Resistance to Volatile Anesthetics by Mutations Enhancing Excitatory Neurotransmitter Release in *Caenorhabditis elegans*

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Manuscript received April 24, 2004
Accepted for publication June 23, 2004

ABSTRACT

The molecular mechanisms whereby volatile general anesthetics (VAs) disrupt behavior remain undefined. In *Caenorhabditis elegans* mutations in the gene *unc-64*, which encodes the presynaptic protein syntaxin 1A, produce large allele-specific differences in VA sensitivity. UNC-64 syntaxin normally functions to mediate fusion of neurotransmitter vesicles with the presynaptic membrane. The precise role of syntaxin in the VA mechanism is as yet unclear, but a variety of results suggests that a protein interacting with syntaxin to regulate neurotransmitter release is essential for VA action in *C. elegans*. To identify additional proteins that function with syntaxin to control neurotransmitter release and VA action, we screened for suppressors of the phenotypes produced by *unc-64* reduction of function. Loss-of-function mutations in *slo-1*, which encodes a Ca²⁺-activated K⁺ channel, and in *unc-43*, which encodes CaM-kinase II, and a gain-of-function mutation in *egl-30*, which encodes Gqα, were isolated as syntaxin suppressors. The *slo-1* and *egl-30* mutations conferred resistance to VAs, but *unc-43* mutations did not. The effects of *slo-1* and *egl-30* on VA sensitivity can be explained by their actions upstream or parallel to syntaxin to increase the level of excitatory neurotransmitter release. These results strengthen the link between transmitter release and VA action.

DESPITE longstanding efforts, volatile anesthetic (VA) mechanisms remain undefined. At a cellular level, the synapse is generally accepted as the site of action (POCOCK and RICHARDS 1991; FRANKS and LIEB 1994). In vertebrate nervous systems, both presynaptic (ZORYCHTA and CAPEK 1978; TAKENOSHITA and TAKAHASHI 1987; KULLMANN *et al.* 1989; MIAO *et al.* 1995; PEROUANSKY *et al.* 1995; SCHLAME and HEMMINGS 1995; MACIVER *et al.* 1996; NISHIKAWA and MACIVER 2000) and postsynaptic anesthetic effects (JONES *et al.* 1992; MIHIC *et al.* 1997; PATEL *et al.* 1999; YAMAKURA *et al.* 2001) have been observed that might contribute to a state of general anesthesia. However, establishing a causal link between VA effects *in vitro* and *in vivo* has been problematic. Genetics is one approach for making such a link. For example, a point mutation in the β3 subunit of the GABA_A receptor eliminates the action of the intravenously administered general anesthetics etomidate and propofol against response to a painful stimulus in a mouse knockin strain (JURD *et al.* 2003). Thus, potentiation of GABA_A receptors is the major relevant action for these anesthetics. However, for VAs, no mutations of a vertebrate gene have resulted in a highly resistant

animal. Screens in *Drosophila* have isolated mutant strains that are modestly VA resistant to some anesthetic endpoints (KRISHNAN and NASH 1990; TINKLENBERG *et al.* 1991; LEBOVITCH *et al.* 1995; GAMO *et al.* 2003); however, highly resistant mutants have not been uncovered.

In *Caenorhabditis elegans*, two sorts of approaches have been taken. Using supraclinical concentrations of VAs to produce immobilization as an anesthetic endpoint, Morgan, Sedensky, and co-workers have isolated novel mutants that are VA hypersensitive or that are suppressors of the VA-hypersensitive phenotype (MORGAN and CASCORBI 1985; SEDENSKY and MENEELY 1987; MORGAN *et al.* 1988; MORGAN *et al.* 1990; MORGAN and SEDENSKY 1994). One of the mutations producing VA hypersensitivity was found to lie in the gene *gas-1*, which encodes the 49-kD subunit of mitochondrial NADH:ubiquinone-oxidoreductase (complex I of the respiratory chain). Suppressors of the hypersensitivity were found among mutations in the *unc-1*, *unc-7*, *unc-8*, and *unc-9* genes. *unc-1* encodes a stomatin homolog (RAJARAM *et al.* 1998); *unc-8* encodes an epithelial sodium channel (TAVERNARAKIS *et al.* 1997); *unc-7* and *unc-9* encode innexins, which are thought to be structural components of invertebrate gap junctions (STARICH *et al.* 1996; BARNES and HEKIMI 1997; PHELAN *et al.* 1998; PHELAN and STARICH 2001). The suppression of VA hypersensitivity by loss-of-function mutations in innexins suggests that mutations in

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gas-1 and the other genes producing VA hypersensitivity may directly or indirectly sensitize to VA immobilization through a mechanism requiring gap junctions. Allele-specific interactions between *unc-1* and *unc-8* also suggest the possibility that these gene products interact and form a complex that regulates the binding or activity of VAs at supraclinical concentrations (RAJARAM *et al.* 1999).

At lower concentrations similar to those used for human anesthesia, VAs do not immobilize *C. elegans*; rather their locomotion becomes uncoordinated and sluggish (CROWDER *et al.* 1996). Mutations in genes that alter neurotransmitter release in *C. elegans* have been found to control these VA behavioral effects (VAN SWINDEREN *et al.* 1999, 2001, 2002). These mutations do not alter sensitivity to supraclinical VA concentrations in the immobilization assay (HUMPHREY *et al.* 2002). Thus, the mechanisms underlying the effects of VAs at clinical and supraclinical concentrations are distinct. In the *C. elegans*, *Drosophila*, and vertebrate nervous systems (ZORYCHTA and CAPEK 1978; TAKENOSHITA and TAKAHASHI 1987; KULLMANN *et al.* 1989; MIAO *et al.* 1995; PEROUANSKY *et al.* 1995; SCHLAME and HEMMINGS 1995; MACIVER *et al.* 1996; NISHIKAWA and KIDOKORO 1999; VAN SWINDEREN *et al.* 1999; NISHIKAWA and MACIVER 2000), VAs have been shown to reduce excitatory neurotransmitter release, an action that could be responsible for at least some behavioral effects of anesthetics. In support of this hypothesis, some VA-resistant mutants in *C. elegans* and *Drosophila* antagonize the effect of VAs on acetylcholine and glutamate release, respectively (NISHIKAWA and KIDOKORO 1999; VAN SWINDEREN *et al.* 1999).

The presumptive presynaptic VA target(s) in *C. elegans* is unknown. Direct screens for high-level VA resistance have been difficult and thus far unsuccessful. However, testing of existing *C. elegans* mutants found that mutations in *unc-64*, which encodes the presynaptic protein syntaxin 1A, produced large allele-specific differences in VA sensitivity. In particular, an unusual neomorphic syntaxin allele, *unc-64(md130)*, was able to dominantly antagonize VA action, producing complete resistance to VAs at clinical concentrations (VAN SWINDEREN *et al.* 1999). Syntaxin 1A is an integral membrane protein that interacts with SNAP-25 and synaptobrevin (VAMP), forming a ternary complex (the SNARE complex) that mediates fusion of the synaptic vesicle with the presynaptic membrane (CHEN and SCHELLER 2001). Multiple other proteins function along with syntaxin and the SNARE complex to regulate the fusion process (JAHN and SUDHOF 1999; MOCHIDA 2000; RIZO and SUDHOF 2002). Our genetic data are consistent with the hypothesis that a protein physically interacting with syntaxin is the VA target responsible for effects of VAs on transmitter release. Alternatively, a syntaxin-interacting protein is not the target but is essential for the action of VAs on their target. In either case, identification of this hypothesized protein and more generally proteins that

regulate VA action on transmitter release is required to define the VA mechanism. Reduction-of-function mutations exist in some of these syntaxin interactors; however, they generally produce severe behavioral deficits that preclude testing of their VA sensitivity. To circumvent this problem, here we test the VA sensitivity of mutations isolated in a screen for enhancers of transmitter release. The screen was performed in the background of a syntaxin reduction-of-function mutation. The syntaxin mutant background facilitated screening and potentially enriched for mutations that directly enhance syntaxin function. Analysis of the synaptic but not the anesthetic phenotypes of mutations in one of the genes identified in this screen was reported in WANG *et al.* (2001).

MATERIALS AND METHODS

Strains and strain constructions: *C. elegans* strains were obtained from multiple laboratories referenced herein, from the *Caenorhabditis* Genetics Center funded by the National Institutes of Health National Center for Research Resources, and from our own mutagenesis screens as described below. All strains were grown on nematode growth media (NGM) agar seeded with OP50 bacteria (BRENNER 1974); well-fed young adults were used for all assays. Strains used in this work include the wild-type strain N2 (var. Bristol; BRENNER 1974) and mutant strains by linkage group as follows:

LGI: *egl-30(js126, tg26, n686), dpy-5(e61)*.

LGII: *bli-2(e768)*.

LGIII: *dpy-19(e1259), unc-64(e246, md130, js21)*.

LGIV: *bli-6(sc16), unc-43(js125, js383, e755)*.

PS2284: *dpy-20(e1282); syEx125[pMH86+ pLB2+ pBS]* (BRUNDAGE *et al.* 1996). PS2284 is referred to in the text as Ex[Gq(+)]. pMH86 contains a full-length genomic clone of *dpy-20(+)*, which rescues *dpy-20(e1282)*, inserted into pBluescript SK⁺ and as such was used as a transformation marker; pLB2 contains a full-length *egl-30(+)* genomic clone inserted into pBluescript SK⁺ (pBS; BRUNDAGE *et al.* 1996).

PS2554 is referred to in the text as GqHS_(Q205L) = *dpy-20(e1282); syIs38[pMH86+ pLB24+ pBS]*; pLB24 contains a mutant *egl-30* genomic fragment (GqQ205L), predicted to produce a constitutively active EGL-30 protein, inserted into pPD49.78, which has a heat-shock-inducible promoter from the hsp16-2 gene (STRINGHAM *et al.* 1992; MELLO and FIRE 1995). pLB24 was injected at 50 ng/μl along with pMH86 and pBS, and the resultant array was integrated with a 38-Gy X-ray source and outcrossed eight times (C. BASTIANI, L. BRUNDAGE and P. STERNBERG, personal communication). PS2284 and PS2554 were kindly provided by Carol Bastiani and Paul Sternberg.

LGV: *slo-1(js100, js118, js379, js380, js381, js382, eg24, eg161, eg73) dpy-11(e224), egl-8(n488), unc-34(e566), unc-60(e677), dpy-11(e224)*.

LGX: *lon-2(e678), dgk-1(nu199)*. Note that *dgk-1(nu199)* is also known as *sag-1(nu199)*.

TY1657: *dpy-5(e61) I; rol-6(e187) II, lon-1(e185) III, bli-6(sc16) IV; unc-23(e324) V*.

Construction of double mutants: Four double mutant strains were constructed: *egl-30(js126); unc-64(md130)*, *egl-30(js126); unc-64(js21)*, *egl-30(js126); dgk-1(nu199)*, and *egl-30(js126); egl-8(n488)*. To build *egl-30(js126); unc-64(md130)*, *egl-30(js126); unc-64(js21)*, and *egl-30(js126); dgk-1(nu199)*, *dpy-5(e61)* was used as a visible

marker *in cis* with *egl-30(js126)*. *egl-30(js126) dpy-5(e61)/+* males were crossed into *unc-64(md130)*, *unc-64(js21)*, and *dgh-1(nu199)*. In the F₂ generation, non-Dpy animals displaying the Unc (*md130* and *js21*) or loopy (*nu199*) phenotype were selected and Dpy F₃ progeny were chosen as presumptive double mutants. *egl-30(js126);egl-8(n488)* was built by crossing *egl-30(js126)/+* males into *unc-34(e566) unc-60(e677) dpy-11(e224)*, a balancer for *egl-8(n488)*. The male progeny were mated with *egl-8(n488)*. Animals in the F₁ progeny that displayed the *js126* semidominant weak loopy phenotype were picked singly to plates and strong loopy F₂ animals were chosen from plates that segregated the balancer phenotypes. F₃ animals not segregating the balancer were presumed to be the double mutant. Genotypes of all double mutants were confirmed by outcrossing and segregation of both mutant phenotypes and by sequencing *egl-30* to confirm homozygosity of the *js126* mutation.

Mutagenesis screen for suppressors of *unc-64(rf)*: *unc-64(e246rf)* L4 larvae were exposed to 50 mM EMS in M9 buffer for 4 hr with agitation and then washed three times with M9. The F₂ progeny were screened visually for improved locomotion compared to *unc-64(e246)*. Progeny of putative suppressor mutants were similarly screened and one animal that clearly moved better than *e246* was kept to establish each strain. Overall, 24,000 genomes were screened and 14 confirmed suppressors were isolated. Each suppressor strain was outcrossed against N2 at least three times and outcrossed strains with and without *e246* in the background were kept for further characterization. Suppressor mutations crossed out of the *e246* background were examined for behavioral, morphological, anesthetic-sensitivity, and aldicarb-sensitivity phenotypes (see below). Any additional phenotypes of the suppressor mutations after outcrossing were confirmed to cosegregate with suppression of *e246*.

Genetic characterization and mapping: Prior to mapping, mutants were analyzed for dominance of their phenotypes and, if recessive, were placed into complementation groups on the basis of their suppression of *unc-64(e246)*. Of the mutations presented here, only *js126* exhibited dominant phenotypes. The mutations fell into at least five complementation groups; representatives from three complementation groups are analyzed in this report. For placement of mutant loci onto chromosomes, the following visible marker mutations were used: *dpy-5(e61)* I; *bli-2(e768)* and *rol-6(e187)* II; *unc-64(e246)* and *lon-1(e185)* III; *dpy-19(e1259)* and *bli-6(sc16)* IV; *dpy-11(e224)* and *unc-23(e324)* V, and *lon-2(e678)* X. The general method was to cross homozygous or heterozygous test mutant males into the mapping strain and then pick F₂ progeny expressing the phenotype(s) of the test mutant [e.g., loopy, aldicarb hypersensitivity, or suppression of *unc-64(e246)*]. The F₃ progeny were scored for the marker phenotype and homozygosity of the test mutation.

***slo-1* alleles:** Genetic characterization, mapping, and identification of the six *slo-1* mutants isolated in this screen (*js100*, *js118*, *js379*, *js380*, *js381*, and *js382*) were described previously (WANG *et al.* 2001).

***egl-30(js126)*:** For mapping of *e246* suppression by *js126*, males with the genotype *e246/+;marker/+* were crossed with *js126;e246* and the brood of suppressor;*e246* animals was scored for frequency of marker segregation. Twelve of 51 Unc Sup animals produced Dpy progeny, indicating loose linkage of *js126* to *dpy-5* on chromosome I. *szT1* balances the left arm of chromosome I and was found to balance the *js126 dpy-5* interval. Candidate genes within this interval consistent with the map distance from *dpy-5* included *egl-30*. Overexpression of *egl-30* had been shown to exhibit loopy locomotion similar to *js126* (BRUNDAGE *et al.* 1996). The entire *egl-30* coding region in *js126* genomic DNA including exon/intron bound-

aries was sequenced and was found to have a single mutation, a G-to-A transition, resulting in a valine to methionine at position 180. For examination of cosegregation of *js126*'s various phenotypes, *egl-30(js126) dpy-5(e61)* was outcrossed, and homozygous loopy non-Dpy F₂ hermaphrodites with the presumed genotype of *js126 +/js126 dpy-5* were allowed to self. The brood of loopy non-Dpy F₃'s that segregated all loopies and no Dpy's were tested for their VA and aldicarb sensitivities. In 53 of 53 independent recombinants, the loopy locomotion, the aldicarb-hypersensitive, and the VA-resistance phenotypes cosegregated.

***unc-43* alleles:** *js125* and *js383* failed to complement for suppression of the aldicarb-resistance phenotype of *unc-64(e246)*. The Sup phenotype of *js125* was placed onto chromosome IV on the basis of limited recombination with *dpy-13*. Only 5/49 animals of the genotype *unc-64(e246); dpy-13 +/+ js125* segregated Dpy Unc Sup progeny placing *js125* near *dpy-13*. Both *js125* and *js383* were noted to exhibit a shrinker phenotype, the simultaneous contraction of all four body-wall muscle quadrants in response to anterior touch, which is characteristic of reduced GABAergic neurotransmission (McINTIRE *et al.* 1993). Two genes that when mutated produced the shrinker phenotype were within the mapped interval, so complementation was performed with both. *js125* fully complemented the shrinker phenotype of *unc-30(e191)* but failed to complement *unc-43(e408)*. The *unc-43* coding region along with exon/intron boundaries was sequenced in *js383* and G-to-A transition in the splice acceptor site before exon 15 was found. In *js125*, a 17-kb region extending from 10 kb upstream of the transcription initiation site through exon 10 was refractory to PCR amplification, indicative of a deletion of this region.

Behavioral assays: All assays were performed at room temperature (22°–23°) on young adults. Two assays were used to quantify locomotion.

Velocity assays: The speed of spontaneous and stimulated movement was measured by digital video imaging. One-day post-L4 young adults were picked onto plates seeded with OP50 *Escherichia coli*. Movies (60 sec) of single animals were taken immediately before and immediately after dropping a weight from a fixed height onto the lid of the Petri dish. The average forward movement of the midbody in each 1-sec frame was measured. For each strain, >20 animals were assayed to calculate the speed (micrometers per second) for that strain.

Body bend assays: The body bend assay was used to measure the VA sensitivity of strains. Without transferring bacteria (either by transferring first to an unseeded plate or by picking animals that were off of the bacterial lawn), young adults were transferred via platinum wire to an unseeded NGM plate. After a 29-min incubation period, the plate was vigorously shaken (inside a clear glass chamber containing volatile anesthetic; see below) for ~5 sec. After one more minute, body bends were counted for 2 min. One body bend consisted of a complete period of the waveform in the nematode's forward sinusoidal motion. Backward body bends were not counted. The body bends of at least 10 animals were counted per strain and condition.

Heat-shock experiments: Young adult animals on agar plates were placed at 30° for 4 hr. Then, the body bend assay was performed as described above. Non-heat-shock controls were kept at 20° during the heat-shock incubation.

Drug assays: **Volatile anesthetics:** After transfer of animals to assay plates, the plates were placed immediately into small glass chambers as previously described (MORGAN and CASCORBI 1985; CROWDER *et al.* 1996). The chambers were sealed except for a small sideport and a measured volume of anesthetic in its liquid phase was deposited through the sideport onto the roof of each chamber using a Hamilton syringe and the sideport was quickly sealed. The anesthetic completely volatil-

izes within 30 sec and produces a steady-state behavioral effect within 10 min. The volume of anesthetic placed in the chamber was calculated to produce a desired gas phase concentration according to the ideal gas equation given the volume of the chamber and the density and molecular weight of the liquid anesthetic. At the end of each assay, the actual VA

concentrations in the chambers were measured by gas chromatography and expressed as volume % (vol%). The predicted and actual concentrations typically differed by <10%. The measured VA concentrations were plotted against the body bends per minute using the sigmoidal equation $y = \min + (\max - \min) / (1 + (x/x^{50})^{-k})$ where y is the body bends per

minute, x is the [VA] concentration in vol%, $\max$ is the body bends per minute with no VA added, $\min$ is the body bends per minute where it is at the lowest (*i.e.*, at the highest VA concentrations), x^{50} is the EC_{50} (VA concentration at which the body bends per minute is half maximal), and k is the slope of the curve. The EC_{50} 's were used as the measure of VA sensitivity. A minimum of 10 points was used to generate each concentration/response curve. The VA sensitivities of strains were statistically compared by simultaneous curve fitting (WAUD 1972; DELEAN *et al.* 1978).

Aldicarb assays: Two methods (kinetic and steady state) were used to determine aldicarb sensitivity. Kinetic assays were used for mapping of *egl-30(js126)* and for the determination of the native aldicarb sensitivity. A total of 20–25 animals were transferred onto a plate containing aldicarb and the fraction of animals paralyzed at multiple time points was measured. Animals were counted as paralyzed when they failed to move spontaneously or upon stimulation with a platinum wire. The steady-state assay (VAN SWINDEREN *et al.* 1999, 2001) was used for all aldicarb experiments performed with volatile anesthetics. Aldicarb sensitivities were measured after a 4-hr incubation on an aldicarb plate so that a concentration of drug inside the animal approximating that at steady state could be achieved. After this 4-hr preincubation, ~30 animals were moved into a 0.5-cm-diameter circle delineated on the back of the aldicarb plate. After one additional hour, the fraction of animals that were outside the circle was scored as the movement index (MI). Because this assay did not require touching the animals and the aldicarb effect was not varying significantly with time, the steady-state assay was better suited for measuring effects of anesthetics (where the animals are enclosed in a gas tight chamber) on aldicarb sensitivity (VAN SWINDEREN *et al.* 1999, 2001).

Phorbol ester assays: Phorbol 12-myristate 13-acetate dissolved in 100% ethanol was added to liquid NGM agar cooled to 60° to produce a 0.5 μ g/ml final concentration. Plates containing an identical volume of ethanol were prepared as a control. A total of 40–50 healthy wild-type adults were transferred to and incubated on seeded phorbol ester and control plates for 2 hr and 20 min. Animals were then transferred onto unseeded phorbol ester and control plates, and a body bends per minute assay was performed as described above.

Arecoline assay: The VA sensitivity of animals was measured on plates containing the muscarinic agonist arecoline hydrobromide (AVERY 1993; LACKNER *et al.* 1999). A sterile 142 mg/ml arecoline stock solution was made and stored at 4°; 167 μ l of a sterile 142 mg/ml arecoline solution in water was added to the surface of freshly poured unseeded 10-ml NGM plates to produce a concentration of 2.4 mg/ml. Then, a fraction of the plates were seeded with OP50. Test animals were incubated on the seeded arecoline plates for 30 min and then transferred to an unseeded arecoline plate and a body

bend assay in the presence and absence of VA was performed as described above.

RESULTS

Isolation and characterization of suppressors of syntaxin reduction-of-function mutants: We performed a mutagenesis screen in *C. elegans* for mutations suppressing the aldicarb-resistance and lethargic locomotion phenotype of the syntaxin reduction-of-function (rf) mutant, *unc-64(e246rf)* (Figure 1). Aldicarb is an acetylcholinesterase inhibitor that is widely used as an indirect measure of the level cholinergic neurotransmission in *C. elegans* (RAND and NONET 1997). Mutations that reduce cholinergic neurotransmission, such as *unc-64(e246rf)*, are resistant to aldicarb, and mutations that increase cholinergic neurotransmission are hypersensitive. A total of 24,000 haploid genomes were screened in the F_2 generation, and 14 suppressor mutations were isolated that fell into at least five complementation groups. Representatives of three of the complementation groups were chosen for characterization. The other mutants were set aside for later analysis. Consistent with a role of the suppressor mutations in regulation of transmitter release, the mutations suppressed both the sluggish locomotion and the aldicarb resistance of *unc-64(e246rf)* (Figure 1, B–D). Outcrossing of suppressor mutations away from *unc-64(e246rf)* revealed that all suppressor mutations conferred hypersensitivity to aldicarb (Figure 1, E and F; WANG *et al.* 2001). Thus, the suppressor mutations are capable of enhancing cholinergic neurotransmission of both wild-type and reduction-of-function syntaxin alleles; that is, the effects on neurotransmission are not syntaxin allele specific.

Loss of *slo-1* function confers VA resistance: As previously reported, the largest complementation group isolated in our screen consisted of loss-of-function mutations in the *slo-1* locus, which codes for a calcium-activated potassium channel (BK channel) negatively regulating the duration of transmitter release in *C. elegans* (WANG *et al.* 2001). After outcrossing the suppressor mutations away from *unc-64(e246)*, all *slo-1(lf)* alleles tested were found to be resistant to the VA halothane

FIGURE 1.—Isolation of mutations suppressing syntaxin reduction-of-function phenotypes. (A) Strategy for isolation of suppressors of syntaxin reduction of function. (B) Example of suppression of lethargy of *unc-64(e246)* by *egl-30(js126)*. Shown in each row are three frames taken at the times noted from 60-sec movies of a representative N2, *unc-64(e246)*, or *egl-30(js126);unc-64(e246)* animals. The animals were allowed to move freely on agar pads with a freshly seeded lawn of bacteria. Note different times for each strain. Bar, 1 mm. (C) Quantification of suppression of *unc-64(e246)* lethargy. Velocities were calculated from serial images of at least 20 animals moving on agar plates seeded with *E. coli*. Velocities just prior to (open bars) and immediately after stimulation (solid bars) by dropping a weight from a fixed height onto the lid of the Petri dish are shown. (D) Suppression of *unc-64(e246)* aldicarb resistance. The percentage paralyzed was plotted *vs.* incubation time on agar plates containing 1.0 mM aldicarb. Animals were considered paralyzed if no spontaneous or touch-evoked movement was observed. (E) Aldicarb sensitivity of *unc-43* single mutants. Assay was exactly as in D except on plates containing 0.5 mM aldicarb. *unc-43(e755)* is a loss-of-function mutant described previously (REINER *et al.* 1999). (F) Aldicarb sensitivity of *egl-30* single mutants. Assay was exactly as in D except on plates containing 0.25 mM aldicarb. *egl-30(n686)* is a partial reduction-of-function mutant (BRUNDAGE *et al.* 1996). All values are expressed as mean $\pm$ SEM.

FIGURE 2.—Sensitivity of suppressor mutants to the volatile anesthetic halothane. (A) Concentration/response curve of halothane against the locomotion rate of *slo-1(js380)* and N2. The rate of body bends was measured for each animal over a 2-min period. Each data point represents the mean $\pm$ SEM of at least 10 animals. (B) Concentration/response curve of halothane against the locomotion rate of *unc-43(js383)* and N2. (C) Concentration/response curve of halothane against the locomotion rate of *egl-30(js126)* and N2. (D) Halothane sensitivities of all suppressor mutants compared to N2. Bars represent EC₅₀'s plus or minus the standard error of the estimate. * $P < 0.05$ compared to N2 by simultaneous nonlinear regression (DELEAN *et al.* 1978; WAUD 1972).

(Figure 2, A and D). *slo-1(lf)* mutations have also been isolated in a screen for resistance to ethanol (DAVIES *et al.* 2003). Three *slo-1* alleles isolated in this screen (*eg24*, *eg161*, and *eg73*) were tested for VA sensitivity. All three were resistant to halothane (Figure 2D). Loss of *slo-1* function has been shown to enhance cholinergic neurotransmission through a prolongation of acetylcholine transmitter release at *C. elegans* motor neuron synaptic terminals (WANG *et al.* 2001). Although SLO-1 could be a direct target of VAs, the VA resistance of *slo-1(lf)* mutants can be explained by an indirect antagonism of the reduction in excitatory neurotransmitter release produced by VAs. Resistance to ethanol, however, does not appear to be the indirect consequence of an alteration in the level of transmitter release; rather, ethanol was shown to directly potentiate the SLO-1 channel

(DAVIES *et al.* 2003). While supraclinical concentrations of VAs have been shown to inhibit vertebrate SLO-1 orthologs, clinical concentrations of VAs have little effect (PANCRAZIO *et al.* 1993; HONG *et al.* 1994). Moreover, to inhibit transmitter release, VAs would be predicted to augment the SLO-1 channel as ethanol does.

Loss of *unc-43* function suppresses syntaxin reduction of function without producing VA resistance: *js125* and *js383* formed a second complementation group of *unc-64* (rf) suppressors. When outcrossed from *unc-64(e246)*, both mutants were found to have VA sensitivities not significantly different from those of wild type (Figure 2, B and D). *js125* and *js383* were also behaviorally quite distinct from the other suppressor mutants. While the other suppressors outcrossed from *e246* moved in a hyperactive manner, both *js125* and *js383* moved sluggishly

and exhibited a shrinker phenotype, the simultaneous contraction of all four body-wall muscle quadrants in response to touch. Shrinking is a relatively uncommon mutant phenotype, found in strains with reduced GABAergic neurotransmission (McINTIRE *et al.* 1993) and in mutants in the *unc-43* gene, which lie within the genetic interval to which *js125* mapped. *unc-43* encodes calcium/calmodulin-dependent protein kinase II (CaM-kinase II; REINER *et al.* 1999; RONGO and KAPLAN 1999). Sequencing of *unc-43* revealed mutations in both *js125* and *js383*. *js125* deletes the first 10 exons of *unc-43*; *js383* had a G-to-A transition in the splice acceptor prior to exon 15 (see MATERIALS AND METHODS). Thus, *js125* likely produces a complete loss of function of *unc-43*. Gain-of-function and loss-of-function *unc-43* alleles have been isolated previously (REINER *et al.* 1999). As expected given that the mutation deletes a large portion of the coding sequence, *js125* resembled *unc-43(e755)*, a previously isolated severe loss-of-function mutant. *js125* and *e755* as well as *js383* are sluggish, shrinkers, and aldicarb hypersensitive (REINER *et al.* 1999; ROBATZEK and THOMAS 2000; Figures 1E and 2B). *unc-43(gf)* alleles do not shrink and are aldicarb resistant (ROBATZEK and THOMAS 2000). *unc-43(e755)* but not *unc-43(gf)* alleles suppressed the phenotypes of *unc-64(rf)* (data not shown). Thus, we conclude that both *js125* and *js383* are loss-of-function alleles of *unc-43* and that *unc-43* negatively regulates syntaxin function but not VA action.

Gq α signaling antagonizes VA action: Unlike the *slo-1* and *unc-43* mutants, the *js126* mutation produced dominant phenotypes, including loopy hyperactive locomotion, aldicarb hypersensitivity, and VA resistance (Figures 1F and 2, C and D). The syntaxin suppressor phenotype of *js126* mapped to a region containing the *egl-30* gene (see MATERIALS AND METHODS), which encodes the α -subunit of the G protein Gq shown to positively regulate locomotion, feeding, egg laying, and cholinergic neurotransmission (BRUNDAGE *et al.* 1996). Sequencing of *js126* identified a mutation in *egl-30*, a G-to-A transition, resulting in a valine-to-methionine substitution at position 180 of EGL-30. While valine 180 has not itself been directly implicated in Gq α function, this region in vertebrate orthologs has been shown to bind to the γ -phosphate of GTP and ADP-ribosylation of the adjacent arginine 179 inhibits GTPase activity (NOEL *et al.* 1993). Thus, a reasonable hypothesis to explain the phenotype of *js126* is alteration of GTPase activity. Indeed, expression of a GTPase-defective EGL-30 results in animals with loopy hyperactive locomotion, constitutive egg laying, and aldicarb hypersensitivity similar to the phenotypes of *js126* (BRUNDAGE *et al.* 1996; LACKNER *et al.* 1999). On the other hand, *egl-30* reduction-of-function mutants such as *n686* (Figure 1F) are sluggish and aldicarb resistant (BRUNDAGE *et al.* 1996; LACKNER *et al.* 1999; MILLER *et al.* 1999). Thus, *js126* behaves as an *egl-30(gf)* allele and not as an *egl-30(lf)* allele. Testing of *egl-30(tg26)*, a behaviorally similar *egl-30* allele

thought to produce a gain of function (DOI and IWASAKI 2002), showed that this allele also suppressed *unc-64(e246)*, increasing its mean velocity nearly fourfold (data not shown), and was VA resistant (Figure 2D). *egl-30(n686)*, a weak reduction-of-function allele, was halothane hypersensitive ($EC_{50} = 0.178 \pm 0.047$ vol%). On the basis of these data, we conclude that *js126* produces a gain of function in *egl-30*, which antagonizes VA action.

Both *js126* and *tg26* could produce a novel activity not normally present in the wild-type *egl-30* product. To test whether wild-type *egl-30* antagonizes VA activity, we measured the VA sensitivity of a strain overexpressing wild-type *egl-30* (BRUNDAGE *et al.* 1996; LACKNER *et al.* 1999). Animals transformed with extra copies of *egl-30(+)* were significantly halothane resistant with EC_{50} 's greater than twofold that of wild-type animals (Figure 3B). Similarly, heat shock of animals transformed with a GTPase-defective *egl-30* transgene fused to a heat-shock-inducible promoter (LACKNER *et al.* 1999) resulted in an even higher level of halothane resistance (Figure 3C). To confirm that the transformed genes were conferring a gain of *egl-30* function, aldicarb sensitivity was measured, and as expected both strains were aldicarb hypersensitive (Figure 3D). These data are consistent with antagonism of VAs by the wild-type activity of EGL-30 and argue against the hypothesis that the *js126* mutation produces a novel function not normally present in *egl-30*.

Pathway for EGL-30's regulation of VA sensitivity: To begin to define the pathway whereby EGL-30 regulates VA sensitivity, we measured the effect of drugs previously shown to activate the EGL-30 signaling pathway. The muscarinic acetylcholine agonist, arecoline, produces hypercontraction of the *C. elegans* pharynx and aldicarb hypersensitivity, both effects of which are suppressed by *egl-30(rf)* (BRUNDAGE *et al.* 1996; LACKNER *et al.* 1999). These results have led to a model where muscarinic receptors are coupled to EGL-30 and activation of muscarinic receptors stimulates EGL-30, which positively regulates acetylcholine release (BRUNDAGE *et al.* 1996; LACKNER *et al.* 1999). If the muscarinic receptor is coupled to EGL-30 in the cells where VAs act, then activation of muscarinic receptors should antagonize VAs. Indeed, the muscarinic agonist arecoline produced halothane resistance (Figure 4A). Downstream of EGL-30, phorbol esters augment acetylcholine release from *C. elegans* motor neurons (LACKNER *et al.* 1999; MILLER *et al.* 1999). Both genetic and biochemical evidence implicate UNC-13 as the phorbol ester receptor acting downstream of EGL-30 to enhance acetylcholine release (MARUYAMA and BRENNER 1991; AHMED *et al.* 1992; LACKNER *et al.* 1999). Moreover, UNC-13 has been shown to interact with the N terminus of syntaxin, making this pathway an attractive candidate for modulation of VA sensitivity by syntaxin mutants (BETZ *et al.* 1998). Indeed, phorbol ester-treated animals were significantly resistant to halothane (Figure 4B).

FIGURE 3.—Gain of function in *egl-30* confers resistance to halothane. (A) Schematic of the constructs used for transformation to produce *egl-30* gain-of-function strains. The Ex[Gq(+)] is a genomic *egl-30* fragment including the native promoter, the entire coding sequence, and 3' noncoding sequence (BRUNDAGE *et al.* 1996). In GqHS_(Q205L), the native promoter was replaced with the hsp-16-2 heat-shock-inducible promoter and the codon for aspartate 205 was mutated to produce a lysine. The Q205L lesion is predicted to disrupt the GTPase activity of EGL-30, producing a constitutively active protein. (B) Halothane sensitivity of animals transformed with Ex[Gq(+)] compared to N2. The rate of body bends was measured for each animal over a 2-min period. Each data point represents the mean $\pm$ SEM of at least 10 animals. EC₅₀ of Ex[Gq(+)] was greater than that of N2, $P < 0.01$ by simultaneous nonlinear regression. (C) Comparison of halothane EC₅₀'s of *egl-30* gain-of-function strains. A 4-hr heat-shock induction was performed on adult animals just prior to anesthetic testing. * $P < 0.05$ compared to N2 by simultaneous nonlinear regression (WAUD 1972; DELEAN *et al.* 1978). (D) Comparison of aldicarb sensitivity of *egl-30* gain-of-function strains. Animals were incubated on plates containing 0.1 mM aldicarb for 4 hr and then the fraction of animals that moved over the next hour was scored as the movement index. Heat-shock induction was performed on adult animals just prior to anesthetic testing. * $P < 0.05$ compared to N2 two-tailed test; † $P < 0.05$ compared to non-heat-shocked control.

To define genetically the pathway whereby *egl-30* regulates volatile anesthetics, we tested strains carrying mutations in genes previously shown to lie downstream of *egl-30* in its regulation of transmitter release. Modulation of neuromuscular transmission by *egl-30* requires, at least in part, the activity of *egl-8* (LACKNER *et al.* 1999; MILLER *et al.* 1999). *egl-8* encodes a phospholipase C β homolog that positively regulates neuromuscular synaptic transmission (LACKNER *et al.* 1999; MILLER *et al.* 1999). *egl-8(lf)* fully suppressed the VA-resistance, aldicarb-resistance, and hyperlocomotion phenotypes of *egl-30(js126)* (Table 1). Thus, as for locomotion and transmitter release, we conclude that EGL-30 regulates VA sensitivity by stimulating EGL-8 PLC β activity. However, *egl-8(n488lf)* itself

was normally sensitive to halothane, indicating that VAS act downstream of or parallel to EGL-8.

Diacylglycerol (DAG) is a major product of PLC β and positively regulates excitatory neurotransmission in vertebrates (BROSE *et al.* 2000) and in *C. elegans* (LACKNER *et al.* 1999; MILLER *et al.* 1999). Diacylglycerol kinase inactivates DAG by converting it to phosphatidic acid. If EGL-30 regulates VA sensitivity through accumulation of DAG, loss-of-function mutations in *dgk-1*, which encodes a *C. elegans* diacylglycerol kinase that inactivates DAG (LACKNER *et al.* 1999; MILLER *et al.* 1999; NURRISH *et al.* 1999), should be VA resistant. *dgk-1(nu199lf)* was halothane resistant at a level similar to *egl-30(js126)* (Table 1). The double mutant combination of *dgk-1(nu199)*

FIGURE 4.—Pharmacologic activation of the Gq signaling pathway confers resistance to halothane. (A) Halothane sensitivity of animals treated with the muscarinic agonist arecoline. Body bends per minute at 0 vol% and $0.3 \pm$ vol% halothane were counted on plates containing 2.4 mg/ml arecoline. Body bends per minute (BBM) values in ~ 0.3 vol% halothane were normalized to the average BBM with no added halothane. * $P < 0.05$, two-tailed t -test, significantly different from buffer. $n = 10$ animals for each condition. (B) Halothane sensitivity of phorbol ester-treated animals. After preincubation on agar plates containing phorbol ester or ethanol solvent, animals were transferred to unseeded phorbol ester or solvent plates and body bends were counted in the presence of various concentrations of halothane. BBM were normalized to the mean BBM without added halothane and then plotted against halothane concentration. * $P < 0.05$ by simultaneous curve fitting, EC_{50} significantly different from solvent. Each data point represents 1 animal.

and *egl-30(js126)* was not significantly more resistant than either single mutant. This lack of additivity is consistent with *egl-30* acting exclusively through DAG to regulate VA sensitivity. Notably, in a different locomotion assay (the dispersal assay) where the task requires moving a fixed distance within the assay period, *dgk-1(lf)* mutants are not VA resistant (VAN SWINDEREN *et al.* 2001). The different results in the two types of assays

presumably reflects an action of anesthetics on locomotion that is measured by the dispersal but not body bends assay and that is not effectively antagonized by *dgk-1(lf)*. Nevertheless, VA resistance in the body bends assay does demonstrate that *dgk-1* regulates at least some components of VA action on the neurons controlling locomotion and is consistent with *egl-30* antagonizing this VA action.

TABLE 1
Anesthetic and behavioral phenotypes of double mutants

Genotype	Body bends per minute ^a	Halothane EC_{50} (vol%) ^b	MI on aldicarb ^c	
			0.1 mM	0.35 mM
N2 (wild type)	23.1 $\pm$ 0.87	0.30 $\pm$ 0.05	0.51 $\pm$ 0.07	0.07 $\pm$ 0.02
<i>egl-30(js126)</i>	23.4 $\pm$ 0.87	0.67 $\pm$ 0.05 ^d	0.08 $\pm$ 0.04 ^d	0.00 $\pm$ 0.00
<i>egl-8(n488)</i>	17.5 $\pm$ 1.60 ^d	0.34 $\pm$ 0.15	ND	0.32 $\pm$ 0.08 ^d
<i>egl-30(js126);egl-8(n488)</i>	18.4 $\pm$ 0.8 ^e	0.23 $\pm$ 0.02 ^e	ND	0.30 $\pm$ 0.07 ^e
<i>dgk-1(nu199)</i>	20.5 $\pm$ 1.17	0.53 $\pm$ 0.08 ^d	0.06 $\pm$ 0.04 ^d	0.00 $\pm$ 0.00
<i>egl-30(js126);dgk-1(nu199)</i>	26.8 $\pm$ 1.33 ^f	0.64 $\pm$ 0.10 ^d	0.02 $\pm$ 0.02 ^d	0.00 $\pm$ 0.00
<i>unc-64(md130)</i>	19.7 $\pm$ 1.66 ^d	1.27 $\pm$ 0.16 ^d	ND	0.95 $\pm$ 0.00 ^d
<i>egl-30(js126);unc-64(md130)</i>	22.4 $\pm$ 2.73	1.52 $\pm$ 0.23 ^d	ND	0.45 $\pm$ 0.05 ^{d,g}
<i>unc-64(js21)</i>	12.5 $\pm$ 0.28 ^d	0.30 $\pm$ 0.07	ND	0.58 $\pm$ 0.02 ^d
<i>egl-30(js126);unc-64(js21)</i>	18.0 $\pm$ 1.52 ^{d,h}	0.68 $\pm$ 0.15 ^{d,h}	ND	0.28 $\pm$ 0.02 ^{d,h}

ND, not determined.

^a Body bends per minute $\pm$ SEM, no anesthetic ($n > 2$ trials, 5–10 animals per trial).

^b EC_{50} 's $\pm$ SE ($n > 2$ trials, 10–14 animals per trial).

^c Movement index $\pm$ SEM on aldicarb ($n > 6$ trials, 90 animals per trial).

^d Different from N2; $P < 0.05$.

^e Different from *js126*; $P < 0.05$.

^f Different from *nu199*; $P < 0.05$.

^g Different from *md130*; $P < 0.05$.

^h Different from *js21*; $P < 0.05$.

The final mediators of synaptic vesicle fusion and neurotransmitter release are thought to be the SNARE proteins, syntaxin, SNAP-25, and VAMP. To examine whether EGL-30 acts through syntaxin to regulate VA sensitivity, we tested the VA sensitivity of *egl-30(js126)* in the presence of two different *unc-64* syntaxin mutations. *unc-64(md130)* has a mutation in the splice donor site of the sixth intron (SAIFEE *et al.* 1998), resulting in the synthesis of truncated syntaxin products and a reduced amount of full-length product. The reduced level of syntaxin is responsible for all of the syntaxin reduction-of-function phenotypes; the truncated products confer VA resistance. *unc-64(js21)* carries a missense mutation that simply reduces syntaxin function (SAIFEE *et al.* 1998) and like other mutants, with the exception of *unc-64(md130)*, with reduced transmitter release is VA hypersensitive. The *egl-30(js126);unc-64(md130)* double mutant was more halothane resistant than *js126* but not significantly different from *md130*. However, the aldicarb resistance of *md130* was significantly reduced by *js126*, indicating that *egl-30* can still positively regulate transmitter release in the presence of the *md130* mutation. Given that *md130* is a reduction-of-function but not null mutation in syntaxin, the ability of *js126* to augment transmitter release in *md130* is not surprising. The lack of additivity of the VA resistances of *js126* and *md130* is more informative and suggests that the effect of the *md130* mutation on VA sensitivity is not modulated by the output of EGL-30 and is not dependent on the general level of cholinergic transmission. In contrast, the anesthetic phenotype of the *js126;unc-64(js21)* double mutant resembled the *js126* single mutant and not *js21*; however, the aldicarb and locomotion phenotypes are intermediate between that of the single mutants. Thus, neither hyperactivity nor increased levels of synaptic acetylcholine are essential for the VA resistance of *js126*. The genetic interactions between *unc-64* and *egl-30(gf)* with respect to locomotion, aldicarb, and VA sensitivities are identical to those between *unc-64* and *goa-1(lf)*, which encode a *C. elegans* Go α homolog (VAN SWINDEREN *et al.* 2001). These results are consistent with *goa-1* acting antagonistically to EGL-30 to regulate cholinergic neurotransmitter release and VA sensitivity (HAJDU-CRONIN *et al.* 1999; LACKNER *et al.* 1999; MILLER *et al.* 1999; CHASE *et al.* 2001; ROBATZEK *et al.* 2001; VAN DER LINDEN *et al.* 2001; VAN SWINDEREN *et al.* 2001).

DISCUSSION

Our previously reported results have shown that a truncated syntaxin produced by an intron splice donor site mutation in *unc-64(md130)* is capable of completely antagonizing the effects of clinical concentrations of volatile anesthetics on *C. elegans* locomotion (VAN SWINDEREN *et al.* 1999). This remarkable finding suggested two important conclusions: first, that volatile anesthetics

were not the nonspecific drugs that many had proposed but in fact their action could be blocked by mutation of a single gene and, second, that at least in *C. elegans* the relevant anesthetic target was likely a molecule that interacted with or is regulated by the syntaxin. The experiments reported here were designed to better understand syntaxin's role in anesthetic action and potentially identify the putative anesthetic target interacting with syntaxin.

Suppressors of syntaxin reduction of function were expected to fall into one of three classes. First, informational tRNA suppressor mutations could revert the mutation in the *e246* protein. However, none of the identified suppressors fall into this class. A second class would be composed of mutations in proteins that complex with syntaxin and suppress *e246* by having complementary mutations in residues involved in interaction with the mutated syntaxin. The *e246* mutation substitutes a valine for alanine at position 248 of syntaxin (SAIFEE *et al.* 1998). This residue has been shown by crystallography and electron spin resonance studies to lie on the hydrophobic face of the H3 helical domain of syntaxin-1A when in a complex with itself and SNAP-25 (a t-SNARE complex) or with VAMP and SNAP-25 (the final ternary SNARE complex; POIRIER *et al.* 1998; SUTTON *et al.* 1998; XIAO *et al.* 2001; BRUNGER and ERNST 2002). Thus, mutations in complementary residues in SNAP-25 and/or VAMP might restore function in *e246* mutated syntaxin. Besides SNAP-25 and VAMP, several additional proteins have been shown to interact with syntaxin in the H3 domain (RIZO and SUDHOF 2002); however, unlike the SNARE complex components, it is unclear if valine 248 interacts with or is required for the interaction with these proteins. In any case, this sort of complementing protein suppressor mutant would act allele specifically. None of the suppressor mutations act on only specific syntaxin alleles; each increases cholinergic neurotransmission of the wild-type syntaxin allele and at least one *unc-64(rf)* allele. In addition, none of the suppressor genes encode known syntaxin-interacting proteins. Therefore, the isolated suppressor mutants are unlikely to belong to this second class of suppressors.

The third class of suppressors could act by enhancing cholinergic neurotransmission regardless of the nature of the syntaxin allele. On the basis of the aldicarb-hypersensitive phenotype of the suppressor mutants in a wild-type syntaxin background, each suppressor appears to fall into this category. Indeed, in the case of *slo-1*, direct electrophysiological evidence demonstrates that *slo-1* (lf) enhances acetylcholine release (WANG *et al.* 2001), and selective expression of SLO-1 shows that it controls cholinergic neurotransmission by acting in neurons not muscle. Thus, SLO-1 acts presynaptically to inhibit transmitter release. The role of EGL-30 in transmitter release has not been confirmed by electrophysiological methods. However, genetic and pharmacological data pre-

sented here and elsewhere indicate that EGL-30 acts in motor neurons by activating phospholipase C β , producing DAG, which directly activates UNC-13, a syntaxin binding protein (LACKNER *et al.* 1999; MILLER *et al.* 1999; BROSE *et al.* 2000).

Among the suppressor mutants, the *unc-43(lf)* alleles are distinct. They were sluggish, had a shrinking response to touch, and had normal sensitivity to VAs. UNC-43 CaMKII has been shown to regulate locomotion, defecation, and glutamate receptor density in the *C. elegans* nervous system (REINER *et al.* 1999; RONGO and KAPLAN 1999). The phenotypes of *unc-43* gain-of-function mutations are suppressed by loss-of-function mutations in HERG-type K $^{+}$ channel for defecation phenotypes and by mutations in Go α and in a cyclic-nucleotide-gated channel for locomotion and transmitter release (REINER *et al.* 1999; ROBATZEK and THOMAS 2000). The genetic interaction of *goa-1* and *unc-43* was explained by a model where UNC-43 acts in motor neuron terminals to activate GOA-1 and thereby inhibit excitatory neurotransmitter release (ROBATZEK and THOMAS 2000). However, in *Drosophila* CaMKII has been shown to act in muscle to regulate transmitter release by inhibiting a retrograde signal (HAGHIGHI *et al.* 2003). Inhibitors of *Drosophila* CaMKII in muscle increased transmitter release from motor neurons while constitutive activation of CaMKII reduced it. The regulation of transmitter release by CaMKII in *C. elegans* could also be explained by a retrograde signal coming from muscle. However, why this action would not produce VA resistance is unclear. Selective expression of UNC-43 in muscle and motor neurons coupled with electrophysiological measurements with and without VAs will be required to resolve this question.

One of the goals of characterizing the anesthetic response of our suppressor mutants was to find the putative syntaxin-interacting anesthetic target. Neither SLO-1 nor Gq α is known to interact with syntaxin. Moreover, in the case of SLO-1, if it were the sought-after target, then *slo-1*(null) would be expected to be at least as resistant as *unc-64(md130)*. It is not. Given that a gain of function in *egl-30* produces resistance and *egl-30*(null) mutations are lethal, discounting EGL-30 as a target is less definitive. However, it seems unlikely. Considering this and previous data, the most parsimonious model is that *slo-1* (lf) and *egl-30*(gf) produce VA resistance by increasing the level of excitatory neurotransmitter release, thereby indirectly antagonizing VA-induced reduction in transmitter release. The VA target(s) remains to be defined.

We thank Laura Metz for help with the anesthetic assays. We thank Kouichi Iwasaki, Lorna Brundage, and Paul Sternberg for sharing of unpublished data and reagents. Some of the strains used in this work were supplied by the *Caenorhabditis* Genetics Center funded by the National Institutes of Health (NIH) National Center for Research Resources. This work was supported by NIH grants to C.M.C. (National Institute of General Medical Sciences) and M.L.N. (National Institute of Neurological Disorders and Stroke) and a Public Health Service grant to O.S.

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Communicating editor: B. J. MEYER