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Pharming for Genes in Neurotransmission: Combining Chemical and Genetic Approaches in *Caenorhabditis elegans*

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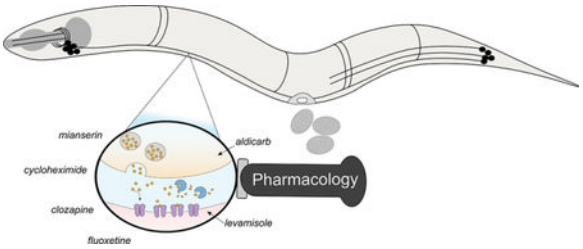
Abstract

Synaptic transmission is central to nervous system function. Chemical and genetic screens are valuable approaches to probe synaptic mechanisms in living animals. The nematode *Caenorhabditis elegans* is a prime system to apply these methods to discover genes and dissect the cellular pathways underlying neurotransmission. Here, we review key approaches to understand neurotransmission and the action of psychiatric drugs in *C. elegans*. We start with early studies on cholinergic excitatory signaling at the neuromuscular junction, and move into mechanisms mediated by biogenic amines. Finally, we discuss emerging work toward understanding the mechanisms driving synaptic plasticity with a focus on regulation of protein translation.

Graphical Abstract



Keywords

Pharmacology; neuromuscular junction; neuronal circuit; acetylcholine; synaptic vesicle release; ion channels; protein synthesis; psychotic drugs; serotonin; neuromodulation; dopamine

INTRODUCTION

Highly organized molecular and electrical events allow neurons to communicate to their targets across anatomically defined structures at cellular junctions known as chemical and electrical synapses. These mechanisms ensure speed, precision, and plasticity of

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S.B. and Y.J. conceived the review topic, selected key research papers, and wrote the manuscript. S.B. prepared the figures and table with critical input from Y.J.

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neurotransmission by varying the type, quantity, and frequency of neurotransmitter release, as well as the responsiveness of target cells. Disruptions of this process at multiple levels have a profound impact on the pathology of nearly all neurological disorders. Thus, research efforts at the cellular and molecular levels have focused on the following general questions: When and where are particular neuro-transmitters released? What are their effects on postsynaptic target cells? How does variation in this process direct behavioral outputs? A powerful way to address these questions has come from the use of neuroactive drugs that perturb synaptic transmission in model organisms that are amenable to genetics.

The free-living nematode *Caenorhabditis elegans* has been a valuable laboratory organism to study synaptic biology owing to its body transparency, defined neuronal anatomy, gene conservation to human, and amenability to genetic and pharmacological manipulations. Sydney Brenner pioneered the genetic studies of *C. elegans,* setting in motion decades of mechanistic investigation in developmental biology and neuro-biology.[1] *C. elegans* reproduce primarily as self-fertilizing hermaphrodites, convenient for laboratory handling, yet males arise at low frequencies and are used to transfer genetic information. The utility of *C. elegans* as a prime experimental model in neuroscience was accelerated by the efforts of John White, Sydney Brenner, and colleagues when they determined the full circuitry of its 302-cell nervous system at the electron microscopy level.[2] Furthermore, Brenner isolated abundant mutants in his pioneering genetic screen based on visually detectable phenotypes. Among them is a large class of mutants exhibiting defective movement, under the general classification of uncoordinated (*unc*), studies of which have led to the discovery of numerous genes that encode conserved proteins mediating synaptic transmission. Brenner also made the first attempt to couple pharmacological approaches with genetic screens. Subsequent work from others then integrated the use of antipsychotic drugs to discover genetic pathways that likely mediate their clinical effects.

In this review, we focus on the major advances gained from pharmacological approaches in *C. elegans*. We begin with early screens focused on cholinergic transmission at the neuromuscular junction using the anthelmintic drugs levamisole and aldicarb. We follow with a discussion of drugs altering behaviors controlled by biogenic amine signaling, with a particular emphasis on recent work leveraging *C. elegans* genetics to identify side effects of psychotropic drugs. Finally, we highlight emerging work using protein translation inhibitors to study gene expression mechanisms that underlie synaptic plasticity in a number of experimental paradigms.

OVERVIEW OF PHARMACOLOGICAL METHODOLOGIES IN *C. elegans*

In the laboratory, *C. elegans* are normally cultured on agarcontaining Petri plates seeded with a thin layer of bacteria. In most studies, chemicals or drugs are added to agar media supplemented with proper solvents. The cuticle of *C. elegans* is largely impenetrable to small molecule compounds.[3] How drugs get into *C. elegans* remains unclear, although drug uptake through the cuticle, gut, or amphid neurons have all been observed.[3] Some chemicals can yield greater effect when *C. elegans* are cultured in liquid media composed of appropriate salts. While success of such approaches is widely reported, pharmacological screening still represents a gigantic effort, often with very small hit rates (below 5%).[4–6]

Further, inherent to pharmacology, many experiments can produce different results, even among compounds belonging to the same drug class. For example, the dihydropyridine analog nemadipine A was shown to target the calcium channel EGL-19, yet other FDA-approved dihydropyridines fail to produce a phenotype.[6] Thus, it is highly advisable to cautiously interpret observed effects or negative results; and effective concentrations of new compounds are always determined empirically. Nevertheless, as described below, drugs displaying specific effects are especially useful when coupling with additional genetic manipulation, such as forward mutagenesis screens or testing candidate genes for altered response to drug induced effects (Figure 1). Studies using penetrable drugs or using *C. elegans* mutants with leaky cuticles have revealed numerous signaling processes that mediate drug activity and their underlying genes. However, like many clinical neuroactive pharmaceuticals, many common chemical compounds are presumed to exert effects through off-target interactions. The advantage of *C. elegans* is that its defined neuronal circuit coupled with single-cell manipulation can help to tease apart direct and indirect contributions.

USE OF LEVAMISOLE AT THE NEUROMUSCULAR JUNCTION: UNDERSTANDING POSTSYNAPTIC ACETYLCHOLINE RECEPTOR BIOLOGY

The *C. elegans* locomotory circuit is an expedient system to reveal the genetic effectors of synaptic transmission at the neuromuscular junction (NMJ). Locomotory behavior relies on coordinated synaptic innervation from excitatory cholinergic neurons that stimulate body muscle contraction while simultaneously activating inhibitory GABAergic neurons, which relax muscle cells on the opposing side of the worm. Disruption in this circuit causes a variety of movement defects nicknamed coiler, kinker, fainter, twitcher, or shrinker, with their gene names generally under the category of *unc*. The simple anatomy of this circuit, coupled with sensitive read-out provided by these phenotypes, makes it an especially tractable platform to probe cholinergic neurotransmission.

In his seminal 1974 paper,[1] Sydney Brenner recognized the potential of *C. elegans* for systematically identifying genes that underlie synaptic transmission by screening for mutants resistant to the insecticides methomyl, a cholinesterase inhibitor, and levamisole, an acetylcholine receptor agonist. Both drugs evoke muscle hypercontraction resulting from either decreased breakdown of acetylcholine at the synaptic cleft (methomyl) or the addition of an exogenous acetylcholine receptor agonist (levamisole) and eventually paralyze the animal. Hence, mutants resistant to these drugs are defective in genes that function in synaptic transmission (Figure 2).

The pharmacokinetics of levamisole was thoroughly examined in dissected worms.[7] Early reports suggested that the drug was a cholinergic agonist, since its effect on muscle was similar to nicotine, and that it was blocked by antagonists like mecamylamine.[7] Levamisole appears to act directly on postsynaptic muscles, as its induced effects persist even in conjunction with anesthetic treatments that block endogenous neurotransmitter release. Reasoning that resistant mutants would likely bear mutations in genes encoding its target

receptors, their biosynthesis, or factors required for their assembly, an expansion of Brenner's early levamisole screens was carried out.[8] The first such screen yielded 11 genetic loci, nicknamed "*lev-Unc*"[7] (Table 1A). An important observation was that *lev-unc* mutants are not completely deficient in motor activity,[7] suggesting levamisole only acts on a subclass of cholinergic receptors. Additionally, studies of mutants that conferred variable levamisole resistance pointed to their possible cellular functions. For example, it was suspected that mutants conferring strong resistance were defective in cholinergic receptors, while mutants showing weak resistance may likely have defects downstream of receptor signaling.[8] Remarkably, subsequent molecular cloning of five levamisole resistant genes revealed that they encode proteins belonging to the family of ionotropic pentameric acetylcholine receptors.[9] Based on the Cys-Loop classification, three of the five *lev-unc* genes, *unc-63*, *unc-38*, and *lev-8* encode ligand-binding α subunits and *unc-29* and *lev-1* encode non-α subunits. All five subunits showed predominant expression in the body muscle where they likely form a postsynaptic receptor complex (Figure 2).[10]

A technological breakthrough in characterizing the physiology of these receptors came from electrophysiology recording at the NMJ.[11] These studies identified two acetylcholine receptors (AChRs) expressed in muscles of *C. elegans*, a levamisole sensitive channel requiring *unc-29* and *unc-38*, and another that responds to nicotine and requires *acr-16* receptor.[11b] Electrophysiological recording on body muscle showed that levamisole evoked current amplitude was significantly reduced in *lev-1* or *unc-63* loss-of-function mutants.[10] Interestingly, *lev-8* or *lev-1* null mutants are not completely levamisole resistant, suggesting that they encode nonessential subunits or alternatively, each is expressed in different muscle AChR.[12] The former hypothesis is supported by single-channel recording experiments in cultured primary muscle cells derived from receptor mutants, which showed that while *unc-29*, *unc-38*, and *unc-63* are required for ACh receptor activity, *lev-1* is dispensable.[13]

Other levamisole resistant mutations were later determined to affect genes essential for levamisole receptor assembly, transport, and ultimately their function at the synapse (Figure 2). *lev-10* encodes a conserved CUB-domain containing transmembrane protein, and *lev-9* encodes an extracellular protein containing a conserved SUSHI domain. Together, these two proteins regulate levamisole receptor clustering at the NMJ.[14,15] *unc-50* encodes a transmembrane Golgi protein that mediates proper trafficking of assembled receptor subunits to the plasma membrane,[16] and *unc-74* encodes a conserved thioredoxin protein that makes disulfide bonds during protein folding in the ER.[17,18] A third assembly factor, *ric-3*, was not identified from initial levamisole resistance screens but was instead isolated by its resistance to the cholinesterase inhibitor aldicarb.[19] The clue for *ric-3* function came from a genetic suppression screen for neuronal degeneration caused by an AChR mutant called *deg-3(u662)*, which carries a missense mutation in the pore forming domain that produces a non-desensitizing channel.[20] All *ric-3(lf)* mutants isolated from this screen exhibited DEG-3 receptor mislocalization within cell bodies instead of to cell processes.[20] *ric-3* belongs to a conserved family of genes important for acetylcholine (ACh) neurotransmission.[21] Subsequent studies have demonstrated its role in the maturation of multiple AChRs, including levamisole receptors. Among the *lev-unc* mutants, only one gene, *lev-11*, does not directly participate in channel activity; *lev-11* encodes a muscle expressed tropomyosin gene that may regulate tissue structure needed for optimal AChR activity.[22]

With the channel subunits and assembly factors in hand, the next *tour de force* experiment was reconstitution of channel activity in *Xenopus* oocytes. When eight components were expressed in the proper molar ratio, electrophysiology experiments demonstrated a functional receptor with specific levamisole sensitivity.[17] Importantly, *unc-50*, *unc-74*, and *ric-3* are required for reconstitution,[17] underscoring the importance of the assembly factors in expressing functional receptor complexes and their indirect yet crucial role in synaptic transmission.

It is worth noting that all three accessory proteins are broadly expressed and that the Lev-R subunits are also expressed in non-overlapping subsets of neurons, in addition to muscles.[10] Indeed, additional studies revealed that three subunits, UNC-38, UNC-63, and ACR-12, can form another heteromeric ACh receptor complex with ACR-2 and ACR-3 in the motor neurons.[23] Morever, reconstitution studies suggest that these neuronal AChR are not levamisole sensitive.[23]

Other screens have isolated mutants with partial levamisole resistance. Studies of these mutants revealed additional factors regulating levamisole AChR assembly and function, such as the secreted IG domain protein OIG-4, which associates with LEV-9 and LEV-10 to mediate receptor clustering,[24] and an assembly factor called EMC-6, which stabilizes protein folding of nascent receptors.[25] A notable finding derived from these screens is the only known levamisole AChR auxiliary factor, called MOLO-1, which directly associates with these receptors and regulates channel gating activity when reconstituted in *Xenopus* oocytes.[26]

In summary, the genetic and subsequent molecular studies on genes displaying selective sensitivity to levamisole demonstrate the power of saturation genetic screens in defining biological signaling events at NMJ postsynaptic sites, as well as the specificity of drug action.

POWERFUL USE OF ACETYLCHOLINESTERASE INHIBITOR ALDICARB: DISCOVERY OF PRESYNAPTIC RELEASE COMPONENTS

Acetylcholinesterase (AChE) breaks down acetylcholine in the synaptic cleft, thereby terminating neurotransmitter action. Various AChE inhibitors cause ACh accumulation in the synaptic cleft and induce time-dependent muscle paralysis, making them particularly useful to assay presynaptic function. Animals with increased presynaptic ACh release are highly sensitive to these drugs, whereas mutants with defective ACh signaling are comparatively resistant. Therefore, the dose-response analysis can be used to assay presynaptic changes that alter release kinetics, such as those impacting the synaptic vesicle cycle (Figure 2). Mutants resistant to such drugs are usually defective in genes that induce cholinergic neurotransmission while hypersensitive mutants are often compromised in genes that regulate presynaptic ACh release.

Aldicarb has been the primary AChE inhibitor used to probe synaptic transmission in *C. elegans*. Early genetic screens isolated few mutants resistant to aldicarb induced paralysis.[27–31] A large-scale aldicarb resistance screen carried out by the Rand laboratory[19,32] yielded

165 mutants of 21 genes named *ric* (resistant to inhibitors of cholinesterase), some of which are previously known *unc* genes (Table 1B).[19] Importantly, these screens used transposon-induced mutagenesis, facilitating rapid cloning of genes affected. The timely molecular identification of the first set of *ric* genes revealed key components of calcium-triggered synaptic vesicle exocytosis (Figure 2). For example, loss of function mutants of synaptotagmin (*snt-1*), the calcium sensor for fast neurotransmitter release,[33] were strongly aldicarb resistant. The identification of acetylcholine vesicular transporter (*unc-17*) led to the discovery of the VAChT vesicular transporter family. Molecular cloning of *unc-13*, *unc-18*, *unc-64*, and *unc-10*, all played pivotal roles in our understanding of the conserved synaptic protein family for Munc13, Munc18, Syntaxin, and Rim in vesicle exocytosis. Additionally, several genes including *unc-11/AP180*,[34] *unc-57/endophilin*,[35] and *unc-26/synaptojanin*[36] were shown to define genes involved in vesicle endocytosis.

With the technological advancement in dsRNAi mediated gene knockdown, genome-wide high-throughput screening became feasible. A large number of genes were tested for sensitivity to aldicarb,[37] which identified 132 additional genes with broad function in synaptic transmission and neuronal circuit development and modulation. A benefit of RNAi-based approaches is the identification of genes that instead confer hypersensitivity to aldicarb (*hic*). *hic* mutants typically have increased cholinergic synaptic transmission, and aldicarb induces paralysis much faster than in wild-type animals. Not surprisingly therefore, *hic* genes are often involved in pathways regulating ACh release.

An interesting example has been the discovery of competing pathways downstream of G-protein-coupled receptors, which modulate ACh release by regulating vesicle exocytosis at presynaptic termini.[38] Serotonin signaling pathway activates G-protein α subunit GOA-1, which negatively regulates synaptic vesicle exocytosis by restricting the vesicle priming factor UNC-13 away from the presynaptic terminal, thereby reducing ACh release.[38a] Mutants deficient in the serotonin biosynthesis factor *cat-4* block this inhibitory pathway and are *hic*.[38a] Likewise, mutants deficient in *goa-1* or its downstream effector *dgk-1* are also *hic*.[38a,b] Interestingly, a parallel yet opposing pathway operating through another G-protein α subunit, EGL-30, instead stimulates ACh neurotransmission by inducing synthesis of diacylglycerol (DAG), which actively recruits UNC-13 to release sites.[38c] Gain-of-function mutations in *egl-30* therefore also confer hypersensitivity to aldicarb.

Two other highly conserved genes, complexin *cpx-1* and tomosyn *tom-1*, negatively regulate vesicle release. The complexins interact with the SNARE complex.[39] In the *C. elegans* motor circuit, *cpx-1* opposes stochastic synaptic vesicle release yet supports evoked release by maintaining a sufficient pool of primed vesicles at the synaptic terminal.[40,40b] Tomosyn, TOM-1, antagonizes the active zone protein UNC-13 to restrict the size of the primed vesicle pool.[41,41b] Both *cpx-1* and *tom-1* mutants are *hic* and exhibit unfettered vesicle release.

Other *hic* genes are required in pathways that support GABA inhibitory neurotransmission. Mutants defective in the GABA transporter *unc-47*, for example, are aldicarb hypersensitive.[42] An RNAi screen targeting genes with predicted synaptic roles identified over 70 *hic* genes,[43] including genes required for GABAergic neuron development, such as TGF-β and

Wnt signaling, and the MAP kinase signaling pathways that regulate GABA neurotransmission.[43] Thus, aldicarb hypersensitivity can also be an effective phenotype leveraged to study inhibitory neurotransmission.

In summary, aldicarb sensitivity mutants identified from both forward and reverse genetic approaches are enriched in presynaptic genes, consistent with the reliance of effects of aldicarb on presynaptic ACh release (Table 1B). All have either reflected on known models of synaptic transmission in other species or uncovered previously unknown components and pathways. For example, the conserved gene *ric-8* was described as a novel regulator of neurotransmitter release through a G-protein-coupled receptor pathway through regulation of DAG levels. Many aldicarb resistance mutants remain poorly understood, and further studies will expand the gene network and advance our knowledge of synapse regulation.

BIOGENIC-AMINE SIGNALING: ACTIONS OF PSYCHIATRIC DRUGS ON CIRCUIT MODULATION

Psychoactive drugs of various classes are commonplace for the treatment of brain disorders. While generally effective as mood suppressors, a major downside is their variable and unpredictable clinical efficacy and wide ranging side effects.[44] Although designed to mitigate biogenic amine signaling at multiple levels, their clinical side effects are postulated to stem from largely unknown secondary targets and extraneous pathways. A critical interest has therefore focused on identifying the corresponding molecular pathways leading to these undesired effects.

The well-defined neural circuitry and powerful genetics of *C. elegans* sparked a surge of research tailored to address these knowledge gaps in psychiatric pharmacology. As with anthelmintics, *C. elegans* responds to many psychotropic drugs, allowing genetic screens to identify mutants with altered drug response.

In *C. elegans*, serotonin (5-HT) and dopamine were first identified by use of formaldehyde-induced fluorescence (FIF) staining,[45] revealing their presence in the head, ventral nerve cord, and tail neurons.[45b] This led to the finding that these neurotransmitters play an important modulatory role in *C. elegans*, affecting egg-laying, foraging behavior, and other activities. Below, we will discuss selective studies addressing cellular targets of antidepressants.

Mapping Antidepressant Side Effects: Serotonin Reuptake Inhibitors.

Serotonin reuptake inhibitors (SSRIs), including imipramine (Tofranil), clomipramine (Anafranil), and fluoxetine (Prozac) are a common class of clinical antidepressants. Analogous to the action of AChEs at the neuromuscular junction, these drugs inhibit serotonin reuptake transporters (SERTs) that reabsorb 5-HT at the synaptic cleft, causing its accumulation and stimulating serotonin receptor signaling. In clinical practice, these drugs induce a range of side effects,[46] with poorly understood cellular mechanisms.

Early studies of egg-laying behavior in *C. elegans* provided the first evidence for serotonin-independent effects of SSRIs and offered an experimental direction to dissect their cellular

pathways. FIF staining revealed serotonin expressing neurons near the vulva tissues required for egg-laying.[45a] Vulva muscles are coordinately innervated by cholinergic motor neurons and hermaphrodite specific motor neurons (HSNs).[47] Rate of egg-laying depends on both serotonin and acetylcholine neurotransmitters, since laser ablation of the HSN neurons causes an egg-laying defective (*egl*) phenotype independent of serotonin[48] and exogenously supplied serotonin does not stimulate egg-laying in ACh neurotransmission deficient mutants.[49] To much surprise, the SSRIs imipramine and clomipramine never-the-less stimulate egg-laying in mutants deficient in serotonin biosynthesis enzyme and lacking HSNs.[49] Also supporting independent pathways for imipramine and clomipramine, mutants that completely lack serotonin were found to exhibit even greater sensitivity to low doses of these drugs.[49]

Do serotonin independent effects of SSRIs involve SERT targeting? Answers to this question came from experiments using mutants defective in MOD-5, the *C. elegans* serotonin reuptake transporter.[50] The SSRI fluoxetine (Prozac) stimulates egg-laying and contraction of nose muscles even in *mod-5* null mutants, suggesting SERT independent pathways mediated by this drug.[50] The effects of SSRIs on SERTs are likely distinct, since *mod-5* mutants remain sensitive to fluoxetine induced egg-laying but inhibit the egg-laying induction by imipramine.[50,51] Importantly, while SSRIs would be expected to accumulate serotonin in the synaptic cleft, SERTs also import exogenous serotonin to neurons lacking serotonin biosynthesis. Fluoxetine eliminates serotonin from these neurons, likely by blocking MOD-5.[52] Therefore, SSRIs may exert their effects through 1) stimulating serotonergic signaling by promoting its accumulation in the synaptic cleft, 2) depletion of serotonin from neurons deficient in serotonin biosynthesis, or 3) SERT-independent pathways.

Identification of serotonin receptors in HSN neurons permitted targeted genetic experiments that clarified potential receptor targets mediating SSRI-induced egg-laying.[51] Importantly, these studies showed that imipramine and fluoxetine act through two SERT and 5-HT independent receptor pathways (Figure 3). While 5-HT stimulates egg-laying through the *ser-1* receptor on vulva muscle, fluoxetine acts independently of either *ser-1* or 5-HT. However, both require the downstream G-protein α subunit *egl-30*, indicating a parallel pathway whereby 5-HT and fluoxetine stimulate egg-laying converging on *egl-30*.[51] The fluoxetine receptor upstream of *egl-30* remains unknown, however signaling likely involves the HSN, since ablation of this cell prevents fluoxetine induced egg-laying.[53] Although imipramine also requires this neuron,[53] it regulates egg-laying through a pathway independent of *ser-1* and *egl-30* and instead requires the *ser-4* receptor expressed in head neurons.[51] These distinct receptor pathways for both drugs could explain their different clinical side effects.

Fluoxetine may also affect neurotransmitter signaling outside of serotonin as it causes muscle paralysis concurrent with reduction in ACh expression in the cholinergic motor neurons, dependent on the AMPA-type glutamate receptor (*glr-1*).[52] This action also trends with serotonin receptor activity, as paralysis is partially dependent on *ser-7* and appears to be opposed by *ser-5* receptor signaling as *ser-5* null animals are fluoxetine hypersensitive.[52] Together, these studies demonstrate that multifaceted mechanisms mediate fluoxetine

phenotypes and are not restricted to SERT inhibition. Future work will probe its effects among different neural circuits and thoroughly delineate its molecular targets in each receptor signaling pathway.

Forward genetic screens for fluoxetine-resistant mutants have led to the identification of novel molecular components of fluoxetine action.[54] These mutants were devoid of the serotonin-independent rapid nose muscle contraction phenotype caused by fluoxetine[54] and affected seven different genes, generally named *nose resistant to fluoxetine* (*nrf*). Double mutant analysis suggests that these genes likely act in the same pathway.[54] Interestingly, these mutants also exhibit a pale-egg (*peg*) phenotype resulting from failure to accumulate yolk, suggesting that fluoxetine may alter lipid function. *nrf-5* is shown to be homologous to a family of lipid-binding proteins.[54] Other genes may act synchronously as lipid proteins required for fluoxetine intercellular transport. Alternatively, lipid modifications by these genes could change their membrane properties and alter neuronal excitability.[54] *peg* genes appear to be required in the intestine to mediate the *nrf* phenotype.[55] Therefore, the *peg* and non-*peg* genes likely constitute distinct pathways mediating fluoxetine resistance. Further experiments will need to address how each pathway mediates fluoxetine side effects and their mutual roles.

Linking Neurotransmission to Aging: Unexpected Roles of Antidepressants.

An intriguing side-effect mediated by certain antidepressants in *C. elegans* is that of lifespan extension (LE). The short development time scale of *C. elegans* makes lifespan assays feasible in large screens that allow the systematic delineation of LE determinant pathways. These pathways, such as those mediating the positive effect of dietary restriction on lifespan,[56] are often highly conserved in mammals.

The initial observation that antidepressants alter *C. elegans* lifespan came from a large-scale chemical screen of 88 000 known compounds,[4,57] revealing 20–30% LE from each of the serotonin receptor antagonists mianserin, methiothepin, mirtazapin, and cyproheptadine.[4] Interestingly, only low doses of mianserin support LE, which also requires the G-protein-coupled serotonin receptor *ser-4*, and the octopamine receptor *ser-3*.[57]

How could mianserin's antagonistic effect on serotonin improve lifespan? At least part of the mechanism involves the well described caloric restriction LE pathways since restricting *C. elegans* diet did not enhance mianserin induced LE.[57] Since serotonin signals the presence of food, antagonizing this process with mianserin could virtualize starvation, thereby mimicking dietary restriction. Another part of mianserin's mechanism likely protects worms from oxidative stress by activating superoxide dismutases.[58] Neurotransmission mutants *snt-1* and *snb-1* are not resistant to oxidative stress in the presence of mianserin, suggesting that this protection requires neuroactivity. A model therefore postulates that mianserin activates an oxidation protective stress response by promoting neurotransmission.[58]

Genomic level insights on the mianserin LE mechanism came from an age-progression transcriptome study, finding misregulated mRNA stoichiometry correlated with worm age caused by a phenomenon named transcriptional drift. This phenomenon, in which genes

belonging to the same functional category change their expression in opposing directions,[59] is significantly reduced in mianserin treated worms and linked to the superoxide detoxification pathway.[59] Interestingly, this effect requires *ser-5*, but not *ser-3* or *ser-4*, which were formerly shown to mediate mianserin LE. Evidence of this phenomenon in humans and mice suggests this mechanism is conserved.[59]

Subsequent work discovered overlap between genes that change in expression in response to mianserin and genes enriched in clinical depression from genome-wide association studies (GWASs).[59] One of these was the Ankyrin ortholog *unc-44*, which increases in expression through age, where its eventual high expression in older animals is detrimental.[59] Mianserin is thought to extend lifespan by maintaining youthful low levels of Ankyrin to counterbalance oxidative stress.[59] Extending this to human GWASs located an allele of ANK3 linked to individuals with depression and is associated with longer lifespan in males.[59] Other genes from this study will be interesting to examine the full spectrum of mianserin induced LE mechanisms.

Target Spectra of Other Antipsychotic Drugs.

Anti-psychotic drugs, thought to act as dopamine antagonists by blocking the D2 receptor, are effective in the treatment of mental illnesses such as schizophrenia and bipolar disorder.[60] Like SSRIs, these drugs are also promiscuous through poorly understood pathways[60] that produce widespread developmental abnormalities, particularly in neonatal infants and young children.[61,62] The tissue-level nature of these effects is reflected by their cytotoxicity in human neuronal cell lines.[63] Rats exposed to antipsychotics *in utero* also develop with fewer proliferating neurons.[64]

First generation antipsychotics, which preferentially antagonize dopamine receptors, also interfere with *C. elegans* larval development, producing a dose-dependent reduction in body length.[65] However, most second-generation drugs with the exception of clozapine, have minimal impact.[65] Importantly, this effect was not rescued by adding dopamine or serotonin together with the drugs, suggesting they may target pathways independent of these neurotransmitters.[65]

Many of these antipsychotics, including calmidazolium, trifluoperazine, and chlorpromazine are likely inhibitors of calmodulin, a neuronal Ca^{2+} binding protein.[65] Worms exposed to the drugs or calmodulin inhibitors developed at the same slow rate, suggesting that calmodulin inhibition pathways may in part be responsible for the developmental defects caused by antipsychotics.[65] Both drugs also interfere with mechanosensory neuron development, leading to abnormal axonal extension,[66] also independent of serotonin or dopamine.

The clinical benefits of antipsychotics may not derive solely from pathway inhibition but could also restore the activity of aberrant signaling pathways. An example of this has been with the second-generation antipsychotic clozapine, which induces an early larval arrest phenotype that is dependent on the highly conserved insulin (Akt) signaling pathway.[67] The Akt signaling cascade leads to the phosphorylation of the FOXO transcription factor DAF-16, restricting its localization to the cytoplasm and reducing its nuclear transcription transactivation activity.[68] Clozapine-induced larval arrest is suppressed in mutants deficient

in multiple components of the insulin-signaling (Akt) pathway, including phosphatidyl inositol 3-kinase (*age-1*) and insulin receptor (*daf-2*).[67] An important downstream effect of clozapine treatment was shown to be unrestrained Akt signaling resulting in the mislocalization of DAF-16.[67]

Soon after, it was realized that nearly all major antipsychotics increase signaling through the insulin pathway, with a clear downstream effect of cytoplasmic DAF-12 accumulation. This results in phenotypes like dauer formation and shortened lifespan, that are well documented consequences of enhanced AKT signaling and require both the AKT-1 and insulin receptor (DAF-2).[69] Although the protein phosphatase calcineurin is a target of calmodulin that regulates DAF-16 activity and is inhibited by several antipsychotics, calcineurin mutants did not restore DAF-16 translocation to the nucleus.[69] Therefore, future work will need to address if the activation of Akt signaling is related to calmodulin inhibition or if each represents two independent secondary effects of antipsychotics that affect animal development.

Clozapine typically has a broader range of therapeutic applications and is often considered the first line of defense for correcting forms of mental illness.[70] An interesting convergence between Akt signaling and the varying clinical efficacy between antipsychotics has also been established. The downstream insulin signaling factor β-arrestin, which activates SGK-1 to ultimately restrict DAF-12 localization, is uniquely required for clozapine effects driven through this pathway.[71] However, it is not clear how clozapine promotes SGK-1 activation through β-arrestin, especially since SGK-1 mRNA expression remains unchanged after treatment.[71] These studies suggest that there may be multiple different pathways induced by antipsychotics that somehow converge on deactivating DAF-12. These insights are significant, since low-levels of Akt signaling are observed in untreated schizophrenics, implying that antipsychotics may exert their benefits through restoring pathway activity.[72,73]

Powerful RNAi screening approaches have isolated suppressors of clozapine-induced larval arrest (*scla*), identifying 40 genes that may be involved in clozapine's mechanism. One of these was the nicotinic acetylcholine receptor subunit *acr-7*,[74] which is likely stimulated by clozapine since both inhibit pumping of the pharynx muscle used for foraging. Another *scla* gene called *sms-1* encodes a sphingomyelin synthetase that is highly expressed in the pharynx and is also required for the clozapine-induced pumping defect. Further experiments provided evidence that clozapine activates *sms-1*, which boosts glucosylceramide levels which in turn reduce cellular protein aggregates by inducing autophagy.[75]

Analysis of a null mutation in the cation-selective transient potential receptor channel *gtl-2* that suppresses larval arrest provided evidence for multiple tissues involved in clozapine action, as restoring *gtl-2* expression only in the excretory canal cell is sufficient to rescue suppression.[76] Although still unclear, suppression appears to be mediated through regulation of Mg^{2+} homeostasis specifically in this cell.[76] This result points to mechanisms underlying not only clozapine action, but also mental illnesses, since TRPM channels are widely expressed in mammalian brains and implicated in bipolar disorders.[77,78] It will be interesting to further address the function of *scla* genes in the apparently many divergent pathways of clozapine action. Similar genetics approaches will be a powerful means to dissect target

repertoires of antipsychotic drugs to build on current models and to provide a blueprint for pharmaceuticals with greater clinical efficacy.

INHIBITORS OF PROTEIN TRANSLATION: UNDERSTANDING SYNAPTIC PLASTICITY

Modulation of neurotransmission driven by relative increases (potentiation) or decreases (depression) in synaptic strength allows nervous systems to store and process information, such as that needed for learning and memory. Regulating gene expression at multiple levels is an essential component of this process and tight dosage of protein levels via translation control has long been appreciated for its key role in the potentiation and depression of synapses (for a review, see ref [79]). Drugs that interfere with various steps of protein synthesis disrupt long-term memory in many experimental models[80,81] and have been useful for defining the temporal requirements of translation during memory. Cycloheximide is a widely used drug for these studies, since its effect on neuronal electrical activity is detectably inert compared to other inhibitors.[82] While its precise mechanism is still poorly understood, cycloheximide is thought to block the elongation step of translation,[83] which early experiments showed produces an amnesic effect in rats due to changes of neurons in the cerebrum without affecting neuron morphology.[84] The intracellular changes to synapses influencing memory are yet to be completely understood but represent a growing area of inquiry.

C. elegans responds to an array of transcription and translation inhibitors, offering a powerful platform to investigate mechanisms that couple control of gene expression with synaptic changes driving memory processes. Several key experiments have recently highlighted both transcription and translation as integral components of *C. elegans* long-term associative memory (LTAM) using dedicated assays for the ability to relate chemoattractants with food availability (Figure 4). A key demonstration of this approach is that following a seven-interval assay using the odorant butanone, associative memory was retained for 16 h. [85] LTAM was blocked in worms treated with the translation inhibitor cycloheximide or transcription inhibitor actinomycin-D, demonstrating that gene expression at both levels is required.[85] LTAM requires the CREB transcription factor[85] and declines as worms age. Consistently, LTAM is improved in *daf-2* mutants, which exhibit extended lifespan via defective insulin-signaling. The expression level of CREB seems to be an indicator of LTAM performance as its expression increases during training sessions and declines with age.[85] These observations are likely to be general to all associative learning processes as aversive olfactory association assays corroborate these results.[86,87] In a variation of these experiments, it was shown that *C. elegans* can also learn to avoid a chemoattractant (1-propanol) when simultaneously provided with an aversive odorant. This learned behavior also requires transcription, translation, and the CREB transcription factor.[86,87]

How does CREB-induced transcription support associative memory? A recent study provided some answers to this question through transcriptome profiling of naive (pre-training) and memory-conditioned (post-training) animals in wild-type and CREB null backgrounds.[88] These experiments identified a developmental requirement for basal CREB

transcription in non-neuronal tissues. However, memory conditioning activates specialized neuronal CREB transcription that alters over 1000 genes related to memory conditioning.[88] This gene set consists of synaptic components including vesicle docking genes, ion channels, seven-pass transmembrane receptors, kinases, and neuropeptide signaling genes.[88] It will be interesting to further address how differential regulation of these genes induces synaptic changes. Together, these studies have helped reveal LTAM genes and a correlation between aging, CREB transcriptional activity, and memory loss.

Variations of the associative learning assay have been employed to study short-term (STAM) and intermediate-term associative memory (ITAM). In these cases, worms are trained for 1 h with butanol, and attraction is tested immediately after exposure (for STAM) or 1 h after training (for ITAM).[89] In contrast to LTAM, short-term memory does not rely on transcription or translation, as worms achieve STAM even when exposed to either actinomycin D or cycloheximide during the training period.[89] However, cycloheximide reduces ITAM indicating that translation is important for retaining memory for slightly longer periods.[89] Remarkably, treating worms with cycloheximide after the training period increased the length of the attraction period, seeming to decelerate memory loss and suggesting that “forgetting” is an active process requiring the translation of new proteins.[89] Additional molecular insights on this model came from the revelation that the conserved *musashi* RNA-binding protein (*msi-1*) regulates forgetting through a pathway involving actin-cytoskeletal rearrangements.[90] *msi-1*(*lf*) animals are strongly impaired in forgetting, which is rescued by wild-type *msi-1* expressed solely in the AVA interneuron responsible for *C. elegans* memory.[90] Immunoprecipitation and sequencing of MSI-1 bound RNA revealed that MSI-1 binds and represses the translation of three Arp2/3 protein complex transcripts that induce actin branching. Cycloheximide mimics the effects of *msi-1* and can substitute its activity in *msi-1*(*lf*) mutants by blocking Arp2/3 translation. This translational repression directed by *msi-1* is specific to the forgetting process, whereas cycloheximide may also interfere with memory formation if applied during conditioning.[90] GFP-tagged glutamate receptor (GLR-1) that labels synapses at AVA was dramatically increased following training. This increase was reduced in an actin-remodeling dependent manner, coinciding with MSI-1 activity after the training period.[90] These results showed that the forgetting process is likely due to a refractory period where enlarged post-learning synapses are gradually reduced to their pre-learning size through actin remodeling.[90] Additional experiments are needed to address how the dynamics of actin skeleton remodeling, which are apparently regulated by translation, regulates memory at different stages of learning.

These studies from *C. elegans* reinforce those from other organisms that protein synthesis is an essential part of the memory dynamics, promoting memory along long and intermediate-terms as well as the active process of memory loss. Future work will focus on the specific genes targeted for translation during memory formation, specific components of the translation machinery that are engaged, and their effect at the synapse. To do so, it will be important to develop new tools with improved resolution that allow quantification of individual translation events. Methods like SUN-Tag[91–93,91b] and the TRICK assay[94] have already shown promise in other systems for visualizing translation in real-time. Luminescent proteins[95] and low half-life fluorescent reporters[96] should allow visualization of protein dynamics. Integrating these techniques with *C. elegans* genetics, pharmacology, and imaging

tools will continue to bring major prospects to our understanding of translation regulation schemes in the nervous system.

CONCLUDING REMARKS

As the only metazoan to-date with anatomically defined neural circuitry and virtually complete expression maps for neuronal receptors and other molecules, *C. elegans* offers great advantages in current research. Confluent with these features, chemical screens in this model have made it possible to link gene function to neurotransmission and neurotransmission to circuit level activity within a living organism. Of note, the mechanisms discovered via these approaches are exceptionally conserved in mammals, making not only the genes but the function of their underlying pathways a foundation to understand the generally larger, more complicated mammalian nervous systems. While productive, lessons from the past 40 years indicate that we have really only scratched the surface leveraging the unique capabilities of this model, and there are likely many more surprises in store.

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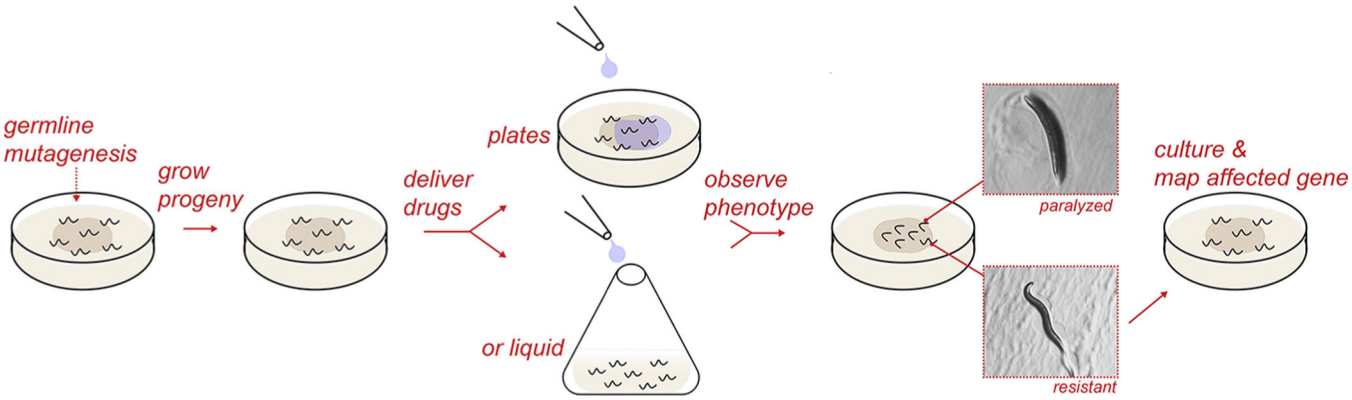

Figure 1.

Pharmacology screens in *C. elegans*. The germ cells of preadult worms are mutagenized with chemicals or radiation on agar plates. After culturing for two generations to allow recessive phenotypes to arise, worms are treated with a chosen neuroactive drug on agar plates or in liquid. Drug-resistant animals are then isolated and cultured, and their mutations are mapped to genes conferring drug-resistance.

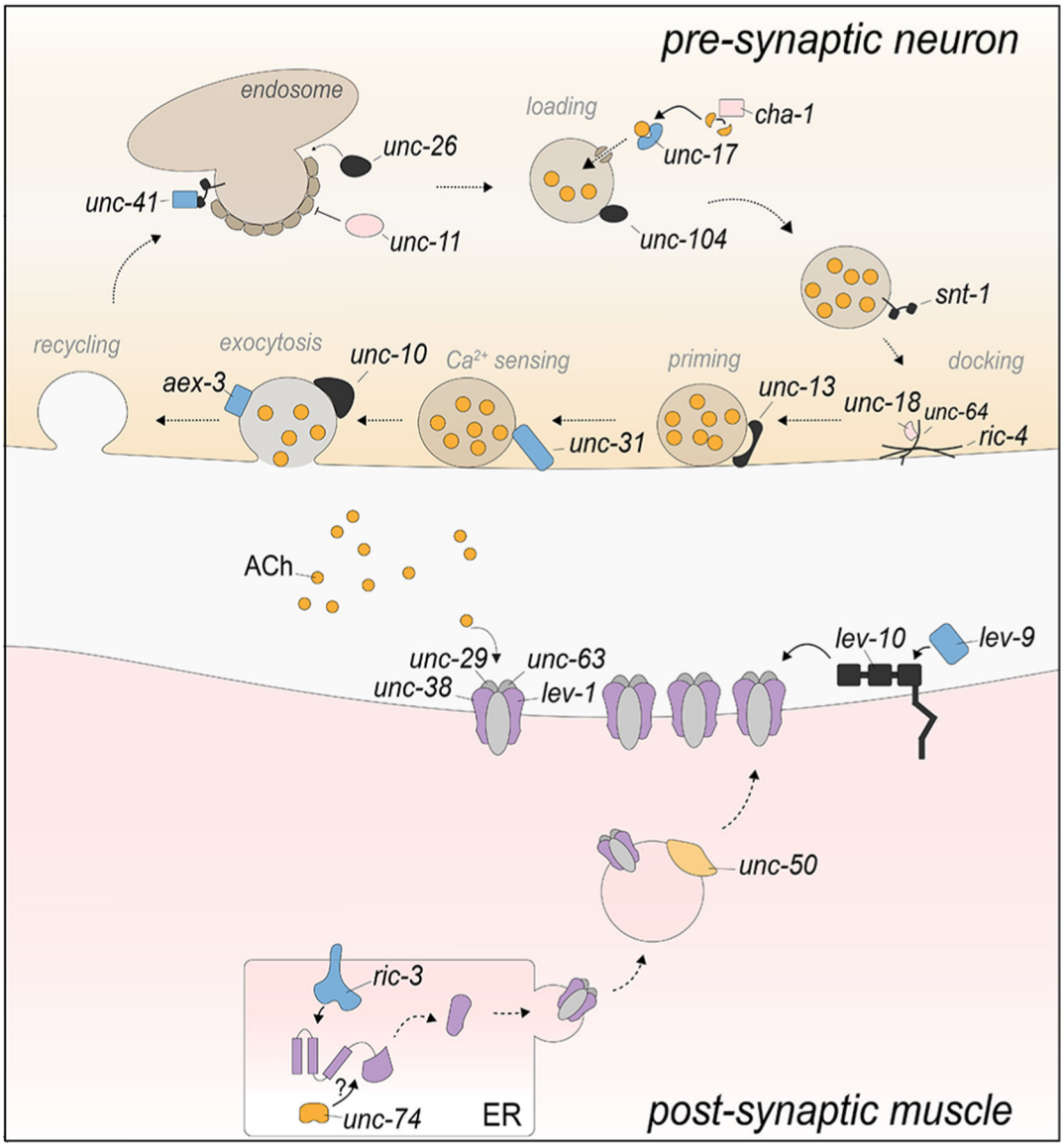

Figure 2.

Genes operating in post- and presynaptic cells of the neuromuscular junction identified in levamisole and aldicarb resistance screens. Levamisole resistance mutations primarily affect genes operating in the postsynaptic muscle, including subunits of the levamisole sensitive pentameric ACh receptor, their assembly factors in the endoplasmic reticulum (ER), vesicular transporters, and receptor clustering proteins. A major class of genes identified in aldicarb resistance (*ric*) screens are synaptic vesicle cycle factors located in the presynaptic terminal.

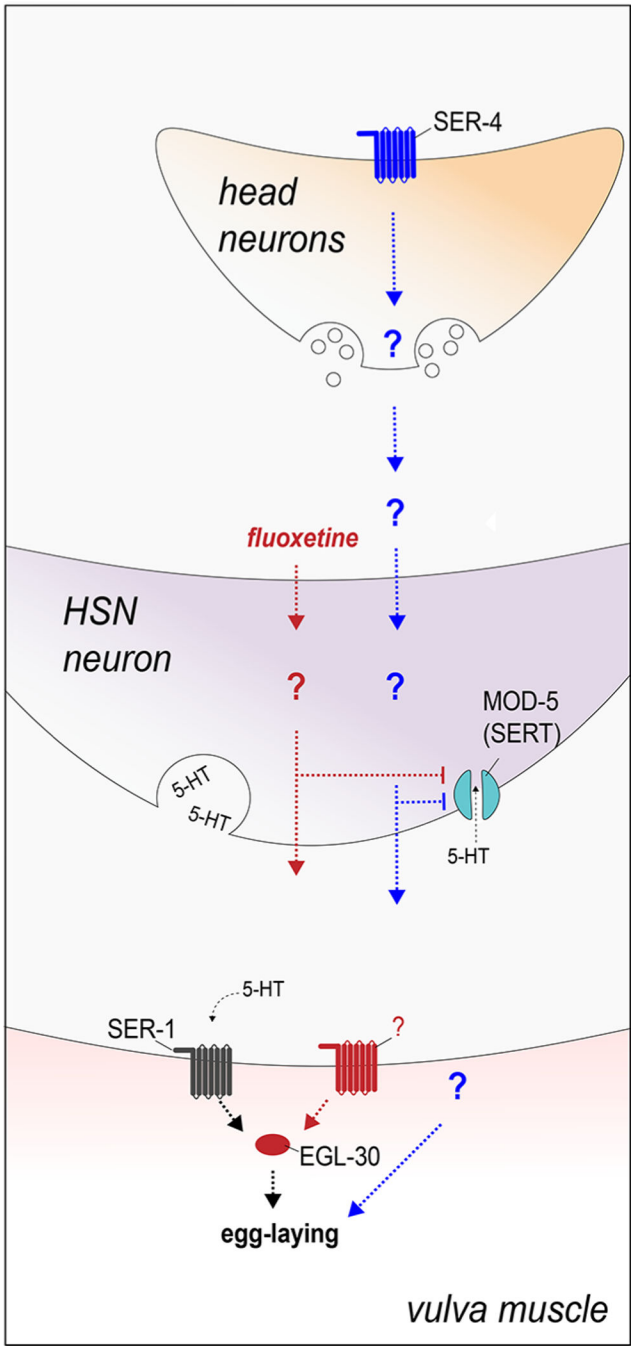

Figure 3.

Distinct pathways of fluoxetine and imipramine induced egg-laying. The imipramine pathway (blue) requires the SER-4 receptor in the head neurons and the HSN neuron to stimulate egg-laying in the vulva muscle, but its intermediate effectors and interneuron linking signals from the head neurons have not been determined. The fluoxetine pathway (red) also requires the HSN neuron and signals parallel to the 5-HT receptor SER-1, converging on EGL-30, through unknown receptor(s). In addition, both drugs inhibit the *C. elegans* SERT homologue MOD-5.

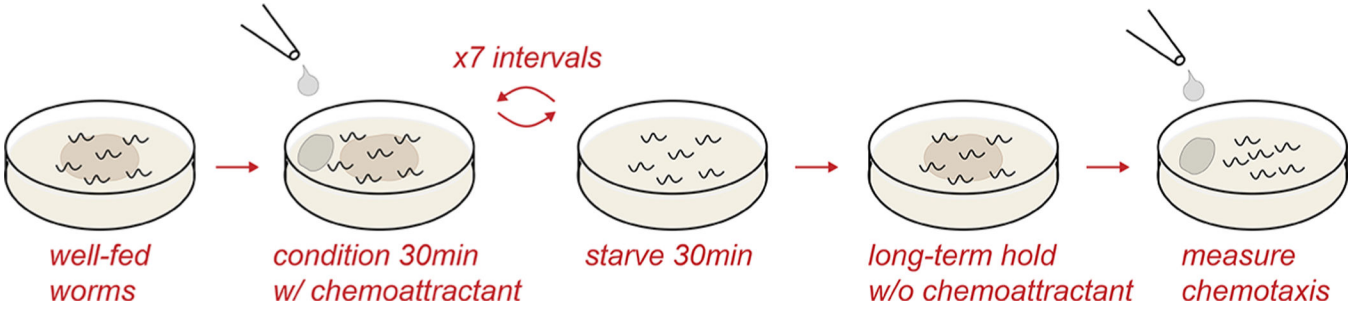

Figure 4.

Long-term associative memory (LTAM) assay scheme. In these experiments, worms are "trained" by alternating exposure to a chemoattractant in the presence of food and starvation over a number of intervals. The trained worms are cultured on plates with food lacking the chemoattractant for a long period and subsequently assayed for improved taxis toward the chemoattractant.

Table 1.

List of Synaptic Transmission Genes Identified in Levamisole and Aldicarb Resistance Screens[a]

A. Major Levamisole-Resistance Genes			
molecular function	***C. elegans* gene**	**identity/homology**	**Selected reference**
postsynaptic nAChR receptor subunits	*lev-1*	non-α-nAChR subunits	9
	unc-29		9
	unc-63	α-nAChR subunits	10
	unc-38		9
	lev-8		12
nAChR assembly, transport, and regulation	*lev-9*	SUSHI domain containing protein	15
	lev-10	CUB domain containing protein	14
	lev-11	tropomyosin homologue	22
	unc-50	golgi membrane protein	16a
	unc-74	thioredoxin	17
	ric-3	transmembrane and coiled-coil domain protein	20
	oig-4	secreted single IG-domain protein	24
	emc-6	endoplasmic membrane protein complex subunit 6	25
	molo-1	levamisole AChR auxiliary factor	26
regulation of muscle contraction	*unc-22*	Titin homologue	97
B. Select Aldicarb Sensitive Genes			
molecular function	*C. elegans* gene	homologues	Selected reference
synaptic vesicle cycle	*unc-13*	synaptic priming factor (mUNC13)	98
	snt-1	synaptotagmin 1 (SYT1)	31
	unc-18	syntaxin binding protein 1 (STXBP1)	99
	unc-11	Adaptor protein (AP180)	34
	ric-4	synaptosome associated protein 25 (SNAP25)	100
	unc-104	kinesin 1A (KIF1A)	101
	unc-10	Rab3 interacting molecule (RIM)	102
	aex-3	rab-3 guanine exchange factor	103
	unc-64	syntaxin 1A (STX1A)	104
	unc-26	synaptojanin 1 (SYNJ1)	36
	unc-31	calcium-dependent secretion activator (CAPS)	105
	unc-41	stonin 2 (STN2)	106
mRNA splicing factor	*unc-75*	CUGBP Elav-like family member (CELF)	107
Ach biosynthesis and transport	*unc-17*	vesicle acetylcholine transporter (VAChT)	28
	cha-1	choline O-acetyltransferase (CHAT)	108
regulation of Ach transmission	*egl-10*	regulator of G protein signaling 7 (RGS7)	109
	egl-30	G protein subunit alpha q (GNAQ)	110
	unc-2	calcium voltage-gated channel subunit(CACNA1A)	111
	ric-8	guanine nucleotide exchange factor (RIC8A)	112
	ric-1	MFSD6	113
	cpx-1	complexin	40a

A. Major Levamisole-Resistance Genes			
molecular function	***C. elegans* gene**	**identity/homology**	**Selected reference**
	tom-1	tomosyn	41a

[a] *unc* = uncoordinated, *lev* = levamisole resistant, *ric* = resistant to aldicarb, nAChR = nicotinic acetylcholine receptor, *emc* = endoplasmic membrane protein complex, *oig* = one IG domain, *molo* = modulator of levamisole-sensitive receptor, *snt* = synaptotagmin, *aex* = Aboc EXpulsion defective, *cha* = choline O-acetyltransferase, *egl* = egg-laying defective, *cpx* = complexin, *tom* = tomosyn.