

Eight genes are required for functional reconstitution of the *Caenorhabditis elegans* levamisole-sensitive acetylcholine receptor

Thomas Boulina^{a,b,1}, Marc Gielen^{a,c,1}, Janet E. Richmond^d, Daniel C. Williams^e, Pierre Paoletti^{a,c,2}, and Jean-Louis Bessereau^{a,b,3}

^aEcole Normale Supérieure, Biology Department, 75005 Paris, France; ^bINSERM U789, Biologie Cellulaire de la Synapse, Paris 75005, France; ^cCNRS UMR 8544, Laboratoire de Neurobiologie, Paris 75005, France; ^dDepartment of Biology, University of Illinois, Chicago, IL 60607; and ^eDepartment of Biology, University of Utah, Salt Lake City, UT 84112

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Levamisole-sensitive acetylcholine receptors (L-AChRs) are ligand-gated ion channels that mediate excitatory neurotransmission at the neuromuscular junctions of nematodes. They constitute a major drug target for anthelmintic treatments because they can be activated by nematode-specific cholinergic agonists such as levamisole. Genetic screens conducted in *Caenorhabditis elegans* for resistance to levamisole toxicity identified genes that are indispensable for the biosynthesis of L-AChRs. These include 5 genes encoding distinct AChR subunits and 3 genes coding for ancillary proteins involved in assembly and trafficking of the receptors. Despite extensive analysis of L-AChRs in vivo, pharmacological and biophysical characterization of these receptors has been greatly hampered by the absence of a heterologous expression system. Using *Xenopus laevis* oocytes, we were able to reconstitute functional L-AChRs by coexpressing the 5 distinct receptor subunits and the 3 ancillary proteins. Strikingly, this system recapitulates the genetic requirements for receptor expression in vivo because omission of any of these 8 genes dramatically impairs L-AChR expression. We demonstrate that 3 α - and 2 non- α -subunits assemble into the same receptor. Pharmacological analysis reveals that the prototypical cholinergic agonist nicotine is unable to activate L-AChRs but rather acts as a potent allosteric inhibitor. These results emphasize the role of ancillary proteins for efficient expression of recombinant neurotransmitter receptors and open the way for in vitro screening of novel anthelmintic agents.

anthelmintic drug | recombinant receptor expression

Soil-transmitted helminth infections are a public health problem of great importance. In the developing world, >1 billion people are infected by various intestinal nematode parasites (1). Among the different anthelmintic drugs, cholinergic agonists such as levamisole are widely used against intestinal nematodes (2). They activate ligand-gated acetylcholine receptors present on muscle cell membranes and cause spastic paralysis of the parasites. Levamisole can be used in mammals because it does not activate the AChRs of the infected host (3), yet the molecular basis of this specificity remains unknown.

Molecular identification of the levamisole target was achieved in the nonparasitic nematode *Caenorhabditis elegans* (4). Acetylcholine is the main excitatory neurotransmitter in *C. elegans*, and >29 genes encoding AChR subunits are predicted from its genome sequence (5). Despite this complexity, genetic screens were able to identify the genes coding for the subunits of levamisole-sensitive AChRs (L-AChRs). Levamisole causes body-wall muscle hypercontraction, paralysis, and ultimately death of *C. elegans* at high concentrations. By screening for mutant animals that survive exposure to levamisole, mutations in 5 genes encoding AChR subunits were found to confer partial or complete insensitivity to levamisole (4). These include 2 non- α -subunits (LEV-1 and UNC-29) and 3 α -subunits (LEV-8, UNC-38, UNC-63) as defined by the presence of a vicinal dicysteine in the primary sequence (6–8). Consistently, electrophysiological analysis demonstrated a drastic reduction of levamisole-elicited currents in the muscle cells of these

mutants. In addition, these experiments identified a second subtype of muscle AChR activated by nicotine (N-AChR) but insensitive to levamisole (9). This receptor contains the subunit ACR-16, which is closely related to the vertebrate $\alpha 7$ gene. ACR-16 forms functional homomeric AChRs when expressed in *Xenopus* oocytes (10). L-AChRs and N-AChRs are partially redundant because disruption of either receptor causes no or weak locomotory defects, whereas disruption of both receptors causes almost complete paralysis of the animal (11, 12).

In addition to AChR subunits, genetic screens identified 3 ancillary proteins, RIC-3, UNC-50, and UNC-74, that are absolutely required for the expression of L-AChRs in vivo. RIC-3 is an endoplasmic reticulum transmembrane protein required for the expression of at least 4 distinct AChRs in *C. elegans*, including L-AChRs and N-AChRs in muscle (13, 14). RIC-3 is thought to act as a chaperone promoting AChR folding, assembly, or maturation (reviewed in ref. 15). *unc-74* was identified in early screens for resistance to levamisole (4). It is predicted to encode a thioredoxin closely related to the human TMX3 protein and seems to be solely required for the expression of L-AChRs (D.C.W. and E. M. Jorgensen, unpublished data; and ref. 16). *unc-50* encodes a transmembrane protein that localizes mostly to the Golgi apparatus and interacts with an ARF-GEF (guanine nucleotide exchange factor for ADP-ribosylation factor GTPases) (17). In the absence of UNC-50, L-AChRs but not N-AChRs are targeted to lysosomes after they exit the endoplasmic reticulum and are degraded. *unc-50* is evolutionarily conserved in most eukaryotes, including yeast, plants, and mammals. However, its role for AChR expression has not been tested so far in nonnematode species.

Despite extensive study of the L-AChR in *C. elegans*, this receptor remains poorly characterized at the pharmacological level, and the molecular basis for the action of levamisole is still unknown. Such analysis was complicated by the inability to express recombinant L-AChRs in a controlled heterologous system. Here, we demonstrate that L-AChRs can be expressed in *Xenopus* oocytes by providing not only the 5 receptor subunits but also the 3 ancillary factors RIC-3, UNC-50, and UNC-74. This expression system was

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¹T.B. and M.G. contributed equally to this work.

²To whom correspondence may be addressed at: Ecole Normale Supérieure, Laboratoire de Neurobiologie, CNRS UMR 8544, 46, rue d'Ulm, 75005 Paris, France. E-mail: paoletti@biologie.ens.fr.

³To whom correspondence may be addressed at: Ecole Normale Supérieure, Département de Biologie, INSERM U789, 46, rue d'Ulm, 75005 Paris, France. E-mail: jlbess@biologie.ens.fr.

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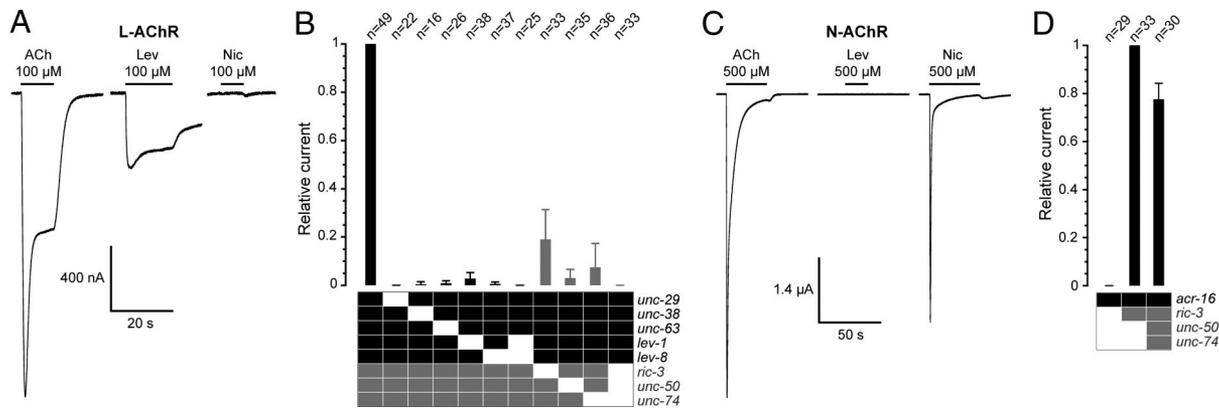


Fig. 1. Expression of functional AChRs from *C. elegans* in *X. laevis* oocytes. (A) A single oocyte coinjected with the 8 cRNAs *unc-29*, *unc-38*, *unc-63*, *lev-1*, *lev-8*, *ric-3*, *unc-50*, and *unc-74* displays large inward currents elicited by ACh (100 μ M) or levamisole (Lev, 100 μ M) but not by nicotine (Nic, 100 μ M). (B) Coexpression of 5 receptor subunits (black squares) and 3 ancillary factors (gray squares) is mandatory for robust expression of the L-AChR. Currents were measured at the peak. Average peak current for coinjection of all 8 cRNAs was $4.1 \pm 3.7 \mu$ A ($n = 49$). (C) A single oocyte coinjected with the *acr-16* and *ric-3* cRNAs displays large transient inward currents elicited by ACh (500 μ M) or nicotine (500 μ M), but not by levamisole (500 μ M). (D) Functional expression of the homopentameric ACR-16 nicotine-sensitive AChR requires the ancillary factor RIC-3 but not UNC-50 or UNC-74. Currents were measured at the peak. Average peak current for coinjection of *acr-16* and *ric-3* cRNAs was $6 \pm 4.2 \mu$ A ($n = 33$). All recordings were made with 1 mM external CaCl_2 . Numbers above bars represent the number of oocytes recorded for each condition.

used to characterize the biophysical and pharmacological properties of the L-AChR.

Results

Eight Genes Are Required to Reconstitute L-AChRs in *Xenopus* Oocytes.

Because 8 genes are required *in vivo* for L-AChR expression, we reasoned that the same set of genes may be necessary for functional expression in a heterologous system. *Xenopus* oocytes are particularly well suited to express multimeric receptors because complex cRNA mixtures can be directly injected into the oocyte cytoplasm. We injected *in vitro*-transcribed cRNAs corresponding to the 5 L-AChR subunit genes *lev-1*, *lev-8*, *unc-29*,

unc-38, and *unc-63* and the 3 ancillary factors *ric-3*, *unc-50*, and *unc-74*. Robust expression of L-AChR was obtained 1 or 2 days after the injection. Perfusion of 100 μ M acetylcholine elicited large inward currents in the 100 nA to several μ A range, with fast activation and deactivation kinetics (limited by the speed of the solution exchange), as expected for a receptor containing a ligand-gated ion channel (Fig. 1A). The initial fast-desensitizing component of the response was likely caused by the activation of calcium-activated chloride channels endogenously expressed in *Xenopus* oocytes (see below and Fig. 2A). Levamisole elicited responses similar to ACh although of lower amplitude, whereas nicotine was surprisingly ineffective, a feature that we later

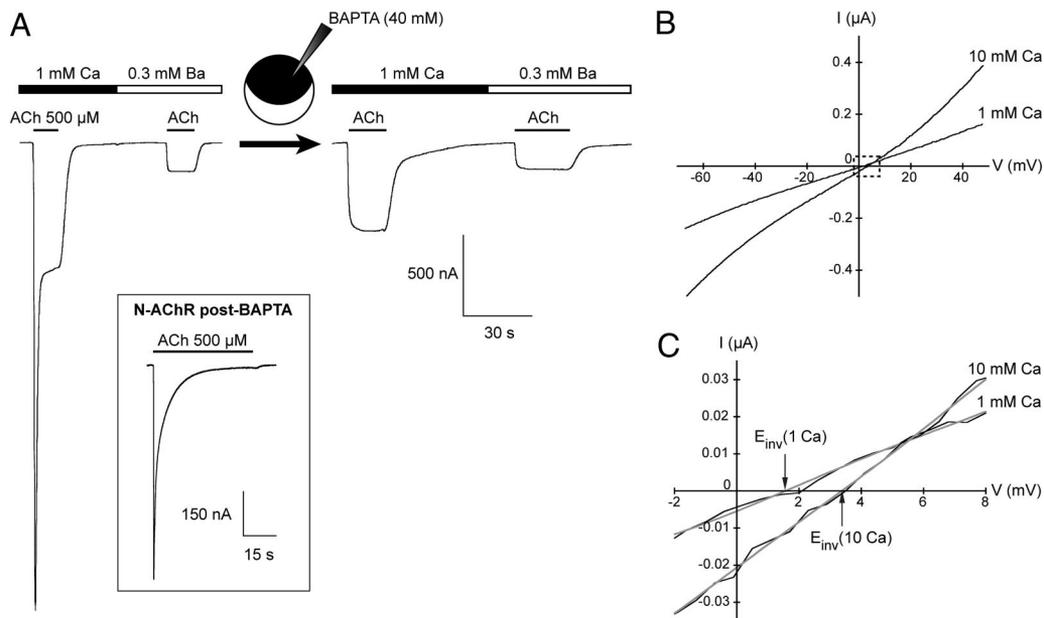


Fig. 2. The L-AChR is permeable to calcium and shows no macroscopic desensitization. (A) All traces are from a single oocyte expressing L-AChRs. In 1 mM external CaCl_2 , the response to ACh displays a preeminent inward peak current. Chelating intracellular calcium by injection of BAPTA or replacing extracellular calcium by a low amount of barium (0.3 mM) eliminates this peak and results in stable responses upon continuous application of ACh. (Inset) N-AChRs display profound macroscopic desensitization upon continuous application of ACh (500 μ M) even after BAPTA injection ($n = 6$). (B) Comparison of the I/V relationships of L-AChR responses elicited by 100 μ M ACh in the presence of 1 mM or 10 mM extracellular CaCl_2 . Note that the L-AChR is potentiated by 10 mM extracellular calcium and that this potentiation occurs over the whole voltage range ($n = 5$, BAPTA-loaded oocytes). (C) Magnified view of the dash-boxed region in B showing the rightward shift of the reversal potential induced by switching from 1 mM to 10 mM external Ca^{2+} (1.6 mV to 3.4 mV for this cell).

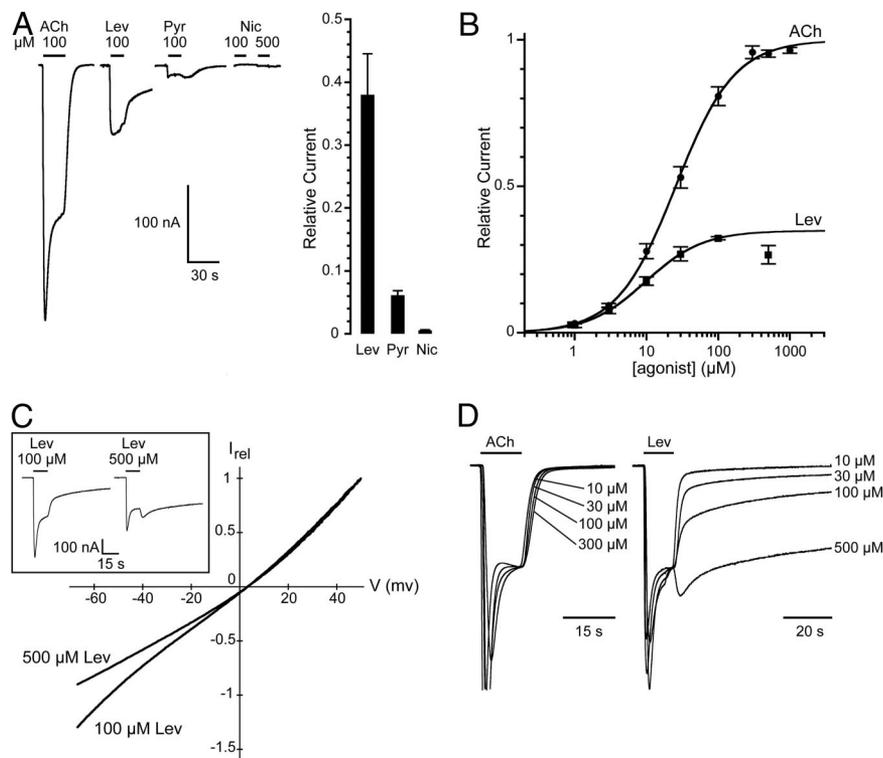


Fig. 3. Agonist pharmacology of the L-AChR. (A) (Left) Acetylcholine, levamisole, and pyrantel, but not nicotine, activate the L-AChR. Concentrations of agonist are indicated above each application. Traces are from a single oocyte. (Right) Current relative to 100 μ M ACh (plateau values): 100 μ M levamisole $38 \pm 6\%$ ($n = 6$), 100 μ M pyrantel $6.1 \pm 0.7\%$ ($n = 6$), 500 μ M nicotine $0.55 \pm 0.21\%$ ($n = 4$). (B) Dose-response curves for ACh and levamisole. The value at 500 μ M levamisole has been excluded from the fit because of voltage-dependent block at this concentration. (C) High concentrations of levamisole cause voltage-dependent channel block of L-AChR. BAPTA-injected oocytes were subjected to voltage ramps in the presence of 100 μ M or 500 μ M levamisole ($n = 5$). (Inset) Representative traces of L-AChR responses evoked by 100 μ M and 500 μ M levamisole. The rebound of the current after washout of 500 μ M levamisole probably occurs because unbinding of levamisole from its pore-blocking site is faster than unbinding of levamisole from its agonist site. (D) Concentration-dependent washout kinetics of levamisole-evoked responses. ACh or levamisole traces obtained from a single oocyte at different agonist concentrations were first normalized to the current level measured just before agonist washout and then superimposed. Note that although ACh-evoked responses display classical concentration-independent washout kinetics, the washout time course of levamisole-evoked responses increases with increasing levamisole concentrations.

hexamethonium (Hex) each inhibited ACh-triggered responses (Fig. 4). In contrast, α -bungarotoxin (α -BgTx) and dihydro- β -erythroidine (DH β E) had very little antagonistic effects, similar to endogenous L-AChRs *in vivo* (9). Even long applications of α -BgTx (100 nM, 30-min incubation), which may be required to reach binding equilibrium, were ineffective at inhibiting L-AChR responses [$7.6 \pm 1\%$ ($n = 7$)].

These results indicate that the L-AChR has a unique pharmacological profile and that levamisole has very distinct agonist properties compared with the natural agonist acetylcholine.

Nicotine Is an Allosteric Inhibitor of L-AChRs. In the experiments presented above, we observed that nicotine was unable to activate recombinant L-AChRs (Figs. 1A and 3A). To test whether this was an intrinsic characteristic of L-AChRs and not a peculiar feature of our recombinant receptors, we analyzed the nicotine response of L-AChRs on *C. elegans* muscle cells. We used a double-mutant strain containing an *acr-16*-null mutation, which removes nicotine-sensitive N-AChRs (12), and a mutation in the *unc-13* gene, which blocks presynaptic vesicle release (27). We expect that in such a double-mutant strain, any current recorded upon nicotine application can only be attributed to postsynaptic L-AChR activation and not to activation of presynaptic AChRs. When muscle cells of double-mutant animals were recorded, no measurable response was detected after

nicotine application (Fig. 5A). In contrast, the response to levamisole application was indistinguishable from the levamisole response in control animals (Fig. 5B; see also refs. 11 and 26). These results demonstrate that native and recombinant L-AChRs share the inability to be activated by nicotine.

We next tested the possibility that nicotine might act as an L-AChR antagonist. Strikingly, 500 μ M nicotine had a potent inhibitory effect on currents evoked by ACh applications (Fig. 5C). This inhibition appears to be almost entirely voltage-independent (Fig. 5D), suggesting that it is not the result of direct channel block. Alternatively, nicotine might act as a competitive antagonist of ACh. However, performing acetylcholine dose-response experiments in the presence of 300 μ M nicotine revealed that nicotine has little effect on the apparent affinity for ACh (<2-fold increase in ACh EC₅₀ by 300 μ M nicotine; Fig. 5E). Taken together, these results indicate that nicotine inhibits L-AChRs mainly through a negative allosteric mechanism.

Discussion

We demonstrate here that the *C. elegans* heteromeric levamisole-sensitive AChR can be readily expressed in *Xenopus* oocytes by providing 3 ancillary *C. elegans* proteins in addition to 5 distinct AChR subunits. Our experiments led to the surprising conclusion that 5 different subunits assemble into the same receptor complex because removal of any of these 5 subunits reduces expression by

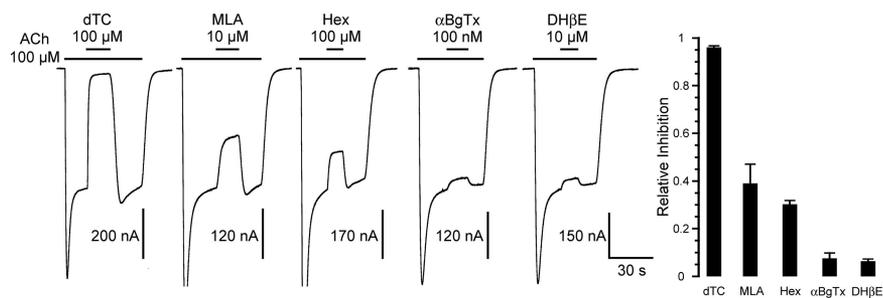


Fig. 4. Antagonist pharmacology of the L-AChR. L-AChRs activated by 100 μ M ACh are inhibited by 100 μ M dTC ($96 \pm 1\%$; $n = 7$), 10 μ M MLA ($39 \pm 8\%$; $n = 5$), and 100 μ M Hex ($30 \pm 2\%$; $n = 5$). In contrast, 100 nM α -BgTx and 10 μ M DH β E produce only very weak inhibition ($7 \pm 1\%$, $n = 5$; and $6 \pm 1\%$, $n = 5$; respectively).

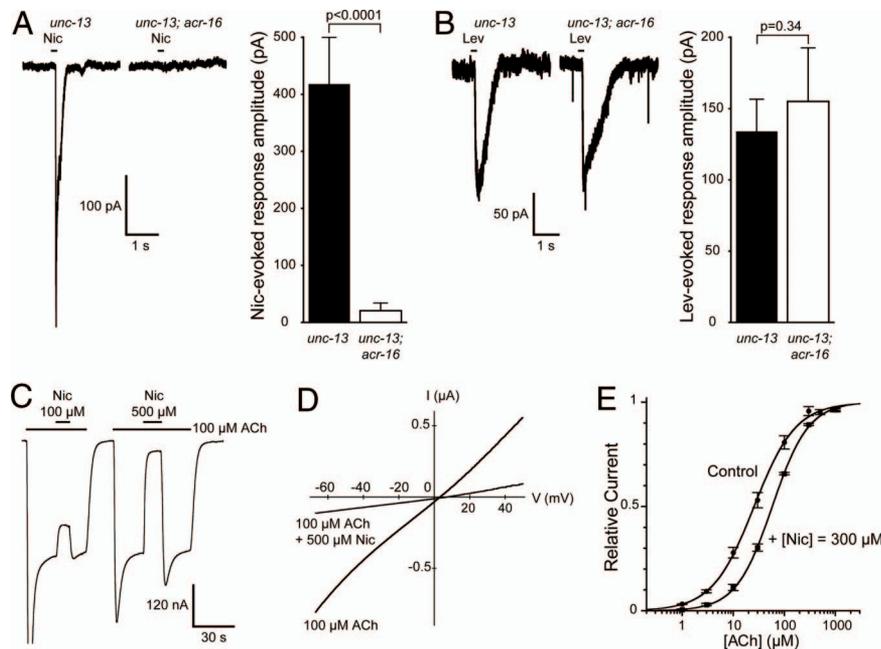


Fig. 5. Nicotine acts mainly as an allosteric inhibitor of the L-AChR. (A) Nicotine fails to activate L-AChR significantly in vivo. In whole-cell recordings from *C. elegans* body-wall muscles, a strain lacking both *acr-16* and *unc-13* shows no response to nicotine, unlike *unc-13* mutants [mean amplitudes of the response to 500 μ M nicotine were 21 ± 11 pA ($n = 4$) and 420 ± 80 pA ($n = 4$), respectively]. (B) In contrast, responses to levamisole are indistinguishable between these 2 strains [mean amplitudes of the response to 500 μ M levamisole were 155 ± 37 pA ($n = 4$) and 134 ± 22 pA ($n = 4$), respectively]. (C) Responses elicited by ACh are inhibited by nicotine in a dose-dependent manner. Percentage inhibition of responses elicited by 100 μ M ACh were $26 \pm 2\%$ ($n = 6$) for 100 μ M nicotine and $93 \pm 3\%$ ($n = 6$) for 500 μ M nicotine. (D) Nicotine inhibition is not voltage-dependent. Voltage ramps (-70 to $+50$ mV) in 100 μ M ACh with or without 500 μ M nicotine. Note that nicotine inhibition occurs over the whole voltage range (nic = 10, BAPTA-injected oocytes). (E) Nicotine has a modest effect on ACh sensitivity. ACh dose-response curves were performed with or without 300 μ M nicotine. In the absence of nicotine, EC_{50} and n_H for ACh are 26 ± 3 μ M and 1.05 ± 0.06 ($n = 6-13$), respectively. In the presence of nicotine, EC_{50} and n_H for ACh are 58 ± 3 μ M and 1.23 ± 0.06 ($n = 5$), respectively.

>97%. In contrast to earlier attempts that only used a subset of the subunits and none of the ancillary proteins (7), L-AChR expression was efficient and robust in our system. ACh-elicited currents, ranging from hundreds of nA to several μ A, could be measured in almost all injected oocytes.

C. elegans RIC-3, UNC-50, and UNC-74 are critical for the expression of L-AChRs in *Xenopus* oocytes. Although these 3 proteins are evolutionarily conserved, our results suggest they may be absent or expressed at insufficient levels in oocytes. Alternatively, species-specific determinants contained in L-AChRs may require the presence of nematode ancillary proteins for efficient expression. The orthologs of RIC-3 have been characterized in insects and vertebrates. They are proposed to act as chaperones that directly interact with various AChR or 5-HT_{3A} subunits and promote maturation and assembly of the receptors (28). The human hRIC-3 is able to enhance the expression of the *C. elegans* DES-2/DEG-3 AChR, demonstrating interspecies functionality (14). Hence, endogenous expression of RIC-3 in *Xenopus* oocytes might explain why low but significant levels of L-AChRs could be expressed when the 2 other factors UNC-50 and UNC-74 were overexpressed. UNC-50 was also conserved during evolution, and an UNC-50 ortholog can be readily identified in *Xenopus laevis* (17). Yeast, nematode, and human UNC-50 proteins are all able to interact with the yeast ARF-GEFs Gea1p, yet the human *unc-50* ortholog is unable to rescue an *unc-50* mutant in *C. elegans* (S. Eimer and J.-L.B., unpublished data). Because *C. elegans* UNC-50 promotes L-AChR expression in oocytes, it might be able to interact with the vertebrate trafficking machinery but provide a nematode-specific interface required for proper L-AChR trafficking to the plasma membrane. The requirement of such ancillary factors might be worth considering when recombinant receptor expression fails. For example, there is no system reported so far to express native insect AChRs [except very inefficient expression of the locust α L1 (29)]. The fact that certain *Drosophila* AChR α -subunits can only be expressed in *Xenopus* when they are coinjected with vertebrate β -subunits (30, 31) may indicate that vertebrate β -subunits are needed to recruit vertebrate ancillary factors and promote receptor expression in the absence of *Drosophila*-specific ancillary proteins.

In *C. elegans*, both levamisole- and nicotine-sensitive AChRs are found at neuromuscular junctions [(11, 12, 32); M. Gendrel and J.-L.B., unpublished data]. Based on the EC_{50} estimated from our dose-response experiments, L-AChRs and N-AChRs seem equally sensitive to their endogenous ligand ACh (26 μ M and 31 μ M, respectively). However, their biophysical and pharmacological differences are dramatically different. First, L-AChRs remain fully active during our longest agonist application (40 s), whereas ACR-16 homopentamers desensitize rapidly and profoundly (>97% within 30 s). Second, even though L-AChRs contain 3 α -subunits that could contribute 3 ACh binding sites per receptor molecule, there is no (or little) apparent ACh binding cooperativity as suggested by the Hill coefficient close to 1. This contrasts with the Hill coefficient of 2.4 for ACR-16 homomers. Third, the calcium permeability of L-AChRs is modest ($P_{Ca}/P_{Na} = 0.6$), a value slightly higher than that found for embryonic muscle AChRs at vertebrate neuromuscular junctions. The calcium permeability of N-AChRs has not been measured. However, ACR-16 resembles the neuronal $\alpha 7$ -like homomer-forming subunits of vertebrates that are much more permeable to calcium than heteromeric channels (19, 22, 24). Fourth, L-AChRs are activated by levamisole, inhibited by nicotine, and insensitive to DH β E, whereas N-AChRs are activated by nicotine, partially inhibited by levamisole, and blocked by DH β E. Finally, in vivo, both receptor subtypes colocalize at neuromuscular junctions, yet distinct molecular machineries are involved in the synaptic clustering of L- and N-AChRs (11, 32). It is tempting to hypothesize that these receptors might contribute different neuromuscular signaling depending on locomotory regimes, but this still needs to be experimentally demonstrated.

Expression of L-AChRs provides a means to analyze the action of levamisole in greater detail. This drug has been described as a potent agonist of nematode AChRs. However, our dose-response experiments demonstrate that levamisole is only a partial agonist, ≈ 3 -fold less efficient than ACh. At 500 μ M, levamisole behaves as an open-channel blocker, as recently suggested by single-channel recording of L-AChRs from *C. elegans* muscle membranes (ref. 26; see Fig. 3C). However, the effectiveness of levamisole on parasitic nematodes might be explained by at least 2 reasons. First, L-AChRs do not desensitize and should stay open as long as the drug is

present in the extracellular environment. Second, at high levamisole concentrations, washout kinetics are very slow, complete recovery taking minutes compared with a few seconds for ACh-evoked responses. Because levamisole is membrane-permeable, it is possible that these very slow deactivation kinetics do not result from an inherently tight ligand–receptor interaction but rather from slow levamisole departure from a nonaqueous reservoir. Such prolonged effects are reminiscent of the activation of GABA_A receptors by neurosteroids. These compounds are thought to partition and accumulate into the plasma membrane from where they access a binding site on the receptor. The rate-limiting factor for deactivation may then be reservoir emptying rather than the intrinsic kinetics of ligand dissociation (33). We propose that similar mechanisms may underlie some of the distinctive effects of levamisole on L-AChRs. Interestingly, the very slow-washout kinetics that we observed are consistent with earlier reports that demonstrated that levamisole caused a much more prolonged contraction of isolated *Ascaris* muscle than ACh (34). This property likely contributes to the anthelmintic efficacy of the drug.

Analysis of recombinant L-AChRs also revealed that nicotine, the prototypical agonist of ionotropic acetylcholine receptors, is an antagonist of L-AChRs. Only vertebrate $\alpha 9^*$ AChR have been reported to be nicotine-insensitive (35). On these receptors, nicotine actually behaves as a potent competitive antagonist. The situation is different with L-AChRs because high concentrations of nicotine do not dramatically affect ACh EC₅₀. We also demonstrated that this inhibition is not caused by open-channel block. Hence, we concluded that nicotine is mainly a noncompetitive L-AChR antagonist, possibly acting through allosteric inhibition. Such a mechanism has been proposed for picrotoxin at GABA_A receptors (36), and for some AChR noncompetitive antagonists such as the steroid promegestone that efficiently inhibits *Torpedo*

AChRs most probably by increasing the fraction of desensitized receptors (37).

In conclusion, the expression system that we have characterized here will provide a means to identify the molecular determinants of the unique L-AChR pharmacology. Understanding the molecular basis for the agonist selectivity of levamisole on nematode but not vertebrate AChRs might open the way for the rational design of novel anthelmintic drugs.

Materials and Methods

For more detailed information, see *SI Materials and Methods*.

General Procedures and Strains. *C. elegans* strains were grown on nematode growth medium by using standard conditions according to (38). The following strains were used for electrophysiological recordings: BC168 *unc-13(s69)* and EN1645 *unc-13(s69);acr-16(ok789)*V.

Electrophysiological Studies in *X. laevis* Oocytes. *X. laevis* oocytes were prepared, injected, voltage-clamped, and superfused as described in ref. 39 except that gentamycin was omitted from the conservation medium because prolonged treatments with this antibiotic can inhibit AChRs expressed in *Xenopus* oocytes (40).

Electrophysiological Studies in *C. elegans*. Electrophysiological recordings on *C. elegans* muscle cells were performed according to ref. 12.

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Supporting Information

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SI Materials and Methods

Reagents. ACh, α -BgTx, DH β E, Hex, MLA, (-)-nicotine hydrogen tartrate, pyrantel citrate, (-)-tetramisole hydrochloride (levamisole), dTC, BAPTA, and BAPTA-AM were purchased from Sigma-Aldrich.

Expression Plasmids. cDNA sequences were amplified by using the following primer combinations.

acr-16 oTB361-AAACTCGAGatgtctgtctgcacccttc/oTB362-TTTGGGCCcttaggcacaagatcaggtg; *lev-1* oTB363-AAACTCGAGatgtgttaggaggtg/oTB364-TTTGGGCCctcagaaaataccaagaact; *lev-8* oTB386-AAACTCGAGatgtggataccacaacgg/oTB387-TTTGGGCCctcaggtgtaagaacgttg; *ric-3* oTB365-AAACTCGAGatgcacaaaactgaacggcg/oTB366-TTTGGGCCctcaagtcttttaggtctc; *unc-29* oTB367-AAACTCGAGatgag-gaccaaccgactatc/oTB368-TTTGGGCCctcaggaatattggatgctg; *unc-38* oTB369-AAACTCGAGatgcgctcttttggttat/oTB370-TTTGGGCCctcagaaaactaattggattag; *unc-50* oTB371-AAACTCGAGatgagttcacagccgcgag/oTB372-TTTGGGCCctaaagaccgctgttg; *unc-63* oTB373-AAACTCGAGatggac-caaatgaccacg/oTB374-TTTGGGCCctaaagcaagagccggcgtg; *unc-74* oTB375-AAACTCGAGatgcaaaaatattctta/oTB376-TTTGGGCCctcactcagcttttctg.

PCR fragments were then digested with XhoI and Bsp120I restriction enzymes and cloned into pTB207, an expression vector suitable for in vitro transcription and containing the 3'-UTR of the *Xenopus laevis* β -globin gene. The resulting vectors are: pTB210 *unc-29*, pTB211 *unc-38*, pTB212 *unc-63*, pTB213 *lev-1*, pTB214 *acr-16*, pTB215 *ric-3*, pTB216 *unc-74*, pTB217 *unc-50*, and pTB226 *lev-8*.

Oocyte Electrophysiology. **BAPTA.** In some experiments (voltage ramps and Fig. 2A), we used BAPTA-injected or BAPTA-loaded oocytes to avoid contamination by endogenous Ca^{2+} -dependent chloride currents. After BAPTA injection [injection of 50 nL of 40 mM K-BAPTA (pH 7.0)], oocytes were incubated in Barth solution at least 30 min (at 19 °C) before recording. For

BAPTA-AM preloading, each oocyte was incubated for at least 4 h in 200 μL of Barth solution containing 100 μM BAPTA-AM (stock solution prepared at 100 mM in DMSO).

Voltage ramps. All current-voltage (I - V) curves were obtained with slow-voltage ramps (2-s duration) from -70 to $+50$ mV performed on BAPTA-injected or -loaded oocytes. For all I - V curves shown, leak currents recorded in the absence of agonist were subtracted from the currents recorded in the presence of agonist.

Measurement of calcium permeability. Measurements of reversal potentials were performed on oocytes preloaded with BAPTA-AM. Switching from the 1 mM CaCl_2 to the 10 mM CaCl_2 Ringer solution resulted in a voltage offset in the reference electrode because of the increase in chloride concentrations. This offset was measured for each oocyte (2.3 ± 0.2 mV, $n = 4$) and subtracted from the measured voltage in I - V ramps performed in 10 mM CaCl_2 .

For calculations of $P_{\text{Ca}}/P_{\text{Na}}$, P_{Na} and P_{K} were assumed to be equal, and the internal Ca^{2+} concentration was considered negligible because of BAPTA chelation. Ionic activities rather than concentrations were used (activity coefficient of 0.72 for Na^+ and K^+ and of 0.56 for Ca^{2+} ions).

Dose-responses curves. For dose-response curves, the experimental data points of each cell were fitted with the following Hill equation: $I_{\text{rel}} = I_{\text{max}}/(1 + (\text{EC}_{50}/[A])^{n_{\text{H}}})$, where I_{rel} is the mean relative current, I_{max} is the relative current obtained at saturating agonist concentrations, $[A]$ is the concentration of agonist, n_{H} is the Hill coefficient, and EC_{50} is the concentration of agonist producing 50% of the maximal current. EC_{50} , I_{max} , and n_{H} were fitted as free parameters. For ACh, data points were then divided by I_{max} to have a maximum relative current normalized to 1. Resulting values obtained for each individual cell and for each concentration of agonist were then averaged, and the mean data points were then fitted with the same Hill equation as above but with I_{max} fixed to 1. For levamisole, data points were normalized in the same way to the mean value of I_{max} instead of 1.

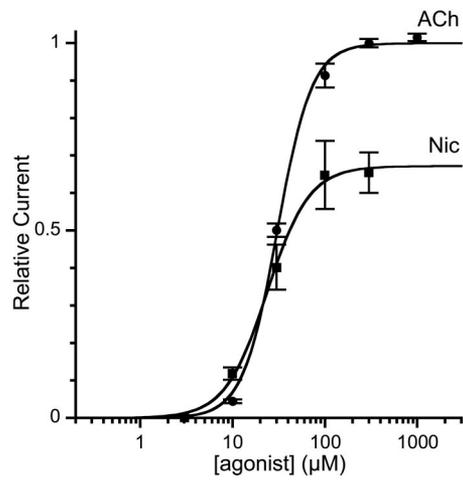


Fig. S1. ACh and nicotine dose–response curves on N-AChRs. Dose–response curves for ACh and nicotine on oocytes coinjected with the two *acr-16* and *ric-3* cRNAs are shown. EC_{50} and n_H for ACh are $31 \pm 0.5 \mu\text{M}$ and 2.4 ± 0.3 ($n = 6$). EC_{50} and n_H for nicotine are $24 \mu\text{M}$ and 1.9. For nicotine, each point is the mean of 5 experimental data points, normalized to a prior application of 1 mM ACh. The maximal currents evoked by nicotine were on average $67 \pm 6\%$ of those elicited by saturating ACh concentrations.