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An Evolutionarily Conserved Presynaptic Protein Is Required for Isoflurane Sensitivity in *C. elegans*

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Abstract

Background—Volatile general anesthetics inhibit neurotransmitter release by an unknown mechanism. A mutation in the presynaptic SNARE protein syntaxin-1A was previously shown to antagonize the anesthetic isoflurane in *C. elegans*. The mechanism underlying this antagonism may identify presynaptic anesthetic targets relevant to human anesthesia.

Methods—Sensitivity to isoflurane concentrations in the human clinical range was measured in locomotion assays on adult *C. elegans*. Sensitivity to the acetylcholinesterase inhibitor aldicarb was used as an assay for the global level of *C. elegans* neurotransmitter release. Comparisons of isoflurane sensitivity (measured by the EC_{50}) were made by simultaneous curve-fitting and F-test as described by Waud.

Results—Expression of a truncated syntaxin fragment (residues 1-106) antagonized isoflurane sensitivity in *C. elegans*. This portion of syntaxin interacts with the presynaptic protein UNC-13, suggesting the hypothesis that truncated syntaxin binds to UNC-13 and antagonizes an inhibitory effect of isoflurane on UNC-13 function. Consistent with this hypothesis, overexpression of UNC-13 suppressed the isoflurane resistance of the truncated syntaxins, and *unc-13* loss-of-function mutants were highly isoflurane resistant. Normal anesthetic sensitivity was restored by full-length UNC-13, by a shortened form of UNC-13 lacking a C2 domain, but not by a membrane-targeted UNC-13 that might bypass isoflurane inhibition of membrane translocation of UNC-13. Isoflurane was found to inhibit synaptic localization of UNC-13.

Conclusions—These data show that UNC-13, an evolutionarily-conserved protein that promotes neurotransmitter release, is necessary for isoflurane sensitivity in *C. elegans* and suggest that its vertebrate homologs may be a component of the general anesthetic mechanism.

Summary Statement: Mutations in *unc-13*, which encodes an evolutionarily conserved presynaptic protein, abolish isoflurane sensitivity. Genetic and cell biological evidence indicate that isoflurane blocks membrane targeting of the UNC-13 protein thereby inhibiting its promotion of neurotransmitter release.

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Introduction

At clinical concentrations, volatile general anesthetics (VAs) like isoflurane have multiple electrophysiological effects that depress overall nervous system activity and likely contribute to their mechanism of action [1]. By clinical concentrations, we mean concentrations of anesthetic that are in the range used in human clinical practice; 2 minimal alveolar concentration (MAC) of isoflurane produces an aqueous concentration of 0.62 mM. Thus, we operationally define anesthetic concentrations less than 0.6 mM as clinical concentrations. One of the actions of clinical concentrations of VAs is inhibition of neurotransmitter release [2]. The mechanism of this inhibition is poorly understood. Release of glutamate and gamma-amino butyric acid (GABA) from rat cortical synaptosomes is inhibited by VAs and inhibition of sodium channels blocks the effect of VAs on 4-aminopyridine-evoked release but not on basal release [3,4]. VAs more efficaciously inhibit glutamate release compared to GABA release from synaptosomes. Sodium channel blockade does not explain the differential inhibition by VAs of the basal release of glutamate and GABA [3]. In rat hippocampus, VAs have been shown to inhibit glutamatergic transmission by a primarily presynaptic mechanism [5]. Subsequent studies confirmed a presynaptic VA action in the hippocampus and attributed approximately a third of the inhibition of glutamate release to a reduction in the action potential and by default the remainder of the effect to downstream targets such as the transmitter release machinery [6,7]. As was found with synaptosomes, VAs selectively inhibited glutamate versus GABA release [6].

Consistent with a presynaptic anesthetic mechanism in the nematode *C. elegans*, we found in a screen through existing *C. elegans* mutants that mutations reducing levels of neurotransmitter release conferred hypersensitivity to the VAs halothane and isoflurane [8] and mutations increasing transmitter release conferred resistance [9-11]. These results could be explained if VAs inhibited excitatory neurotransmitter release. Indeed, isoflurane and halothane both produced behavioral and pharmacological effects resembling mutants with reduced excitatory neurotransmission [8] in that anesthetized animals moved sluggishly and were resistant to the acetylcholinesterase inhibitor aldicarb. Aldicarb increases the steady state level of acetylcholine at the neuromuscular junction and thereby produces a depolarizing neuromuscular blockade; mutations or drugs that reduce acetylcholine release confer resistance to aldicarb [12].

In testing all available viable alleles of genes known to regulate neurotransmitter release in *C. elegans*, we found one mutation in the *C. elegans* syntaxin-1A gene *unc-64* that had an unexpected phenotype. *unc-64(md130)* (indicates a strain carrying the *md130* mutation in the *unc-64* gene) had reduced excitatory neurotransmission by behavioral, aldicarb sensitivity, and electrophysiological measurements [8,13], yet it was highly VA resistant [8]. For example, its isoflurane EC_{50} was more than 5-fold that of wild type, making *unc-64(md130)* fully resistant to isoflurane concentrations in the clinical range. *unc-64(md130)* is 30-fold less sensitive to isoflurane than other *unc-64* reduction-of-function alleles; thus, isoflurane resistance is not a general property of reduction of syntaxin function. The *md130* mutation disrupts an intron donor splice sequence, resulting in a reduced level of full-length syntaxin and the production of novel truncated syntaxins [8]. By Western blot, the relative ratio of full-length to truncated syntaxin is approximately 4:1 (Barbara Scott, B.S and CM Crowder, M.D., Ph.D, Dept. of Anesthesiology, Washington University, St. Louis, MO, USA, written communication, March, 2007); thus, if truncated syntaxins are actually responsible for the anesthetic resistance, a relatively low total concentration of truncated syntaxin can antagonize anesthetic action.

Despite our previous report of isoflurane binding to syntaxin [14], several genetic results argue against the most direct model where the isoflurane resistance of the *md130* mutation is due to deletion of the isoflurane binding sites on syntaxin. First, the isoflurane resistance phenotype of *md130* is semidominant [8]. Second, an *unc-64* null mutation has no anesthetic phenotype as

a heterozygote [8]. In other words, unlike with the *md130* mutation in the background, one copy of wild type syntaxin confers normal isoflurane sensitivity; thus, the *md130* mutation does not behave genetically as if the isoflurane resistance is due to loss of a binding site. Third, structural and cell biological studies strongly support a model where the portion of syntaxin deleted by the *md130* mutation is absolutely required for the normal function of syntaxin [15]. Thus, in *unc-64(md130)*, which expresses both wild type and truncated protein, the loss of binding of isoflurane to the truncated form would have no consequence since it cannot serve as a functional syntaxin anyway. Further, the remaining wild type syntaxin in the mutant would still bind isoflurane as usual and should be affected normally. Rather than the *md130* mutation deleting an isoflurane binding site, the most parsimonious hypothesis is that these truncated syntaxins act essentially as VA antagonists against the anesthetic target. Here we show that truncated syntaxins do in fact antagonize isoflurane action, identify the likely protein target for truncated syntaxin, find that this protein is necessary for VA sensitivity, and show that VAs act to alter its synaptic localization. Thus, this protein fits criteria for a functional VA target.

Materials and Methods

C. elegans strains and transformants

A list of strains used in the work is given in Table 1. N2 *var* Bristol was the wild type strain and the genetic background for all mutants [16]. *tom-1(ok285)* was obtained from the *C. elegans* knockout consortium; *ok285* is a 1580 bp *tom-1* deletion, which removes all or part of 4 exons in the center of the gene [17]. *tom-1(ok285) unc-13(e376);unc-64(js115);oxIs34* was constructed by selecting Unc non-Dpy progeny segregating from *dpy-5(e51) + e376/+ ok285 +* hermaphrodites and homozygosing for *ok285 e376*. *unc-64(js115);oxIs34*[*unc-64*(L166A/E167A);P*myo-2*::GFP][18] males were then crossed with *ok285 e376* and the best moving Unc Green second generation progeny were clonally passaged. The presence of the homozygous*tom-1(ok285)* deletion was confirmed by polymerase chain reaction amplification of the mutant gene; the homozygous *js115* mutation was confirmed by outcrossing from *oxIs34* and observing segregation of dead larval progeny on all second-generation broods. *tom-1(ok285) unc-13(s69);unc-64(js115);oxIs34* was constructed similarly and was a gift from Janet Richmond, Ph.D., Department of Biological Sciences, University of Illinois Chicago, Chicago, Illinois, USA. For heterozygous *unc-64(md130)* animals, non-Unc non-Bli animals segregating from *md130+/+bli-5* were used. *mdIs3*[*unc-13(+);snb-1*::GFP] is an integrant of *mdEx43*19 and was a gift from Kenneth G. Miller, Ph.D. (Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). *mdIs3;gcEx45* [pTX_{md130}] and *mdIs3;gcEx55*[$pTX_{1–107}$] were constructed by crossing *gcEx45 or gcEx55* males into *mdIs3* and passaging progeny with both Green Fluorescent Protein (GFP) cotransformation markers until homozygous for *mdIs3*. *unc-13(s69);oxIs78* [*myr::unc-13*S::GFP;ccGFP] was a gift from Erik Jorgensen, Ph.D. and Kim Schuske, Ph.D., Department of Biology, University of Utah, Salt Lake City, Utah, USA and was generated by injection and integration of KP280 [20]; the presence of the myristoylation consensus sequence was confirmed by sequencing the integrated transgene. The truncated syntaxin transformants were generated by gonad injection of 50 – 100 ng/μl of the the particular pTX plasmid along with 40 ng/μl of the hypodermal GFP coinjection marker pPHgfp-1 [21] into N2 and selection of stably transformed lines.

Plasmids constructs

The parent plasmid for all syntaxin plasmid constructs was pTX21, which contains a 12 kb *Pst*I – *Mfe*I rescuing genomic fragment from pTX20 [13] inserted into pBluescript (KS^{-}) and includes all known *unc-64* transcripts and transcriptional regulatory regions. pTX^{md130} was generated by polymerase chain reaction amplification from *unc-64(md130)* genomic DNA and insertion of an *Nhe*I – *Nsi*I fragment spanning the *md130* mutation into *Nhe*I – *Nsi*I cut pTX21.

$pTX_{1–258}$, $pTX_{1–227}$, $pTX_{1–158}$, $pTX_{1–106}$, $pTX_{1–86}$, and $pTX_{1–64}$ were all made by one or two rounds of oligonucleotide-directed mutagenesis (Stratagene Quickchange, Stratagene, Inc, La Jolla, CA, USA) of pTX21 to generate a stop codon at the desired site. All plasmid constructs were sequenced to confirm the mutation.

Behavioral and drug assays

Locomotion was measured on 2% agar plates as the fraction of animals that dispersed in 40 minutes from the center of a 9.5 cm plate to the edge that was seeded with bacteria (the dispersal index) [10], by the rate of body bends [9], or by the speed of movement across an agar plate as described previously [22]. *unc-64(md130)* is resistant in all three assays (Laura B. Metz, B.S., Dept. of Anesthesiology, Washington University, St. Louis, MO, USA, written communication, March, 2007). However, the slow and slowest strains did not move well enough to disperse to the edge of the dispersal plates, and the slowest strains had weak and infrequent body bends that produce excessively variable results in body bend assays. Thus, we used the relatively easy body bends assay for slow strains and the highly quantitative but tedious speed assay for the slowest strains. The dispersal assay was used for normally moving strains. Aldicarb sensitivity was measured by the rate of paralysis on 0.35 mM aldicarb-containing agar plates [23]. Isoflurane were delivered and the concentrations measured as described previously [24]. Locomotion as measured by one of the three assays was plotted against anesthetic concentration; concentration/response data were fitted to a modified Hill equation to calculate EC_{50} (the anesthetic concentration at which the reduction in locomotion was half maximal) and slope [24].

Scoring of UNC-13::GFP puncta density

L4 larval stage animals were exposed on agar plates to 0 or 1 μg/ml phorbol-12-myristate-13-acetate (PMA) for 1 hour. Subsequently, the plates were placed into glass chambers containing 0 or 2 vol% isoflurane for an additional 1 hr then transferred rapidly by platinum wire onto a thin agarose pad into a fresh drop of M9 buffer [16] containing 0 or 1mM isoflurane (≈ 1.9 vol % isoflurane) and 10 mM azide. Agar pads were rapidly sealed with a glass coverslip. The dorsal nerve cord was imaged within five minutes of removal of worms from the glass chamber. Preliminary experiments showed that isoflurane without azide remained effective at producing sluggish movement under these conditions for at least five minutes (CM Crowder, M.D., Ph.D., Dept. of Anesthesiology, Washington University, St. Louis, MO, USA, written communication, March, 2007). The Images were obtained on a Zeiss Axioskop 2 (Carl Zeiss Microimaging, Inc, Thornwood, NY, USA) using a 63X Plan-Apo 1.4na oil immersion lens and a Chroma 41017 Endow GFP filter set and captured using a Retiga Exi CCD camera coupled to Q-Capture Pro software (QImaging, Inc, Surrey, BC, Canada) with identical capture settings for all animals. Image files were coded and scored using Scion NIH image software (Scion, Corporation, Frederick, MD, USA) by an observer blinded to condition. For each animal, puncta were manually counted along the longest length of dorsal nerve cord in the focal plane. The distance of dorsal nerve cord scored was measured by conversion of pixel number to micrometers. Puncta density was then expressed for each animal as puncta/100 μm of dorsal nerve cord.

Statistical analysis

All statistical comparisons were made using GraphPad Prism 4 software (GraphPad, Software, Inc., San Diego, CA, USA). EC_{50}s were compared for statistical differences by simultaneous curve fitting as described by Waud [25] using GraphPad Prism 4. For a particular strain, EC_{50}s were estimated by pooling all of the data for that strain; the error values following the EC_{50} values are the error of the fit. Locomotion rates were compared by two-sided t-test. GFP puncta

density was compared by two-sided t-test. The threshold for statistical significance for all tests was set at $p < 0.01$.

Results

To test whether the truncated syntaxin was responsible for the VA resistance phenotype and, if so, to define the structural requirements for its antagonism, we generated a series of plasmids expressing truncated syntaxins under the native *unc-64* promoter , and tested the isoflurane sensitivity of the transformed animals (fig. 1). As expected, transformation of wild type *C. elegans* with pTX_{md130}, which contains the original *md130* mutation, conferred isoflurane resistance (fig. 1B,I). $pTX_{1–258}$, which expresses a truncated syntaxin longer than that produced by *md130* and without the additional amino acids produced by read through into the intron, also conferred isoflurane resistance (fig. 1C,I). Wild type animals transformed with $pTX_{1–227}$, which introduces a stop codon immediately 5' to the splice donor site mutated by *md130*, were also isoflurane resistant (fig 1D,I). The isoflurane resistance produced by both $pTX_{1–258}$ and $pTX_{1–227}$ demonstrates conclusively that truncated *unc-64* syntaxin does antagonize VA action and that the novel amino acids produced by the original *md130* mutation are not required for VA antagonism. Transformation with plasmids expressing increasingly smaller truncated syntaxins, $pTX_{1–158}$, $pTX_{1–106}$, showed that a fragment encoding only the H_{AB} domains of syntaxin was sufficient to produce resistance (fig. 1E,F,I). However, further truncation removing all or half of $H_{B,}$ $pTX_{1–64}$ or $pTX_{1–86}$, abolished the VA resistance-conferring activity (fig. 1G,H,I). Thus, C-terminally truncated syntaxin does indeed antagonize VA action and only a relatively small N-terminal fragment is sufficient for this action. This syntaxin fragment does not include the Soluble NSF Attachment Protein Receptor (SNARE) domain, encoded by residues 185 – 255, that is thought to interact with the majority of syntaxin-interacting proteins.

We have previously shown that mutations that increase transmitter release in *C. elegans* produce resistance to VAs [9,11]. Thus, an alternative to the hypothesis that the truncated syntaxin is a direct VA antagonist is that it indirectly antagonizes VA action by increasing synaptic transmitter release. We tested this hypothesis by measuring the locomotion and aldicarb sensitivity of three truncated syntaxin-expressing constructs that confer isoflurane resistance. Hyperactive locomotion and aldicarb hypersensitivity are indicative of increased neurotransmitter release [12]. None of the transformants were significantly hyperactive or hypersensitive to aldicarb (fig. 2A,B). Movement data for the hyperactive mutant, *goa-1 (sy192)* is included as a positive control for hyperactivity [11]. Data for the original *unc-64 (md130)*, which is aldicarb resistant due to reduced levels of wild type syntaxin[8,13], and *slo-1 (js379)*, which has a loss of function mutation in the BK Ca^{++}-activated K^+ channel that negatively regulates transmitter release [9,26], are included for positive controls for aldicarb resistance and hypersensitivity, respectively. Thus, we conclude that increased release of neurotransmitter is unlikely to be the mechanism whereby the truncated syntaxins produce VA resistance. We hypothesize that the truncated syntaxin interacts with another protein that regulates VA sensitivity and thereby antagonizes VAs.

With what might the truncated syntaxin interact to antagonize VA action? We first considered whether the putative interacting protein also interacted with wild type syntaxin. If so, wild type syntaxin should compete with truncated syntaxin and restore VA sensitivity. Indeed, animals with one copy of the *md130* mutation and one wild type allele were less resistant than homozygous *md130* animal (fig. 3A,B). In addition, transformation of *unc-64(md130)* with a plasmid containing full-length syntaxin (pTXfl) partially rescues the VA resistance of *md130*. Thus, wild type and truncated syntaxin appear to compete for interaction with the putative protein controlling VA sensitivity.

In vertebrates, two proteins that have been shown to interact with the N-terminal two helices of syntaxin are mUNC-13 and N-type calcium channels (fig. 4A) [27,28]. *C. elegans* has one mUNC-13 homolog (UNC-13) and one N-type calcium channel (UNC-2). If one of these is the relevant VA target, a null mutant should be VA resistant. Both UNC-2 and UNC-13 promote transmitter release in *C. elegans* by a mechanism at least partially conserved in mammals [29-34]. UNC-2 is thought to promote transmitter release by supplying calcium to the transmitter release machinery [29]. The molecular mechanisms whereby UNC-13 promotes transmitter release are more complex. UNC-13 acts in steps after docking of synaptic vesicles with the presynaptic membrane and somehow prime vesicles so that they are competent for fusion [31]. UNC-13's activity to promote vesicle fusion is partially dependent on interaction with syntaxin and may involve conversion of syntaxin from a closed conformation where the H3 helix containing the SNARE domain interacts tightly with its H_{ABC} domains to an open conformation where the H_3 helix is free from its self interactions and available for binding to the SNARE domains of other SNARE proteins [18]. However, catalyzing the transformation of syntaxin from a closed to open conformation does not appear to be the only function of UNC-13 [35]. We tested two severe *unc-13* alleles[19] and one null *unc-2* allele[29,36]. In order to test the essentially paralyzed *unc-13*(lf) animals, we placed the *unc-13* mutations in the background of three other mutations, *tom-1(ok285* null), *unc-64*(L166A/E167A), and *unc-64(js115* null). Both the tomosyn loss-of-function mutation, *tom-1(ok285)*, and the *unc-64*(L166A/E167A) mutation, which favors the open conformation of syntaxin[18], have been found to suppress partially the paralyzed phenotype of *unc-13*(lf) and when combined suppress the paralyzed *unc-13*(lf) phenotype adequately for anesthetic testing [17,37,38]. Tomosyn is thought to form a complex with syntaxin, preventing its binding to the vesicular SNARE synaptobrevin/vesicle-associated membrane protein (VAMP) and thereby reducing neurotransmitter release [17]. Open syntaxin may partially suppress *unc-13*(lf) by supplying the product of one of the normal functions of UNC-13, which is to convert closed syntaxin to open syntaxin [18]. The *unc-64 (js115)* mutation is included in the strain so that normal UNC-64 syntaxin does not compete with open syntaxin and thereby diminish the positive effect on locomotion of the *unc-13* mutants [18].

Both *unc-13* alleles were highly isoflurane resistant (fig. 4B,C). The locomotion of the null *unc-13* allele *s69* was not significantly decreased by isoflurane at concentrations up to 4 vol %, a concentration that maximally reduced wild type locomotion. The partial loss-of-function *e376* allele was less resistant than *s69* but still highly resistant compared to the wild type strain or to the *tom-1(ok285);unc-64(js115);oxIs34* genetic background control strain (fig. 4B,C). The *unc-2* null mutant was fully sensitive to isoflurane with an isoflurane EC_{50} not significantly different from wild type (fig. 4B,C). These results shown that UNC-13 is required for isoflurane sensitivity and that the UNC-2 calcium channel is not.

Thus, UNC-13 is required for normal VA sensitivity, but is it the target of the truncated syntaxin as originally proposed? To examine this hypothesis, we tested the ability of overexpressed wild type UNC-13 (produced by *mdIs3*) to suppress the VA resistance of the truncated syntaxin (fig. 5), the presumption being that UNC-13 when in excess of truncated syntaxin should restore VA sensitivity. Overexpression of full-length UNC-13 did fully suppress the isoflurane resistance of both pTX_{md130} and $pTX_{1–107}$ (fig. 5A,B). However, full-length UNC-13 overexpression (*mdIs3*) did not significantly change isoflurane sensitivity in a wild type background (fig. 5B). These results are most directly explained if truncated syntaxin physically interacts with UNC-13 to block VA sensitivity and when UNC-13 is expressed in excess of truncated syntaxin, it becomes available for inhibition by VAs. Alternatively, UNC-13 overexpression might prevent interaction of the truncated syntaxin with some other unknown protein that is essential for VA sensitivity.

If UNC-13 is a VA target in *C. elegans*, how might binding of VAs disrupt UNC-13 function? mUNC-13 (mammalian UNC-13) has been shown to bind to Rab3a-interacting molecule (RIM) [39], calmodulin [40], and syntaxin [27] (fig. 5A), and these interactions promote synaptic transmission [34,35,40,41]. Loss of function mutants of mouse RIM or the *C. elegans* homolog UNC-10 drastically reduces neurotransmitter release, and mammalian RIM has been implicated in cerebellar long term potentiation [42-45]. In mice, calmodulin binding to mUNC-13 has been implicated in activity-dependent synaptic facilitation [40]. Whether this function of calmodulin is conserved in *C. elegans* is unknown. In terms of VA action in *C. elegans*, a reasonable hypothesis is that VAs disrupt RIM or calmodulin binding to UNC-13 or directly alter RIM/calmodulin function in an UNC-13-dependent fashion. As for *unc-13*, *unc-10* null mutants move slowly, and as for *unc-13* mutants, this sluggishness is suppressed by open syntaxin [45], allowing testing of their anesthetic sensitivity. Both the *unc-10(md1117* null);open syntaxin double mutant and the open syntaxin mutant alone were fully sensitive to isoflurane (fig. 5C). These results demonstrate that UNC-10 RIM is not required for isoflurane action and that isoflurane does not act by inhibiting the interaction of UNC-10 with UNC-13. Additionally, the normal sensitivity of open syntaxin-bearing animals argues against a mechanism where VAs inhibit UNC-13's promotion of the open form of syntaxin [18]. Calmodulin null mutants are lethal in *C. elegans*. To test calmodulin's role, we measured the isoflurane sensitivity of animals transformed with a shortened form of UNC-13, UNC-13S (Fig. 5A), that lacks the calmodulin- and RIM-binding domains and partially rescues locomotion and transmitter release [20,23]. Animals expressing UNC-13S were normally sensitive to isoflurane (fig. 5D,F), demonstrating that neither the UNC-10- nor calmodulin-binding domains are necessary for VA sensitivity.

Based on four previous observations, we considered the hypothesis that VAs might antagonize diacyl glycerol (DAG) binding to and/or activation of UNC-13. First, DAG enhances the transmitter release promoting activity of UNC-13 in *C. elegans* and in higher organisms [20, 23,32]. Second, VAs and anesthetic alcohols have been shown to antagonize DAG-binding to and activation of mammalian Protein kinase C (PKC), which contains a C1 domain homologous to that binding DAG in UNC-13 [46,47]. Third, anesthetic alcohols have been shown to bind to the C1 domain near the DAG binding pocket [46]. Finally, we have previously shown that phorbol ester, a C1 domain agonist, antagonizes VAs in *C. elegans* 9.

To test whether VAs might act to block DAG activation of UNC-13, we made use of the ability of myristoylated UNC-13S (myr-UNC-13S) to promote transmitter release in *C. elegans* in a DAG-independent manner [20,23]. If VAs act on UNC-13 to disrupt DAG binding or activation, myr-UNC-13S by targeting itself to the membrane in a DAG-independent manner should bypass the effect of VAs on UNC-13 and should therefore be VA resistant. Indeed, animals expressing myr-UNC-13S were highly isoflurane resistant compared to those expressing UNC-13S or full-length UNC-13 - *unc-13(s69* lf);*mdIs3* (fig. 5E,F). We considered the possibility that myr-UNC-13S is VA resistant because it has increased levels of neurotransmitter release compared to wild type. To test this hypothesis, we compared the aldicarb sensitivity of wild type, *unc-13*S, and *myr-unc-13*S strains (fig. 5G). *myr-unc-13*S animals had wild type aldicarb sensitivity whereas *unc-13*S animals were aldicarb resistant. Thus, a general increase in neurotransmitter release does not explain the resistance of *myr-unc-13*S. Rather, the most direct explanation of these results is that myristoylation of UNC-13S confers isoflurane resistance by bypassing isoflurane antagonism of DAG-mediated membrane targeting of UNC-13.

To test directly whether isoflurane acts on UNC-13 to antagonize DAG-mediated membrane targeting, we observed the effect of isoflurane on localization of an UNC-13S::GFP fusion protein. This fusion protein has previously been shown to concentrate at synapses in a DAG-dependent manner, where it is visible as distinct puncta that co-localize with other presynaptic

proteins in *C. elegans* dorsal and ventral nerve cords [20,23,38]. Thus, the density of puncta along the nerve cord is an indicator of the relative amount of presynaptic membrane-localized UNC-13. Isoflurane significantly reduced both basal and phorbol-ester stimulated UNC-13S::GFP puncta in the dorsal nerve cord (Fig. 6, Table 2). Thus, we conclude that isoflurane decreases DAG-mediated synaptic localization of UNC-13. Alternatively, isoflurane could decrease overall levels of UNC-13; our data do not exclude this possibility.

Discussion

We have shown that a relatively short N-terminal syntaxin-1A fragment can fully antagonize the behavioral effects of clinical concentrations of the volatile anesthetic isoflurane in *C. elegans*. The antagonism is direct in that the truncated syntaxin had no apparent effect on locomotion or synaptic concentrations of acetylcholine as measured by aldicarb sensitivity. The specificity of the truncated syntaxin coupled with its dominant genetic behaviour argues that the truncated syntaxin is acting essentially as a pharmacological antagonist against the VA target. Working under this assumption, the question becomes to what is the truncated syntaxin binding to antagonize VAs? The number of proteins which have been shown to bind to this N-terminal segment of syntaxin is few. Testing of mutants of two such candidates showed that UNC-13 but not the non-L type Ca^{++} channel was essential for sensitivity to VAs in the clinically relevant concentration range. Consistent with UNC-13 as the target of the antagonistic activity of truncated syntaxin, overexpression of UNC-13 suppressed the VA resistance phenotype of truncated syntaxin. Finally, isoflurane appears to decrease synaptic localization of UNC-13, and animals with membrane targeted UNC-13 are isoflurane resistant. Thus, UNC-13 is required for VA sensitivity in *C. elegans*, is likely the protein to which the truncated syntaxin is binding, and its localization is inhibited by and important for isoflurane action. A schematic of our working model based on these data is shown in figure 7.

The questions now posed by our findings are several including: why has UNC-13 not been previously implicated in anesthetic action, is UNC-13 a direct anesthetic target, and what might be the role, if any, of UNC-13 orthologs in vertebrate anesthesia? Multiple reasonable explanations can be posited as to why no direct evidence till now has implicated UNC-13/mUNC-13 in anesthetic action. First, the primary methodology for most anesthetic mechanism studies is electrophysiology. While multiple electrophysiological studies have shown that volatile anesthetics do inhibit neurotransmitter release in various preparations [2], no specific mUNC-13 inhibitors are available that might have implicated mUNC-13. Moreover, inhibition of UNC-13/mUNC-13 does not produce a distinct electrophysiological phenotype that would clearly implicate these proteins. Likewise, binding studies that might have identified UNC-13/mUNC-13 as a VA target are severely limited by the low affinity and high membrane partitioning of VAs. Indeed, no synaptic protein has been specifically identified in binding studies on crude membrane preparations despite the presence of relatively abundant proteins such as $GABA_A$ receptors, which are very likely to be direct VA targets [1].

UNC-13 has several properties suggesting that it is a direct and relevant anesthetic target in *C. elegans*. First, animals lacking UNC-13 are VA resistant. Resistance is neither a necessary nor sufficient feature of an anesthetic target because of the possibility of multiple targets, genetic redundancy, and indirect effects. Nevertheless, the high level resistance of the *unc-13* mutants is consistent with the VA target being UNC-13. Second, a mutation in UNC-13 (a myristoylation sequence) that otherwise does not disrupt UNC-13 function confers isoflurane resistance, and UNC-13 synaptic localization appears to be decreased by isoflurane. These results are difficult to explain by an indirect model. One reasonable indirect model to consider is that VAs act to prevent production of DAG and thereby inhibit the function of UNC-13. Such an anesthetic mechanism would depend on UNC-13, be circumvented by myr-UNC-13S, and decrease UNC-13 synaptic localization. However, we have previously shown

that a probable null mutation in *egl-8*, which encodes the only known phospholipase Cβ acting upstream of UNC-13 to stimulate transmitter release, is not as VA resistant as the *unc-13* or truncated syntaxin mutants [9]. Moreover, this mechanism does not explain the resistance produced by the truncated syntaxin. On the other hand, a model where the truncated cytoplasmic syntaxin competes with anesthetics for binding to UNC-13 is plausible and consistent with the existing data.

Is there any evidence that VAs bind to UNC-13 or its vertebrate homologs? While no binding experiments have been reported with UNC-13/mUNC-13, volatile anesthetics and anesthetic alcohols have been shown to inhibit PKC [48], which has a C1 domain structurally similar to the C1 domain in UNC-13. Subsequent studies found that anesthetic alcohols do bind to the C1 domain of bovine PKCα and compete for diacyl glycerol binding [47]. More recently, a photoaffinity anesthetic alcohol labelled tyrosine 236 of the C1 domain of PKCδ [46]. The X-ray crystal structure of the C1B domain of PKCδ places tyrosine 236 approximately 10Å from the diacyl glycerol binding pocket [46] and provides a structural explanation for how anesthetics might inhibit diacyl glycerol binding to the C1 domain. The structure of mUNC13-1 C1 domain has been compared to that of PKCδ by solution nuclear magnetic resonance spectroscopy and found to be generally similar with conservation of the location of the homologous tyrosine [49]. However, a tryptophan residue was found to overlie the DAG-binding pocket in mUNC13-1 but not in PKCδ. This structural difference was proposed to account for the lower affinity of mUNC13-1 for phorbol esters. Thus, while anesthetic binding to the C1 domain of PKCα and PKCδ suggests that anesthetics are likely to also bind to the C1 domain of UNC-13/mUNC-13, the structural differences between the proteins offer the possibility that the affinities and/or effect of binding may differ between the homologous domains.

What might be the role of vertebrate UNC-13 homologs in general anesthesia? This question can be divided into two parts. First, in general, how might inhibition of neurotransmitter release contribute to general anesthesia? Second, specifically, what role might inhibition of vertebrate UNC-13 homologs play in the overall presynaptic anesthetic mechanism in vertebrates? Clearly, inhibition of excitatory neurotransmitter release could lead to an overall depression in nervous system function and contribute to a general anesthetic state. On the other hand, reducing inhibitory neurotransmitter release would counteract this effect and might actually increase arousal. In particular for volatile anesthetics, a block of inhibitory release would likely reduce anesthetic efficacy because of the well-established postsynaptic VA action of potentiation of ligand gating of inhibitory $GABA_A$ and glycine receptors. However, as outlined in the introduction, at clinical concentrations volatile general anesthetics including isoflurane selectively inhibit neurotransmitter release, reducing glutamate release significantly more than GABA release. Thus, VAs could reduce excitatory neurotransmission by its presynaptic mechanism while at the same time, because of minimal effect on inhibitory neurotransmitter release, potentiate postsynaptic inhibitory GABA and glycine currents and thereby synergistically depress central nervous system activity. Besides this potential for synergy, in particular brain regions such as the hippocampus, presynaptic excitatory inhibition appears to be a predominant effect [5,6,50]. Thus, presynaptic effects could be particular critical for the amnestic effects of VAs.

As to what is the role of UNC-13 orthologs in presynaptic VA action in vertebrates, one must consider that VAs almost certainly act on other presynaptic targets besides UNC-13 homologs. Sodium channels have been strongly implicated as essential for a significant portion of VA presynaptic inhibition[3,4,51]. Thus, unlike in *C. elegans*, which lacks sodium channels, UNC-13 orthologs are unlikely to be the sole presynaptic VA target in mammals. However, the mammalian UNC-13 homologs, mUNC13-1, 2, and 3 have interesting distinct functional roles that could account for the synapse selective effects of VAs. Specifically, mUNC-13 isoforms are differentially expressed in GABA versus glutamate terminals. Release from the majority

of glutamatergic terminals in mouse hippocampus requires the mUNC13-1 isoform whereas mUNC13-1 and mUNC13-2 function redundantly in GABAergic release at least in the cerebral cortex and hippocampus [52]. In rat brain, mUNC13-1 is expressed throughout the central nervous system whereas mUNC-13-2 expression is restricted to the cerebral cortex and hippocampus. mUNC-13-3 appears to be expressed exclusively in the cerebellum [53]. Intriguingly, mUNC13-1- and mUNC13-2-mediated release differ in their potentiation by DAG; mUNC13-1 is less efficaciously potentiated [52]. These previous observations coupled with the results reported here suggest that mUNC13-1, the closest homolog to *C. elegans* UNC-13, may be more sensitive to VAs because its weak DAG potentiation is more efficaciously blocked by VAs compared to that of mUNC13-2. The availability of mouse knockout strains for each of the mUNC13 isoforms will allow testing of this hypothesis.

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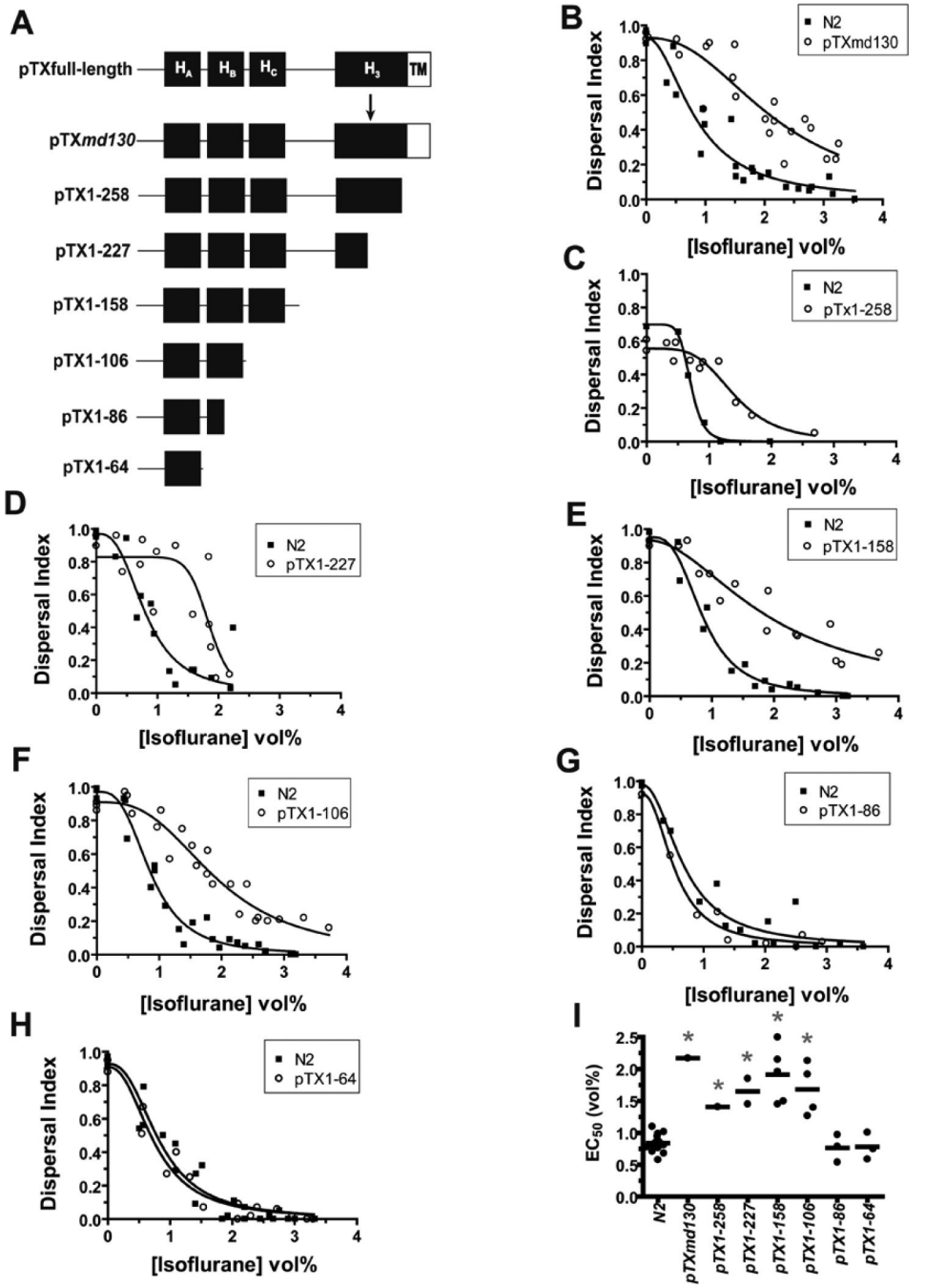

Fig. 1.

Syntaxin structural requirements for isoflurane antagonism. (**A)** Predicted syntaxin products generated from transforming plasmid constructs. The relative locations of the four helical domains, H_A, H_B, H_C, and H_3, and the C-terminal transmembrane domain, TM, are shown. pTX*md130* contains the g-a splice donor mutation in the 6th intron following codon 228 (position denoted by arrow) found in *unc-64(md130)* **(B)** Isoflurane sensitivity of locomotion (dispersal index) of a pTX_{md130} transformed strain vs that of the concurrently tested wild-type strain N2. EC_{50}s: N2 – 0.84 ± 0.09, pTX_{md130} – 2.17 ± 0.18 - $p < 0.0001$. **(C)** Isoflurane sensitivity of $pTX_{1–258}$ vs N2. EC_{50}s: N2 – 0.71 ± 0.02, $pTX_{1–258}$ – 1.41 ± 0.09 - $p < 0.0001$. **(D)** Isoflurane sensitivity of $pTX_{1–227}$ vs N2. EC_{50}s: N2 – 0.81 ± 0.10, $pTX_{1–227}$ – 1.85 ± 0.09

- $p < 0.0003$. **(E)** Isoflurane sensitivity of $pTX_{1–158}$ vs N2. EC_{50}s: N2 – 0.86 ± 0.05, $pTX_{1–158}$ – 1.96 ± 0.20 - $p < 0.0001$. **(F)** Isoflurane sensitivity of $pTX_{1–106}$ vs N2. EC_{50}s: N2 – 0.87 ± 0.05, $pTX_{1–106}$ – 1.92 ± 0.09 - $p < 0.0001$. **(G)** Isoflurane sensitivity of $pTX_{1–86}$ vs N2. EC_{50}s: N2 – 0.68 ± 0.09, $pTX_{1–86}$ – 0.54 ± 0.06 - $p = 0.24$. (**H)** Isoflurane sensitivity of $pTX_{1–64}$ vs N2. EC_{50}s: N2 – 0.75 ± 0.06, $pTX_{1–65}$ – 0.83 ± 0.07 - $p = 0.45$. (**I)** Summary scatter plot of all transformants. Each point represents the EC_{50} derived from the pooled concentration response data for each independently transformed strain except for N2 where each point represents the EC_{50} for a particular experiment. * $p < 0.01$ for each transformed strain versus the concurrently measured N2 EC_{50}. All comparisons by shared-parameter simultaneous curve-fitting.

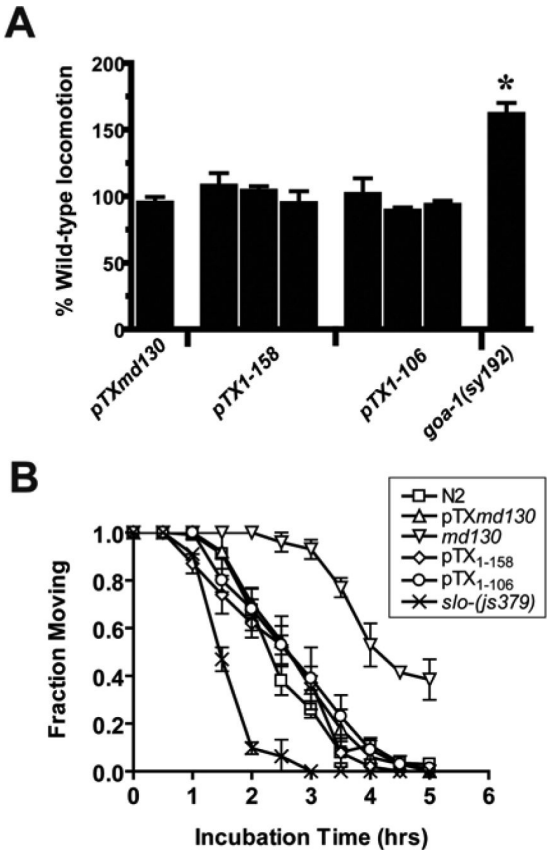

Fig. 2.

VA resistance not due to enhancement of locomotion or neurotransmitter release. (**A)** Locomotion rates of age synchronized young adults from strains transformed with pTX_{md130} (MC168), $pTX_{1–158}$ (MC150, MC151, and MC152), and $pTX_{1–106}$ (MC158, MC159, and MC139) was measured by the number of body bends/minute and normalized to concurrent wild-type N2 values. Values are mean ± sem of > 10 animals. The hyperactive locomotion mutant, *goa-1(sy192)*, is shown as a positive control [1]. * - significantly different from 100% @ $p < 0.01$, two-tailed t-test. (**B)** Aldicarb sensitivity of a subset of truncated syntaxin transformants. The fraction of animals moving after various incubation times on agar plates containing 0.35 mM aldicarb. Each point represents the mean ± sem of triplicate measurements of at least 30 animals/measurement. The aldicarb hypersensitive/isoflurane resistant strain *slo-1(js379)* and the aldicarb resistant/isoflurane resistant *unc-64(md130)* strain are shown for comparison [2-4] .

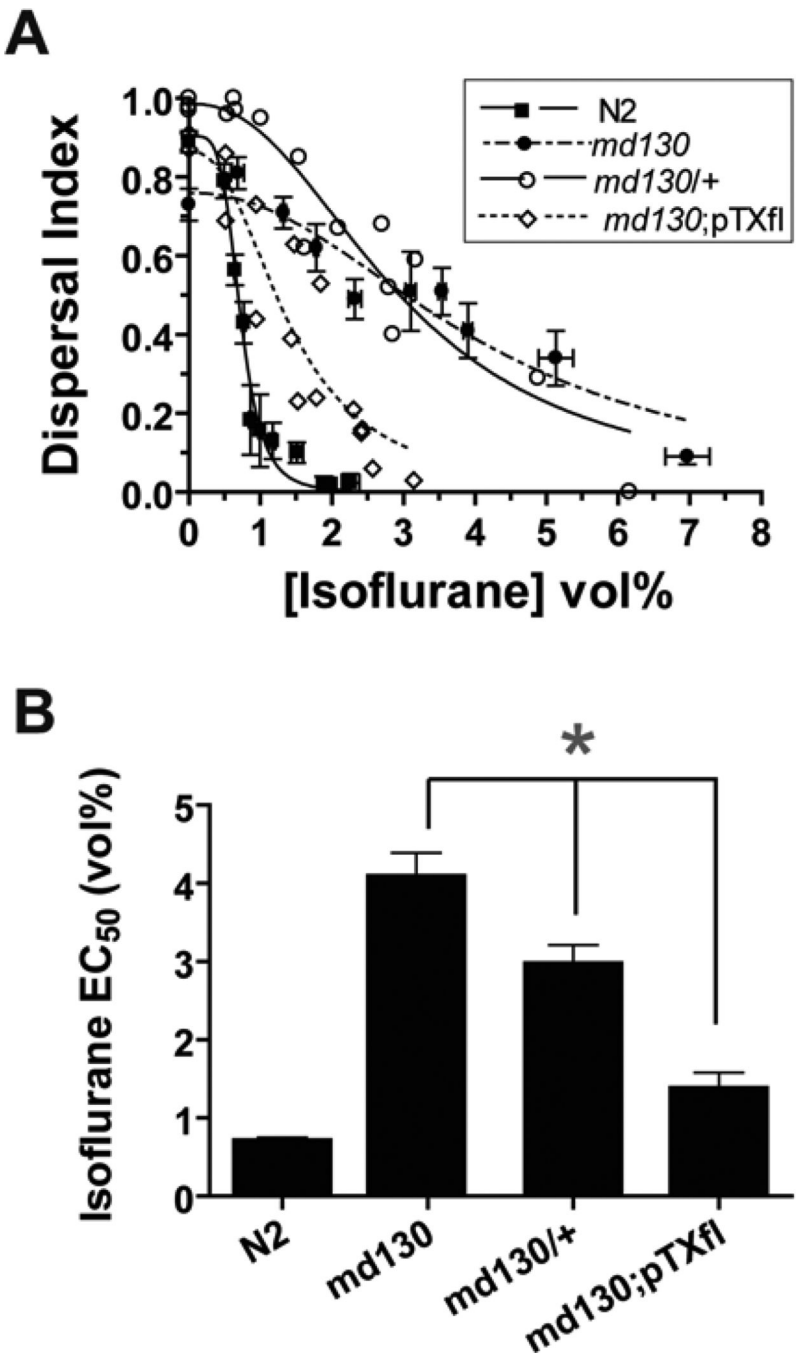

Fig. 3.

Wild type syntaxin suppresses the VA resistance of truncated syntaxins. **(A)** Semidominance and rescue of the *md130* isoflurane resistance. Locomotion measured by dispersal index is plotted as a function of [Isoflurane]. *md130*;pTX(fl) (MC72 – Table 1) is *unc-64(md130)* transformed with a full-length syntaxin genomic construct. *md130/+* is heterozygous for the *md130* mutation. **(B)** Summary of Isoflurane EC_{50}'s ± SE of the fit from **A**. * - $p < 0.01$

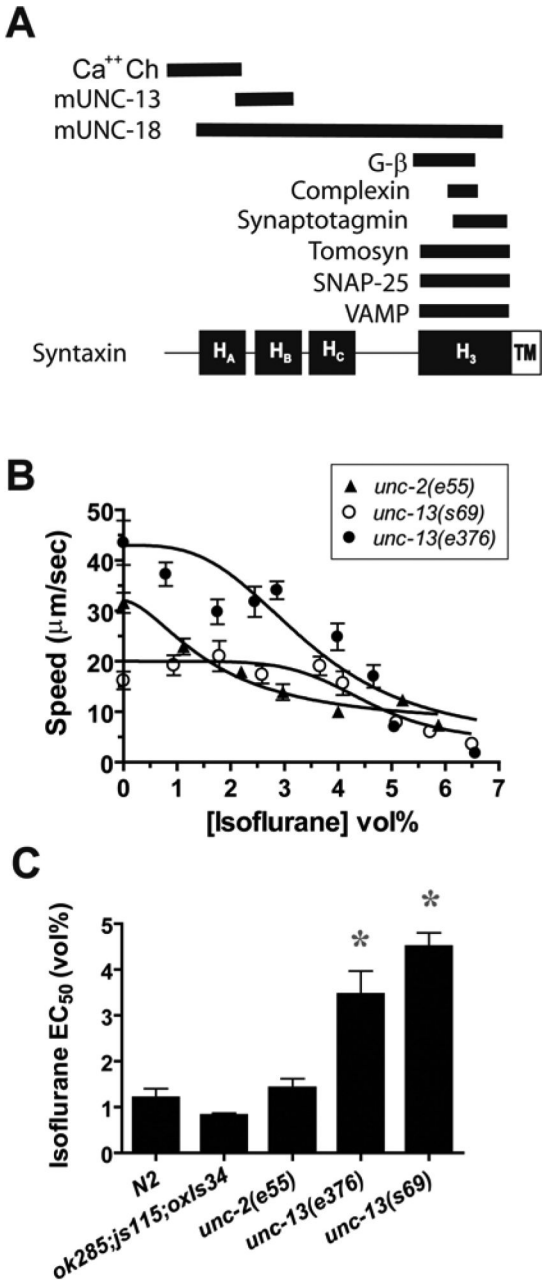

Fig. 4.

UNC-13 is required for isoflurane sensitivity. (**A**) Proteins known to bind to syntaxin. The proteins are aligned with the syntaxin region, with which it binds. Ca^{++} Ch – N- and P- type calcium channels [5,6], mUNC-18 – mammalian UNC-18 [7,8], mUNC-13 – mammalian UNC-13 [9], Gβ – β-subunit of a G-protein [5], complexin [10,11], synaptotagmin [7], tomosyn [12], SNAP-25 [13], Vesicle-associated Membrane Protein (VAMP) [13,14] **(B)** Isoflurane sensitivity of the locomotion of strains with loss-of-function mutations in *unc-13* and *unc-2*. The full genotypes are: *tom-1(ok285*null*) unc-13(s69*lf*);unc-64(js115* null);*oxIs34*[*unc-64*(L166A/E167A);P*myo-2*::GFP] , *tom-1(ok285*null*) unc-13(e376*lf*);unc-64(js115* null);*oxIs34*[*unc-64*(L166A/E167A);P*myo-2*::GFP]/+, and *unc-2(e55* null*)*. Each point represents the mean ± sem of 10 animals. **(C)** Summary of Isoflurane EC_{50}'s ± SE of the fit from **B**. *tom-1(ok285);unc-64*

(js115);oxIs34 is the genetic background for the *unc-13* mutants and is shown for comparison along with the wild type strain N2. * - $p < 0.01$ vs N2 and *ok285;js115;oxIs34*

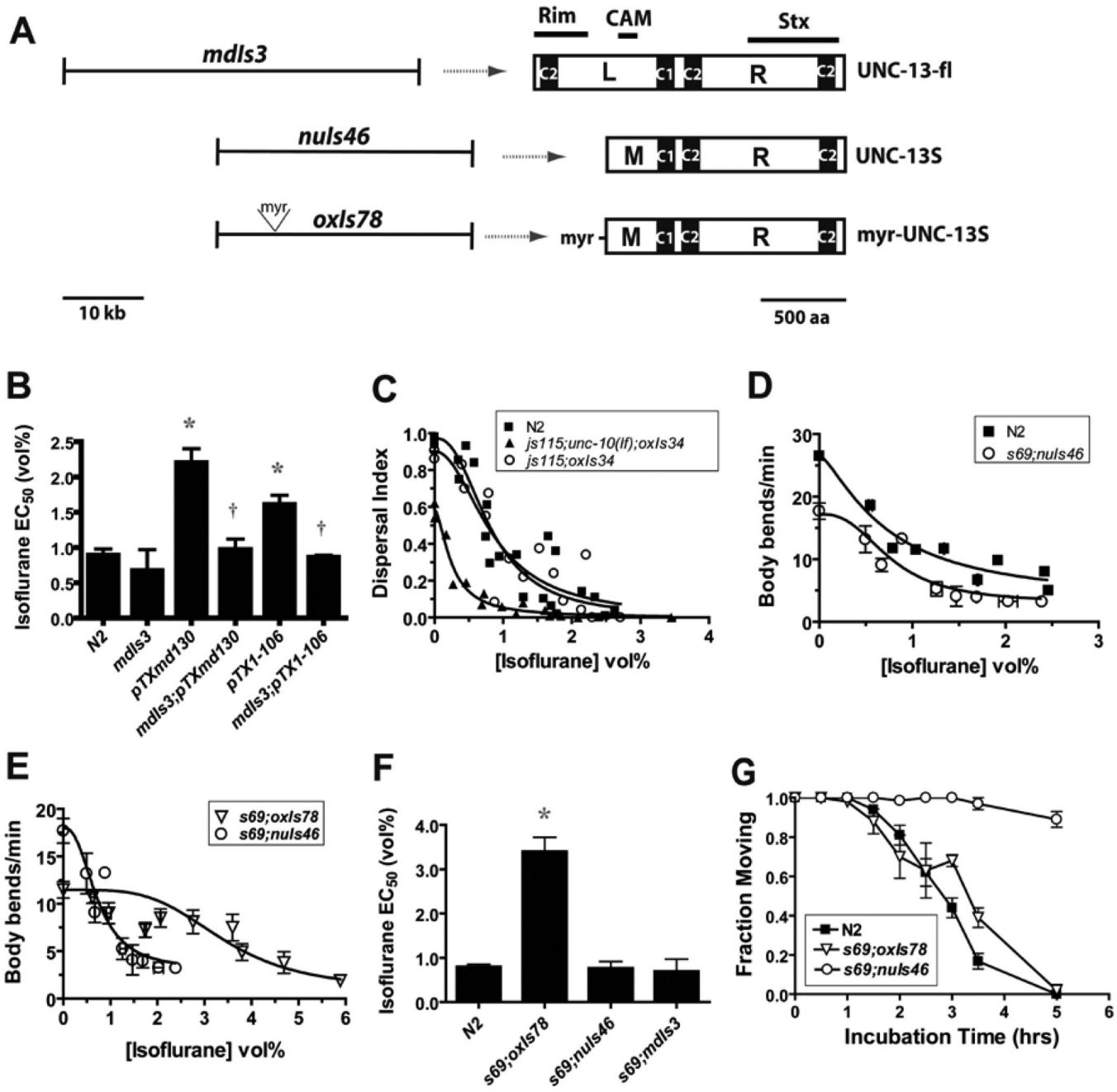

Fig. 5.

UNC-13 domains required for rescue of isoflurane sensitivity. **(A)** *unc-13* constructs used to transform *unc-13(s69)* and the resultant protein products. *mdIs3* is an integrated array of a genomic fragment encoding the entire *unc-13* L + R coding sequence [15] the predicted locations of the Rab3a-interacting molecule (RIM) [16], calmodulin (CAM) [17], and syntaxin (Stx) [9] binding sites are indicated. *nuIs46* is an integrated array encoding the M + R shortened form of UNC-13 – UNC-13S – that partially rescues locomotion and transmitter release [18]. *oxIs78* is an integrated array otherwise identical to *nuIs46* except with a 51-nucleotide insertion at the 5' end of the *unc-13S* coding sequence that encodes the N-terminal myristoylation sequence of the *C. elegans* Goα protein [19]. **(B)** Suppression of isoflurane antagonism of truncated syntaxin by overexpression of UNC-13. Isoflurane EC_{50}s measured by the body bend assay.

mdIs3[*unc-13*(+)] is an integrant of the *mdEx43* array [15] that overexpresses wild type UNC-13. * - $p < 0.01$ vs N2; † - $p < 0.01$ vs corresponding truncated syntaxin strain alone. **(C)** A null mutant of *unc-10* RIM is not isoflurane resistant. The full genotype is *unc-64(js115*null*); unc-10*(*md1117*null); *oxIs34*[*unc-64*(L166A/E167A);P*myo-2*::GFP] [20]. The isoflurane sensitivity of *unc-64(js115* null);*oxIs34* is shown for comparison. EC_{50}s (vol%): N2 – 0.82 ± 0.09, *js115;md1117;oxIs34* – 0.25 ± 0.03, *js115;oxIs34* – 0.85 ± 0.16 **(D)** UNC-13S restores isoflurane sensitivity to *unc-13(s69)*. EC_{50}s: N2 = 0.72 ± 0.13, *s69;nuIs46* = 0.77 ± 0.15 **(E)** animals expressing myr-UNC-13S are isoflurane resistant. EC_{50}s: *s69;nuIs46* = 0.77 ± 0.15, *s69;oxIs78* = 3.40 ± 0.32. **(F)** Summary of isoflurane EC_{50}'s ± SE of the fit of the curves in D, E, and the body bend data for *unc-13(s69);mdIs3*. * - $p < 0.01$ vs N2. **(G)** *myr*-UNC-13s has wild-type sensitivity to aldicarb. The fraction of animals moving after various incubation times on agar plates containing 0.35 mM aldicarb. Each point represents the mean ± sem of triplicate measurements of at least 30 animals/measurement.

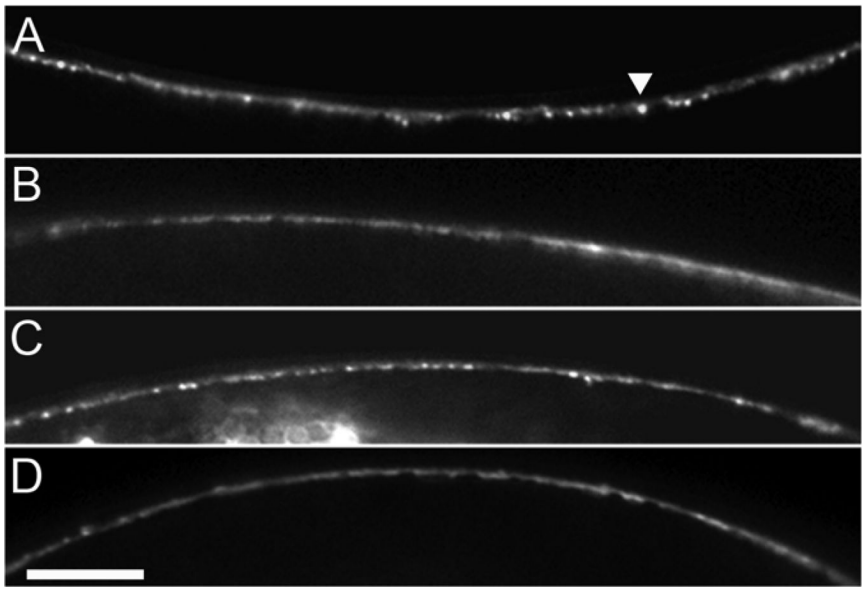

Fig. 6.

Isoflurane reduces synaptic UNC-13::GFP puncta. Representative images of segments of dorsal nerve cord from *unc-13(s69);nuIs46*[UNC-13S::GFP;ccGFP] L4 larval animals treated with **(A)** Air, no PMA (phorbol-12-myristate-13-acetate), **(B)** isoflurane (2 vol%), no PMA, **(C)** Air, 1 μg/ml PMA, **(D)** isoflurane (2 vol%), 1 μg/ml PMA. Note synaptic puncta (example denoted by arrowhead) decreased in **B** and **D** relative to their respective air controls. scale bar = 20 μm.

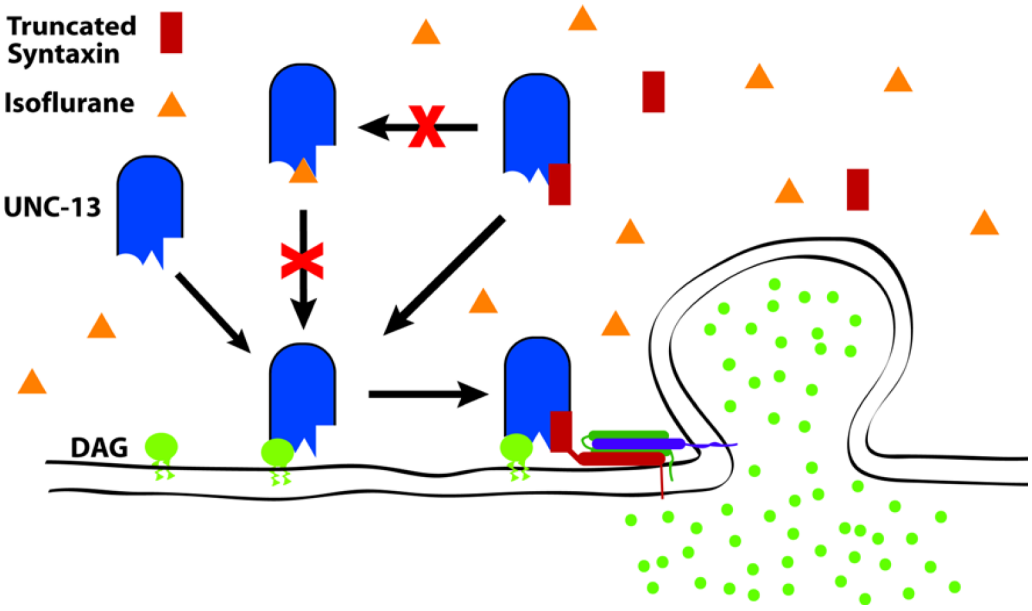

Fig. 7.

Working model of presynaptic mechanism of isoflurane in *C. elegans*. Diacyl glycerol – DAG (green circle) is known to bind to UNC-13 (blue rounded rectangle) and thereby increase the local concentration of UNC-13 at the presynaptic membrane [18,19,21,22]. Membrane translocation of UNC-13 is thought to promote interaction with syntaxin and thereby promote fusion of synaptic vesicles and transmitter release. Isoflurane (orange triangle) antagonizes DAG-mediated membrane translocation of UNC-13, and myristoylated UNC-13, which promotes transmitter release in a DAG-independent manner [18,19], confers isoflurane resistance (Figs. 5, 6 & Table 2). Animals without UNC-13 are highly isoflurane resistant (Fig. 4). These three results can be explained by isoflurane binding to UNC-13 and antagonizing DAG binding or the effect of binding (indicated by crossed out arrow). Truncated syntaxin (red rectangle), which lacks its transmembrane domain, blocks isoflurane sensitivity (Fig. 1), presumably by competing with isoflurane for binding to UNC-13 (indicated by crossed out arrow) since UNC-13 overexpression can suppress the isoflurane resistance of truncated syntaxin.

Table 1

Strains list

Strain	Genotype	Mutation	Transforming plasmid	Transforming protein	Refs.
N2	wild type	None	None	None	16
MC339	*unc-64(md130)*	Truncated syntaxin1-227 +few novel aa + reduced wild type syntaxin	None	None	8,13
MC270	*md130 +/+ bli-5*	heterozygous md130 products	None	None	8,13
MC72	*unc-64(md130); gcEx5*	Truncated syntaxin1-227 +few novel aa + reduced wild type syntaxin	pTXfull-length = pTX21; pPH::GFP-1	Full-length wild type UNC-64; hypodermal GFP	8,13
MC185	*gcEx85*	None	pTXmd130; pPH::GFP-1	*md130* product; hypodermal GFP	21
MC105	*gcEx95*	None	pTX1-258; pPH::GFP-1	Truncated syntaxin residues 1-258; hypodermal GFP	21
MC153, MC155	*gcEx53, gcEx55*	None	pTX1-227; pPH::GFP-1	Truncated syntaxin residues 1-227; hypodermal GFP	21
MC150, MC151, MC152, MC184, MC187	*gcEx50, gcEx51, gcEx52, gcEx84, gcEx87*	None	pTX1-158; pPH::GFP-1	Truncated syntaxin residues 1-158; hypodermal GFP	21
MC139, MC158, MC159, MC232	*gcEx91, gcEx58, gcEx59, gcEx90*	None	pTX1-106; pPH::GFP-1	Truncated syntaxin residues 1-106; hypodermal GFP	21
MC233, MC234, MC271	*gcEx92, gcEx93, gcEx94*	None	pTX1-86; pPH::GFP-1	Truncated syntaxin residues 1-86; hypodermal GFP	21
MC176, MC177, MC178	*gcEx76, gcEx77, gcEx78*	None	pTX1-64; pPH::GFP-1	Truncated syntaxin residues 1-64; hypodermal GFP	21
NM1968	*slo-1(js379* null*)*	Loss of SLO-1 BK channel	None	None	9,26
PS1762	*goa-1(sy192* lf*)*	Dominant negative Go-alpha protein	None	None	11
VC223	*tom-1(ok285* null*)*	Loss of tomosyn	None	None	17
EG1985	*unc-64(js115* null*); oxIs34*	Loss of syntaxin	Punc-64(L166A/E167A); Pmyo-2::GFP	Open Syntaxin; pharynx GFP	18
MC272	*tomo-1(ok285* null*); unc-64(js115* null*); oxIs34*	Loss of tomosyn; Loss of syntaxin	Punc-64(L166A/E167A); Pmyo-2::GFP	Open Syntaxin; pharynx GFP	17,18
MC261	*tomo-1(ok285* null*) unc-13 (e376);unc-64(js115* null*);oxIs34*	Loss of tomosyn; Reduction of UNC-13; Loss of syntaxin	Punc-64(L166A/E167A); Pmyo-2::GFP	Open Syntaxin; pharynx GFP	17,18,19
MC275	*tomo-1(ok285* null*) unc-13(s69); unc-64(js115* null*); oxIs34*	Loss of tomosyn; Loss of UNC-13; Loss of syntaxin	Punc-64(L166A/E167A); Pmyo-2::GFP	Open Syntaxin; pharynx GFP	17,18,19
MC344	*unc-10(md1117* null*); unc-64(js115* null*); oxIs34*	Loss of UNC-10 - RIM; Loss of syntaxin	Punc-64(L166A/E167A); Pmyo-2::GFP	Open Syntaxin; pharynx GFP	45
CB55	*unc-2(e55* null*)*	Loss of non-L-type Ca^{++} Channel	None	None	29,36
BC168	*unc-13(s69* null*)*	Loss of UNC-13	None	None	19
CB376	*unc-13(e376* lf*)*	Reduction of UNC-13	None	None	19
KP3299	*unc-13(s69* null*); nuIs46*	Loss of UNC-13	KP268[UNC-13S::GFP]; ccGFP	UNC-13S::GFP; coelomocyte GFP	19,23
EG2840	*unc-13(s69* null*); oxIs78*	Loss of UNC-13	KP280[myr::unc-13S::GFP]; ccGFP	Myristoylated UNC-13S::GFP; coelomocyte GFP	19,20
RM2333	*mdIs3*	None	C44E1; Psnb-1::GFP - integrant of *mdEx43*	wild type UNC-13; neuronal GFP	19
MC273	*mdIs3;gcEx37*	None	C44E1; Psnb-1::GFP; pTX_{md130}; pPH::GFP-1	wild type UNC-13; neuronal GFP; *md130* truncated syntaxin; hypodermal GFP	19,21
MC274	*mdIs3;gcEx58*	None	C44E1; Psnb-1::GFP; pTX_{1-106}; pPH::GFP-1	wild type UNC-13; neuronal GFP; truncated syntaxin 1-106; hypodermal GFP	19,21

More than one strain in a row represents multiple transformants with the same plasmid; If - loss-of-function but not necessarily null *Is* - indicated a chromosomally integrated array, *Ex* - indicates an extrachromosomal array; aa = amino acid

Table 2

Effect of isoflurane on UNC-13 localization

Condition	Puncta/100 μm	Animals	p-value vs Air	p-value vs No PMA
Air, No PMA	11.2 ± 0.5	43	—	—
Iso, No PMA	8.2 ± 0.5	54	0.00006	—
Air, PMA	14.2 ± 0.5	51	—	0.00004
Iso, PMA	10.8 ± 0.6	42	0.00002	0.0006

[Iso]=2 vol%, PMA = phorbol-12-myristate-13-acetate[PMA] = 1 μg/ml; values are mean ± sem; Scorer blinded to condition; p-value by two-tailed test