Published in final edited form as:
Nat Neurosci. 1999 September ; 2(9): 791–797. doi:10.1038/12160.

One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction

Janet E. Richmond and **Erik M. Jorgensen**
Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, Utah 84112-0840, USA

Abstract

We describe an electrophysiological preparation of the neuromuscular junction of the nematode *C. elegans*, which adds to its considerable genetic and genomic resources. Mutant analysis, pharmacology and patch-clamp recording showed that the body wall muscles of wild-type animals expressed a GABA receptor and two acetylcholine receptors. The muscle GABA response was abolished in animals lacking the GABA receptor gene *unc-49*. One acetylcholine receptor was activated by the nematocide levamisole. This response was eliminated in mutants lacking either the *unc-38* or *unc-29* genes, which encode alpha and non-alpha acetylcholine receptor subunits, respectively. The second, previously undescribed, acetylcholine receptor was activated by nicotine, desensitized rapidly and was selectively blocked by dihydro-β-erythroidine, thus explaining the residual motility of *unc-38* and *unc-29* mutants. By recording spontaneous endogenous currents and selectively eliminating each of these receptors, we demonstrated that all three receptor types function at neuromuscular synapses.

Sydney Brenner originally proposed developing the nematode *Caenorhabditis elegans* as a model system for understanding the nervous system, and the uncoordinated mutants which he isolated have helped identify a large number of molecules which function in the nervous system. However, understanding how these genes function requires electrophysiological characterization of their mutant phenotypes. Such techniques in *C. elegans* have been restricted by the difficulty of exposing synapses in an organism measuring only one millimeter in length[2]. To date, electrophysiological studies of synaptic function in *C. elegans* have been limited to extracellular recordings from the pharynx[3], in which a suction pipette placed around the head of the nematode is used to examine synaptic activity by measuring capacitative transients from the whole organ[3,4]. However, extracellular recordings cannot resolve very small currents, such as those generated by the release of a single synaptic vesicle, nor can they measure voltage-sensitive currents. Here we report the development of an electrophysiological preparation of *C. elegans* that permits patch-clamp recordings of postsynaptic currents in the muscle, which we have used to identify the receptor composition of this polyinnervated cell.

Central neurons are polyinnervated, receiving and processing synaptic inputs from multiple excitatory and inhibitory neurons[5]. Polyinnervated cells segregate receptors and localize them to specific synapses or regions of synapses[6–8]. Distinct mechanisms independently regulate the activity and expression levels of these receptors to modulate the strength of individual synaptic inputs[9–13]. Knowledge of the processes governing receptor localization and modification at synapses is still limited, in part because of the difficulties of accessing identified neurons and establishing receptor identities of the postsynaptic cells.

Correspondence should be addressed to J.E.R. (richmond@biology.utah.edu).

The neuromuscular junction of *C. elegans* provides a simple and accessible model of a polyinnervated synapse[2]. Genetic analysis in *C. elegans* could address questions about the mechanisms underlying the organization, maintenance and regulation of a polyinnervated cell. The first step toward this goal is to identify the postsynaptic receptor composition of the *C. elegans* neuromuscular junction. Analysis of mutants and studies of neuronal connectivity indicate that the muscle is innervated by both γ-aminobutyric acid (GABA)[14,15] and acetylcholine motor neurons[16]. Genetic screens[14] identified a gene, *unc-49*, encoding three full-length GABA receptor subunits[17]. Two of these subunits, which are present in body wall muscle, form a functional GABA receptor in heterologous expression systems[17]. The acetylcholine receptor genes *unc-38* and *unc-29* were also identified in genetic screens and have been implicated in muscle function[16]. Mutations in either of these genes strongly impair locomotion in *C. elegans*[18]. Furthermore, mosaic analysis and expression studies demonstrate that the UNC-29 subunit is required and expressed in body wall muscles[19,20]. Residual motility in *unc-29* and *unc-38* mutants suggests that additional excitatory receptors may also be present in the muscle[21]. To functionally characterize the polyinnervation of the body wall muscle, we asked whether these genetically defined GABA and acetylcholine receptors operate at synapses on the muscle and whether additional, genetically unidentified receptors account for the residual motility of *unc-38* and *unc-29* mutants. From electrophysiological, genetic and pharmacological techniques, we conclude that body wall muscle synapses use one GABA receptor and two acetylcholine receptors.

Results

A new dissection technique was developed to examine the electrophysiological properties of the *C. elegans* neuromuscular junction. Individual worms were immobilized by applying a cyanoacrylic glue (Histoacryl Blue) along the dorsal side of the body. A lateral incision in the cuticle of immobilized animals exposed the ventral muscle quadrants and the innervating ventral nerve cord (Fig. 1). Stable, whole-cell, voltage-clamp recordings could be obtained from several ventromedial muscle cells in each preparation. Because these cells are adjacent to the ventral nerve cord and do not extend long muscle arms, distortions caused by inadequate space clamp are minimized. Specifically, the rapid activation of voltage-sensitive currents and the reproducible current–voltage relationships indicated that these cells are relatively isopotential. Robust voltage-gated outward currents typical of potassium currents were observed at potentials greater than −30 mV (Fig. 2a and **b**). The potassium channel blocker tetraethylammonium chloride (TEA, 20 mM), which is selective for delayed rectifier potassium channels, reduced the outward currents and revealed a fast inactivating current (Fig. 2a and **b**). This remaining potassium current was blocked by 4-aminopyridine (3 mM), which is selective for type A potassium channels (Fig. 2a and **b**). Residual outward current was eliminated by replacing internal KCl with CsCl and including 5 mM TEA in the recording pipet solution. Under these conditions, an inward current was revealed with a current–voltage relationship consistent with a high-voltage-activated Ca^{2+} current (Fig. 2c and **d**). Peak inward current was observed at 0 mV. Consistent with a Ca^{2+} current, this inward current was abolished in 0 Ca^{2+} and 50 μM Cd^{2+} external Ringer's.

GABA receptors

Genetic and pharmacological data suggest that GABAergic motor neurons innervate body wall muscles[14,15]. To confirm that body wall muscles express functional GABA receptors, we pressure-ejected GABA (100 μM) from a pipette positioned close to voltage-clamped muscle cells. At a holding potential of −80 mV and a recording pipette chloride concentration of 128.5 mM, GABA evoked a robust inward current (n = 25; Fig. 3a). This response could be mediated directly by GABA receptors on the muscle or indirectly by GABA receptors on motor neurons, which in turn release a neurotransmitter, such as acetylcholine, onto the muscle. To test whether

GABA acted directly on the muscle or through neurons, we applied GABA while inhibiting synaptic transmission with 0 Ca^{2+} and 0.5 mM EGTA. The GABA-evoked current amplitudes were greater than or equal to (106 ± 4%, $n = 7$) those obtained in the same cells in the presence of Ca^{2+}, indicating that GABA acts directly on body wall muscles.

To test whether the muscle GABA receptor was a chloride-permeant channel, we determined the reversal potential of the GABA response. Measurements of the current–voltage relationship produced a linear fit with the reversal potential at +17 mV ($n = 4$), close to the predicted chloride reversal potential of +24 mV for the solutions used (Fig. 3c). Replacing external acetate with chloride shifted the reversal potential to −10 mV, near the predicted reversal potential of −8 mV. Thus, the GABA receptor in *C. elegans* body wall muscle seems to be a chloride-permeant channel. *C. elegans* is sensitive to the GABA agonist muscimol[14]. Muscimol (100 μM) produced an inward current in the body wall muscle (Fig. 3b) with a reversal potential of +20 mV ($n = 3$), similar to the GABA response in the same solutions (Fig. 3c).

The *unc-49* gene encodes several GABA receptor subunits produced through differential splicing, and at least two of these subunits are expressed in body wall muscle[17]. To test whether *unc-49* encodes the functional GABA receptor in this muscle, we applied GABA to the muscles of *unc-49(e407)* mutants. In contrast to the robust GABA response of muscles in wild-type animals (Fig. 3a), the *unc-49(e407)* muscle showed no response ($n = 5$, Fig. 3d). These muscle cells were healthy, because *unc-49(e407)* muscle cells that failed to respond to GABA generated large inward currents in response to subsequent acetylcholine application (100 μM; data not shown). The muscimol response was also eliminated in *unc-49(e407)* muscles ($n = 9$, Fig. 3e). These data suggest that *unc-49* encodes the GABA receptors responsible for all the GABA-evoked ionic current in the body wall muscle.

Levamisole-sensitive acetylcholine receptor

Genetic and pharmacological analyses suggest that acetylcholine is the excitatory neurotransmitter at the *C. elegans* neuromuscular junction[16,18,21,22]. As expected, acetylcholine pressure-ejected onto voltage-clamped body wall muscles elicited a robust inward current (Fig. 4a). The reversal potential for this response was calculated to be +11 mV from a linear fit of measurements made at voltage steps between −80 mV and +10 mV ($n = 4$; Fig. 4c), consistent with the expected reversal potential for a non-selective cation-permeant channel. D-tubocurare (dTBC) is a potent inhibitor of many nicotinic acetylcholine receptors. At 500 μM, dTBC reversibly eliminated the acetylcholine response in body wall muscle (Fig. 4a). To test the specificity of this action, we also examined the effects of dTBC on the GABA response of the body wall muscles. As expected, dTBC failed to inhibit the GABA response (data not shown).

Levamisole, a potent nematocide, is thought to act as an acetylcholine agonist at nematode muscle receptors, producing muscle paralysis and ultimately death[21,23]. Levamisole (100 μM) pressure-ejected onto voltage-clamped muscles produced an inward current with a reversal potential similar to that produced by acetylcholine ($n = 3$, Fig. 4b and **c**). To demonstrate that the levamisole response was due to a direct action on the muscle, we applied levamisole in 0 Ca^{2+} and 0.5 mM EGTA. The levamisole response persisted under these conditions, although the amplitude of the response was reversibly reduced (50 ± 5% of amplitudes in 5 mM Ca^{2+}, $n = 4$). This reduction could suggest that 50% of the response to levamisole is caused by stimulation of motor neurons; alternatively, a substantial component of the muscle current could be due to calcium flux. To distinguish between these possibilities, we applied levamisole onto the muscles of an *unc–13(s69)* mutant that lacks evoked responses (J.R. and E.J., unpublished observation). These mutants showed a similar reduction in levamisole response in the absence of calcium (data not shown), suggesting that the levamisole current in the muscle has a substantial calcium component. The levamisole response of the

muscle was also completely inhibited by dTBC in 0 Ca^{2+} and 0.5 mM EGTA, demonstrating that dTBC directly blocks the muscle response.

Mutations in several putative acetylcholine receptor genes confer resistance to levamisole and produce uncoordinated locomotion phenotypes[16]. Two of these genes encode alpha (*unc-38*) and non-alpha (*unc-29*) nicotinic acetylcholine receptor subunits[20]. UNC-29 is expressed in body wall muscles and forms a functional acetylcholine receptor when expressed heterologously in *Xenopus* oocytes with UNC-38 and another non-alpha nicotinic acetylcholine receptor subunit, LEV-1 (ref. 20). Unlike *unc-38* and *unc-29* mutants, *lev-1* mutants show wild-type motility, detectable levamisole binding and only weak levamisole resistance, suggesting that UNC-29 can substitute for LEV-1 subunits to form functional acetylcholine receptors *in vivo*[20]. To test whether *unc-38* and *unc-29* are required for the levamisole response of body wall muscles, we applied levamisole to *unc–38(e264)* and *unc-29 (e1072)* mutants. The levamisole response was eliminated in both mutants (n = 5 for *unc-38 (e264)*, n = 7 for *unc-29(e1072)*; Fig. 4d and **e**). These data suggest that levamisole-sensitive receptors in body wall muscles require the expression of both UNC-38 and UNC-29.

Nicotine-sensitive acetylcholine receptor

Although locomotion in *unc-38(e264)* and *unc-29(e1072)* mutants is impaired, the muscles still contract, suggesting that a second pharmacological class of acetylcholine receptor may be present[21]. To test this prediction, we pressure-ejected acetylcholine onto the muscles of *unc-38 (e264)* and *unc-29(e1072)* mutants. Although these muscles failed to respond to levamisole, subsequent application of acetylcholine elicited a robust inward current in both *unc-38 (e264)* (n = 6) and *unc-29(e1072)* (n = 4) mutants (Fig. 5a). The average current amplitudes were smaller in mutant (445 ± 88 pA, n = 14, in *unc-38(e264)*; 555 ± 62 pA, n = 19, in *unc-29 (e1072)*) than in wild-type muscles (774 ± 79 pA, n = 22), consistent with the loss of one of the two receptors in these mutants. The *unc–29(e1027)* mutants also responded to acetylcholine in 0 Ca^{2+} and 0.5 mM EGTA, demonstrating that these responses were due to a direct action on the muscle, although the amplitudes were reversibly decreased to 57 ± 3% (n = 3) of response amplitudes in 5 mM Ca^{2+}. Together, these data indicate that in addition to the levamisole-sensitive receptor, there is a second acetylcholine receptor expressed by the body wall muscles.

Desensitization rates were also different between the two muscle acetylcholine receptors. Desensitization was measured as the percentage of the peak current (I_{max}) remaining after a one-second acetylcholine application (I_{1s}). The currents remaining in the mutants *unc-38 (e264)* (25.4 ± 4.4%, n = 8) and *unc-29(e1072)* (23.1 ± 2.8%, n = 8) were significantly smaller than in wild-type animals (74.9 ± 3%, n = 15; Fig. 5a and **b**). These data suggest that the levamisole-resistant acetylcholine receptor desensitizes rapidly.

The acetylcholine agonist nicotine (100 μM) was also applied to the body wall muscles. A one-second nicotine application to wild-type muscle produced a rapidly desensitizing response (31.25 ± 2.5%, n = 4) indistinguishable from the acetylcholine responses of *unc-38(e264)* and *unc-29(e1072)* (Fig. 5c and **d**). A one-second nicotine application also produced a rapidly desensitizing inward current in both *unc-38(e264)* (23.7 ± 3%, n = 3) and *unc-29(e1072)* (28.5 ± 4.6%, n = 8; Fig. 5c). In these mutants, levels of desensitization to nicotine and to acetylcholine were nearly identical (Fig. 5). These data suggest that the second acetylcholine receptor is more nicotine sensitive than the levamisole-sensitive acetylcholine receptor. Because nicotine application to wild-type body wall muscle also produced a fast-desensitizing response indistinguishable from that of *unc-38(e264)* and *unc-29(e1072)* (Fig. 5c and **d**), this nicotine-sensitive receptor must normally be present in wild-type animals and cannot be a compensatory response to the absence of the levamisole-sensitive acetylcholine receptors in *unc-38* and *unc-29* mutants.

Dihydro-β-erythroidine (DHβE) is a competitive antagonist of nicotinic acetylcholine receptors[24]. At a concentration of 5 μM, DHβE selectively inhibited the acetylcholine responses attributable to the nicotine-sensitive receptor. Specifically, in mutants expressing only the nicotine-sensitive receptor (*unc–38(e264)* and *unc-29(e1072)*), DHβE reversibly inhibited acetylcholine responses by 80 ± 9% (n = 3) and 88 ± 2% (n = 5), respectively (Fig. 6a and **b**). By contrast, the levamisole-sensitive receptor was largely unaffected by DHβE, which only weakly reduced the levamisole response in wild-type animals (15 ± 9%, n = 3; Fig. 6c). The acetylcholine response of wild-type animals was reduced by 43 ± 8% (n = 4), consistent with a mixed population of two acetylcholine receptors (Fig. 6d) and suggesting that these two receptors contribute equally to the wild-type response. Muscle nicotine responses were blocked by DHβE, with statistically indistinguishable effects on wild-type (80 ± 3%, n = 4), *unc-38 (e264)* (83 ± 4%, n = 3) and *unc-29(e1072)* (91 ± 4%, n = 4; Fig. 6e–g) animals. In 0 Ca^{2+} and 0.5 mM EGTA, DHβE blocked 91 ± 4% of *unc–29(e1072)* muscle acetylcholine responses (n = 3), indicating direct inhibition of the muscle nicotine-sensitive receptor.

Synaptic localization of receptors

The application of exogenous neurotransmitters demonstrated that distinct classes of GABA and acetylcholine receptors are present on the muscle membrane. However, this method does not demonstrate whether the receptors are specifically localized at the neuromuscular junction. To identify the functional complement of receptors at the neuromuscular synapse, we measured endogenous synaptic activity. At a holding potential of −80 mV, whole-cell, voltage-clamp recordings from individual muscle cells revealed endogenous synaptic events as small inward currents (Fig. 7). In the *unc-49(e407)* mutant, which lacks the muscle GABA receptor, synaptic currents should represent activity at acetylcholine receptors. As expected, bath application of the acetylcholine antagonist dTBC completely abolished synaptic responses in *unc-49(e407)* muscles (n = 8; Fig. 7a). Thus, this antagonist can be used to uncover GABA-dependent synaptic events in wild-type muscles. Bath application of dTBC to wild-type muscles reduced the frequency of synaptic events no 54 ± 10%, from 19.4 ± 3.8 Hz before dTBC to 10.2 ± 3.3 Hz with dTBC (n = 5; Fig. 7b). These synaptic GABAergic currents are of variable amplitude and are likely to include events resulting from stimulation of the motor neurons by neuronal activity. Calcium-independent miniature postsynaptic currents represent spontaneous fusions of synaptic vesicles without neuronal activity. In 0 Ca^{2+} and 0.5 mM EGTA, the frequency of synaptic events in wild-type animals was greatly reduced, from 19.4 ± 3.8 Hz to 4.3 ± 1.8 Hz. Addition of dTBC further reduced the synaptic event frequency by only 11 ± 14%. These results suggest that most calcium-independent spontaneous synaptic events are GABA responses, consistent with the observation that the amplitude of muscle acetylcholine responses but not GABA responses was greatly reduced in 0 Ca^{2+}.

To demonstrate that the nicotine-sensitive receptor functions at the synapse, we made recordings in *unc-29(e264)* and *unc-49(e407)* double mutants. The double mutant lacked responses to pressure-ejected levamisole (n = 3) and GABA (n = 3) but retained responses to nicotine (n = 5) and acetylcholine (n = 3; data not shown). Synaptic activity in the double mutant had an average frequency of 7.4 ± 4.1 Hz and was abolished by either dTBC (n = 2) or DHβE (n = 3; Fig. 7c). To demonstrate that the levamisole-sensitive receptor is also active at the synapse, we treated *unc-49(e407)* mutants, which lack the GABA receptor, with DHβE to eliminate the nicotine-sensitive receptor. The synaptic activity in these mutants was reduced 48 ± 9% by DHβE, from 24.4 ± 2.4 Hz to 12.3 ± 1.1 Hz, consistent with loss of the nicotine-sensitive component of the synaptic response. The DHβE-insensitive synaptic events that could be attributed to the levamisole-sensitive receptor were eliminated by perfusion of dTBC (n = 3; Fig. 7d). Through the use of pharmacological agents and specific receptor mutants, these results support the conclusion that all three classes of receptor, the GABA receptor, the

levamisole-sensitive acetylcholine receptor and the nicotine-sensitive acetylcholine receptor, may be functional at the neuromuscular junction.

Discussion

We have combined a new *C. elegans* electrophysiological preparation with genetic and pharmacological manipulation of candidate receptors to identify three postsynaptic ionotropic receptors (one GABA receptor and two distinct acetylcholine receptors) functional at the neuromuscular junction. The *C. elegans* body wall muscles respond to GABA, triggering a chloride-mediated current. Under physiological conditions, this chloride current would be predicted to produce muscle hyperpolarization. In the nematode *Ascaris*, GABAergic inhibition of body wall muscles is thought to underlie contralateral muscle relaxation, contributing to the establishment of sinusoidal body posture[25]. Furthermore, we demonstrated that this response requires the GABA receptor subunits encoded by *unc-49*, as it is completely eliminated in the null mutant, *unc-49(e407)*.

C. elegans body wall muscle has two functional acetylcholine receptors: a receptor activated by levamisole, and a previously unknown receptor activated by nicotine. We have shown that the levamisole-sensitive receptor requires the expression of subunits encoded by both *unc-38* and *unc-29*; the levamisole response is completely eliminated in null mutants of either gene, consistent with their original identification in levamisole-resistance screens[16]. Thus, we now provide electrophysiological evidence for this genetically defined receptor in *C. elegans*.

Levamisole is a potent nematocide that causes hypercontraction in sections of dissected *C. elegans* body[16]. However, the presynaptic versus postsynaptic locus of this response at the neuromuscular junction was previously unknown. Mosaic analysis demonstrates that *unc-29* expression is required in body wall muscles[19]. However, both UNC-29 (ref. 20) and UNC-38 (J. L. Bessereau and E. J. unpublished data) are also expressed in a subset of neurons. Our data demonstrate that levamisole produces a direct excitatory response in body wall muscles, thus confirming that these muscles represent at least one of the targets of levamisole.

The second acetylcholine receptor in the muscle is pharmacologically distinguishable from the levamisole-sensitive receptor; it is sensitive to nicotine but not to levamisole and is selectively blocked by DHβE. This receptor was identified because there is a residual rapidly desensitizing acetylcholine response when the levamisole-sensitive receptor is eliminated by mutation of *unc-38* and *unc-29*. This receptor contributes roughly half of the acetylcholine response of the adult body wall muscle. There are about 30 candidate genes for this receptor, whose molecular identity remains unknown, in the *C. elegans* genome.

Why are there two classes of acetylcholine receptors in body wall muscles? First, the multiple acetylcholine receptors could provide kinetically and pharmacologically diverse responses[26–28]. For example, in chicken sympathetic neurons, multiple acetylcholine receptor subtypes with distinct single-channel conductances and kinetics[29] form homogeneous clusters, which are spatially segregated in the same neuron[26]. Miniature synaptic responses recorded from these neurons tend to follow complex, non-quantal amplitude distributions, which are proposed to reflect variations in the postsynaptic receptor composition. Thus, synaptic efficacy may depend on the postsynaptic distribution of diverse receptor subtypes[30]. Second, receptor diversity also provides a basis for the differential modulation of receptor subtypes by kinases. For example, receptor subtypes within the same neuron may be differentially regulated by calcium/calmodulin kinase[13]. Similarly, both protein kinase A and protein kinase C can modulate nicotinic acetylcholine receptors[10,31]. Kinase activity influences desensitization[10,11] as well as the clustering[9] and expression levels of specific nicotinic acetylcholine receptor subunits[13]. Thus, synaptic receptor diversity may impose differential

susceptibility to short-term modulation. Third, receptor diversity may arise as a result of changing synaptic requirements during development. Interestingly, locomotion in *unc-29* and *unc-38* mutants is only moderately impaired in adults but is severely affected at larval stages[20]. It is possible that the two acetylcholine receptors identified on *C. elegans* muscle have different developmental expression patterns, such that the levamisole-sensitive receptor predominates during larval stages and the nicotine-sensitive receptor has an increased role in adult worms. Similar developmental changes in acetylcholine receptor composition occur at vertebrate central and neuromuscular synapses[26,27].

In summary, this study establishes an electrophysiological preparation in which synaptic function can be studied at a relatively simple polyinnervated synapse. Genetic studies in *C. elegans* have demonstrated that the proteins that mediate synaptic transmission are highly conserved across both invertebrate and vertebrate species[2]. In this regard, the *C. elegans* neuromuscular junction is an attractive genetic model system for the more complex and often inaccessible polyinnervated synapses of vertebrate central neurons.

Methods

The strains and corresponding alleles used in this study were as follows: CB904*: unc-38* (e264) *I*, CB1072:*unc-29(e1072) I* and CB407: *unc-49(e407) III*. All strains are available from the *C. elegans* Genetic Center.

Dissection

The N2 Bristol strain of *C. elegans* was grown on agar nematode growth media seeded with HB101 bacteria. Adult nematodes were glued along the dorsal cuticle (Hisotacryl Blue, B. Braun, Melsungen, Germany) to the Sylgard-coated surface of a coverslip under recording solution. A lateral incision was made in the nematode cuticle using a borosilicate needle (World Precision Instruments, Sarasota, Florida). The viscera were cleared from the area of the incision by mouth pipet, and the cuticle flap was glued back to expose the body wall muscles and nerve cord (Fig. 1). In preparation for patch-clamp recording, the basement membrane overlying the muscles was enzymatically removed using a mixture of 0.23 mg per ml protease (protease XIV, Sigma) and 0.62 mg per ml of collagenase (type A, Boehringer-Mannheim) applied for 30 seconds.

Electrophysiology

Nematodes were viewed for recording under a 40X water emersion objective of a Zeiss Axioskop. Whole-cell, voltage-clamp recordings of individual ventral medial body wall muscles were made using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany) and digitized at 2.9 kHz via an ITC-16 interface (Instrutech, Great Neck, New York). Voltage-clamp recordings were typically maintained for two to five minutes, although seals could be held for more than ten minutes if desired. Muscle cells with leak currents up to 200 pA following whole-cell voltage clamp were included in the data set. Voltage clamping and data acquisition were controlled by Pulse software (HEKA) run on a Power Mac 6500/225. Recordings were maintained in the whole-cell, voltage-clamp configuration at a holding potential of −80 mV. Modified *Ascaris* Ringer's, with or without receptor antagonists, was bath applied to the preparation by gravity flow at a rate of 2 ml per minute. Pressure ejection of receptor ligands from pipets of 2–4 μ;m tip diameter was computer triggered. Fire-polished recording pipets with resistances ranging from 5–9 MΩ were pulled from borosilicate glass (World Precision Instruments). Series resistance was compensated by more than 50% in most recordings. Optimal recording conditions were obtained using a modified *Ascaris* Ringer's[32] containing 23 mM NaCl, 110 mM NaAc, 5 mM KCl, 6 mM $CaCl_2$, 5 mM $MgCl_2$, 11 mM glucose and 5 mM HEPES, pH 7.2, ~330 mOsm. The pipet solution contained 120 mM KCl, 20 mM KOH,

4 mM $MgCl_2$, 5 mM (*N*-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid), 0.25 mM $CaCl_2$, 4 mM NaATP, 36 mM sucrose and 5 mM EGTA, pH 7.2, ~315 mOsm.

Voltage-gated currents were obtained using a series of 200-ms command potentials from −60 mV to +60 mV at 10-mV increments. Leak subtraction was performed automatically by the software using a *P*/4 protocol, from a holding potential of −60 mV. The intracellular solution used to isolate calcium currents contained 140 mM CsCl, 4 mM MgATP, 5 mM tetraethyl-ammonium chloride, 5 mM (*N*-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid), 0.3 mM $CaCl_2$ and 5 mM CsBAPTA, pH 7.2, ~315 mOsm. Subsequent analysis and graphing were done using Pulsefit (HEKA) and Igor Pro (Wavemetrics, Lake Oswego, Oregon). All statistically derived values are given as mean ± s.e. All drugs were obtained from Sigma.

Acknowledgements

The authors thank K. Broadie, T. Fergestad and D.E. Featherstone for technical assistance, L. Avery and A.V. Maricq for discussions, M. Hammarlund for providing the F21D12.3-GFP strain, K. Knobel for confocal images and K. Broadie and D.E. Featherstone for reading the manuscript. This work was supported by NIH grant RO3 MHS9820-01 (J.E.R.) and the Damon Runyon Fund (E.M.J.).

References

1. Chalfie M, Jorgensen EM. *C. elegans* neuroscience: genetics to genome. Trends Genet 1998;14:506–512. [PubMed: 9865157]
2. Jorgensen EM, Nonet ML. Neuromuscular junctions in the nematode *C elegans*. Dev Biol 1995;6:207–220.
3. Raizen DM, Avery L. Electrical activity and behavior in the pharynx of *Caenorhabditis elegans*. Neuron 1994;1:2, 483–495.
4. Nonet ML, et al. Caenorhabditis elegans rab-3 mutant synapses exhibit impaired function and are partially depleted of vesicles. J Neurosci 1997;17:8061–8073. [PubMed: 9334382]
5. Tempia F, Bravin M, Strata P. Postsynaptic currents and short-term synaptic plasticity in Purkinje cells grafted onto an uninjured adult cerebellar cortex. Eur J Neurosci 1996;8:2690–2701. [PubMed: 8996819]
6. Eshhar N, Petralia RS, Winters CA, Niedzielski AS, Wenthold RJ. The segregation and expression of glutamate receptor subunits in cultured hippocampal neurons. Neuroscience 1993;57:943–964. [PubMed: 8309554]
7. Nusser Z, Sieghart W, Somogyi P. Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 1998;18:1693–1703. [PubMed: 9464994]
8. Sillar KT, Roberts A. Segregation of NMDA and non-NMDA receptors at separate synaptic contacts: evidence from spontaneous EPSPs in *Xenopus* embryo spinal neurons. Brain Res 1991;545:24–32. [PubMed: 1650276]
9. Nimnual AS, Chang NS, Ross AF, Gelman MS, Prives JM. Identification of phosphorylation sites on AChR delta-subunit associated with dispersal of AChR clusters on the surface of muscle cells. Biochemistry 1998;37:14823–14832. [PubMed: 9778356]
10. Nishizaki T, Sumikawa K. Effects of PKC and PKA phosphorylation on desnsitization of nicotinic acetylcholine receptors. Brain Res 1998;812:242–245. [PubMed: 9813350]
11. Paradiso K, Brehm P. Long-term desensitization of nicotinic acteylcholine receptors is regulated via protein kinase A-mediated phosphorylation. J Neurosci 1998;18:9227–9237. [PubMed: 9801362]
12. Liao D, Hessler NA, Malinow R. Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. Nature 1995;375:400–404. [PubMed: 7760933]
13. De Koninck P, Cooper E. Differential regulation of neuronal nicotinic ACh receptor subunit genes in cultured neonatal rat sympathetic neurons: specific induction of alpha 7 by membrane depolarization through a Ca^{2+}/calmodulin-dependent kinase pathway. J Neurosci 1995;15:7966–7978. [PubMed: 8613734]

14. McIntire SL, Jorgensen E, Horvitz HR. Genes required for GABA function in *Caenorhabditis elegans*. Nature 1993;36:4, 334–337.
15. McIntire SL, Jorgensen E, Kaplan J, Horvitz HR. The GABAergic nervous system of *Caenorhabditis elegans*. Nature 1993;36:4, 337–341.
16. Lewis JA, Wu CH, Levine JH, Berg H. Levamisole-resistant mutants of the nematode *Caenorhabditis elegans* appear to lack pharmacological acetylcholine receptors. Neuroscience 1980;5:967–989. [PubMed: 7402460]
17. Bamber BA, Beg AA, Twyman RE, Jorgensen EM. The *C. elegans unc-49* locus encodes multiple subunits of a heteromultimeric GABA receptor. J Neurosci 1999;19:5348–5359. [PubMed: 10377345]
18. Lewis JA, et al. Cholinergic receptor mutants of the nematode *Caenorhabditis elegans*. J Neurosci 1987;7:3059–3071. [PubMed: 3668616]
19. Lackner MR, Kornfeld K, Miller LM, Horvitz HR, Kim SK. A MAP kinase homolog, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. Genes Devel 1994;8:160–173. [PubMed: 8299936]
20. Fleming JT, et al. *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. J Neurosci 1997;17:5843–5857. [PubMed: 9221782]
21. Lewis JA, Wu CH, Berg H, Levine JH. The genetics of levamisole resistance in the nematode *Caenorhabditis elegans*. Genetics 1980;9:5, 905–928.
22. Brenner S. The genetics of *Caenorhabditis elegans*. Genetics 1974;7:7, 71–94.
23. Robertson SJ, Martin RJ. Levamisole-activated single-channel currents from muscle of the nematode parasite *Ascaris suum*. Br J Pharmacol 1993;10:8, 170–178.
24. Zoli M, Lena C, Picciotto MR, Changeux JP. Identification of four classes of brain nicotinic receptors using β2 mutant mice. J Neurosci 1998;18:4461–4472. [PubMed: 9614223]
25. Walrond JP, Stretton AO. Excitatory and inhibitory activity in the dorsal musculature of the nematode *Ascaris* evoked by single dorsal excitatory motonerons. J Neurosci 1985;5:16–22. [PubMed: 3965640]
26. Moss BL, Role LW. Enhanced ACh sensitivity is accompanied by changes in ACh receptor channel properties and segregation of ACh receptor subtypes on sympathetic neurons during innervation in vivo. J Neurosci 1993;13:13–28. [PubMed: 7678641]
27. Hartman DS, Claudio T. Coexpression of two distinct muscle acetylcholine receptor alpha-subunits during development. Nature 1990;343:372–375. [PubMed: 2300185]
28. Owens JL, Kullberg R. Three conductance classes of nicotinic acetylcholine receptors are expressed in developing amphibian skeletal muscle. J Neurosci 1989;9:2575–2580. [PubMed: 2746339]
29. Gardette R, Listerud MD, Brussard AB, Role LW. Developmental changes in transmitter sensitivity and synaptic transmission in embryonic chicken sympathetic neurons innervated *in vitro*. Dev Biol 1991;14:7, 83–95.
30. Hume RI, Honig MG. Physiological properties of newly formed synapses between sympathetic preganglionic neurons and sympathetic ganglion neurons. J Neurobiol 1991;22:249–262. [PubMed: 1653822]
31. Role LW. Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels. Curr Opin Neurobiol 1992;2:254–262. [PubMed: 1643408]
32. Byerly L, Masuda MO. Voltage-clamp analysis of the potassium current that produces a negative-going action potential in *Ascaris* muscle. J Physiol (Lond) 1979;288:263–284. [PubMed: 469718]

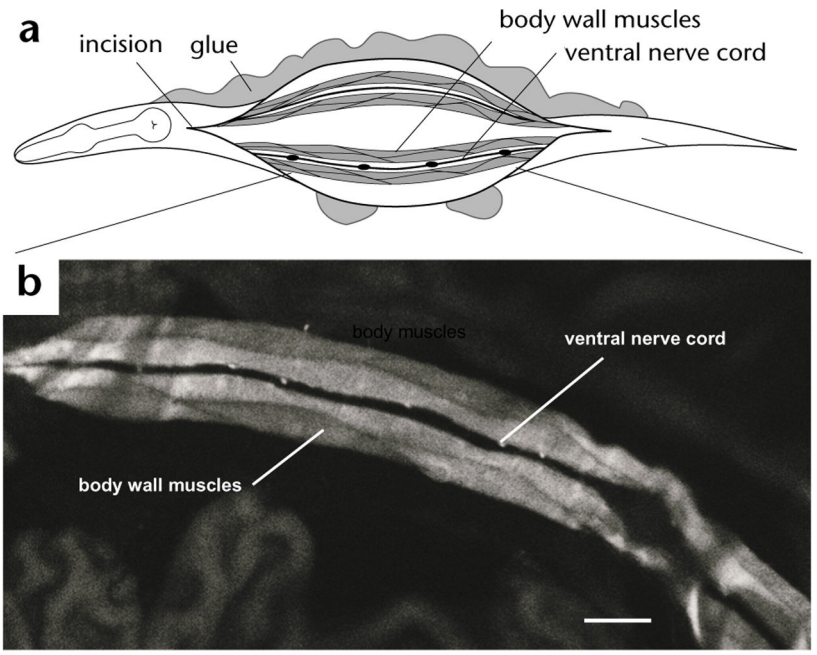

Fig. 1.

C. elegans neuromuscular preparation**. (a)** Schematic of the dissected *C. elegans* adult. The ventral body wall muscle quadrants are exposed following incision of the cuticle and removal of internal organs. **(b)** The ventral muscle quadrants and ventral nerve cord are intact after dissection. Green fluorescent protein is expressed in the body wall muscles and ventral nerve cord (cell bodies between the muscle quadrants) under the control of the F21D12.3 promoter (gift of M. Hammarlund). Scale bar, 50 μM.

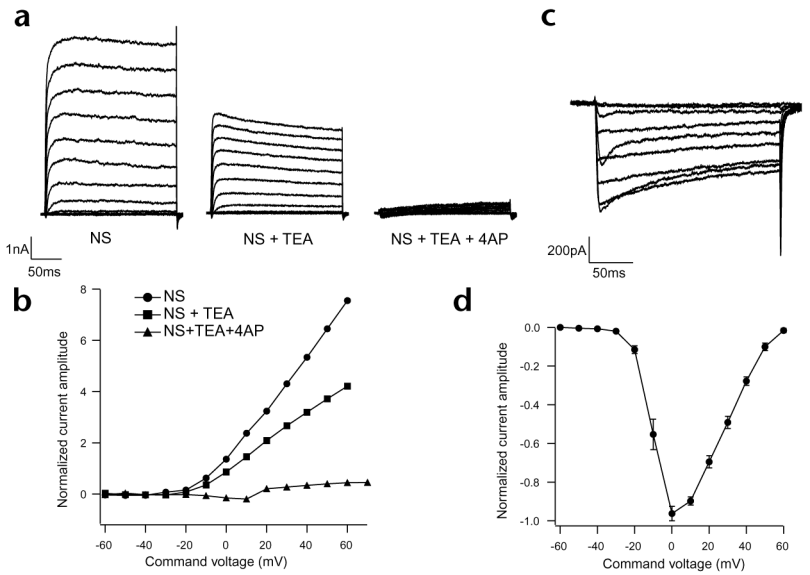

Fig. 2.

Voltage-gated potassium and calcium currents are present in body wall muscles. Command voltages (200 ms) from −60 to +60 mV in 10 mV increments were applied to whole-cell voltage-clamped muscles. **(a)** K^+ currents (I_K) were elicited at voltages above −30 mV. Addition of 20 mM TEA to the external solution, reduced I_K at all voltages. The remaining I_K was blocked by 3 mM 4-AP. **(b)** Typical current–voltage relationships for I_K in normal Ringer's, and in the presence of TEA and 4-AP. **(c, d)** Calcium currents were observed at potentials above −40 mV and peaked at 0 mV.

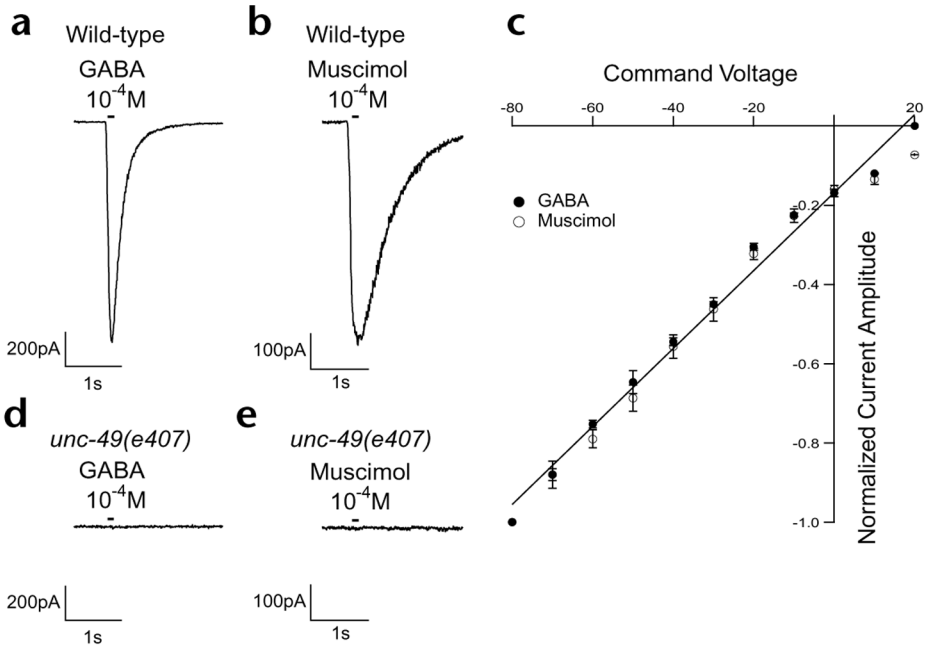

Fig. 3.

A functional GABA receptor is expressed in the muscle and encoded by the *unc-49* gene. **(a, b)** Pressure-ejected pulses (100 ms) of GABA **(a)** and the GABA agonist muscimol **(b)** produced large inward currents in individual whole-cell voltage-clamped body wall muscles. Holding potential in this and all subsequent figures is −80 mV, and the preparation was constantly perfused with *Ascaris* Ringer's throughout the experiments. **(c)** Current–voltage (*I–V*) relationships for the GABA (●) and muscimol (○) responses were generated at potentials ranging from −80 mV to +20 mV in 10 mV increments (mean and s.e.). The linear fit shown is to the GABA *I–V* relationship. **(d, e)** Both the GABA **(d)** and muscimol **(e)** responses are abolished in the body wall muscles of *unc-49(e407)* null mutants. Note that GABA produces an inward current in our solutions because intracellular chloride concentration is higher than external chloride concentration, resulting in an outward flow of chloride ions through GABA receptors.

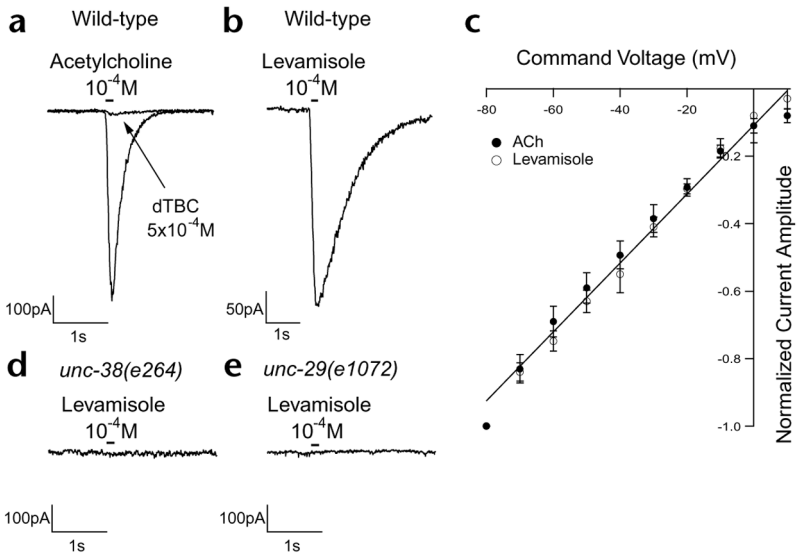

Fig. 4.

The levamisole-sensitive current is abolished in *unc-38(e264)* and *unc-29(e1072)* mutants. **(a, b)** The body wall muscles produced large inward currents in response to 100-ms pressure-ejected pulses of acetylcholine **(a)** and the nematode acetylcholine receptor agonist levamisole **(b)**. The acetylcholine response was eliminated by 500 μM d-tubocurare (dTBC) **(a). (c)** The *I–V* relationships for acetylcholine (●) and levamisole (○) (mean and s.e.), with a linear fit to the acetylcholine response (same protocol as described in Fig. 2). **(d, e)** There was no response to 100-ms levamisole application in *unc-38(e264)* **(d)** or *unc-29(e1072)* **(e)** mutants.

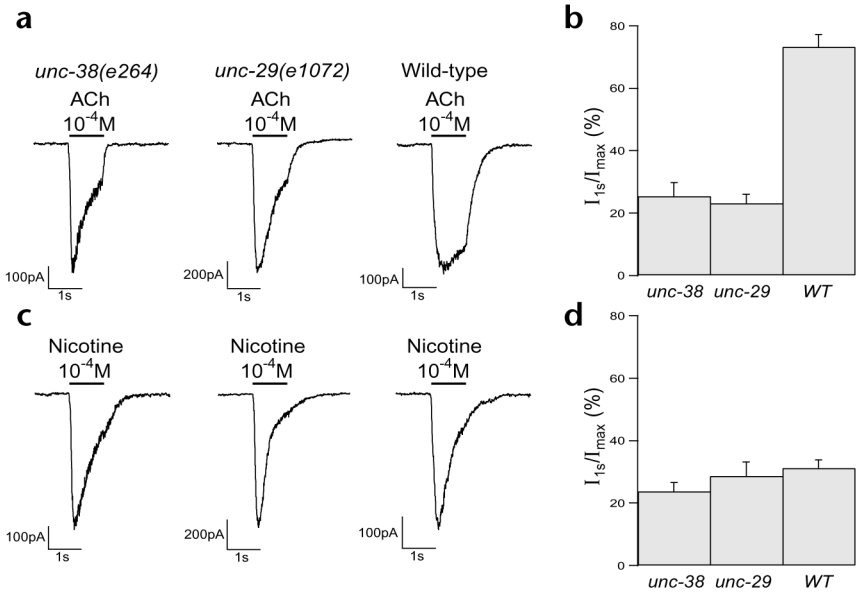

Fig. 5.

A nicotine-sensitive, rapidly desensitizing acetylcholine receptor is expressed in the muscle. **(a, c)** The body wall muscles of *unc-38(e264)*, of *unc-29(e1072)* and of wild-type animals respond to acetylcholine **(a)** and nicotine **(c)**. A one-second agonist application was used to quantify the level of desensitization. **(b, d)** Desensitization to acetylcholine **(b)** and nicotine **(d)**, plotted as the percentage of the peak current (I_{max}) remaining after a one-second agonist application (I_{1s}).

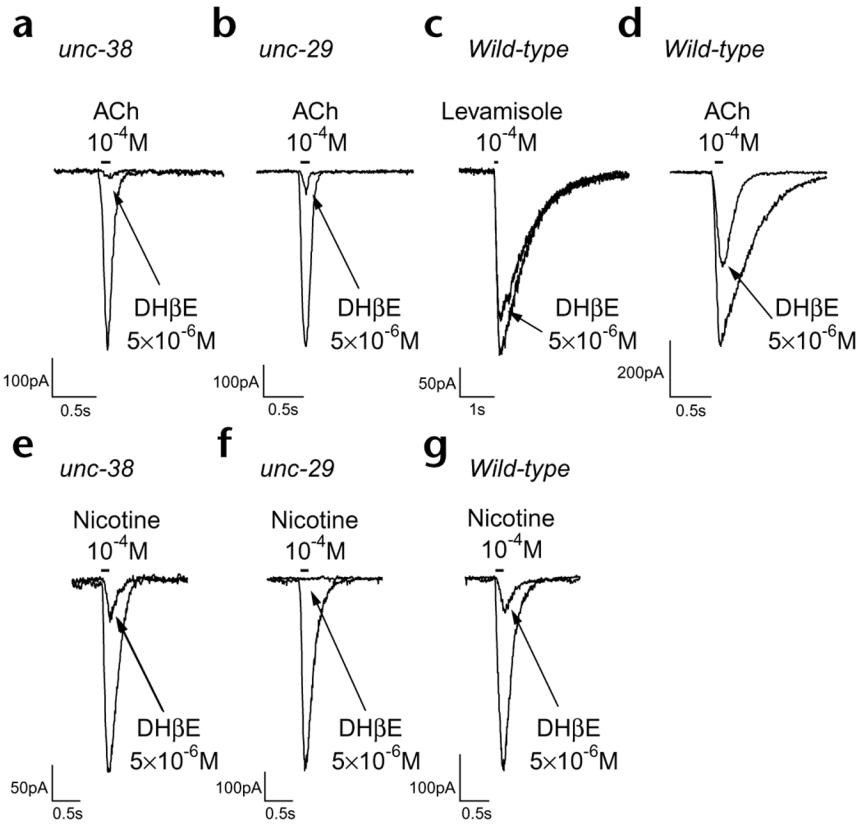

Fig. 6.

The nicotine-sensitive acetylcholine receptor is selectively blocked by the nicotinic antagonist dihydro-β-erythroidine (DHβE, 5 μM). **(a–c)** The acetylcholine responses of both *unc–38 (e264*) **(a)** and *unc-29(e1072)* **(b)** were severely inhibited by DHβE, whereas the wild-type levamisole response was only slightly blocked **(c). (d)** The wild-type acetylcholine response was partially blocked by DHβE. **(e–g)** The nicotine responses of *unc-38(e264)* **(e)**, *unc-29 (e1072)* **(f)** and wild-type animals **(g)** were all extensively blocked by bath-applied DHβE. Acetylcholine and nicotine were pressure ejected for 100 ms in each trace.

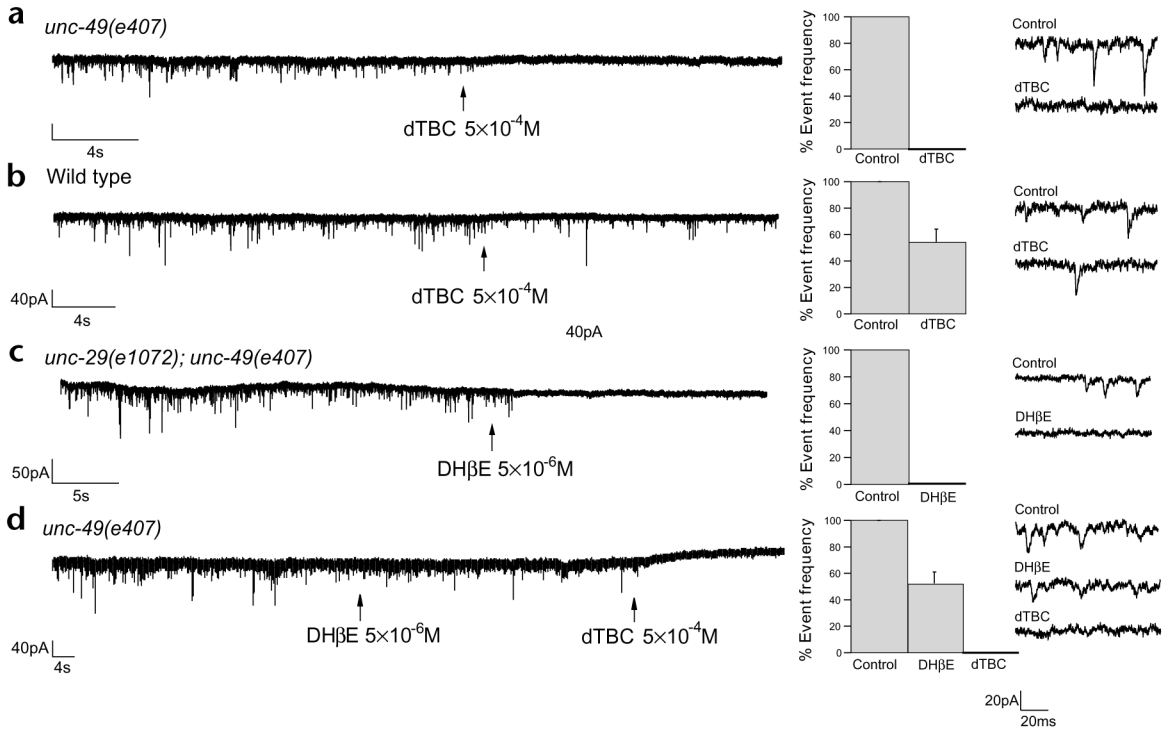

Fig. 7.

All three receptors are functionally active at the synapse. Endogenous synaptic activity was recorded in *Ascaris* Ringer's at a holding potential of −80 mV. **(a)** In the GABA receptor null mutant *unc-49(e407)*, all synaptic activity is blocked by the acetylcholine receptor antagonist D-tubocurare (dTBC). Left panels are typical recordings of synaptic activity, middle panels show average percent decrease in event frequency following antagonist application, and right panels contain a sample of the recordings at higher temporal resolution to show event characteristics. **(b)** In wild-type muscle, endogenous synaptic activity is reduced by dTBC, leaving the GABA-responsive component. **(c)** In *unc-29(e1072)* and *unc-49(e407)* double mutants, all synaptic activity can be attributed to the nicotine-sensitive receptor and is abolished by DHβE. **(d)** In the GABA receptor null mutant *unc-49(e407)*, synaptic activity has a DHβE-sensitive component (the nicotine-sensitive receptor) and a DHβE-insensitive component (the levamisole-sensitive receptor), both of which can be blocked by the acetylcholine antagonist dTBC.