Fluoxetine-Resistant Mutants in C. elegans Define a Novel Family of Transmembrane Proteins

Robert K. M. Choy* and James H. Thomas*†‡

*Program in Molecular and Cellular Biology
University of Washington and
Fred Hutchinson Cancer Research Center
†Department of Genetics
University of Washington
Seattle, Washington 98195

Summary

Fluoxetine (Prozac) is an antidepressant that is thought to act by blocking presynaptic reuptake of the neurotransmitter serotonin. Despite widespread clinical use of fluoxetine, direct evidence for this mechanism has been difficult to obtain in vivo. We have determined that fluoxetine has an additional neuromuscular effect on C. elegans that is distinct from inhibition of serotonin reuptake. By screening for mutants resistant to this effect, we have identified seven genes. We report that two of these genes are homologous to each other and define a novel gene family that encodes over a dozen multipass transmembrane proteins. Our findings may have clinical implications for the mechanism of action of fluoxetine.

Introduction

Fluoxetine is the defining member of the selective serotonin reuptake inhibitor (SSRI) class of antidepressants (Wong et al., 1995). Clinical depression has been proposed to be caused by a deficit in serotonergic neurotransmission (for review, see Maes and Meltzer, 1995). The primary evidence for this model is that two classes of antidepressants, SSRIs and tricyclic antidepressants, inhibit the serotonin reuptake transporter both in vitro and in vivo (Hyttel, 1994; Malagie et al., 1995). This inhibition is presumed to lead to an increase in synaptic serotonin levels and a subsequent alteration in serotonin signaling that ultimately alleviates depression. However, several pieces of evidence suggest that other targets are relevant to the action of antidepressant drugs. First, tianeptine, a drug that enhances serotonin reuptake in vitro and in vivo, is also an efficacious antidepressant (Defrance et al., 1988; Mocaër et al., 1988). Second, desipramine, an inhibitor of norepinephrine reuptake, and bupropion, a drug that has no established pharmacological target at physiological concentrations, are both efficacious antidepressants (Soroko et al., 1977; Dahl et al., 1982). Third, SSRIs cause increased synaptic serotonin levels within a few hours of beginning drug therapy, yet require 2-4 weeks to be effective in reducing depression (Allain et al., 1995; Komhuber et al., 1995). Similar delays have been observed for other classes of antidepressants. Taken together, these observations raise the possibility that other targets are responsible for the ability of fluoxetine to alleviate depression.

Although fluoxetine has a more favorable side effect profile than several other classes of antidepressants, insomnia, muscle tension, nausea, and sexual dysfunction are often reported as adverse reactions by patients taking fluoxetine (Preskorn, 1995). The molecular targets that are responsible for these side effects are poorly characterized. In particular, it is unclear whether they are a result of serotonin reuptake block or the action of fluoxetine on additional targets. Fluoxetine and other SSRIs have been shown to bind and inhibit various liver cytochrome P450 isoenzymes involved in the metabolism and excretion of hydrophobic compounds (for review, see Riesenman, 1995). This demonstrates that in vivo, fluoxetine has at least one physiologically relevant interaction other than inhibition of the serotonin reuptake transporter, and it may have others that are relevant to either side effects or to the antidepressant mechanism itself.

Genetics offers an alternative to traditional approaches to identifying drug targets and characterizing mechanisms of action. By isolating mutants with an altered response to a particular drug, one can identify genes that are involved in this response. The primary advantage of such genetic screens is that they make no prior assumptions about the targets or the mechanism of action of the drug in question. C. elegans has a variety of easily assayed behaviors, many of which can be altered by pharmacological agents (Lewis et al., 1980a; Trent et al., 1983; Avery and Horvitz, 1990). We have used genetic and pharmacological analysis of C. elegans to understand better the mechanism of fluoxetine and related antidepressants. We have determined that fluoxetine has physiological effects on C. elegans that are not related to its effects on serotonin signaling, and we have identified seven genes that are involved in this nonserotonergic effect. We have cloned two of these genes and found that they encode homologous, novel transmembrane proteins expressed in the hypodermis and intestine.

Results

The Effects of Fluoxetine on C. elegans

In order to determine the effects of fluoxetine on C. elegans, we observed the behavior of wild-type adult animals bathed in solutions of fluoxetine at various concentrations. Adult animals incubated in 1.0 mg/ml fluoxetine rapidly hypercontracted their nose muscles (Figures 1A and 1B), became rigidly paralyzed, and began to lay eggs (Weinshenker et al., 1995). Ten-fold lower doses had no acute effect on nose muscles, body muscles, or egg laying (data not shown). The high doses of drug required are probably because of the impermeability of the C. elegans cuticle. The concentration of drug at the relevant target sites is unknown, but is likely to be much lower (Lewis et al., 1980a). In mammals, fluoxetine inhibits the serotonin reuptake transporter. Egg-laying
Table 1. Nrf Mutants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of Alleles</th>
<th>Chromosome</th>
<th>Other Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>nrf-1</td>
<td>4</td>
<td>III</td>
<td>Gonad migration defect, moderate resistance</td>
</tr>
<tr>
<td>nrf-2</td>
<td>1</td>
<td>II</td>
<td>None</td>
</tr>
<tr>
<td>nrf-3</td>
<td>1</td>
<td>IV</td>
<td>None</td>
</tr>
<tr>
<td>nrf-4</td>
<td>1</td>
<td>I</td>
<td>Pale eggs, no yolk, accumulation</td>
</tr>
<tr>
<td>nrf-5</td>
<td>1</td>
<td>V</td>
<td>Pale eggs, accumulates yolk, embryonic lethality</td>
</tr>
<tr>
<td>nrf-6</td>
<td>4</td>
<td>II</td>
<td>Pale eggs, accumulates yolk, embryonic lethality</td>
</tr>
<tr>
<td>ndg-4</td>
<td>4</td>
<td>III</td>
<td>Pale eggs, accumulates yolk, embryonic lethality</td>
</tr>
</tbody>
</table>

Figure 1. A Serotonin-Independent Effect of Fluoxetine on C. elegans

(A and B) Nomarski photographs of noses of wild-type adult animals (A) with no drug treatment and (B) after incubation in 1.0 mg/ml fluoxetine for 20 min. The rounded nose after fluoxetine treatment is caused by contraction of muscle fibers that run anterior-posterior. The nose muscle hypercontraction is easily observed at 50× magnification using a dissecting microscope.

(C, D, and E) Fluoxetine dose response curves of wild type and cat-4 mutant. The x axis is time in minutes; the y axis is the percentage of animals with contracted noses. cat-4 mutants are serotonin deficient and are fully sensitive to fluoxetine-induced nose contraction. Similar results were obtained with cat-1 (VMAT) mutants (data not shown). Plot lines for cat-4 mutants at 1.0 and 0.75 mg/ml fluoxetine do not continue to 40 min because cat-4 animals are hypersensitive to fluoxetine; 25%-50% of animals died before the end of the assay at these concentrations, and, therefore, scoring was abbreviated. Each plot represents the average of at least six trials of ten animals each. Error bars show SEM. Some error bars appear to be missing because they are smaller than the plot symbols.

Genetic Screen for Fluoxetine-Resistant Mutants

To investigate the mechanism of fluoxetine-induced nose muscle contraction, we carried out a genetic screen for mutants that were nose resistant to fluoxetine (Nrf). After EMS mutagenesis, we isolated 15 Nrf mutants. Genetic mapping and complementation tests determined that the 15 mutations are recessive and correspond to seven different genes (Table 1). Six of these were not previously identified, and the remaining one had not been extensively characterized (see below). In the absence of fluoxetine, the Nrf mutants exhibited no obvious neuromuscular defects, such as uncoordinated movement, failure to lay eggs, or feeding or defecation defects. Interestingly, all 15 of the mutants we isolated were only partially resistant: at high doses (1.0 and 0.75 mg/ml) of fluoxetine, a small percentage of all mutant induction by fluoxetine in C. elegans is dependent on serotonin, suggesting that inhibition of serotonin reuptake by fluoxetine is conserved in C. elegans and leads to an increase in serotonin transmission (Weinshenker et al., 1995).

To test whether nose muscle contraction caused by fluoxetine could be explained by inhibition of serotonin reuptake, we assayed serotonin transmission-deficient mutants cat-1 and cat-4. cat-1 encodes a C. elegans vesicular monoamine transporter (VMAT) that loads biogenic amines such as serotonin into synaptic vesicles (Duerr et al., 1999). The cat-1 allele we tested is an early stop mutation that completely eliminates VMAT immunostaining and is therefore presumed to be null. cat-4 is not yet cloned, but serotonin immuno-staining is completely absent in cat-4 mutants (Weinshenker et al., 1995). If fluoxetine induces nose muscle contraction by inhibiting serotonin reuptake, then these serotonin transmission-deficient mutants should be resistant to this effect. Instead, we found that both mutants were fully sensitive to nose contraction by fluoxetine (Figures 1C-1E and data not shown). Similarly, excess serotonin should have the same effect as inhibition of serotonin reuptake. Exogenous serotonin has several behavioral effects on C. elegans, demonstrating that it can reach synaptic targets (Trent et al., 1983; Avery and Horvitz, 1990; Weinshenker et al., 1995), but we found it did not cause nose contraction, even at its solubility limit (data not shown). Taken together, these results indicate that fluoxetine causes nose contraction in C. elegans by a mechanism other than inhibition of serotonin reuptake.
Fluoxetine-Resistant Mutants in *C. elegans*

Figure 2. Pharmacological Characterization of Nrf Mutants

(A and B) Time courses of fluoxetine-induced nose contraction of the wild type compared to Nrf mutants nrf-2, nrf-3, nrf-6, and ndg-4. (A) 0.5 mg/ml fluoxetine. (B) 1.0 mg/ml fluoxetine. Similar results were obtained with other Nrf mutants described in the text.

(C and D) Time courses of nose contraction induced by the SSRI antidepressant paroxetine (C) and the tricyclic antidepressant clomipramine (D) for Nrf mutants nrf-6 and ndg-4 compared to the wild type. The concentrations shown here are chosen from dose response curves of these two drugs. Similar results were obtained with other Nrf mutants described in the text. Axes, plots, and error bars are as described in the Figure 1 legend.

Fluoxetine and clomipramine were slightly less potent, but the qualitative effects of the three drugs on *C. elegans* nose muscles were indistinguishable. All Nrf mutants were resistant to nose contraction by paroxetine and clomipramine to an extent similar to their resistance to fluoxetine (Figures 2C and 2D; data not shown). These results suggest that SSRIs and tricyles have a common target in *C. elegans* that is distinct from serotonin reuptake block.

Three alleles of one Nrf gene failed to complement a mutation in ndg-4, a gene that was previously identified in a screen for resistance to lethality from the lipoxygenase inhibitor nordihydroguaiaretic acid (NDG) (Shreffler et al., 1995). We found that NDG also caused nose muscle contraction in *C. elegans* in a manner that was similar to fluoxetine and that all the Nrf mutants were resistant to this effect of NDG (data not shown). *C. elegans* has no lipoxygenase homologs, and the target of NDG in *C. elegans* has not been further characterized. These results suggest that the effects of NDG and antidepressants are mediated by a similar pathway in *C. elegans*, but it is also possible that the Nrf genes mediate sensitivity to other classes of drugs.

Because the Nrf mutants were resistant to nose contraction induced by a variety of drugs, one possibility is that they have a generally decreased sensitivity to nose contraction by pharmacological agents. For example, a general defect in drug uptake or in muscle structure should result in resistance to diverse compounds. To test this possibility, we assayed the sensitivity of Nrf mutants to levamisole (nicotinic acetylcholine receptor agonist), aldicarb (acetylcholine esterase inhibitor), and ouabain (Na⁺, K⁺-ATPase inhibitor). All three of these drugs induce nose and body muscle contraction in *C. elegans* (Lewis et al., 1980a, 1980b; Nonet et al., 1993). We found that Nrf mutants were fully sensitive to nose contraction by all three drugs, even at the lowest concentrations that were effective on wild type (Figures 3A and 3B; data not shown). In fact, we observed a slight hypersensitivity to these drugs in all Nrf mutants. These results indicate that the Nrf mutants are not resistant to fluoxetine because of a nonspecific defect in nose contraction or a general decrease in drug transport or uptake.

To determine whether defects in synaptic transmission could confer resistance to fluoxetine-induced nose muscle contraction, we tested mutants in unc-29, unc-38, and lev-1 (nicotinic acetylcholine receptors; Fleming et al., 1997), cha-1 (ACh biosynthetic enzyme; Alfonso et al., 1994), unc-17 (vesicular ACh transporter; Alfonso et al., 1993), unc-31 (CAPS, calcium activated protein for secretion; Livingstone, 1991; Walent et al., 1992), and unc-64 (syntaxin; Saifee et al., 1998). We found that at 1.0 mg/ml fluoxetine, all these mutants contracted their noses in a manner that was similar to wild-type animals (Figure 3C and data not shown, compare to Figure 2B). At 0.5 mg/ml fluoxetine, these mutants all had weak defects in fluoxetine-induced nose contraction but, in general, were distinguishable from the Nrf mutants that we isolated (Figure 3D and data not shown, compare to Figure 2A). It seems most likely that the weak resistance of these mutants results from an indirect effect of reduced cholinergic muscle excitation in these mutants, rather than from any direct effect on the mechanism of...
Figure 3. The Nrf Mutants' Response to Other Muscle Agonists and Synaptic Transmission-Defective Mutants' Response to Fluoxetine

(A and B) Time course of nrf-6 and ndg-4 mutants response to nose muscle contraction induced by the nicotinic acetylcholine agonist levamisole (A) and the acetylcholine esterase inhibitor aldicarb (B). Similar results were seen at 3 μM and 12 μM levamisole, 0.5 mM and 2.0 mM aldicarb, with the Na\(^+\), K\(^+\)-ATPase inhibitor ouabain at 5, 10, and 15 mM (data not shown), and with other Nrf mutants described in the text.

(C and D) Time course of nose contraction induced by fluoxetine in synaptic transmission-defective mutants lev-1, cha-1, and unc-31. nrf-6 mutants are shown for comparison as representative Nrf mutants. Similar results were obtained with unc-17, unc-29, unc-38, and unc-64 mutants. Axes, plots, and error bars are as described in the Figure 1 legend.

fluoxetine-induced nose muscle contraction. Based on these results, it is not surprising that we did not isolate these or other synaptic transmission-defective mutants in our screen.

nrf-5, nrf-6, and ndg-4 Define a Subclass of Nrf Mutants

During our characterization of the Nrf mutants, we observed that nrf-5, nrf-6, and ndg-4 mutants share a secondary phenotype of producing unusually pale eggs. Eggs produced by wild-type animals appear dark when viewed with a dissecting microscope due to a large number of light scattering yolk granules. nrf-5, nrf-6, and ndg-4 eggs have fewer yolk granules and accumulate large globules of yolk in the pseudocoelomic space (data not shown). These yolk globules themselves are not directly responsible for the fluoxetine-resistant phenotype of these animals because males of these genotypes lack yolk and are also Nrf (data not shown). In C. elegans, yolk proteins are synthesized in the intestine, secreted into the pseudocoelomic space, and then taken up by developing oocytes (Kimble and Sharrock, 1983). We have not yet determined in which of these steps nrf-5, nrf-6, and ndg-4 mutants are defective. Lack of yolk is presumably related to the slow development and incompletely penetrant embryonic lethality in the progeny of these mutants (data not shown). Therefore, yolkless embryos of nrf-5, nrf-6, and ndg-4 mutants also lack yolk proteins in their pseudocoelomic space and do not produce a high percentage of dead embryos. It is possible that this results from a partial loss of function of nrf-4 or that the pale eggs of nrf-4 are due to an additional linked mutation that is not relevant to its fluoxetine resistance.

Cloning of nrf-6 and ndg-4 Defines a Novel Gene Family

The common secondary phenotype of nrf-5, nrf-6, and ndg-4 suggests that these three genes function in a complex or pathway and that mutations in these genes confer the fluoxetine-resistant phenotype by a similar mechanism. We cloned nrf-6 and ndg-4 by transgenic rescue of the Nrf and pale egg phenotypes with candidate cosmids clones and subclones (see the Experimental Procedures) and sequencing of mutant alleles. nrf-6 and ndg-4 mutants were rescued by cosmids C08B11 and F56F3 and had mutations in the predicted genes C08B11.4 and F56F3.2, respectively. We found two nonsense mutations in nrf-6 (sa367 and sa525) and one in ndg-4 (sa529) that are expected to truncate substantial portions of the protein and are, thus, likely to be null. Other alleles were missense mutations, a late nonsense mutation, and a mutation of an invariant splice acceptor nucleotide (Figure 4A). The missense alleles nrf-6(sa526) and ndg-4(sa510) have Nrf and pale egg phenotypes that were slightly weaker than the nonsense alleles (data not shown).

We obtained three nrf-6 EST cDNAs from the C. elegans Genome Project and completed their sequence to determine the exon-intron boundaries (see the Experimental Procedures). From the sequence of the nrf-6 cDNAs and the predicted F56F3.2 gene product, we found that nrf-6 and ndg-4 both encode predicted transmembrane proteins that are homologous to each other (Figures 4A and 4B). Both NRF-6 and NDG-4 contain an
Fluoxetine-Resistant Mutants in C. elegans

Figure 4. Alignment, Hydropathy Plots, and Dendrogram of the nrf-6 and ndg-4 Gene Family

(A) Alignment of NRF-6, NDG-4, and GM06434 amino acid sequences. Black boxes denote identical amino acids; gray boxes denote similar amino acids. GM06434 is a partial Drosophila cDNA that we identified from the Genbank EST database. No mutations or map data are known for this gene. Mutations for nrf-6 and ndg-4 are indicated by arrows. nrf-6(sa525), nrf-6(sa367), and ndg-4(sa529) are putative null mutations. Lines labeled TM indicate predicted transmembrane domains. The sixth TM is questionable because it is missing from GM06434. Asterisks indicate conserved cysteine residues in the predicted N-terminal extracellular domain that may form disulfide bonds.

(B) Hydropathy plots of NRF-6 and NDG-4 (Kyte and Doolittle, 1982). Both proteins are predicted to have an N-terminal extracellular domain, approximately 12 transmembrane domains, and a short C-terminal tail. Predicted transmembrane domains are aligned to demonstrate the similar topology.

(C) Dendrogram showing the relationships among nrf-6/ndg-4 family members. All genes other than nrf-6, ndg-4, and GM06434 are predicted by the C. elegans Genome Project and have no known mutations.

N-terminal hydrophilic domain of 200–300 amino acids, followed by approximately 12 hydrophobic predicted transmembrane domains and a short C-terminal tail of fewer than 50 amino acids. The location and orientation of transmembrane domains were predicted by the method of Sonnhammer et al. (1998). We predict that the N-terminal hydrophilic domains of NRF-6 and NDG-4 are extracellular based on the following two observations. First, NRF-6 and NDG-4 have cleavable signal sequences for membrane insertion consisting of a basic residue followed by several hydrophobic residues (Nielsen et al., 1997), and, second, there are several conserved cysteine residues before the first predicted transmembrane domain, with the potential to form the disulfide bonds that are characteristic of extracellular domains (Figure 4A). The predicted topology of NRF-6 and NDG-4 is unlike any previously characterized multi-pass transmembrane protein family, including neurotransmitter transporters, ion channels, and ATP-binding cassette-containing proteins.
Figure 5. Expression Pattern of nrf-6

(A and B) Fluorescence photomicrograph and corresponding Nomarski image of nrf-6::gfp expression in an adult animal in hyp 3 (thin arrows) and hyp 5 (thick arrow), the anterior-most cells in the hypodermis. Lower nrf-6::gfp expression can be seen in more posterior hypodermal cells (arrowheads).

(C and D) Fluorescence photomicrograph and corresponding Nomarski image of nrf-6::gfp expression in intestinal cells of an adult animal.

Sequence database searches with NRF-6 and NDG-4 identified a family of over a dozen other predicted \textit{C. elegans} proteins with no significant homology to any other proteins of known function (Figure 4C). Members of this novel gene family all have similar predicted topologies, but their sequences are more similar in their transmembrane domains than in their N-terminal predicted extracellular domains. By sequence database searches, we also identified a \textit{Drosophila} EST, GM06434, with homology to this novel gene family. In order to better determine this relatedness, we obtained and sequenced the GM06434 cDNA and found that it is a partial cDNA with homology extending from approximately the third transmembrane domain to the C terminus of NRF-6 and NDG-4 (Figure 4A). Recently, we have identified six additional \textit{Drosophila} ESTs and two from \textit{Bombyx mori} that also have homology to the nrf-6/ndg-4 gene family.

Because we isolated multiple Nrf mutations in nrf-6 and ndg-4, it is unlikely that the other members of this family can mutate readily to confer resistance to fluoxetine-induced nose contraction. nrf-5 has mutant phenotypes that appear identical to nrf-6 and ndg-4, but nrf-5 is apparently not a member of this gene family because its genetic map position does not coincide with any family members. Furthermore, based on our genetic map data, none of the other Nrf mutants are candidates for corresponding to other members of the novel gene family.

Expression Patterns of nrf-6 and ndg-4

In order to determine the expression pattern of nrf-6, we fused the green fluorescent protein (GFP) (Chalfie et al., 1994) coding sequence in-frame to the 3’ end of the nrf-6 cDNA under the control of 2.3 kb of nrf-6 upstream sequence (see the Experimental Procedures). This fusion gene was able to rescue the Nrf and pale egg phenotypes of nrf-6 mutant animals (data not shown) and expressed strong GFP fluorescence in hyp 3 and hyp 5, the most anterior cells in the hypodermis, and in the intestine (Figure 5). This expression began at the L1 stage and continued throughout adulthood. Weaker fluorescence was also observed in other hypodermal cells throughout the animal, particularly in later stages of development. To determine the expression pattern of ndg-4, we fused 2.0 kb of the ndg-4 upstream region to the GFP coding region. In transgenic animals expressing this promoter fusion, we observed fluorescence in a pattern virtually identical to the nrf-6::gfp fusion described above (data not shown). These results suggest that nrf-6 and ndg-4 have highly overlapping, and probably identical, expression patterns.

Nrf-Nrf Double Mutants Do Not Have Enhanced Resistance

One explanation for our observation that nrf-6 and ndg-4 null mutants are only partially fluoxetine resistant (see above) is that they both play similar roles in conferring sensitivity to fluoxetine-induced nose contraction but act in parallel. If this were the case, then nrf-6;ndg-4 double mutants should have increased resistance to fluoxetine-induced nose contraction when compared to the single mutants. Alternatively, because of their similar mutant phenotypes, molecular identities, and expression patterns, nrf-6 and ndg-4 might interact directly to form a complex or act in a common signaling pathway. In order to test these hypotheses, we constructed nrf-6;ndg-4 double mutants using putative null alleles of nrf-6 and ndg-4 and assayed their response to fluoxetine. We found that this double mutant was indistinguishable from the respective single mutants (Figure 6A and data not shown). This result suggests that nrf-6 and ndg-4...
function in the same pathway, possibly in a complex, to confer sensitivity to fluoxetine-induced nose contraction and that elimination of either gene function is sufficient to eliminate the function of the entire complex or pathway. Using similar logic, we constructed nrf-6:nrf-5 double mutants and found that they were also indistinguishable from the respective single mutants (Figure 6B and data not shown). This suggests that nrf-5 also functions in the same pathway or complex as nrf-6 and ndg-4.

Discussion

A Novel Screen for Antidepressant Targets

The screen described here represents a novel approach to identifying targets of antidepressant drugs. Previously, antidepressants have been characterized primarily by their in vitro binding to specific receptors or other signal transduction components. For example, SSRIs were shown to bind and inhibit serotonin reuptake transporters but not dopamine or norepinephrine reuptake transporters. It is widely assumed that the molecular targets identified by these in vitro binding assays represent the targets that are relevant to the action of the drug in vivo. Some evidence has been obtained that fluoxetine has similar serotonin reuptake blocking activity in vivo, but establishing a causal relationship between this activity and the antidepressant action of fluoxetine has been challenging, particularly given the complexity of clinical depression. Many years of clinical use have established that fluoxetine has numerous side effects in addition to relieving depression. One of these side effects, the inhibition of liver cytochrome P450, is known to be due to an effect other than serotonin reuptake inhibition, and it seems likely that other side effects result from additional targets. A complete enumeration of in vivo targets is essential both for understanding the action of fluoxetine as an antidepressant and in future development of antidepressants with fewer negative side effects. The Nrf mutant screen is an attempt to identify antidepressant targets using a genetic screen that makes no assumptions about the activities of the drug in question.

The fact that the serotonin-deficient mutants cat-1 and cat-4 are fully sensitive to fluoxetine-induced nose muscle contraction suggests that fluoxetine has a neuromuscular activity that is distinct from its well-characterized serotonin reuptake inhibition. We have shown that other SSRIs and tricyclic antidepressants have similar effects on the nose muscles of C. elegans and that mutants resistant to fluoxetine are also resistant to these other drugs. This is significant because it suggests that in C. elegans, SSRIs and tricyclics have a target in common other than the serotonin reuptake transporter. Excitable cells in C. elegans have a wide range of the receptor, ion channel, and signal transduction components that have been found in insects and vertebrates (Rand and Nonet, 1997; Bargmann, 1998). Therefore, we think that it is likely that targets of fluoxetine that are identified in C. elegans will have mammalian homologs. In support of this assertion, it has been recently demonstrated that the antidepressant imipramine interacts directly with the eaq voltage-gated K⁺ channel from both C. elegans and mice (D. Weinshenker et al., submitted).

The Function of nrf-6 and ndg-4

Two of the genes identified in our screen for fluoxetine-resistant mutants, nrf-6 and ndg-4, define a novel gene family that encodes multipass transmembrane proteins. In the absence of fluoxetine, nrf-6 and ndg-4 mutants do not have severe neuromuscular defects, suggesting that they are not Nrf because of a general decrease in neurotransmission. Furthermore, we found that mutants with generally decreased neurotransmission are not strongly Nrf.

A nrf-6::gfp fusion protein that rescues all mutant phenotypes and an ndg-4::gfp promoter fusion are both expressed in the hypodermis, particularly at the tip of the nose, and in the intestine. Because mutants for these genes were isolated in a screen for a neuromuscular effect, it is not immediately obvious how the Nrf phenotypes of these mutants can be explained from the expression patterns. It is unlikely that the intestinal expression of nrf-6 and ndg-4 is relevant to their fluoxetine resistance because pharyngeal pumping ceases almost immediately in wild-type animals bathed in fluoxetine (data not shown). Therefore, we predict that very little drug would enter through the intestine. In contrast, the expression of nrf-6 and ndg-4 in the hypodermis surrounding the nose is more plausibly relevant to their nose-resistant mutant phenotypes. Previous pharmacological experiments on C. elegans have established that the cuticle and hypodermis form a relatively impermeable barrier to many pharmacological agents (e.g., Lewis et al., 1980a). One possibility is that nrf-6 and ndg-4 function to transport fluoxetine across the hypodermal barrier to the inside of the animal, where it can then act on neuromuscular targets to induce muscle contraction. nrf-6 and ndg-4 are predicted to have approximately 12 transmembrane domains, a topology that is consistent with a transport function. Double mutant analysis suggests that nrf-6 and ndg-4 act together in a complex; this complex may have the proposed transport function. It is unlikely that nrf-6 and ndg-4 function nonspecifically to transport pharmacological agents across the hypodermis, because nrf-6 and ndg-4 mutants are fully sensitive to low levels of three other drugs that induce nose muscle contraction: levamisole, aldicarb, and ouabain. However, since nrf-6 and ndg-4 were also resistant to nose contraction by two other antidepressants, this proposed transporter would have to transport multiple chemically diverse compounds. Alternatively, fluoxetine and related antidepressants may be agonists of a nrf-6/ndg-4 transporter that transports another compound that directly or indirectly induces nose muscle contraction. These antidepressants all share one target in the serotonin reuptake transporter and may also share nrf-6 and ndg-4 as targets.

An alternative model that may explain the expression of nrf-6 and ndg-4 in the anterior hypodermis is based on an observation from the parasitic nematode Ascaris. In assays of ACh synthesis, Johnson and Str€utn (1985) found that while excitatory motoneurons had high levels of choline acetyltransferase (ChAT) activity, hypodermal tissue also had detectable levels. Although typical hypodermal ChAT levels were 10-fold lower than neurons, they found that hypodermal tissue from the tip of the
nose had much higher ChAT levels, comparable to neuronal tissue. Many similarities have been described between the neurophysiology and neuroanatomy of C. elegans and A. punctulatum (White et al., 1976; Stretton et al., 1978). In particular, AChe is the primary excitatory neurotransmitter for body muscle in both nematodes. Therefore, AChe in the anterior hypodermal cells in C. elegans may be relevant to fluoxetine-induced nose contraction. For example, fluoxetine could stimulate the release of AChe from anterior hypodermal cells, thereby causing nose muscle contraction. Although total loss of AChe signaling is lethal (Alfonso et al., 1994), we found that cholinergic-defective mutants unc-29, unc-38, lev-1, unc-17, and cha-1 had weak defects in response to fluoxetine-induced nose contraction. This result could be explained by residual or redundant AChe function, or the proposed fluoxetine-sensitive hypodermal AChe pathway could be independent of these mutants. If nrf-6 and ndg-4 were involved in hypodermal AChe synthesis, storage, or release, eliminating them by mutation could result in a Nrf phenotype. It will be interesting to determine the biochemical function of nrf-6 and ndg-4, their role in conferring fluoxetine sensitivity, and their relationship to the many related gene products in both C. elegans and other organisms.

Based on the expression of nrf-6 and ndg-4 in the hypodermis and intestine, and their predicted transmembrane topology, one possibility is that these two genes play a role in regulation of membrane transport in C. elegans. Tightly regulated transport across the membranes of brain capillary endothelial cells is the basis of the blood-brain barrier, which controls the passage of substances in and out of the brain (Goldstein and Betz, 1986). However, the molecular mechanisms by which antidepressants cross the blood-brain barrier to reach their presumed sites of action is poorly understood. One possibility is that nrf-6 and ndg-4 have an analogous role in membrane transport in C. elegans. If so, these genes could serve as a valuable model system for studying drug transport across membranes.

**Experimental Procedures**

**Pharmacology**

Stock solutions of fluoxetine (Eli Lilly, Indianapolis, IN), paroxetine (SmithKline Beecham, Philadelphia, PA) and clomipramine (Sigma, St. Louis, MO) in M9 buffer (Brenner, 1974) were prepared by mixing 12-16 hr at 37°C and were stored at room temperature wrapped in aluminum foil for 3-4 weeks with no obvious decrease in potency. For all three drugs, 1.0 mg/ml was at or near the maximum solubility in M9 buffer. Stock solutions of 100 mM levamisole (Sigma) in water and 100 mM alicarb (Chem Service, West Chester, PA) in 70% ethanol were stored at -20°C until use (Lewis et al., 1980a; Nguyen et al., 1995). Stock solutions of 15 mM ouabain were made fresh and sonicated to dissolve completely.

For nose contraction assays using antidepressants and ouabain, adult animals were transferred to microwell plates containing 25 μl of drug in M9 buffer. Nose contraction assays with levamisole and alicarb were performed on standard NEM plates (Brenner, 1974). Levamisole or alicarb was added to the NG agar immediately before plate pouring. Animals were scored for nose contraction using a dissecting microscope at 50× magnification. Animals with any discernible nose rounding were scored as contracted. All strains were grown at 20°C and assayed at room temperature (-22°C), except for cha-1(pJ1866ts), which was grown at 20°C, then shifted to 25°C for 2-5 hr before assaying at 25°C. For most assays, the results of six or more trials of ten animals each were averaged. Statistical analysis was performed using InStat v.2.01 for the Macintosh.

For Nrf screens, F2 progeny of ethylmethane sulfonate (EMS)-mutagenized (Brenner, 1974) N2 animals were incubated in 1.0 mg/ml fluoxetine with agitation. After 20 min, adult animals with uncontracted noses were picked as candidate mutants, and their broods were retested for heritable resistance. Approximately 11,000 mutagenized genomes were screened in total. Although fluoxetine also causes paralysis of body muscles, we found this phenotype to be less robust and more difficult to score accurately, and therefore, we instead screened for resistance to nose contraction.

**Genetics**

The following mutations were used for the pharmacological assays reported here: cat-1(e1111), cat-4(e1411), unc-38(x111), unc-29(1072), lev-1(e121), unc-7(e113), cha-1(pJ1866ts), unc-31(e928), unc-64(e246), nrf-6(sa367), nrf-6(sa525), ndg-4(sa529), nrf-5(sa513), nrf-2(sa366), nrf-3(sa363). Bristol N2 was the wild-type strain, and all mutants used were derived from this background.

Standard genetic mapping techniques were used (Brenner, 1974). All map data are available from the Caenorhabditis Genetics Center. Map data for the most informative crosses are presented below. The numbers in parentheses indicate the number of recombinants detected between the respective genes. nrf-1 mutants were mapped by their gonadal defect: nrf-1 (5 dpy-17(3) unc-32. nrf-2 and nrf-3 mutants were mapped by their Nrf phenotype: unc-85 (5) nrf-2 (5) dpy-10 and unc-24 (2) nrf-3 (21) daf-14, nrf-4, nrf-5, nrf-6, and ndg-4 were mapped by their pale egg phenotype: dpy-5 (17) unc-29 (3) nrf-4; lin-25 (10) nrf-5 (13) unc-76; unc-104 (92) nrf-6 (8) unc-105; and unc-103 (39) ndg-4 (54) dpy-17.

**Molecular Biology**

nrf-6(sa367); lin-15(n765ts) animals were injected with candidate cosmid or cosmid subclones (5 ng/μl) and pbLH 90 lin-15(1) as a marker for transformation (Mello et al., 1991; Clark et al., 1994). Transgenic progeny were scored for rescue of the Nrf and pale egg phenotypes. Cosmid pools containing cosmid C08B11, C08B11 alone, or pRC1 (an 8 kb BsaHI fragment containing the predicted gene C08B11.4) all rescued the Nrf phenotype. It will be interesting to determine the biochemical function of nrf-6 and ndg-4, their role in conferring fluoxetine sensitivity, and their relationship to the many related gene products in both C. elegans and other organisms.

For nose contraction assays using antidepressants and ouabain, adult animals were transferred to microwell wells containing 25 μl of drug in M9 buffer. Nose contraction assays with levamisole and alicarb were performed on standard NG plates (Brenden, 1974).

**GFP Fusions**

For nrf-6:gfp, the GFP coding region was generated by PCR from pPD95.69 (A. Fire) and inserted at the BspBI site (Arg-819) in the nrf-6::gfp plasmid. A 2.8 kb NotI-BglI genomic fragment containing 2.3 kb of nrf-6 promoter, exon 1, intron 1, and part of exon 2 was then fused to the BglI site in exon 2 of the nrf-6 cDNA:gfp fusion. The resulting plasmid was injected with pbLH98 into nrf-6(sa367); lin-15(n765ts) animals, and transgenic lines were scored for fluorescence. For the ndg-4::gfp fusion, 2.0 kb of sequence upstream of the
F56F3.2 predicted ndg-4 ATG was generated by PCR from cosmid F56F3 and fused by overlap extension PCR to GFP generated by PCR from pPD95.69. Bulk PCR product was injected along with pblH98 into lin-15(n765ts) animals, and transgenic lines were scored for fluorescence.

Acknowledgments

We thank Margaret Niedenthal at Eli Lilly and Company for fluoxetine, Wayne Shreffler and Eve Wolinsky for ndg-4(B108), Alan Coulson for cosmids, the Genome Sequencing Centers for sequence information, Andrew Fire for GFP vectors, and Yuji Kohara for cDNAs, and Elizabeth Newton, David Weisnhenker, Leo Pallanck, and Ed Giniger for helpful comments on this manuscript. Some of the strains used in this study were obtained from the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources. R. K. M. C. is supported by a Predoctoral Fellowship from the Howard Hughes Medical Institute. This work was supported by National Institutes of Health grant R01 MH58916 to J. H. T.

Received March 26, 1999; revised June 2, 1999.

References


GenBank Accession Numbers

The sequences reported in this paper have the following GenBank accession numbers: nrf-6, AF173372; ndg-4, AF173373; and GM06434, AF173374.