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Accessibility
A Genome-Wide RNAi Screen in *Caenorhabditis elegans* Identifies the Nicotinic Acetylcholine Receptor Subunit ACR-7 as an Antipsychotic Drug Target

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**Abstract**

We report a genome-wide RNA interference (RNAi) screen for Suppressors of Clozapine-induced Larval Arrest (scla genes) in *Caenorhabditis elegans*, the first genetic suppressor screen for antipsychotic drug (APD) targets in an animal. The screen identifies 40 suppressors, including the α-like nicotinic acetylcholine receptor (nAChR) homolog *acr-7*. We validate the requirement for *acr-7* by showing that *acr-7* knockout suppresses clozapine-induced larval arrest and that expression of a full-length translational GFP fusion construct rescues this phenotype. nAChR agonists phenocopy the developmental effects of clozapine, while nAChR antagonists partially block these effects. ACR-7 is strongly expressed in the pharynx, and clozapine inhibits pharyngeal pumping. *acr-7* knockout and nAChR antagonists suppress clozapine-induced inhibition of pharyngeal pumping. These findings suggest that clozapine activates ACR-7 channels in pharyngeal muscle, leading to tetanus of pharyngeal muscle with consequent larval arrest. No APDs are known to activate nAChRs, but a number of studies indicate that α7-nAChR agonists may prove effective for the treatment of psychosis. α7-like nAChR signaling is a mechanism through which clozapine may produce its therapeutic and/or toxic effects in humans, a hypothesis that could be tested following identification of the mammalian ortholog of *C. elegans acr-7*.

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**Introduction**

Treatment of psychotic disorders has been hampered by the limited efficacy of currently available APDs and the toxic side effects of these drugs [1]. Clozapine is the most effective medication for treatment-refractory schizophrenia but produces toxic side effects such as agranulocytosis, metabolic syndrome, and developmental defects after exposure early in life [2–4]. The molecular mechanisms underlying the therapeutic and toxic side effects of clozapine and other APDs remain poorly understood [5,6]. A better understanding of these mechanisms could facilitate the design of superior drugs and may inform our understanding, not only of drug mechanisms, but also of disease pathogenesis. For example, studies of antidepressant drug mechanisms have provided new insight into the important role of neurogenesis in depression itself [7,8].

The genetic tools of invertebrate models offer new paradigms for drug discovery in schizophrenia [9,10]. Pharmacogenetic experiments in *Caenorhabditis elegans* identify novel and important signal transduction pathways through which APDs exert their biological effects [11–14]. Large-scale genetic screens in *C. elegans* provide an unbiased approach to discover genetic targets of APDs, and such experiments are not possible in knockout or transgenic mice. Using such an unbiased approach, we identified a potential APD target with homology to human brain receptors.

We found that loss-of-function mutations in *acr-7*, which encodes a homolog of the human α7-like nAChRs, suppressed both developmental and behavioral effects of clozapine. Our results indicated that clozapine likely activated the ACR-7 receptor, a novel observation that may be relevant to clinical drug effects. No known APDs have been previously demonstrated to activate nAChRs, and whether APDs might act by this mechanism is an important question. Recent studies in humans have underscored the potential importance of nAChRs in the pathophysiology of schizophrenia owing to: (1) the abnormal expression of nAChRs in post-mortem brains of schizophrenic patients; (2) the striking association between smoking (a nicotine delivery system) and schizophrenia; (3) the improved cognition seen in animals and humans with nicotine exposure; (4) the genetic association between the human α7-nAChR and schizophrenia; and (5) the potential efficacy of nAChR agonists as treatments for schizophrenia [15–17].
Author Summary
Clozapine is the most effective medication for treatment-refractory schizophrenia but produces toxic side effects such as agranulocytosis, metabolic syndrome, and developmental defects after exposure early in life. However, clozapine’s molecular mechanisms of action remain poorly understood. In past studies, we showed that pharmacogenomic experiments in C. elegans identify novel signaling pathways through which clozapine exerts its biological effects. Here, we report the first genetic suppressor screen for antipsychotic (APD) drug targets in an animal and identify 40 suppressors of clozapine-induced larval arrest, including the α7-like nicotinic acetylcholine receptor (nAChR) acr-7. We validate our RNAi result by showing that an acr-7 knockout suppresses clozapine-induced larval arrest and inhibition of pharyngeal pumping. Expression of a full-length translational acr-7:GFP (Green Fluorescent Protein) constructs in the acr-7 mutant rescues suppression of these phenotypes. Clozapine-induced phenotypes are phenocopied by nAChR agonists and blocked by nAChR antagonists. The results suggest that clozapine induces these phenotypes through activation of the ACR-7 receptor. Recent studies have underscored the potential importance of nAChRs in the pathophysiology of schizophrenia. A clearer understanding of APD mechanisms would facilitate the design of improved drugs and may inform our understanding, not only of drug mechanisms, but also of disease pathogenesis.

Results
A chemical genetic screen for APD targets
To identify novel molecular mechanisms of action of APDs, we conducted a genetic suppressor screen for APD targets in an animal [18]. APDs cause larval arrest or developmental delay in C. elegans (Figure 1B). APDs inhibit pharyngeal pumping [12,19,20], indicating that the developmental phenotype has a neuromuscular basis. To identify potential APD targets, we performed a genome-wide RNAi screen for Suppressors of Clozapine-induced Larval Arrest (scla genes). Mutants that escaped clozapine-induced larval arrest grew to adulthood and were easily detected under a dissecting microscope (Figure 1B, [21]). The experimental design is outlined in Figure 1A and involved a primary RNAi screen in liquid culture followed by tripticate testing in agar wells. To ensure adequate knockdown of potential targets, we exposed animals to two rounds of RNAi and tested progeny in the second generation. We also employed the N24256 rrf-3(pk1426) II strain, which is hypersensitive to RNAi and which improves detection of genes with postembryonic mutant phenotypes, to maximize recovery of neuronal genes [22].

Of the 19,968 wells we tested in the primary screen, 1,375 wells or 6.9% displayed suppression of clozapine-induced larval arrest. magi-1, a membrane-associated guanylate kinase gene, and sec-8, an exocyst complex gene, were each identified as positives twice in the primary screen. Subsequent testing of primary screen positives in tripticate identified 40 candidate suppressors, constituting 0.2% of the total wells screened (Table 1). To rule out false positives, we collected viable knockout mutants corresponding to our RNAi suppressors and tested them for suppression of clozapine-induced larval arrest. These knockouts included strains acr-7(tm863), cep-1(op347), cep-1(gk138), cep-1(gk2501), gtl-2(n2618), gtl-2(tm1463), ina-1(gk139), ins-22(ok3616), bnn-6(ok1119), magi-1(gk157), puf-6(ok3044), scla-1(tm4534), scla-1(bw4806), and sms-1(ok2399).

Suppression of clozapine-induced larval arrest was seen in 70% of the RNAi screen positives when tested by knockout rather than by RNAi knockdown, including acr-7, gtl-2, ina-1, magi-1, puf-6, sms-1, and scla-1. The cep-1, ins-22, and bnn-6 knockout strains failed to suppress clozapine-induced larval arrest, suggesting that these suppressors were false positives. Alternatively, suppression may occur in these three cases with partial loss-of-function by RNAi knockdown, but not with complete loss-of-function by knockout.

Chemicals such as paraquat are commonly used to study the genetics of stressor-induced developmental delay or lethality in C. elegans. We asked whether genes recovered in our RNAi screen suppress the effects of stressors, including paraquat, DTT, SDS, and tunicamycin [23–26]. Tunicamycin up to 10 μg/ml and DTT at 5 mM produced no or mild developmental delay in N2 animals (data not shown). More robust effects were seen with 0.01% SDS or 0.2–0.5 mM paraquat (Figure S1). Developmental delay in N2 animals was compared with that in scla-1(ok2399), gtl-1(hx546), scla-1(tm4806), scla-1(tm4534), and acr-7(tm063) mutant strains. None of these mutant strains grew more rapidly in the presence of SDS or paraquat than N2 animals, suggesting that these mutations did not act by suppressing stressor effects (Figure S1). Rather, the suppression seen with these mutations appeared to be specific for the developmental effects of clozapine. Stressor-induced lethality was also scored, and, similar to the results of the developmental delay assay, no suppression was seen with these mutations (data not shown).

The functional classes of the suppressors revealed that a more diverse group of genes was required for the biological effects of clozapine than had been previously appreciated. A fraction of these genes likely suppressed clozapine-induced larval arrest via indirect effects. However, molecules involved in signal transduction significantly outnumbered those in other functional classes, consistent with the idea that clozapine’s developmental effects have a neuromuscular basis (Figure 2, [12,19]). Moreover, the fraction of signal transduction genes from our RNAi screen was higher than that from a screen for all genes with RNAi phenotypes in C. elegans [27]. This difference indicated that our dataset was specific to the effects of clozapine.

acr-7 sequence and homology
We sought to understand the molecular mechanisms of action of the RNAi suppressors in detail and chose to focus initially on acr-7. The rationale for focusing on acr-7, as opposed to other new candidate APD targets, included potential relevance to psychotic disorders [15–17], probability of a human ortholog [28,29], and potential for physiological characterization [30]. Blasting the ACR-7 protein sequence against the human genome using Wormbase BLAT revealed that ACR-7 is homologous to a number of human α7-like nAChRs, but which human gene is the true acr-7 ortholog is not known. Homology comparisons with the human α7-nAChR showed 36% identity overall and greater identity within the first two predicted transmembrane domains of the ion channel (Figure 3A). The pore-lining transmembrane domain II (M2) and neighboring residues play an important role in the ion selectivity filter of nAChRs. Specifically, substitution of the glutamate residue immediately preceding M2 or the glutamate residue at the extracellular mouth of the pore significantly reduces the calcium permeability of various nAChR subunits [31]. We noted that both glutamate residues are conserved in ACR-7, suggesting that, like the human α7-nAChR, ACR-7 is highly permeable to calcium. Our attempts to obtain voltage-clamp recordings of ACR-7 expressed in heterologous systems, including Xenopus laevis oocytes and cultured HEK293T and PC12 cells,
failed, consistent with the possibility that ACR-7 is a subunit of a heteromeric channel [32,33].

Knockout of acr-7 suppressed clozapine-induced larval arrest

We tested growth effects over a range of clozapine concentrations (40–320 μM). N2 animals grown in the presence of 0.1% DMSO alone reached adulthood in 3 days (Figure 1B and Figure 4A). Consistent with our previous study [12], clozapine inhibited the development of N2 animals in a dose-dependent fashion. For example, 98% of control animals were gravid adults on day 3, compared to 36% on 80 μM clozapine and 0% on 320 μM clozapine (Figure 1B and Figure 4A).

The acr-7(tm863) allele is a 625 bp out-of-frame deletion with a 7 bp insertion, which removes exons 6–7 and most of exons 5 and 8 (Figure 3B) and lacks all four transmembrane domains (Figure 3C). Therefore, acr-7(tm863) is predicted to be a null allele. We backcrossed the acr-7(tm863) allele to the wild-type strain six times to generate the strain EAB200, which, like animals exposed to acr-7(RNAi) (Figure 1B), displayed normal development and normal adult morphology. On 320 μM clozapine, 95% of acr-7(RNAi) animals in 0.1% DMSO alone displayed normal development. acr-7(RNAi) suppressed developmental delay caused by 320 μM clozapine. (B5) The acr-7(tm863) knockout suppressed developmental delay caused by 320 μM clozapine. Note that experiments depicted in (B) were performed on NGM plates, not in liquid culture, to allow clear photographs.

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Developmental effects of nAChR agonists and antagonists

To determine the mechanism of action of clozapine, we tested the nAChR agonists nicotine and levamisole [29] and found that they inhibited growth in a dose-dependent fashion, phenocopying the effects of clozapine. Only 3% of N2 animals grew to young adults or beyond by day 3 on 1 mM nicotine, compared to 100% of control animals (Figure 4B). On 10 mM levamisole, all N2 animals were arrested at the L1–L2 stages on day 3 (Figure 4B).

Similar to its suppression of clozapine-induced developmental delay, the acr-7(tm863) mutation suppressed nicotine-induced developmental delay (Figure 4C). This suppression was partially rescued in acr-7(tm863) II mchEx40 animals (Figure 4C). We also found that clozapine-induced larval arrest was blocked by the nAChR antagonists d-tubocurarine (d-TC) and dihydro-beta-erythroidine (DHβE) [29]. In 320 μM clozapine alone, 96% of N2 animals were arrested at the L1–L2 stages on day 3, compared to 25% in the presence of 500 μM d-TC and 41% in the presence of 500 μM DHβE (Figure 4D). N2 animals displayed normal development in the presence of d-TC or DHβE alone, consistent with the normal development seen with acr-7 knockdown or knockout (Figure 4D). Taken as a whole, our results were consistent with the notion that acr-7 encodes a nAChR and that clozapine activates ACR-7. Suppression of the developmental
# Table 1. Summary of *scla* genes recovered in genome-wide RNAi screen.

<table>
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<th>Sequence Name</th>
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<th>Functional Group</th>
<th>Brief Description</th>
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<th>Mm</th>
<th>Dm</th>
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<td>MAP1LC3A</td>
<td>Map1lc3a</td>
<td>ATG8</td>
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<tr>
<td>ZK896.6</td>
<td>clec-187</td>
<td>Cell surface</td>
<td>C-type lectin</td>
<td>+++</td>
<td>MRC2</td>
<td>Mrc2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genes were listed alphabetically by sequence name, followed by their locus, functional group, brief description, and suppression score. *Hs*, *Mm*, *Dm*, and *Sc* refer to human, mouse, fly, and yeast, respectively. Homologs were found by searching the Wormbase and NCBI databases. In some cases, sequences were aligned with ClustalW to confirm assignments. The suppressor *acr-7* (in bold) is homologous to a number of human α-like nAChRs.

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effects of clozapine and nicotine are the first phenotypes to be identified in acr-7 mutants [34].

Mutations in cha-1 or unc-17 failed to suppress clozapine-induced larval arrest

Our results did not exclude the possibility that clozapine might activate ACR-7 by triggering release of acetylcoline (Ach). If clozapine does so, then mutations in genes required for Ach release should have suppressed clozapine-induced developmental delay. We tested two alleles of the choline acetyltransferase gene cha-1 and two alleles of the synaptic vesicle Ach transporter gene unc-17 for suppression of clozapine-induced developmental delay [35,36]. Although both genes are required for Ach release, we found that mutations in cha-1 or unc-17 were not suppressors, suggesting that clozapine did not cause developmental delay by stimulating Ach release (Figure 6).

acr-7 was expressed in pharyngeal muscle

We generated transgenic lines expressing a Pacr-7::GFP transcriptional fusion construct or a Pacr-7::acr-7::GFP translational fusion construct. In both cases, GFP was strongly expressed in the pharyngeal muscles of transgenic animals (Figure 7A). Using pharyngeal pumping and fluorescent bead assays, we previously showed that clozapine caused dose-dependent inhibition of pharyngeal pumping [12]. Inhibition of pharyngeal pumping causes larval arrest in C. elegans [37]. Thus, clozapine likely delayed development, in part, by inhibiting pharyngeal pumping.

What is the mechanism by which clozapine inhibited pharyngeal pumping? The C. elegans pharynx receives cholinergic innervation from three types of pharyngeal motor neurons: M1, M2, and M5 [38]. Nicotine inhibited pharyngeal pumping by causing tetanic contraction of pharyngeal muscle [37]. Therefore, we hypothesized that clozapine or the biologically active clozapine metabolite N-desmethyloclozapine [39] activated pharyngeal muscle ACR-7 channels, causing tetanus with consequent larval arrest. We further hypothesized that acr-7(tm863) and acr-7(RNAi) loss-of-function mutations blocked activation by clozapine and thereby suppressed pharyngeal paralysis and larval arrest.

Knockout of acr-7 suppressed clozapine-induced inhibition of pharyngeal pumping

Clozapine inhibited pharyngeal pumping of wild-type animals in a concentration-dependent manner. On average the pumping rate in the absence of clozapine was 112.7±1.4 pumps/20 sec, whereas the pumping rate in the presence of clozapine was 59.0±4.0 pumps/20 sec (160 µM), 34.4±3.7 pumps/20 sec (320 µM), and 16.8±2.5 pumps/20 sec (500 µM) (Figure 7B, P<0.0001, unpaired t-test). The pharyngeal pumping rate of acr-7(tm863) mutants was slightly decreased compared to wild type. The mean pumping rate of acr-7(tm863) mutants in the absence of drug was 103.1±1.2 pumps/20 sec (Figure 7B, P<0.05, unpaired t-test). That the acr-7(tm863) mutant displays a relatively normal pumping rate argues against a model in which acr-7 is required for pumping and clozapine inhibits this function. However, the acr-7(tm863) deletion suppressed clozapine-induced inhibition of pharyngeal pumping. While the mean pumping rate at 500 µM clozapine was 16.8±2.5 pumps/20 sec for N2 animals, the rate for acr-7(tm863) mutants was 40.9±2.9 pumps/20 sec (Figure 7B, P<0.0001, unpaired t-test). Compared to acr-7(tm863) mutants, acr-7(tm863) mchEx40 animals were partially rescued for this

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**Figure 2. Functional diversity of scla genes.** Genes were classified by biological process based on Gene Ontology terms from Wormbase. The percentages for each functional class of genes required for suppression of clozapine-induced larval arrest are shown. doi:10.1371/journal.pgen.1003313.g002
suppression with a mean pumping rate of 29.3 ± 2.9 pumps/20 sec at 500 μM clozapine (Figure 7B, *P*<0.01, unpaired t-test).

In order to test whether ACR-7 acts specifically in pharyngeal muscle to affect pumping, we generated transgenic lines expressing *Pmyo-2::acr-7* or *Pmyo-2::acr-7::GFP* translational fusion constructs. *myo-2* encodes a myosin heavy chain gene expressed exclusively in pharyngeal muscle. *acr-7(tm863)* animals, expressing the *Pmyo-2::acr-7* transgene, were rescued for suppression of clozapine-induced inhibition of pharyngeal pumping with a mean pumping rate of 17.3 ± 1.4 pumps/20 sec at 500 μM clozapine (Figure 7B, *P*<0.001, unpaired t-test). *acr-7(tm863)* *mchEx207* animals were also rescued for suppression of clozapine-induced developmental delay (Figure 4A). *acr-7(tm863)* *mchEx211* animals, expressing the *Pmyo-2::acr-7::GFP* transgene, displayed strong fluorescence in the pharyngeal muscles and were rescued for both the pharyngeal pumping and developmental phenotypes. Thus, *acr-7* was required in pharyngeal muscle for clozapine-induced inhibition of pharyngeal pumping and for clozapine-induced developmental delay.

Nicotine and levamisole phenocopied clozapine’s inhibition of pharyngeal pumping. The mean pumping rate of N2 animals was 58.1 ± 2.9 pumps/20 sec in 2 mM nicotine (Figure 7C, *P*<0.0001, unpaired t-test) and 0.0 pumps/20 sec in 300 μM levamisole (data not shown). The *acr-7(tm863)* knockouts were also rescued for nicotine-induced developmental delay.

Figure 3. ACR-7 protein sequence alignment and *acr-7* gene structure. (A) The *C. elegans* ACR-7 protein and human α7-nAChR subunit CHRNA7 were aligned using ClustalW. The four putative transmembrane domains, as predicted for the human CHRNA7 protein, were boxed. nAChRs are members of the cyst-loop family of ionotropic neurotransmitter receptors, and this conserved di-cysteine loop is dotted line-boxed. α-nAChR subunits contain a pair of vicinal cysteines within the Ach binding site, and these residues are marked with asterisks. The *C. elegans* and human proteins share 36% identity overall and 41% identity within the pore-lining transmembrane domain II (M2). (B) The *acr-7* gene contains 13 exons, and the region deleted in the *tm863* allele is marked with a line. *tm863* is an out-of-frame deletion which removes exons 6–7 and most of exons 5 and 8. (C) The *tm863* knockouts are predicted to lack all four transmembrane domains and to be a null allele.
not shown). d-TC alone did not change the pumping rate in wild-type C. elegans, similar to the relatively normal pharyngeal pumping rate seen with acr-7 knockout. However, d-TC did block inhibition of pharyngeal pumping by either clozapine or nicotine. The mean pumping rate of wild-type animals in 500 μM clozapine was 25.3 ± 3.1 pumps/20 sec (Figure 7C, P<0.0001, unpaired t-test), compared to 69.5 ± 4.9 pumps/20 sec with co-application of 500 μM d-TC (Figure 7C, P<0.0001, unpaired t-test). The mean pumping rate of wild-type animals in 2 mM nicotine was significantly increased to 68.5 ± 3.7 pumps/20 sec with co-application of 500 μM d-TC, compared to wild-type animals in 2 mM nicotine alone (Figure 7C, P<0.05, unpaired t-test). However, d-TC failed to block the residual inhibition of pharyngeal pumping produced by either clozapine or nicotine in acr-7(tm863) mutants, indicating that acr-7 knockout occludes the effect of d-TC (Figure 7D). These results suggested that clozapine and nicotine inhibit pharyngeal pumping, in part, by activating ACR-7 receptors in the pharynx.

**Discussion**

Clozapine exposure during adulthood modulates several C. elegans behaviors, such as enhancement of egg-laying [11] and inhibition of pharyngeal pumping [12,19] and locomotion. In addition, clozapine exposure during early development produces larval arrest in C. elegans [12,20], an effect that may relate to human developmental abnormalities associated with clozapine treatment during pregnancy [20,40]. High drug concentrations...
are required for penetration of the *C. elegans* cuticle [41], but we used HPLC to show that clozapine levels required to produce larval arrest in *C. elegans* (~18 μg/ml) [12] are close to those expected in brains of human patients treated with clozapine (~11 μg/ml) [42,43]. We employed clozapine-induced developmental delay to perform a genome-wide RNAi screen in *C. elegans* for *sca* genes and identified 40 candidate suppressors of clozapine-induced larval arrest. Several suppressors, such as *magi-1*, *lin-12*,

**Figure 5. Suppression by acr-7(lf) is specific.** A series of 22 nAChR mutants were tested for suppression of clozapine-induced larval arrest, and only *acr-7(lf)* produced robust suppression.

**Figure 6. Ach release was not the mechanism of clozapine-induced larval arrest.** Mutations in genes required for Ach release failed to suppress clozapine-induced developmental delay. Strong loss-of-function of the choline acetyltransferase gene *cha-1* or the synaptic vesicle Ach transporter gene *unc-17* alone produced developmental delay. Therefore, both weak (*n2411* or *e113*) and strong (*p1152* or *e245*) loss-of-function alleles were tested.
and \textit{ma-1}, have human orthologs implicated in the pathogenesis of schizophrenia but have not been previously shown to mediate the biological effects of an APD [44–46].

The molecular mechanisms of action of human disease genes underlying psychotic disorders are largely unclear. The identification of candidate APD targets in a genetic model organism with a nervous system afforded us the opportunity to analyze the mechanism of action of one such target in detail. However, elucidating the mechanisms underlying APD modulation of ACR-7 cannot fully explain the effects of APDs on \textit{C. elegans}. Several lines of evidence indicate that clozapine-induced larval arrest involves multiple genetic pathways. First, the number and diversity of suppressors to emerge from our screen suggest that they may not constitute a single pathway. Second, clozapine’s developmental- and behavioral effects in \textit{C. elegans} are partially, not fully, suppressed by \textit{acr-7(lf)} (Figure 4A and Figure 7B). Third, other pathways, such as the insulin signaling pathway, are known to be involved [11,12,14]. Previously, we used a candidate gene approach to show that mutations in the \textit{C. elegans} insulin/IGF receptor ortholog \textit{daf-2} and its downstream effector the phosphatidyl inositol 3-kinase (PI3K) \textit{age-1} suppressed clozapine-induced larval arrest. Subsequent work confirmed the discovery that clozapine activates insulin signaling in \textit{C. elegans} and showed that other APDs do so as well [13,14]. Of note, \textit{acr-7} and \textit{age-1} may

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Figure 7. ACR-7 acted in the pharyngeal muscle. (A) ACR-7 was highly expressed in the pharynx as indicated by the arrows. A, anterior; P, posterior; D, dorsal; V, ventral. Scattered expression was also observed in the tail and vulval regions. (B) Clozapine inhibited pharyngeal pumping in wild-type animals in a concentration-dependent manner. Suppression of clozapine-induced inhibition of pharyngeal pumping was seen in \textit{acr-7(tm863)} animals, and rescue was observed with expression of ACR-7 in the mutant background. A putative \textit{acr-7} promoter was utilized to drive expression for the \textit{acr-7(tm863)} \textit{mchEx40} strain, whereas the pharyngeal muscle-specific \textit{myo-2} promoter was utilized for the \textit{acr-7(tm863)} \textit{mchEx207} strain. (C) Both nicotine and clozapine inhibited the pumping rate in N2 animals. d-TC partially blocked these effects. Importantly, d-TC alone had no effect on pumping. (D) d-TC failed to block clozapine- or nicotine-induced inhibition of pumping in \textit{acr-7(lf)} animals. * \textit{P} < 0.05; ** \textit{P} < 0.01; *** \textit{P} < 0.0001. doi:10.1371/journal.pgen.1003313.g007
Furthermore, a translational suppressed clozapine-induced inhibition of pharyngeal pumping. PLOS Genetics | www.plosgenetics.org 10 February 2013 | Volume 9 | Issue 2 | e1003313

Developmental delay required behavioral effects of clozapine. Similarly, nicotine-induced developmentally delayed larval arrest [49,50]. To clarify the mechanism whereby clozapine modulates ACR-7, we undertook a series of genetic, pharmacological, developmental, and behavioral studies in C. elegans. C. elegans ACR-7 and Antipsychotic Drug Effects

Although some RNAi suppressors may act indirectly, some may have human homologs that directly mediate either therapeutic or toxic effects of clozapine. Based on the fact that cha-1([q]) and unc-17([q]) alleles failed to suppress clozapine-induced larval arrest, we hypothesized that clozapine does not stimulate Ach release, but rather that clozapine modulates ACR-7 directly. Particularly in whole-organism studies, direct effects can be pharmacologically complex, involving activation, inhibition, or a combination of the two. For instance, clozapine acts as an agonist at the human muscarinic M1 receptor, while its biologically active metabolite, N-desmethylclozapine, acts as an antagonist [39,48]. Such pharmacological complexity is relevant in the case of nAChRs specifically, where, for example, addition of a methyl group at the amine moiety of cocaine changes that drug’s profile from noncompetitive antagonist to agonist [49]. To clarify the mechanism whereby clozapine modulates ACR-7, we undertook a series of genetic, pharmacological, developmental, and behavioral studies in C. elegans.

We showed that that acr-7 mutants suppressed clozapine-induced developmental delay and that a full-length translational Pacr-7::acr-7::GFP fusion construct rescued this effect. acr-7 was strongly expressed in the C. elegans pharynx, and acr-7 knockout suppressed clozapine-induced inhibition of pharyngeal pumping. Furthermore, a translational Pmyo-2::acr-7 fusion construct rescued suppression of the developmental delay and pharyngeal pumping phenotypes, indicating that ACR-7 acts specifically in pharyngeal muscle cells to mediate these phenotypes. These results suggested that clozapine activates ACR-7 to inhibit pharyngeal pumping and, thereby, produce larval arrest. Consistent with this model, nAChR antagonists phenocopied both the developmental and behavioral effects of clozapine. Similarly, nicotine-induced developmental delay required acr-7. However, acr-7(tm063) did not block nicotine-induced inhibition of pharyngeal pumping, suggesting that nicotine may act through multiple nAChRs to inhibit pharyngeal pumping (Figure 7C and 7D).

Also consistent with our model, nAChR antagonists suppressed clozapine-induced developmental delay and clozapine-induced inhibition of pharyngeal pumping. The results indicated that, as predicted on the basis of sequence information, acr-7 encodes a nAChR. Suppression of the effects of nicotine and clozapine on pharyngeal pumping and development are the first phenotypes to be identified in acr-7 mutants [54]. As noted above, clozapine’s effects at the whole-organism level may be pharmacologically complex, involving activation, inhibition, or both, but the simplest model to explain our results is that clozapine activates ACR-7. Thus, our data pointed to a novel finding - the existence of nAChRs that are activated by clozapine and/or the biologically active clozapine metabolite N-desmethylclozapine [39].

A wealth of evidence has implicated nAChR expression and function in general, and the α7-nAChR in particular, in the pathogenesis of schizophrenia [15–17,50], a psychotic disorder for which clozapine appears to be the most effective medication [2–4]. First, decreased nAChR expression has been reported in a number of brain regions of schizophrenics [51–54]. Second, schizophrenics smoke more frequently than the general population, and nicotine exposure has been shown to improve cognition in animals and humans. Therefore, increased smoking in schizophrenics may represent an attempt to self-medicate for cognitive deficits associated with the disease [50]. Third, genetic studies have linked the human α7-nAChR gene (CHRNA7) to defective gating of the P50 auditory evoked response, which is related to attentional abnormalities in schizophrenia [17]. Finally, α7-nAChR agonists are currently in development for the treatment of psychosis, although no known APDs have been demonstrated to act by this mechanism [16].

Previous reports have suggested that clozapine inhibits the function of α7-nAChRs [55,56]. However, inhibition may be specific to the background of the receptor or to species-specific sequence differences [29,57,58]. Moreover, our RNAi screen was designed to identify targets activated by clozapine, since we screened for suppression of the drug’s effects by gene knockdown. Interestingly, clozapine improved a schizophrenia-like endophenotype in mice via stimulation of α7-nAChRs, although this effect was hypothesized to be indirect [59].

C. elegans has at least 29 different nAChR subunits grouped into five subfamilies based on homology: DEG-3-like, UNC-38-like, ACR-8-like, UNC-29-like, and ACR-16-like nAChRs [28,29]. ACR-7 is a member of the ACR-16-like group of nAChR subunits, a group that shows homology to the vertebrate α7-10 nAChRs [28,29]. Importantly, we do not know which human α7-like subunit is the ACR-7 ortholog. For example, although acr-7 showed homology to the vertebrate α7-10 nAChRs, a mouse α4β2 nAChR transgene driven by the acr-16 promoter rescued nicotine-dependent behaviors in the acr-16 mutant background, while a mouse α7 nAChR transgene did not [60].

Mammals have at least 17 different nAChR subunits [28,30], and these nAChRs may assemble in a wide variety of functional combinations [31,61]. Of these, nine different α7-nAChR subunits have been cloned from mammalian tissues to date and have been shown to form homopentamers or heteropentamers [30,61]. Effects of APDs on the spectrum of nAChR subunit combinations have not been tested [62–63]. Therefore, whether APDs can activate one or more of these receptors is an important question, and finding such an interaction would constitute an important breakthrough in APD drug development.

In summary, we conducted the first genetic suppressor screen for APD targets in an animal. We then used the experimental advantages of C. elegans neurobiology and neurogenetics to delineate the novel molecular mechanism of action of a specific
APD target. α-like nAChR signaling is a mechanism through which clozapine may produce its therapeutic and/or toxic effects in humans. This project was designed to translate basic findings in C. elegans into clinical applications. Identification of new mechanisms underlying the actions of APDs may lead to changes in both concepts and better-targeted treatments in psychiatric neurobiology.

Materials and Methods

Strains

We used the C. elegans Bristol strain N2 as the wild-type parent of our mutant strains and grew nematodes under standard culture conditions. Strains were maintained at 20°C. The mutant strains used in our experiments are provided in Table S1. The age-1(he3346) II mutation in strain TJ1052 had been backcrossed to the N2 strain three times, and we performed another three backcrosses to generate strain EAB100. The presence of the mutation was followed by restriction digest and confirmed by DNA sequencing. We backcrossed the following mutations to the N2 strain six times to generate strains: EAB1 sns-1(ok2399) IV, EAB101 scla-1(tm4806) IV, EAB102 scla-1(tm4534) IV, EAB103 ins-22(ok3616) III, EAB200 acr-7(m863) II, and EAB201 acr-16(ok789) V from strains RB1854, F22B3.8(tm4806), F22B3.8(tm4534), RB2594, FX863, and RB918, respectively. The presence of the deletions was followed by PCR.

RNAi screen

We employed an RNAi feeding library (Geneservice Ltd), constructed by J. Ahringer’s laboratory at the The Wellcome CRC Institute, Cambridge, Cambridge, England, to carry out a genome-wide screen in liquid culture for suppressors of clozapine-induced larval arrest [21]. Our protocol for feeding RNAi bacteria to animals in liquid culture was adapted from Nollen et al. [2004] [66]. Each RNAi culture was grown overnight at 37°C in 500 μl of LB with 100 μg/ml of ampicillin. The cultures were induced for four hours the following day by adding 1 ml of LB containing 100 μg/ml of ampicillin and 1 mM isopropylthiogalactoside (IPTG) in a 37°C incubator. We transferred 200 μl of the induced cultures to 96-well plates and centrifuged them at 3000 rpm for 2 minutes. The supernatant was removed, and the induced bacterial pellet was resuspended in 100 μl of S-Basal. We then added 50 μl of solution containing S-Basal, 50 μg/ml ampicillin, 1 mM IPTG, and ~3–5 synchronized L1 rrf-3 animals. These animals were maintained in liquid culture at 20°C on a rotating platform for three days until they reached young adulthood. We then added 1.5 ml of clozapine stock solution (20 mg/ml clozapine powder from Sigma-Aldrich #C6305 in 100% ethanol) to each well for a final concentration of 200 μg/ml (~600 μM) clozapine. Progeny were scored for suppression of clozapine-induced larval arrest on day 8 (Figure 1A).

 Wells containing progeny that had developed to the L4 stage or beyond were scored as positives (Figure 1B). Those wells lacking progeny or containing contamination were retested. Each set included wells with dpy-6 RNAi in the absence of clozapine and blank wells in the presence of clozapine as controls.

Positive wells from the genome-wide RNAi screen in liquid culture were tested in triplicate using feeding RNAi on plates containing Nematode Growth Medium (NGM). Each set included triplicate wells of dpy-6 RNAi and unc-44 RNAi with and without clozapine as controls. Those wells lacking progeny, lacking a lawn, or containing contamination were retested. Mutants that produced suppression in at least 2/3 wells were taken to be positives, and the strength of suppression was scored: weak (+) - few progeny escaped larval arrest, medium (++)- ~25% of progeny escaped larval arrest, robust (+++)- most progeny escaped larval arrest, NA - not scored (Table 1). The identity of triplicate positives was verified by sequencing plasmid DNA (MGH DNA Sequencing Core) using primer M13 (Integrated DNA Technologies, Inc.) and then blasting those sequences against the C. elegans genome using Wormbase BLAT. Triplicate positives without preexisting gene names and validated by knockout testing were assigned to the new gene name class scla for Suppressor of Clozapine-induced Larval Arrest.

Plasmid constructions

To generate a Pacr-7::GFP transcriptional fusion construct, we PCR-amplified the putative promoter region, including 1.3-kb upstream of the acr-7 gene, using DNA prepared from adult animals and using primers listed in Table S2. This clone was digested with PsI and XmaI restriction enzymes and ligated into the GFP vector pPD95.75. To generate a Pacr-7::acr-7::GFP translational fusion construct, we PCR-amplified the same acr-7 promoter sequence along with 4.3-kb of acr-7 genomic sequence, omitting the native stop codon, using DNA prepared from adult animals and using primers listed in Table S2. This clone was digested with PsI and KpnI restriction enzymes, sub-electroporated into pCR-XL-TOPO (Invitrogen, Cat: K4700-10), and then subcloned into pPD95.75. PCR was performed using PrimeSTAR HS DNA Polymerase (Takara Bio Inc.). GFP vector pPD95.75 was obtained from the Fire Lab C. elegans Vector Kit (Addgene).

To generate the Pywo-2::acr-7 and Pywo-2::acr-7::GFP translational fusion constructs, RNA was isolated from mixed-stage worms, and acr-7 cDNA was obtained using SuperScript III First-Strand Synthesis System (Invitrogen) with primers listed in Table S2. We then cloned the cDNA into Fire Lab vectors L2531 or L3790 (Addgene) using Nhel and KpnI restriction enzymes or Sall and EagI restriction enzymes respectively. These Pywo-2 constructs were injected into EAB200 animals using the same protocol described below for injection of the Pacr-7::acr-7::GFP construct.

All plasmid constructions were confirmed by sequencing (MGH DNA Sequencing Core).

Expression analysis

We generated extrachromosomal transgenic strains by microinjecting DNA into the gonads of young adult animals as described [67]. The Pacr-7::GFP construct was injected at 25 ng/μl along with the rol-6(su1006) marker (pRF4) at 50 ng/μl into N2 animals. The Pacr-7::acr-7::GFP construct was injected at 25 ng/μl along with the Paacr-7::Dra2D2 marker (pHC183; a gift from Antony Jose of the Hunter laboratory) at 40 ng/μl into EAB200 animals. Three to four independent extrachromosomal lines were generated for both the Pacr-7::GFP and Pacr-7::acr-7::GFP constructs. ACR-7::GFP expression shown here was studied in the EAB40 strain, but similar results were obtained in studies of the EAB20-24, EAB39, and EAB41 strains. Animals were placed on a 2% agarose pad on a slide in M9 with 50 mM sodium azide, then covered with a slip and studied by DIC and fluorescence microscopy using a Zeiss Axio Image A1 microscope. Photographs were taken using a 40× objective on a confocal laser microscope (Leica TCS, Germany).

Developmental assays

Experiments were performed using Falcon 12-well plates (Becton Dickinson) containing OP50 bacteria on NGM. Clozapine, nicotine, levamisole, DHBE, and d-TC were purchased from Sigma-Aldrich. Olanzapine was purchased from Waterstone Technology. Stock solutions of each drug were prepared in
DMSO and then diluted 1:1000 in water with acetic acid (1:10,000). These diluted solutions were then pipetted onto the agar surrounding the bacterial lawn for final drug concentrations of 40, 80, 160, or 320 μM (0.1% DMSO), and the plates were left to dry overnight. On the next day, ~30 synchronized L1 larvae were loaded into each well. Animals were then scored as L1, L2, L3, L4, young adult, or gravid adult at 24, 48, 72, 96 hours.

For stressor experiments, paraquat dichloride, tunicamycin, and DL-Dithiothreitol (DTT) were purchased from Sigma-Aldrich, and sodium dodecyl sulfate (SDS) was purchased from Fisher Scientific. Stock solutions of 10% SDS, 2 M paraquat, or 0.2 M DTT in water or 2.5 mg/ml tunicamycin in DMSO were diluted in M9 and pipetted onto the agar to achieve the range of drug concentrations. On the next day, 5–10 adult animals were placed in each well of a 12-well plate. Adults were allowed to lay 25–50 eggs and then removed. Plates were scored for developmental delay and lethality each day until the next generation hatched or until all animals died.

Pharyngeal pumping assays

Drug plates were prepared as described above. Synchronized L1 larvae were grown to the L4 stage and picked 24 hours in advance of the experiments. Five adult animals were transferred into drug wells or control wells (0.1% DMSO alone). Two hours later, the number of pumps during 20 seconds was scored. Only animals on the bacterial lawn were scored.

Statistical analysis

Data were expressed as means ± S.E.M. Statistical comparisons were performed using the unpaired, two-tailed Student’s t-test and one-way ANOVA test where appropriate.

Supporting Information

Figure S1 RNAi screen data were not suppressed stressor-induced developmental delay. (A) Developmental delay seen with SDS or paraquat after 24 hours. (B) Developmental delay seen with SDS or paraquat after 48 hours. For control conditions, vehicle alone was applied to the agar. Paraquat at 0.5 mM produced significant lethality at 48 hours (data not shown).

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Author Contributions

Conceived and designed the experiments: TS LH XW BMC EAB. Performed the experiments: TS SED AO GR5 LH XW EAB. Analyzed the data: TS SED AO GR5 LH XW EAB. Wrote the paper: TS AO LH XW BMC EAB.


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