E and F). Quantal content mirrored the effects on excitatory junction potentials (fig. 5, G and H, $P = 0.022$, two-way ANOVA with Sidak's multiple comparisons test), indicating that isoflurane exposure reduced neurotransmitter release, without affecting the postsynaptic responsiveness to released neurotransmitter (because miniature excitatory junction potential amplitudes were unaffected). These experiments confirm that coexpression of syx^{227} affords resistance to isoflurane at synapses, with the faster recovery of quantal content representing a quicker return to normal levels of neurotransmitter release at larval motor nerve terminals.

Is the Truncated Syntaxin1A Protein in the Core SNARE Complex?

How might the truncated syntaxin1A protein be promoting a faster recovery from isoflurane anesthesia at these different levels of investigation (adult responsiveness, larval locomotion, and quantal content)? One key question pertains to whether or not the truncated protein is incorporated into core SNARE complexes. If it is, then the protein might somehow promote vesicle release (thus, quantal content) in the presence of the drug. If it is not in the SNARE complex, then the protein might be promoting the recruitment of different components of the soluble n-ethylmaleimide sensitive factor attachment protein receptor machinery instead (fig. 6A). We were able to address this question by determining whether the HA-tagged syx^{227} and the syx^{FL} proteins were incorporated into the soluble n-ethylmaleimide sensitive factor attachment protein receptor complex (see Materials and Methods). We first used a syntaxin1A antibody to confirm that wild-type syntaxin1A could be detected in all three strains (Canton-S [wild-type], syx^{FL}, and syx^{227}; fig. 6B, left, black arrowhead). We noted that unlike the syx^{FL} protein (fig. 6B, left, red arrowhead), the syntaxin1A antibody did not recognize the smaller syx^{227} protein. The native syntaxin1A protein is also clearly present in the SNARE complex (fig. 6B, left, light red arrowhead). Because our constructs are HA-tagged, we then used an HA antibody to probe specifically for the transgenic syntaxin1A proteins. We found two different results: the full-length protein is clearly incorporated into the SNARE complex, whereas the truncated protein is not (fig. 6B, right, red and orange arrowheads). We found exactly the same result in *Drosophila* brains collected under isoflurane exposure (Supplemental Digital Content 2, http://links.lww.com/ALN/B985).

Discussion

In this study, we found that deletion mutations in syntaxin1A, when coexpressed alongside wild-type syntaxin1A in *Drosophila melanogaster*, significantly reduce the recovery time after isoflurane anesthesia. Whereas resistance to isoflurane was also evident during anesthesia induction, this effect was weaker compared with effects on recovery, in adult flies. This suggests that recovery from isoflurane anesthesia depends at least in part on syntaxin1A function. It was surprising how long adult wild-type flies required to regain normal levels of behavioral responsiveness after the procedure, compared with regaining locomotion; behavioral responsiveness still remained impaired even after two hours. In contrast, the syntaxin1A mutants could recover behavioral responsiveness after 10 min. Delayed recovery of behavioral responsiveness in *Drosophila* may therefore be a promising model for studying cognitive impairments following general anesthesia in humans, which also often follow a longer time course than simply regaining consciousness.[40] A full restoration of presynaptic functions across the brain is probably a complex problem in any animal, and the extremely conserved nature of synaptic release mechanisms suggests this might be a common mechanism.

Our syntaxin1A manipulations improved anesthesia recovery times across entirely different levels of analysis. Examination of effects on neurotransmission at the fly neuromuscular junction corroborated our behavioral findings: recovery of quantal content occurred within 5 min in the syntaxin1A mutant animals. Although it is unknown what

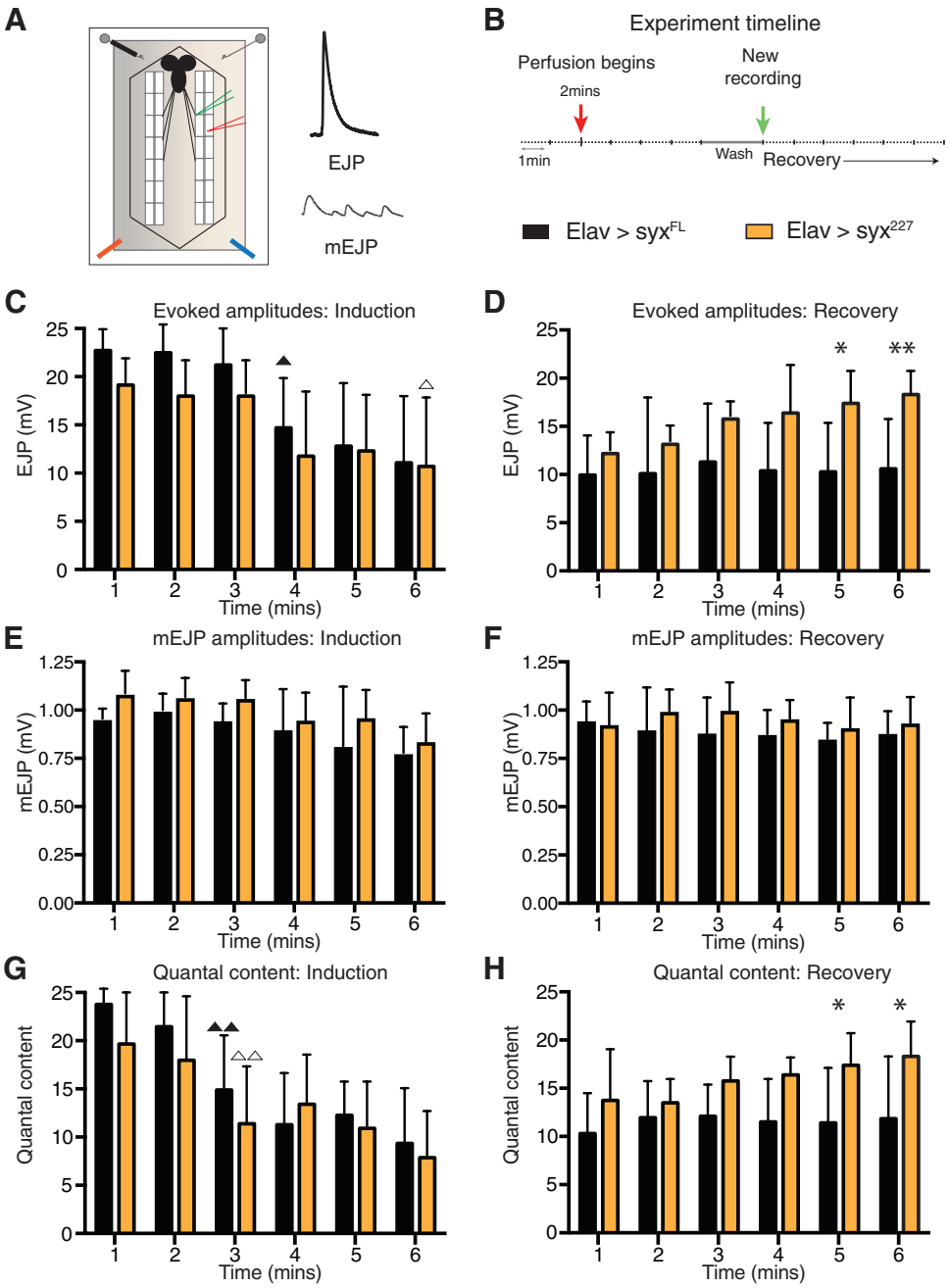

Fig. 5. Neurotransmission at the larval neuromuscular junction. (*A*) Schematic showing larval electrophysiology preparation (*left*). White squares represent body wall muscles with recording electrode shown in red and stimulating electrode shown in green. Example excitatory junction potential (EJP) and miniature excitatory junctional potential (mEJP) traces are shown on the *right*. (*B*) Time course of a neuro-muscular junction electrophysiology experiment, showing duration of the recording (in minutes) with *red arrow* denoting when isoflurane perfusion begins. Isoflurane perfusion stops after 6 min, and the preparation is washed for several minutes with fresh HL3 saline. A new recording begins (*green arrow*) and continues for 6 min. Panels *C* through *H* show control (Elav-Gal4/+; syx^{FL}/+, n = 8) and Elav-Gal4/+; syx^{227}/+ (*black*, n = 8) larvae recording parameters over time. Mean EJPs (mV) during isoflurane induction (*C*) and isoflurane recovery (*D*). The amplitude of spontaneous mEJPs (mV) during isoflurane induction (*E*) and isoflurane recovery is shown (*F*). Quantal content changes over isoflurane induction (*G*) and isoflurane recovery (*H*). Triangles show first instance of significant differences from baseline (1 triangle $P < 0.05$, 2 triangles $P < 0.01$), two-way ANOVA (within-subjects) corrected for multiple comparisons (Dunnett's). *Asterisks* show significant differences between genotypes $^{*}P < 0.05$, $^{**}P < 0.01$, two-way ANOVA (between-subjects) corrected for multiple comparisons (Sidak's). *Error bars* show ± SD.

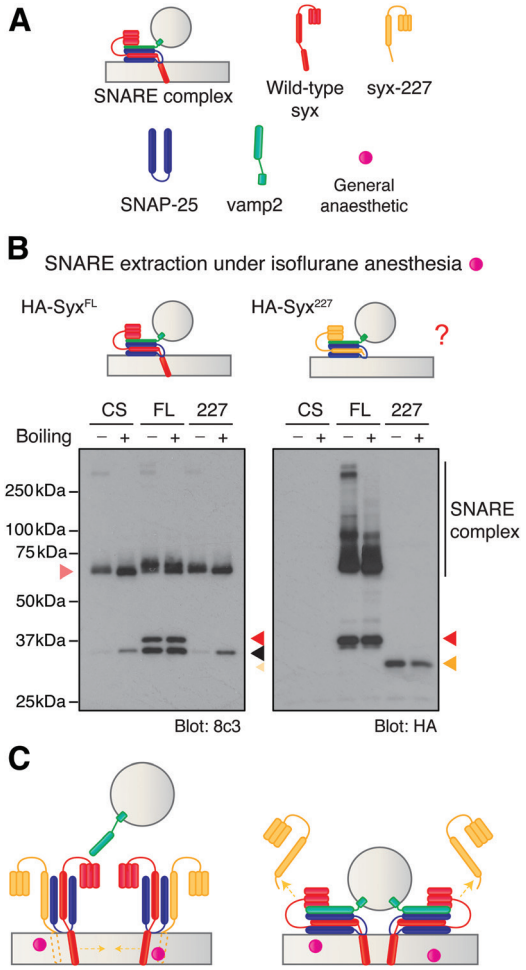

Fig. 6. syx^{227} is absent from soluble n-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complexes. (*A*) Key molecules involved in neurotransmitter release, and their presumed arrangement in the SNARE complex. (*B*) Western blots of fly head samples probed for the presence of wild-type syntaxin1A protein (*Canton-S* strain), the truncated syntaxin1A protein (227), and full-length overexpression (FL) in the SNARE complex. *Left*: A syntaxin1A antibody (8c3) identifies the endogenous protein in all samples (*black arrowhead*) and the longer full-length construct (due to the attached human influenza hemagglutinin (HA) tag, *red arrowhead*). *Light red arrowhead* shows syntaxin1A in SNARE complexes. *Right*: a HA antibody identifies the coexpressed full-length (syx^{FL}, *red arrowhead*) and truncated (syx^{227}, *orange arrowhead*) proteins. HA labeling can be seen in the SNARE complex (~100 kd [kDa] smear) when the full-length protein is expressed, but completely absent when the truncated protein is expressed. (*C*) A model for the role of syx^{227} in conferring resistance to anesthesia at the SNARE. *Left*: syx^{227} binds synaptosomal associated protein 25 (SNAP25) along with endogenous syntaxin1A, providing open syntaxin1A that is ready to interact with other proteins at active zones, to form SNAREs. *Right*: Once a vesicle docks and vesicle-associated membrane protein 2 (VAMP2) binds SNAP25 and wild-type syntaxin1A, it is energetically more favorable, thereby releasing the truncated syntaxin1A, thus why it is undetected within the SNARE in *B*.

neurotransmission recovery dynamics might be like in adult brain synapses, it seems likely that similar effects on recovery might be present because the same syntaxin1A protein is involved in the brain as at the larval neuromuscular junction, or in all animal synapses.[9,27] Mutant *Drosophila* larvae also recovered faster than controls behaviorally (within 5 min), indicating an effect that transcends brains at different levels of complexity (the larval brain has an order of magnitude fewer neurons than the adult fly brain).[19] It remains unknown, however, whether adult brain synapses are affected in the same way as motor synapses. Behavioral recovery dynamics in adult mutant animals remain more sluggish than recovery of quantal content at the larval neuromuscular junction. This suggests that brain synapses might recover function differently than motor nerve terminals in larvae.

One limitation of our study was the use of only female flies for behavioral experiments in adults. Sex-specific effects in *Drosophila* have been observed during recovery from anesthesia,[41,42] although these effects generalize to cold-shock and oxygen deprivation anesthesia, which are likely unrelated to the presynaptic mechanisms described in this study. Given the lack of sexual dimorphism in synaptic proteins, it is unlikely that the phenotypes we describe here would be different using male flies, although this remains to be tested experimentally. In our larval studies we used both male and female animals. Despite any potential sexual differentiation in larval motor nerve terminals,[43] we still found significant effects in our larval isoflurane experiments with expression of the truncated syntaxin1A protein.

How might a coexpressed syntaxin1A truncation construct be conferring a rapid recovery from isoflurane anesthesia? This same manipulation has now been shown to produce resistance to diverse general anesthetics across a variety of systems, *in vitro* and *in vivo*.[1,3,4,10] Because syntaxin1A is a key player in SNARE-mediated exocytosis, it was therefore surprising to find that the HA-tagged truncated protein syx^{227} was not present in soluble n-ethylmaleimide sensitive factor attachment protein receptor complexes, at least in *Drosophila*. Because syx^{227} has been shown to interact with synaptosomal associated protein 25 (SNAP25) and wild-type syntaxin1A,[1] this suggests an effect immediately before SNARE formation, meaning the truncated protein is probably ejected upon full soluble n-ethylmaleimide sensitive factor attachment protein receptor formation (when vesicle-associated membrane protein 2 links with syntaxin1A and SNAP25 to form a release-ready tetrameric complex; fig. 6C). This would imply that the protective effect of this protein is required before SNARE formation, and accordingly that the anesthetic effect on syntaxin1A function is also prior to soluble n-ethylmaleimide sensitive factor attachment protein receptor formation. This view is consistent with our recent findings using super-resolution microscopy to track the mobility of single syntaxin1A molecules under propofol anesthesia.[1] We found that clustering of syntaxin1A

caused by propofol was dependent on an interaction with SNAP25, but not with vesicle-associated membrane protein 2, thereby suggesting a mechanism of action (for propofol) immediately before full SNARE formation. Work in other systems also suggests an interaction between volatile anesthetics and syntaxin1A/SNAP25,[44] suggesting that this might indeed by a general anesthetic target. On the other hand, work in *Caenorhabditis elegans*, where the effect of the truncated syntaxin1A was discovered,[10] points to *unc-13* as a likely mediator of this resistance-promoting mechanism.[45] *unc-13* is understood to be associated with presynaptic active zones, where the SNAREs ultimately reside,[46] so one interpretation of these diverse findings is that the drug-mediated clustering of syntaxin1A/SNAP25 occurs at these active zones, and that these pre-SNARE complexes are prevented from transforming into full SNAREs because *unc-13* is less able to catalyze the next step. In this regard, it will be especially interesting to investigate what role *unc-18* plays in this process; *unc-18* has been shown to keep syntaxin1A in a closed conformation, until interaction with *unc-13* opens syntaxin1A to promote complete full SNARE formation.[47] One hypothesis consistent with this model is that general anesthetics promote a closed syntaxin1A conformation, by for example impairing the capacity of *unc-13* to catalyze SNARE formation. One hypothesis for how syx^{227} affords resistance then is that the truncated (or deletion) proteins might promote open syntaxin1A moieties, and in this way remove an emergent target (the closed syntaxin1A-*unc-18* complex). Interactions with vesicle-bound vesicle-associated membrane protein 2 would then lead to an energetically more favorable ternary complex, effectively ejecting syx^{227} upon SNARE formation (fig. 6C). Future biochemical experiments should determine whether syx^{227} promotes an open syntaxin1A conformation, and to what level *unc-13* and *unc-18* are involved in this process.

One of the most striking observations in this this study is the prolonged duration of recovery from isoflurane anesthesia in wild-type flies, and how syntaxin1A mutations significantly reduce this recovery time. If syx^{227} is acting before SNARE formation, then how might this lead to faster recovery? One possibility following from the hypothesis proposed above is that syx^{227} provides more efficient access to already open pre-SNARE complexes that are ready to be incorporated into fully formed SNAREs. If general anesthetics produce a traffic jam of nonfunctional pre-SNARE nanoclusters, as suggested by our single-molecule imaging experiments,[1] then the time required for dissolving this proteinaceous traffic jam might take longer than clearance of the anesthetic drugs themselves. Consistent with this view, our imaging work showed that expression of syx^{227} in mammalian cells prevented the syntaxin1A clustering effects of another general anesthetic, propofol. In contrast to these sluggish presynaptic recovery effects, the postsynaptic effects of general anesthetics such as isoflurane and propofol are most likely rapid, as they primarily linked to gamma-aminobutyric acid receptor function.[13] General anesthesia induction is a rapid process, as this probably engages potent inhibitory systems in the brain that are designed to promote a rapid loss of consciousness.[12,48] However, a rapid reversal of the effect on gamma-aminobutyric acid receptors after removal of these drugs might have little consequence on recovery from anesthesia until presynaptic processes across the brain have been fully restored. Our data on syntaxin1A fly mutants exposed to isoflurane support this view of general anesthesia, with the largest effects seen for recovery rather than induction. However, the fact that these mutants are also resistant to isoflurane upon induction suggests that presynaptic effects might play a role during anesthesia induction as well. It will be interesting in future experiments to combine genetic manipulations that promote anesthetic resistance at both a pre- and postsynaptic levels, in animals that have both target mechanisms (*i.e.*, sleep/wake pathways and SNAREs).[11] Such experiments will allow us to better dissect the relative contributions of either target process, and to determine whether some circuits or neurotransmitter systems are more affected by the presynaptic mechanisms we have uncovered.

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Competing Interests

The authors declare no competing interests.

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